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Association of plasma clusterin concentration with severity, pathology, and progression in Alzheimer disease

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AddNeuroMed consortium

Abstract

Context—Blood-based analytes as indicators of pathological processes in Alzheimer's disease (AD).

Objective—Combined proteomic and neuroimaging approach to identify plasma proteins associated with AD pathology.

Design—Discovery-phase proteomic experiments to identify plasma proteins associated with correlates of AD pathology including evidence of atrophy using neuroimaging and more rapid clinical progression, followed by replication using quantitative immunoassay. Extension studies in older non-demented humans using ^{11}C-PiB amyloid imaging and transgenic mice with amyloid pathology.

Setting—Multi-center European study, AddNeuroMed, and the Baltimore Longitudinal Study of Aging (BLSA) in United States.

Participants—AD patients, mild cognitive impairment (MCI) subjects and healthy controls with standardized clinical assessments and structural neuroimaging. Plasma samples from non-demented older BLSA participants with brain amyloid imaging by PET.

Main outcome measures—Association of plasma proteins with brain atrophy, disease severity and rate of clinical progression. Extension studies in man and transgenic mice tested 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 beta amyloid (Aβ) burden in the medial temporal lobe. Subjects with AD had increased clusterin mRNA in blood but there was no effect of SNPs in the gene encoding clusterin (CLU) with gene or protein expression. Finally, APP/PS1 transgenic mice showed increased plasma clusterin, age-dependent increase in brain clusterin and amyloid and clusterin co-localisation in plaques.

Conclusions—Clusterin/apolipoprotein-J is a known amyloid chaperone associated with Alzheimer's disease severity, pathology and progression. Increased plasma concentration of clusterin is also associated with greater burden of fibrillar Aβ in the brain. 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 Alzheimer's disease.

INTRODUCTION

Peripheral compartments including blood and cerebrospinal fluid (CSF) exhibit signals reflecting neuropathological changes in Alzheimer's disease (AD)1, 2. In CSF, these include a decrease in Aβ and an increase in total and phosphorylated tau concentrations3, reflecting amyloid sequestration as plaques and neurofibrillary degeneration respectively4, 5. Similarly, while numerous reports suggest that plasma concentrations of several metabolites and proteins might represent responses to neuropathological changes in AD6–11, these findings have not been conclusively replicated12. 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 difficult13,14. 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 slowly15,16.

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), two components of the medial temporal lobe (MTL) that show early pathological changes in AD17. For rate of clinical progression we used both retrospective and prospective measures of cognitive decline. We initially performed two independent discovery-phase studies using proteomic analysis of plasma in separate groups of subjects. In the first, we sought proteins reflecting hippocampal atrophy, in mild cognitive impairment (MCI) and established AD. In the second, we identified proteins differentially expressed in fast 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 ELISA in a large independent cohort of AD, MCI and control subjects.

METHODS

SUBJECTS AND SAMPLES

Samples used came from two studies – the Alzheimer's Research Trust funded cohort at King's College, London (KCL-ART)7 and AddNeuroMed18 studies. The KCL-ART study is a cohort of people with AD, MCI19 and normal elderly started in 2001. All subjects are white UK citizens with grandparents born in the UK and are assessed annually. AddNeuroMed is a cross-European cohort; AD cases are assessed at 3-monthly 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 UK, France, Italy, Finland, Poland and Greece. Standardized assessments include demographic and medical information, cognitive assessment including MMSE (both studies; all subjects), ADAS-Cog (AddNeuroMed only), CERAD battery, and scales to assess function, behavior and global levels of severity including the Clinical Dementia Rating (CDR). Cases with probable AD (NINCDS-ADRDA criteria) and amnestic MCI were identified as previously described7 and evaluated with a standardised assessment shown to have high diagnostic validity20. Cases with amnestic MCI were defined as having subjective memory complaints, CDR score <1 and evidence of objective memory impairment using the CERAD delayed word list recall (−1.5 SD cut off). Normal elderly controls, defined as having no evidence of cognitive impairment (MMSE > 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 described7, 18. Intotal we studied 95 and 689 subjects in discovery and validation studies respectively with a further 60 subjects from the Baltimore Longitudinal Study of Aging21 (supplementary tables 1–4). Ethical approval was obtained in each of the participating countries.

NEUROIMAGING

MRI DATA ACQUISITION—In the KCL-ART study, whole-brain coronal three-dimensional SPGR images (repetition time [TR] = 14 msec, echo time [TE] = 3 msec, 256 × 192 × 124 acquisition matrix, 1.5 mm slices) were obtained on a GE Signa 1.5T Neuro-optimized MR system. In the AddNeuroMed study, whole-brain sagittal three-dimensional MP-RAGE images (TR = 8.6, TE = 3.8, 256 × 192 acquisition matrix, 180 × 1.2mm slices) were obtained on a 1.5T MR system at each of the 6 centers. Quality control was undertaken using the ADNI phantom and two volunteers who visited each of the centers, ensuring compatibility across the study. ERC was calculated with Freesurfer using a cortical reconstruction technique22،23.

^{11}C-PiB PET STUDIES—Dynamic ^{11}C-PiB PET studies (37 time frames over 90 minutes) were acquired in 3D mode on a GE Advance scanner immediately after intravenous bolus injection of approximately 15 mCi. 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 4.5mm FWHM at the center of the field of view. Parametric images of distribution volume ratios (DVR) were calculated by simultaneous fitting of a reference tissue model using linear regression and spatial constraint with the cerebellum as a reference region24،25. SPM5 (Statistical Parametric Mapping 5; Wellcome Department of Imaging Neuroscience, London, UK) was used to investigate the association between clusterin and medial temporal ^{11}C-PiB retention (significance threshold of $p \leq 0.05$, with a spatial extent of 25 voxels). Based on *a priori* hypotheses in the light of our results on association between ERC atrophy and clusterin concentration in AD, a restricted search of the MTL was performed using the regional definition from the WFU Pick-Atlas26.

PROTEOMICS

Two-dimension gel electrophoresis (2DGE) and tandem mass spectrometry (LC/MS/MS) were performed as previously described7. Gels were analysed using image analysis software (either Melanie 2-D or Progenesis SameSpots v3.0 (Nonlinear Dynamics)). Protein spots of interest were excised, washed, in-gel digested with trypsin and analysed by LC/MS/MS7.Mass spectral data were processed into peptide peak lists and searched against the Swiss-Prot Database using Mascot software (Matrix Science, UK).

For validation experiments plasma clusterin concentration was assayed by a commercially available ELISA kit (Human Clusterin ELISA, RD194034200R, Biovendor Laboratory Medicine Inc). 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%, BLSA sub-study 3.1%)

GENOMICS

GENE EXPRESSION OF CLUSTERIN—Approximately 2.5ml of venous blood was collected into a PAXgene tube for each subject at the baseline visit, processed according to 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 manufacturer instructions. Samples were assessed for yield using a spectrophotometer and quality using the RNA 6000 Pico Chip on the Agilent Bioanalyser. Samples with a RNA integrity number>7.0 were used for PCR assays.

Using the Quantitect Reverse Transcription kit (Qiagen), 500ng RNA was reverse transcribed to cDNA in a 40μl reaction, and subsequently diluted to 200μl. RT-PCR reactions were performed in 384 well plates in the 7900HT Fast Real-time PCR machine (Applied Biosystems). geNORM housekeeping selection kit (Primer Design Ltd) was used

to assay 12 housekeeping genes in a subset of the samples. Using NormFinder software, the two most stable genes for normalisation were determined to be splicing factor 3a, subunit 1 (SF3A1) and ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (ATP5B). Samples were assayed in duplicate and a standard curve of known copy number run on each plate for clusterin, SF3A1 and ATP5B. Data was nonparametric, and was therefore log-transformed.

CLUSTERIN GENOTYPING

Tagger software (http://www.broad.mit.edu/mpg/tagger/) identified seven 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).. 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) were genotyped for the seven SNPs.

TASTPM TRANSGENIC MOUSE MODEL EXPERIMENTS

Heterozygote transgenic mice overexpressing hAPP695swe (TAS10) and presenilin-1 M146V mutations (TPM) were generated as previously described27. Western blot analysis of clusterin was performed in plasma samples at 6 months using.an anti-ApoJ mouse polyclonal (Abcam AB349-50; 1:5,000). For immunohistochemistry, antigen retrieval was undertaken as described previously27›28. Primary antibodies were 1E8 (pan-Aβ), 20G10 (Aβ42) (GlaxoSmithKline;1:1000) and anti-clusterin (R&D Systems goat polyclonal AF2747;1:20,000). Images were captured at ×4 magnification on an Axioscope microscope and analysed by Image J software to generate percentage Aβ or clusterin load. Animal experiments were conducted according to the Council of Europe (Directive 86/609) guidelines.

STATISTICS

Discovery phase proteomic data was analysed by Partial Least Squares (PLS) regression using SIMCA-P (v.8.0). Spot data were scaled to unit-variance and log10 transformed where appropriate. Observations with greater than 50% missing values were excluded. Partial least squares discriminant analysis (PLS-DA) was used to derive a panel of protein spots that discriminated between fast and slow declining AD groups.

Validation-phase protein data was examined using SPSS (version 17). Covariates were chosen where such variables were significantly different between the groups of interest or where 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 gender as covariates. In analysing associations between MMSE and plasma clusterin concentration, partial correlation was performed with age as covariate. To test differences in clusterin concentration between rapid and non-rapidly declining AD patients, age and gender were not significantly different between the two groups and were therefore not included as covariates. 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 non-rapid decliners and clusterin concentration between these groups was therefore compared using an independent samples t-test. Linear regression adjusting for disease status, age, gender and APOE ε4 status was performed to investigate the association between CLU SNPs and clusterin plasma levels and to examine the relationship between CLU mRNA and disease. Image analysis is

described in the relevant sections. All other statistical analyses were performed using SPSS (v.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 2DGE and LC-MS/MS with hippocampal atrophy as the independent variable. Here we analysed samples from 44 subjects from the KCL-ART cohort representing a continuum of disease; 27 with mild to moderate AD and 17 with MCI (table-1 Supplementary data). Bivariate correlation of integrated optical densities of spots detected by 2DGE revealed 13 spots that were significantly associated with hippocampal volume ($r \geq +/-0.35$ and $p<0.05$). Subsequently, using PLS regression29, a method suited to analysis of proteomic data where collinearity among predictor variables is common, a model with two components was fitted to the hippocampal volume data. This was constituted by 8 of the 13 spots which, together, explained 34% of the variance in (R2Y) in hippocampal volume. Using LC-MS/MS we identified these eight spots as complement C3, γ -fibrinogen, serum albumin, complement factor-I, clusterin (in two spots), α-1-microglobulin, and serum amyloid-P (Figure-2). We then performed a second discovery-phase experiment in an independent set of samples in 51 carefully matched (age, gender, severity at the time of blood sampling, all subjects on cholinesterase inhibitor treatment) AD subjects from the AddNeuroMed cohort that we could divide into fast (N=22) or slow progressors (N=29) based on their annualized rate of cognitive decline (Table-1 Supplementary data). We defined, *a priori*, fast decline as a fall of 2 or more points on the ADAS-cog scale over a period of 6 months. A PLS-DA model discriminating the fast from slow progressing AD groups was constituted by the integrated optical densities of 27 silver-stained 2DGE spots. Of these, 8 were well-defined, discrete, present in all 51 gels and were identified by LC-MS/MS. These spots contained complement component C4 (in three spots), complement C8, clusterin, apolipoprotein-A1 (in two spots) and transthyretin (Figure-2).

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

Only one 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 (Table-2 supplementary data). 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 two studies.

Confirming the discovery-phase study, we observed a trend towards association between clusterin concentration and ERC atrophy in the combined AD+MCI cohort (n=219, $R= -0.12$ and $p=0.06$) after covarying for age and gender. This relationship was driven primarily by a highly significant association between ERC atrophy and clusterin concentration in AD patients (n=113, $R= -0.30$ and $p=0.001$). 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 ($r=-0.22$; $p<0.001$; age as covariate).

We then compared clusterin levels in fast 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 Table-2, supplementary data). Retrospective decline was estimated from the duration of disease and the MMSE at the point of blood sampling allowing the annualized fall in MMSE to be calculated. We used MMSE as the ADAS-cog score was not available in all subjects and defined fast decline as a fall in 2 points or more over a period of one year relative to the time of blood sampling. Prospective decline was directly measured as the fall in MMSE one year after blood sampling. We observed a significant increase in clusterin concentration in AD patients with accelerated cognitive decline prior to blood sampling (ANCOVA; n=344; t(341)=3.40; p=0.0007; duration of disease as covariate) (Figure-3A) and an increase in clusterin concentration in AD patients with faster cognitive decline subsequent to blood sampling (N=237; independent samples t-test, p=0.01) (Figure-3B). Cox proportional regression analysis showed that higher plasma clusterin concentration was associated with a greater risk of rapid cognitive decline one year after blood sampling (Figure-3c). We then performed an analysis of variance (age and gender 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 (S.D. 25.6; N=336), MCI 77.6 ng/ml (S.D. 22.5; N=222), and control subjects 82.2 ng/ml (SD 23.8; N=385). Finally we compared differences in plasma clusterin concentration between APOEε4 carriers and non-carriers (independent samples t-test) in the combined cohort of AD, MCI and control subjects and did not find any significant difference.

CLUSTERIN IS ASSOCIATED WITH FIBRILLAR AMYLOID BURDEN IN THE ENTORHINAL CORTEX IN NON-DEMENTED OLDER INDIVIDUALS

As 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 amyloid burden with ^{11}C-PiB (N=60; Table-3, supplementary data) 28xx. Although all participants were non-demented at the time of the PiB-PET study, a range of *in vivo* amyloid burden is observed in cognitively normal individuals30 and increased amyloid deposition may represent the earliest phase of AD pathology in these subjects. Measuring plasma clusterin concentration from samples collected ten years before the PiB-PET studies, we investigated associations between clusterin concentration and subsequent development of *in vivo* fibrillar amyloid burden.

A directed search of significant associations between clusterin and MTL PiB values was conducted using the MTL region defined by the WFU Pick-Atlas26 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, higher on the right (right ERC; $p<0.009$ and left ERC; $p<0.034$) (Figure 4A). This suggests that increased plasma concentration of clusterin, even in non-demented older individuals is predictive of greater extent of fibrillar amyloid burden in the ERC - the same region where we also demonstrate 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) (Table-4, supplementary data). Diagnosis had a significant effect on clusterin gene expression (ANCOVA; df=2; $P<0.001$ and age as covariate). Pair-wise comparisons between the three groups showed significantly higher clusterin gene

expression in AD than MCI and control subjects (P=0.008 and P<0.001 respectively; Bonferroni adjustment for multiple comparisons) (Figure 4B). Gender and presence of the apolipoprotein-E (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 or rate of decline in MMSE 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 seven clusterin gene SNPs on either clusterin mRNA expression in blood cells or plasma concentration of clusterin (supplementary data; Table-5 and 6). The SNPs analysed included those reported in the recent large GWAS studies to be associated with risk for sporadic AD[31,32].

PLASMA CONCENTRATION OF CLUSTERIN IS INCREASED 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 over-production of human APP[27] and mimic various hallmarks of AD including amyloid plaques as well as cognitive and behavioural deficits[27,28]. In the light of our MRI data in AD patients and PiB-PET results in non-demented 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=0.02; independent samples t-test) in 6-month old transgenic TASTPM mice (N=10) relative to wild-type littermates (N=10) (Figure 4C). Previous studies have established both marked Aß cerebral deposits as well as cognitive deficits in TASTPM mice at this age relative to wild type littermates[27,28].

BRAIN CLUSTERIN IS CLOSELY ASSOCIATED WITH AMYLOID IN A TRANSGENIC MOUSE MODEL OF ALZHEIMER'S DISEASE

Using double labeling immunohistochemistry we demonstrated that cortical plaques in TASTPM mice contained both Aß and clusterin (Figure-4D). Finally, we established the close association between Aß and clusterin by showing that both cortical Aß burden and clusterin deposition increase with age in TASTPM mice (N=9–11) (Figure-4E) and that there is a highly significant correlation ($F_{1, 37} = 107.57$, $p<0.0001$, adjusted $R^2 = 0.737$) between cortical Aß 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 Alzheimer's disease.

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 MRI images and in the much larger validation phase, 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, associated with an increased risk of conversion from MCI to AD and may even precede the development of cognitive decline33,34. CSF levels of phosphorylated tau (p-tau) correlate with hippocampal volume, indicating that this measure reflects an integral feature of AD pathology35. Moreover, decreased hippocampal volume in AD patients is associated with neuronal loss, confirming its validity as a marker of neurodegeneration35. 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 over a 6-month interval in AD patients. Using this measure, we dichotomised AD patients as fast and slow decliners; an approach previously shown to predict long term prognosis in AD36.

Only clusterin was associated both with hippocampal atrophy, in AD and MCI subjects and with fast progressing, or more aggressive AD. Evidence from human CSF, post-mortem brain and transgenic animal models suggest a plausible link between clusterin and AD pathology37–40. 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, MMSE and rate of progression in AD ($p=0.001$, $p<0.001$ and $p=0.0007$ respectively). 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 subsequent deposition of fibrillar amyloid in the ERC. Our finding of raised plasma clusterin concentration ten years before fibrillar amyloid deposition in brain in normal aged individuals suggests that clusterin is raised very early, possibly as an aetiopathological 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, two groups, including one in which we participated, reported from genome wide studies that polymorphic variation in the CLU gene, encoding clusterin, was associated with AD31,32. 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 six 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 two, perhaps 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 gene 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 two closely related but distinct spots in the discovery-phase 2DGE studies. Proteins are components of multiple spots on 2DGE because of changes in post-translational 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 mutations41 and also show that clusterin is closely associated with cortical amyloid plaques and shows an age-dependent concomitant increase with brain amyloid burden.

Previous studies suggest that clusterin belongs to a family of extracellular chaperones regulating amyloid formation and clearance42. *In vitro* experiments show that clusterin regulates amyloid formation in a biphasic manner with low clusterin:substrate ratios enhancing and higher ratios inhibiting amyloid formation respectively43. In mice, *in vivo* binding of Aβ to clusterin enhances its clearance and efflux through the blood brain barrier44. However, previous studies reporting differences in CSF clusterin concentration between AD patients and controls have been inconclusive39,40. Our findings may have implications for the discovery and characterization of other amyloid chaperone proteins in blood linked to AD pathogenesis. In this context, alpha2-macroglobulin (A2M), has recently been characterized as an amyloid chaperone that inhibits fibril formation45,46. In a previous proteomic analysis of plasma, we reported the differential expression of A2M in AD patients and have also found associations between the plasma concentration of A2M and hippocampal metabolite abnormalities in AD7, 47. In this previous study7, in addition to A2M, we also identified components of the complement pathway associated with AD. We note in the discovery phase of the current study, 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 AD7.

In summary, we have employed a novel proteomic-neuroimaging discovery paradigm where the primary endpoints 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 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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responsibilities; CT, CF, IK, PM, HS, MT, BV, DM, SP and SL were responsible for collection of clinical data; YZ, L-OW, EW, DM, SMR, LF, DFW, YZ, SM, AE, CS were responsible for collection of neuroimaging data; MT, LV, AH, AK, AG, MC, RK, SL, RL, DO'B, MW generated proteomics data from human subjects; MB, FC, DRH, RJW, SIS, CM, JR PTF generated and analyzed protein data from animal models; MT, AH, JC and SL performed statistical analyses on the protein data; MT, AS, YZ, L-OW, EW, SM, AE performed analyses of neuroimaging; PP, JP, KL, AH, GB, SF were responsible for the collection and analysis of genomic data; SL was principal investigator for the project, SL and MT co-wrote the paper with contributions from all authors and SL 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.

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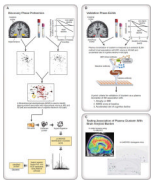

Figure-1. Study design

Schematic diagram of the design of A) Discovery and B) Validation-phase studies for the identification of blood-based 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 non-demented older humans and a transgenic mouse model of AD.

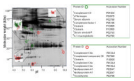

Figure-2. Gel based proteomic discovery phase studies

Proteomic identification of plasma proteins associated with hippocampal volume in AD +MCI subjects (top panel) and those associated with fast AD progressors (bottom panel). A representative 2DGE gel is shown with spots outlined in green denoting proteins associated with hippocampal volume in AD+MCI and those in red highlighting proteins associated with fast AD progression.

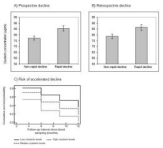

Figure-3. Increased concentration of plasma clusterin is associated with rate of clinical progression in AD

AD patients with a rapid progression rate, measured A) Prior to blood sampling (Rapid progressors; N=219, Slow progressors; N=125) and B) One year after blood sampling (Rapid progressors; N=115, Slow progressors; N=122) 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. AD patients (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. The figure shows the cumulative hazard functions for the effect of the `prognostic factor' (i.e. plasma clusterin concentration) on the `survival probability' i.e. maintaining a non-aggressive clinical course (decline in MMSE≤ 2 points/ year). The cumulative survival functions represent estimated survival probabilities for three representative AD patients with the lowest (5.87ng/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 non-aggressive clinical course one 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%CI (1–1.147), p=0.05.

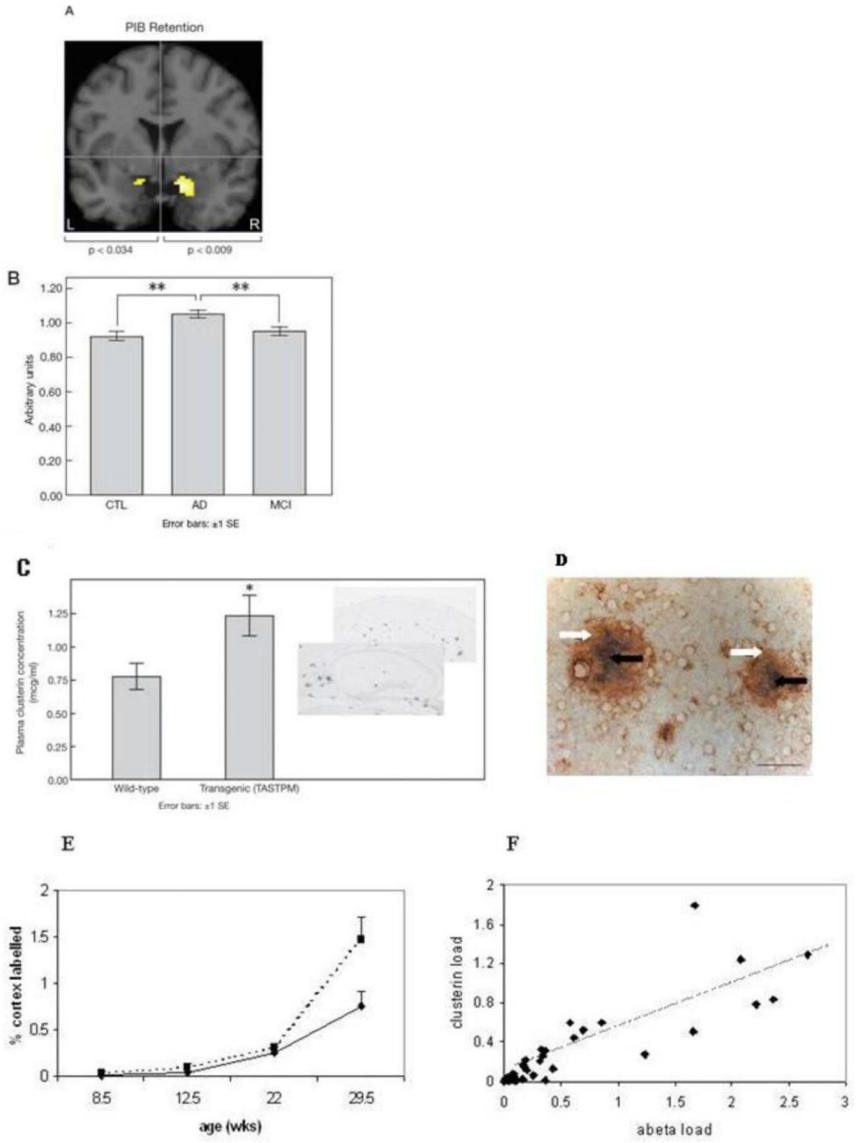

Figure-4. Clusterin expression is associated with amyloid pathology

A) Clusterin is an antecedent biomarker of *in vivo* fibrillar amyloid burden in the entorhinal cortex in non-demented older individuals (N=60). SPM analysis shows correlation between plasma clusterin concentration and ^{11}C-PiB uptake controlling for age and sex, $p < 0.05$; uncorrected. Highlighted areas denote regions in the ERC of both hemispheres that show significant association with plasma clusterin concentration 10 years prior to the PiB-PET scans.

B) Gene expression of clusterin is altered in AD. Clusterin mRNA levels are significantly elevated in blood cells from AD patients (N=182) relative to healthy controls (N=179, ** $p<0.001$) and MCI subjects (N=207, ** $p=0.008$) 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 littermates (N=10) at 6 months of age ($p=0.02$). 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).

D) Representative photomicrograph of cortical amyloid plaques in 6-month old TASTPM mouse. A close association is observed between Aβ within amyloid plaques (black arrows – monoclonal antibody to $A\beta_{42}$; grey-black labelling with diaminobenzidine) and clusterin (white arrows – polyclonal antibody, R&D Systems; brown labelled with Novared). Colours have been slightly enhanced digitally for illustrative purposes. Scale bar represents 25 microns.

E) TASTPM mice show age-dependent increases in cortical Aβ (1E8, pan- Aβ; dashed line) and clusterin (solid line) load as determined by quantitative image analysis of immunohistochemical labelling. N=9–11 at each timepoint, mixed male and female mouse population. Quantitative estimates of amyloid burden and clusterin deposition were derived using Image J software.

F) TASTPM mice demonstrate a highly significant ($p<0.0001$) correlation between Aβ and clusterin load (N=39, male and female mice, 8–30 weeks of age). X and Y axes represent Aβ and clusterin load (percentage area labelled) respectively.