

ORIGINAL ARTICLE

CSF protein biomarkers predicting longitudinal reduction of CSF β -amyloid42 in cognitively healthy eldersN Mattsson^{1,2,3}, P Insel¹, R Nosheny¹, H Zetterberg^{2,4}, JQ Trojanowski⁵, LM Shaw⁵, D Tosun^{1,3} and M Weiner^{1,3} for the Alzheimer's Disease Neuroimaging Initiative

β -amyloid (A β) plaque accumulation is a hallmark of Alzheimer's disease (AD). It is believed to start many years prior to symptoms and is reflected by reduced cerebrospinal fluid (CSF) levels of the peptide A β 1–42 (A β 42). Here we tested the hypothesis that baseline levels of CSF proteins involved in microglia activity, synaptic function and A β metabolism predict the development of A β plaques, assessed by longitudinal CSF A β 42 decrease in cognitively healthy people. Forty-six healthy people with three to four serial CSF samples were included (mean follow-up 3 years, range 2–4 years). There was an overall reduction in A β 42 from a mean concentration of 211–195 pg ml⁻¹ after 4 years. Linear mixed-effects models using longitudinal A β 42 as the response variable, and baseline proteins as explanatory variables ($n = 69$ proteins potentially relevant for A β metabolism, microglia or synaptic/neuronal function), identified 10 proteins with significant effects on longitudinal A β 42. The most significant proteins were angiotensin-converting enzyme (ACE, $P = 0.009$), Chromogranin A (CgA, $P = 0.009$) and Axl receptor tyrosine kinase (AXL, $P = 0.009$). Receiver-operating characteristic analysis identified 11 proteins with significant effects on longitudinal A β 42 (largely overlapping with the proteins identified by linear mixed-effects models). Several proteins (including ACE, CgA and AXL) were associated with A β 42 reduction only in subjects with normal baseline A β 42, and not in subjects with reduced baseline A β 42. We conclude that baseline CSF proteins related to A β metabolism, microglia activity or synapses predict longitudinal A β 42 reduction in cognitively healthy elders. The finding that some proteins only predict A β 42 reduction in subjects with normal baseline A β 42 suggest that they predict future development of the brain A β pathology at the earliest stages of AD, prior to widespread development of A β plaques.

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INTRODUCTION

β -amyloid (A β) plaques is a hallmark of Alzheimer's disease (AD) and are reflected by reduced levels of cerebrospinal fluid (CSF) A β 42.^{1–3} Reduced CSF A β 42 has high diagnostic accuracy for AD as determined by autopsy⁴ and agrees well with the results of positron emission tomography (PET) using A β tracers.^{5–7} Although somewhat controversial, many believe that biomarkers reflecting the accumulation of brain A β in plaques are the earliest detectable biomarker signs of AD in elders, reaching pathological levels several years before the development of cognitive symptoms.⁸ Other hallmarks of AD are axonal degeneration and tangle pathology, which are reflected by increased CSF tau (total-tau, T-tau) and phosphorylated tau (P-tau), respectively.⁹ Reports on both autosomal-dominant AD⁸ and late-onset AD¹⁰ suggest that increased CSF T-tau and P-tau levels occur first after widespread amyloid plaque deposition, evidenced by reduced CSF A β 42 and amyloid imaging. Currently, possibly aside from the Apolipoprotein E (APOE) ϵ 4 variant,^{11,12} there are no biomarkers that are known to predict the development of A β plaques in people without familial AD. Biomarkers predicting A β plaque formation in humans would be important both for determining individual risk of A β pathology and as *in vivo* evidence linking distinct molecular pathways to A β pathology in humans.

Experimental and genetic data have suggested that inflammation (especially microglial activity^{13,14}) and synaptic function^{15,16} may be important for A β production, accumulation and/or toxicity, but there are little *in vivo* data supporting such pathways in humans. However, previous studies have suggested that some proteins in human CSF may reflect changes in such pathways.^{17,18}

To our knowledge, no previous study has attempted to identify protein biomarkers that predict longitudinal A β plaque formation in cognitively healthy people. Therefore, the main goals of this study were to investigate the following *a priori* hypotheses using proteins measured in normal controls in Alzheimer's Disease Neuroimaging Initiative (ADNI): (1) baseline levels of CSF proteins involved in A β metabolism, microglia activity, synaptic/neuronal function or other AD-related processes predict longitudinal CSF A β 42 decrease in cognitively healthy people; (2) baseline levels of some of these proteins may predict CSF A β 42 decrease even in subjects without A β plaque pathology at baseline (reflected by CSF A β 42 levels below an AD-related cutoff⁴) and (3) These proteins are less predictive of P-tau and T-tau changes, according to a model in which P-tau and T-tau become abnormal downstream of A β pathology and associated biomarkers.¹⁹

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SUBJECTS AND METHODS

Study design

This was a longitudinal study on the associations between CSF proteins at baseline and longitudinal changes in CSF A β 42, T-tau and P-tau. Data used in the preparation of this article were obtained from the ADNI database (<http://adni.loni.ucla.edu/>). The ADNI was launched in 2003 by the National Institute on Aging, the National Institute of Biomedical Imaging and Bioengineering (NIBIB), the Food and Drug Administration, private pharmaceutical companies and non-profit organizations, as a \$60 million, 5-year public-private partnership. The primary goal of ADNI has been to test whether serial magnetic resonance imaging, PET, other biological markers, and clinical and neuropsychological assessment can be combined to measure the progression of mild cognitive impairment (MCI) and early AD. Determination of sensitive and specific markers of very early AD progression is intended to aid researchers and clinicians to develop new treatments and monitor their effectiveness, as well as lessen the time and cost of clinical trials. The Principal Investigator of this initiative is Michael W Weiner, MD, VA Medical Center and University of California-San Francisco. ADNI is the result of efforts of many co-investigators from a broad range of academic institutions and private corporations, and subjects have been recruited from over 50 sites across the United States and Canada. The initial goal of ADNI was to recruit 800 subjects but ADNI has been followed by ADNI-GO and ADNI-2. To date, these three protocols have recruited over 1500 adults, aged 55–90 years, to participate in the research, consisting of cognitively normal older individuals, people with early or late MCI and people with early AD. The follow-up duration of each group is specified in the protocols for ADNI-1, ADNI-2 and ADNI-GO. Subjects originally recruited for ADNI-1 and ADNI-GO had the option to be followed in ADNI-2. For up-to-date information, see www.adni-info.org. Written consent was obtained from all subjects participating in the study according to the Declaration of Helsinki, and the study was approved by the institutional review board at each participating site.

Participants

Our study population was 46 ADNI-1 healthy control participants, from whom longitudinal CSF samples were taken three to four times and analyzed for A β 42, T-tau and P-tau in a 4-year follow-up assay run, and in whom baseline samples had been analyzed using multiplex proteomics. Inclusion/exclusion criteria for ADNI-1 subjects are described in detail at www.adni-info.org. Briefly, all the control subjects included in ADNI-1 were between the ages of 55 and 90 years, had completed at least 6 years of education, were fluent in Spanish or English, were free of any significant neurologic disease and had CDR scores of 0. *APOE* genotypes were determined for all subjects. We also tested 16 ADNI-1 AD dementia patients, in whom longitudinal CSF samples (taken three to five times) had been analyzed for A β 42, T-tau and P-tau, and in whom baseline samples had been analyzed using multiplex proteomics in the same assay runs as the samples from the healthy controls.

Procedures

The study procedures included lumbar punctures for CSF sampling at baseline and follow-up approximately every 12 months (the mean total follow-up 3.1 years, range 1.9–4.2). Procedures for CSF sampling, transport and storage have been described previously.⁴ Participants also underwent repeated medical evaluation and neuropsychological testing. For this study, we used total scores for mini mental state examination, Alzheimer's Disease Assessment Scale-Cognitive Behavior section (ADAS-Cog) and a modified 13-item version of ADAS-Cog (ADAS-MOD).²⁰ For six subjects who had undergone ¹¹C-Pittsburgh compound B (PiB)-PET scanning,²¹ we included these data to test the concordance between CSF A β 42 measurements and the brain A β load as estimated by using PiB-PET.

CSF protein measurements

CSF A β 42, T-tau and P-tau were measured at the ADNI Biomarker Core laboratory at the University of Pennsylvania Medical Center, using the multiplex xMAP Luminex platform (Luminex, Austin, TX, USA) with the INNOBIA AlzBio3 kit (Innogenetics, Ghent, Belgium).⁴

CSF multiplex proteomics was carried out using an xMAP multiplex kit developed by Rules Based Medicine (MyriadRBM). Details regarding the assay technology, validation and quality control can be found online (<http://adni.loni.ucla.edu/>). In brief, the kit included 159 analytes, selected for analytes believed to be relevant for a number of different diseases including cancer, autoimmune disorders and AD. Previous versions of the

kit had been used to explore CSF or plasma proteins in AD.^{22–24} Seventy-six analytes were adequately quantifiable in the CSF samples in this study (the other analytes were mostly below the assay-detection limit or had other assay limitations as outlined in the assay documentation found online at <http://adni.loni.ucla.edu/>). For these, we did a systematic literature review to find previous evidence of associations with A β metabolism, microglial activity, synaptic function or other aspects of AD (See Supplementary Table 1 for results, including references). We identified 67 analytes of interest that were included in this study. Some analytes were used after log transformation as described in the assay documentation. Together with A β 42, T-tau and P-tau, this resulted in 69 analytes available for each test described below (baseline A β 42, T-tau or P-tau were excluded in tests of trajectories of A β 42, T-tau and P-tau, respectively).

Statistical analyses

Potentially confounding effects by age, sex, education and *APOE* on explanatory proteins were evaluated by linear regression. Logistic regression was used to test whether any demographic parameter was associated with study drop-out.

Effects of explanatory proteins on longitudinal A β 42, T-tau and P-tau were tested by using linear mixed-effects models, in which A β 42, T-tau or P-tau was used as a response variable, and the time from the baseline visit in years, explanatory protein, the interaction between time and explanatory protein, age, sex, education and *APOE* $\epsilon 4$ status were included as fixed effects (rates of change of A β 42, T-tau and P-tau were tested separately with each explanatory protein). All models included a random intercept and slope (except some models that would not converge with a random slope, and therefore only included a random intercept). After inspection of the correlation within subjects, a compound symmetry structure was assumed. We assessed the applicability of the linear mixed-effect model by evaluating (1) linearity of biomarker concentration over time within subjects, (2) the normality of the model residuals and (3) the difference between models with and without time as a random effect. Owing to the large number of proteins tested, we corrected for multiple comparisons using a false discovery rate correction.

Prognostic discrimination of explanatory proteins for longitudinal A β 42 reduction was also tested by ROC statistics. For this, CSF A β 42 change was calculated as the relative change over 4 years, estimated using individual intercepts and slopes from a mixed effect model, adjusted for age, sex, education and *APOE*. The cutoff defining A β 42 reduction was determined by the 25th percentile, and the cutoff for A β 42 stability by the 75th percentile. Dichotomous A β 42 reduction status was then regressed on each significant protein separately, using logistic regression. Predicted probabilities from these models were then used to calculate each protein's ability to discriminate A β decliners from those with stable A β . This predictive accuracy was estimated as the area under the ROC curve (AUC). Each accuracy was cross-validated using a leave-one-out design. Confidence intervals (CIs) for cross-validated accuracies were calculated using variance estimates from 200 bootstrap resamples. To evaluate the proteins' collective ability to discriminate A β decliners from nondecliners, we used logistic regression with a least absolute shrinkage and selection operator penalty on the coefficients of the proteins. The least absolute shrinkage and selection operator penalty was used to simultaneously estimate the effects of a large number of correlated protein concentrations on A β reduction. Results were summarized with predictive accuracy (AUC) and 95% CIs estimated from 200 bootstrap resamples.

Significance was determined at $P < 0.05$ after correction for multiple comparisons using a false discovery rate correction. All statistics were performed using R (v. 2.15.2, The R Foundation for Statistical Computing). The nlme package v.3.1-105²⁵ was used for linear mixed effect models. The ROCR package v.1.0-2²⁶ was used to calculate AUC.

RESULTS

Table 1 summarizes demographics and clinical characteristics. There were longitudinal reductions in the mini mental state examination ($\beta = -0.18$, s.e. = 0.081, $P = 0.034$) and increases in ADAS-MOD ($\beta = 0.47$, s.e. = 0.23, $P = 0.042$) but no significant changes in ADAS-Cog (linear mixed effect models, adjusted for age, sex, education and *APOE*). Five subjects progressed to MCI during the study.

In the six subjects in whom PiB-PET data of the brain A β were available, CSF A β 42 and PiB-PET measurements were 100% concordant when using previously published cutoffs (Supplementary

Figure 1), supporting the use of CSF Aβ42 to identify brain Aβ accumulation in this study.

Half the study population ($n = 23$, 50%) had three longitudinal samples and the other half had four longitudinal samples over a maximum of 4 years of follow-up. There were no significant associations between the demographic parameters and lack of the 4th sample (age, $P = 0.51$; sex, $P = 0.10$; education, $P = 0.074$; $APOE \epsilon 4$, $P = 0.53$, tested by logistic regression).

Effects of age, sex, education and APOE on CSF proteins

Age, sex, education and $APOE$ were potential confounders of the relationship between the investigated proteins and Aβ42, T-tau and P-tau. We tested for imbalance using linear regression with baseline proteins as dependent variables, and age, sex, education and $APOE$ as independent variables. Higher age was associated with higher levels of monokine induced by gamma interferon ($P < 0.001$), CD40 antigen (CD40, $P = 0.0025$), β-2-microglobulin (B2M, $P = 0.026$) and tumor necrosis factor receptor 2 ($P = 0.045$); female sex with higher levels of follicle-stimulating hormone ($P < 0.001$), T-tau ($P = 0.0081$), P-tau ($P = 0.020$) and leptin ($P = 0.0099$), and lower levels of serum amyloid P-component ($P = 0.001$), α-1-microglobulin ($P = 0.018$), ApoD ($P = 0.024$), immunoglobulin A ($P = 0.033$), monocyte chemoattractant protein 1 ($P = 0.040$) and fibrinogen ($P = 0.042$); longer education with higher levels of tissue inhibitor of metalloproteinases 1 (TIMP1, $P < 0.001$), plasminogen activator inhibitor 1 ($P = 0.0049$), tumor necrosis factor receptor 2 ($P = 0.0073$), P-tau ($P = 0.013$), macrophage colony-stimulating factor 1 (MCSF, $P = 0.017$), interleukin (IL)-16 ($P = 0.018$), von Willebrand factor ($P = 0.020$), B2M ($P = 0.020$), insulin-like growth factor-binding protein 2 ($P = 0.035$) and Clusterin (CLU, $P = 0.035$); $APOE \epsilon 4$ with lower levels of Aβ42 ($P = 0.0070$), T-tau ($P = 0.0014$), Osteopontin ($P = 0.0078$), P-tau ($P = 0.0098$) and chemokine CC-4 ($P = 0.032$). On the basis of these results, we included age, sex, $APOE$ and education as covariates in all subsequent analyses.

Baseline CSF proteins associated with baseline CSF Aβ42, T-tau and P-tau

Linear regression was used to test associations between individual baseline proteins and baseline Aβ42, T-tau and P-tau. No proteins were significantly associated with baseline Aβ42, but several proteins were significantly associated with baseline T-tau, including P-tau ($P < 0.001$), Chromogranin A (CgA, $P < 0.001$) and Axl receptor tyrosine kinase (AXL, $P < 0.001$, Supplementary Table 2).

Proteins predicting longitudinal change in CSF Aβ42

The main goal of the study was to test the prediction by baseline proteins of longitudinal CSF Aβ42 (predictions of CSF T-tau and P-tau were also tested, presented below). See Figure 1 for plots of longitudinal CSF Aβ42, T-tau and P-tau. We tested the prediction by baseline proteins of longitudinal CSF Aβ42 in two ways. First, we used linear mixed effect models with longitudinal Aβ42 as a continuous variable, including all subjects. Second, we used ROC statistics as described in the Patients and methods section, dichotomizing the study population based on relative change in Aβ42 from baseline to 4-year follow-up (using estimated rates from the linear mixed effect model). To clearly separate the subjects compared in the ROC statistics, we only compared subjects with the most and the least Aβ42 change (1st and 4th quartiles of subjects ranked by relative Aβ42 change).

Using linear mixed effect models (with continuous Aβ42, including all subjects), 10 proteins had significant effects (See Table 2 and Figure 2 for the significant proteins, and Supplementary Table 3 for data on all tested proteins). The most significant proteins were angiotensin-converting enzyme (ACE), CgA, AXL, Log TNF-related apoptosis-inducing ligand receptor,

Table 1. Demographics of healthy control subjects

Characteristic	Baseline	Last follow-up
N (% male)	46 (54%)	46 (54%)
Age (year), mean (s.d.)	77 (5)	80 (5)
$APOE \epsilon 4$ allele carriers (%)	24%	24%
Education (year), mean (s.d.)	16 (3)	16 (3)
MMSE, mean (s.d.)	29.4 (0.8)	29.0 (1.5)
ADAS-Cog, mean (s.d.)	6.1 (2.9)	6.6 (4.1)
ADAS-MOD, mean (s.d.)	9.3 (3.9)	10.7 (5.8)
CSF Aβ42 (ng l ⁻¹), mean (s.d.)	211 (57)	195 (56)
CSF T-tau (ng l ⁻¹), mean (s.d.)	73 (30)	83 (30)
CSF P-tau (ng l ⁻¹), mean (s.d.)	25 (11)	36 (11)
N with three timepoints		23
N with four timepoints		23

Abbreviations: ADAS-Cog, Alzheimer's Disease Assessment Scale-Cognitive Behavior section; CSF, cerebrospinal fluid; MMSE, mini mental state examination. CSF Aβ42, T-tau and P-tau calculated using fitted data from linear mixed effect models (adjusted for age, sex, education and $APOE$) at baseline and 4-year follow-up.

CD40 and MCSF. For all these proteins, high baseline protein levels predicted reduced Aβ42 over time.

For the ROC statistics, we compared subjects showing the most Aβ42 reduction (1st quartile >11.5% reduction from baseline) with subjects showing the least reduction (4th quartile <4.0% reduction from baseline). Note that the 11.5% cutoff for Aβ42 reduction exceeds the within-run measurement variability of the xMAP AlzBio3 Aβ42 assay and thereby very likely reflects a biological change.²⁷ By these definitions, 12 subjects had Aβ42 reduction (four $APOE \epsilon 4 +$, six female subjects, mean age 80 years (range 73–88, mini mental state examination 29.3 (28–30), ADAS-Cog 5.7 (1.7–15), ADAS-MOD 8.9 (1.7–18)) and 12 subjects had stable Aβ42 (one $APOE \epsilon 4 +$, six female subjects, aged 78 years (72–87), mini mental state examination 29.6 (20–30), ADAS-Cog 6.8 (2.3–10), ADAS-MOD 10 (4.0–15)). In the ROC analysis, 11 proteins significantly predicted Aβ42 reduction (Figure 3 and Supplementary Table 4). These proteins largely overlapped with the proteins identified by the linear mixed effect analysis described above. Highest AUC were seen for ACE (AUC = 0.87 (95% CI 0.72–1.00)), B2M (AUC = 0.83 (95% CI 0.67–0.98)) and CgA (AUC = 0.83 (95% CI 0.67–0.98)). All identified significant proteins were higher at baseline in subjects with longitudinal Aβ42 reduction, except Cystatin C, which was lower in subjects with longitudinal Aβ42 reduction.

Combination of biomarkers to predict CSF Aβ42 change

We used logistic regression with a least absolute shrinkage and selection operator penalty to test whether a combination of baseline proteins could be used to predict CSF Aβ42 change. When all proteins were included, it resulted in an 81% (95% CI 62–100%) cross-validated accuracy to discriminate between subjects with the most Aβ42 reduction (1st quartile >11.5% reduction from baseline) and subjects with the least reduction (4th quartile <4.0% reduction from baseline). Thus, we could not find evidence that combining biomarkers was superior to using the best individual biomarkers for the prediction of CSF Aβ42 change.

Proteins predicting longitudinal CSF Aβ42 change: effects of baseline Aβ42

The goal of this analysis was to test whether the predictive effect of baseline proteins differed between subjects with or without Aβ plaque pathology at baseline. For this, we dichotomized the subjects into those with normal baseline Aβ42

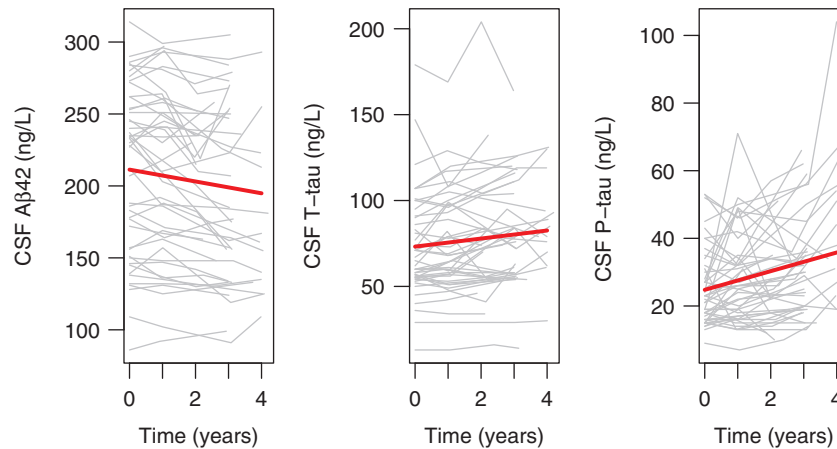


Figure 1. Longitudinal measurements of CSF A β 42, T-tau and P-tau. CSF A β 42, T-tau and P-tau in cognitively healthy controls followed up to 4 years, with three to four samples each. Thick lines are regression curves calculated using linear mixed effect models, adjusted for age, sex, education and *APOE*. The changes over time (in years) were significant for A β 42 ($\beta = -4.11$, s.e. = 0.77, $P < 0.0001$), T-tau ($\beta = 2.34$, s.e. = 0.51, $P < 0.0001$) and P-tau ($\beta = 2.76$, s.e. = 0.49, $P < 0.0001$).

Table 2. Effects of CSF proteins on longitudinal CSF A β 42 reduction in healthy controls

CSF biomarker	Linear mixed effect models			
	β	s.e.	P	Adjusted P
Log angiotensin-converting enzyme (ACE)	-3.09	0.81	<0.001	0.009
Chromogranin A (CgA)	-3.21	0.87	<0.001	0.009
AXL receptor tyrosine kinase (AXL)	-3.14	0.86	<0.001	0.009
Log TNF-related apoptosis-inducing ligand receptor (TRAIL)	-2.91	0.83	0.001	0.011
Log CD40 antigen (CD40)	-3.14	0.91	0.001	0.012
Log macrophage colony-stimulating factor 1 (MCSF)	-2.84	0.91	0.002	0.026
Log beta-2-microglobulin (B2M)	-2.75	0.91	0.003	0.030
Log stem cell factor (SCF)	-2.56	0.89	0.005	0.042
Log clusterin (CLU)	-2.43	0.87	0.006	0.042
Log interleukin-3 (IL-3)	-2.43	0.87	0.006	0.042

Linear mixed effect model data on β -coefficients, s.e. and *P*-values for the interaction terms between proteins and time (years from baseline). Adjusted *P*-values were corrected for multiple comparisons using a false discovery rate correction. Data were adjusted for age, sex, education and *APOE*. Protein levels were centered and standardized.

(A β 42 > 192 ng l $^{-1}$, $n = 27$, 59%, 12 female subjects, 1 *APOE* $\epsilon 4+$, mean age 77 years (range 72–88)) and reduced baseline A β 42 ($n = 19$, 41%, 9 female subjects, 10 *APOE* $\epsilon 4+$, mean age 79 years (range 63–93)). A β 42 decreased with time in both these subgroups (time as years after first sample; normal baseline A β 42, $\beta = -4.9$, s.e. = 1.3, $P < 0.001$; reduced baseline A β 42, $\beta = -3.2$, s.e. = 0.68, $P < 0.001$).

In subjects with normal baseline A β 42, several proteins were significantly associated with longitudinal A β 42 reduction (co-varying for age, sex, education and *APOE*, and adjusting for multiple comparisons), including CgA ($\beta = -5.69$, s.e. = 1.24, $P = 0.001$), CD40 ($\beta = -5.01$, s.e. = 1.46, $P = 0.036$), AXL ($\beta = -4.55$, s.e. = 1.38, $P = 0.036$), ACE ($\beta = -4.38$, s.e. = 1.36, $P = 0.036$), Log TNF-related apoptosis-inducing ligand receptor ($\beta = -4.17$, s.e. = 1.34, $P = 0.039$) and IL-3 ($\beta = -4.12$, s.e. = 1.36, $P = 0.042$). In subjects with reduced baseline A β 42, only TIMP1 ($\beta = -2.2$, s.e. = 0.56, $P = 0.020$) was associated with longitudinal A β 42 reduction.

Proteins associated with longitudinal CSF T-tau and P-tau increase
No protein was significantly associated with the change in T-tau or P-tau (See Supplementary Tables 5 and 6).

Proteins associated with clinical outcome

Five of the 46 healthy control subjects progressed to MCI during the study follow-up. Although the differences were not statistically significant, these five subjects had lower baseline CSF A β 42 (mean 175 (s.d. 43) versus 215 (58) ng l $^{-1}$), and higher baseline CSF T-tau (85 (38) versus 72 (29) ng l $^{-1}$) and P-tau (28 (16) versus 25 (11) ng l $^{-1}$), than the 41 subjects who did not develop MCI. We compared protein levels between stable controls and developers by linear regression, adjusting for age, sex, *APOE* $\epsilon 4$ and education. Matrix metalloproteinase 2 (MMP2) was the only protein that was significantly different between developers and stable controls (reduced in developers, $\beta = -1.64$, s.e. = 0.43, $P = 0.03$ adjusted for multiple comparisons, protein levels centered and standardized).

Proteins associated with longitudinal CSF A β 42, T-tau and P-tau changes in AD patients

To test whether CSF proteins were associated with longitudinal change in CSF A β 42, T-tau or P-tau in subjects with advanced disease, we also examined data from 16 patients with clinical AD diagnosis (10 female patients, 13 *APOE* $\epsilon 4+$, mean age 75 (s.d. 6) years). As expected and as shown previously in the ADNI cohort,⁴

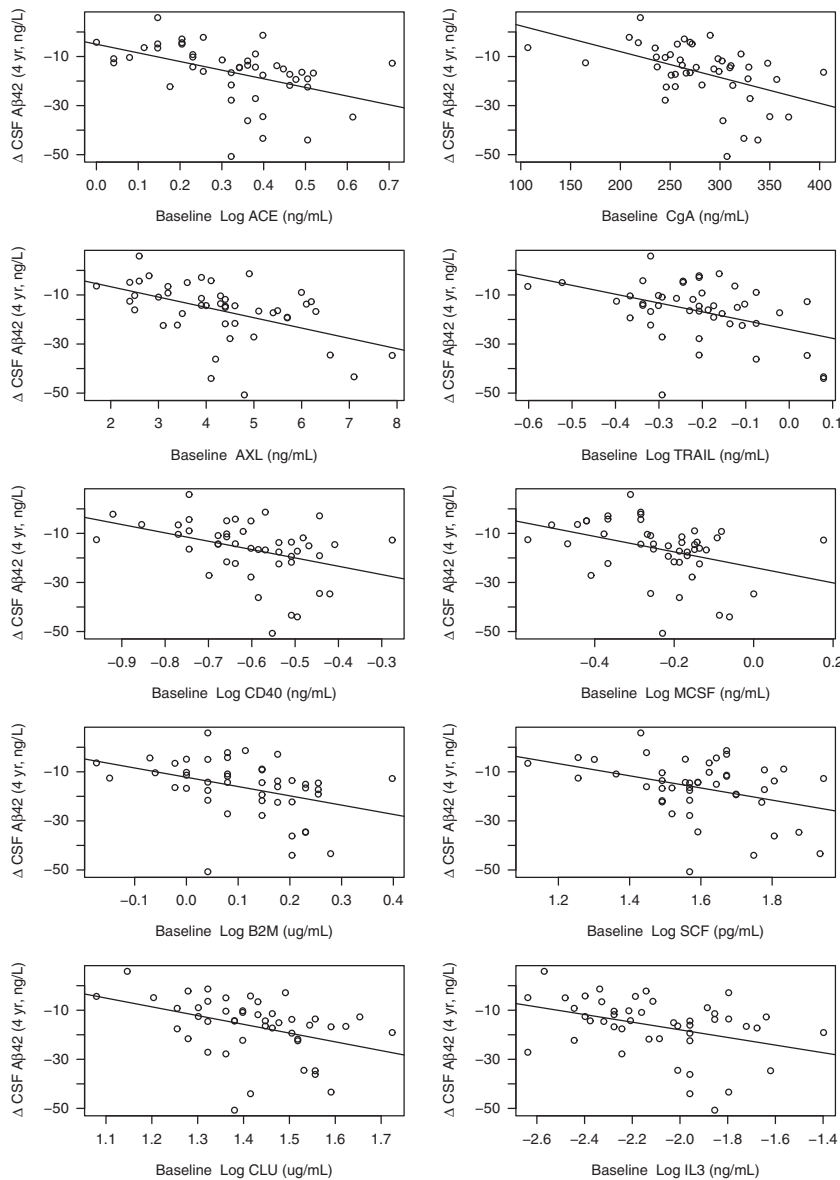


Figure 2. Proteins significantly associated with CSF A β 42 change in healthy controls. The graph includes the baseline proteins (x axes) that were significantly associated with CSF A β 42 rates, as tested using linear mixed effect models. y axes show CSF A β 42 reduction over 4 years (ng l^{-1}), adjusted for age, sex, education and *APOE*. Each dot represents a study participant.

these subjects had reduced baseline CSF A β 42 (mean 133 (s.d. 19) ng l^{-1}), and elevated baseline CSF T-tau (131 (64) ng l^{-1}) and CSF P-tau (42 (13) ng l^{-1}). In contrast to what we found in the healthy controls, there were no significant changes over time in CSF A β 42 ($P=0.90$) or T-tau ($P=0.63$), but there were significant longitudinal increases in P-tau ($\beta=4.6$, s.e.=1.23, $P=0.0007$) in the 16 AD patients (linear mixed effect models, co-varied for age, sex, education and *APOE* ϵ 4). After correcting for multiple comparisons, no tested protein was significantly associated with rates of change for CSF A β 42, T-tau or P-tau (data not shown).

DISCUSSION

The major findings of this study were as follows: (1) there were significant longitudinal AD-like changes in CSF A β 42, T-tau and P-tau—meaning A β 42 fell and T-tau/P-tau rose—in this cohort of cognitively healthy elderly; (2) baseline levels of 10 CSF proteins, primarily related to the A β metabolism, microglial regulation or

synaptic function, significantly predicted longitudinal reduction in A β 42 when using linear mixed effect models (and 11 proteins when using ROC statistics); (3) several baseline proteins were associated with longitudinal A β 42 changes in subjects with normal baseline A β 42 but not in subjects with reduced baseline A β 42 and (4) no baseline proteins predicted changes in T-tau or P-tau. The finding that the predictive effects of baseline proteins on the subsequent decline of A β 42 were different in subjects with normal and reduced baseline CSF A β 42 levels suggests that some proteins predict brain A β plaque accumulation at a very early stage of AD, prior to widespread plaque accumulation. The failure of baseline proteins to predict longitudinal changes of T-tau and P-tau suggests that these proteins are specifically predictive of A β pathology. Taken together, the evidence suggest that changes of CSF proteins primarily related to A β metabolism, microglial regulation or synaptic functions most likely reflect pathological alterations at very early stages of AD, and for some proteins even prior to widespread neuritic plaque formation.

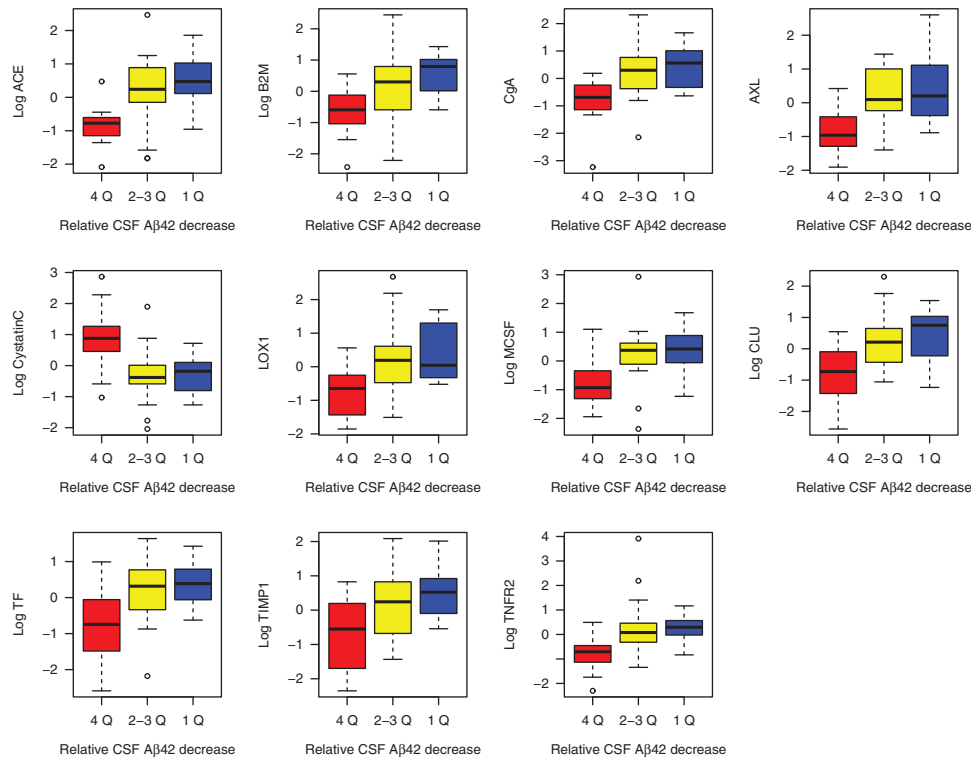


Figure 3. Proteins and quartiles of CSF A β 42 change in healthy controls. The graph includes the baseline proteins that significantly predicted longitudinal reduction in CSF A β 42 in ROC analysis. For this analysis, subjects were ranked according to relative change in A β 42 over 4 years, and subjects with most changes (1st quartile of subjects >11.5% reduction, red boxes) were compared with subjects with least changes (4th quartile of subjects <4.0% reduction, blue boxes). Subjects in the 2nd and 3rd quartiles (representing intermediate reductions) were not included in the ROC analysis but are shown for comparison in yellow boxes (for most proteins, subjects in the 2nd and 3rd quartiles had concentrations comparable to subjects in the 1st quartile). Baseline protein levels were centered and standardized.

The first major finding was that cognitively healthy elderly showed longitudinal changes in A β 42, P-tau and T-tau, partly replicating a previous report using 3-year follow-up data from ADNI-1 (the same subjects in this study) showing that healthy controls manifested longitudinal decreases in A β 42 and increases in P-tau.²⁸ Cross-sectional studies have shown that cognitively healthy subjects (or MCI subjects) at risk for future development of AD dementia have altered A β 42, P-tau and T-tau several years prior to symptoms, with reduced A β 42 appearing first.⁸ A few previous studies have reported longitudinal measurements of A β 42, P-tau and T-tau in cognitively healthy subjects.^{28–34} Most of these have found no or only small changes of CSF A β 42, P-tau and T-tau levels in cognitively healthy subjects,^{30–34} but these have had fewer participants, shorter follow-up or fewer serial samples than the present study. Our finding supports the use of A β 42, T-tau and P-tau to track the development of AD-like pathology prior to clinical symptoms.

Our second major finding was that 10 CSF proteins measured at baseline significantly predicted longitudinal decrease in CSF A β 42 as determined using linear mixed effect models, and 11 proteins as determined using ROC analysis (these sets of proteins largely overlapped). There has been no published previous analysis demonstrating the predictive effect of some CSF proteins on longitudinal changes of CSF A β 42, T-tau or P-tau. The finding that these CSF proteins predicted longitudinal decrease in CSF A β 42 indicate that they may be used as very early biomarkers of A β pathology and thereby possibly also of AD.

Furthermore, the findings that these proteins predict the decrease in CSF A β 42 provide *in vivo* evidence that biological pathways related to the A β metabolism, microglia activity and

synaptic/neuronal function are involved in early A β pathology (see Supplementary Table 1 for references for the proteins described in this paragraph). Several of the identified proteins are associated with A β metabolism in different ways. For example, CLU and Cystatin C may bind A β peptides and influence their aggregation, ACE may degrade A β peptides, and Stem Cell Factor and IL-3 may protect against A β deposits and toxicity. Several of the proteins (including AXL, CD40, CgA, CLU, Cystatin C, MCSF and TIMP1) may modulate microglial activity or have their expression promoted by microglia, and some of the proteins (including ACE, B2M, CgA, IL-3 and TIMP1) have been described to be involved in synaptic or other neuronal functions. For example, CgA has been proposed as an indicator of presynaptic structures, and B2M and TIMP1 are involved in synaptic plasticity. Several of the identified proteins also have other associations with AD. For example, CLU is a major AD risk gene. Some of the proteins have been reported to have altered CSF levels in AD, with increased levels of MCSF and tumor necrosis factor receptor 2, and decreased levels of TIMP1, CgA and stem cell factor. Taken together, the evidence linking these proteins with A β , microglia and synaptic function, and other evidence of their association with AD, provides additional support for the view that these proteins are closely involved, and may reflect causal events, in the pathophysiological events resulting in AD pathology.

The third major finding was that several of the identified proteins (CgA, CD40, AXL, ACE, TNF-related apoptosis-inducing ligand receptor and IL-3) were associated with the future decrease in CSF A β 42 only in subjects without any signs of A β pathology at baseline; however, only one tested protein (TIMP1) was associated with further A β 42 accumulation in subjects in whom A β pathology

was already present. This suggests that these proteins reflect biochemical processes occurring prior to widespread plaque deposition because widespread plaques are reflected by low CSF A β 42.^{1,7} Therefore, we interpret these results to suggest that some of the identified proteins reflect pathophysiological alternations that occur at the very earliest stages of AD. We also tested 16 patients with AD dementia, in whom we found no associations between baseline protein levels and change of CSF A β 42, T-tau or P-tau. Although this result is difficult to interpret because of the small number of subjects, it may support the notion that the identified proteins mainly reflect brain processes involved in the early stages of AD.

The fourth finding of this study was that there were no significant associations between the baseline proteins and longitudinal T-tau/P-tau. This fits with the dynamic biomarker model, in which changes in A β 42 (and thereby associated processes) are more likely to be detected in early stages of the disease than changes in T-tau and P-tau.¹⁹ One intriguing finding was that several proteins were associated with baseline T-tau levels. Among them were CgA, AXL and ACE, which themselves were strong predictors of longitudinal CSF A β 42 reduction. One possible explanation for the association between baseline T-tau and these proteins is that they may share metabolic pathways unrelated to A β pathology. This view is supported by the fact that, although CgA, ACE and AXL were strong predictors of longitudinal CSF A β 42 reduction, baseline T-tau did not predict longitudinal CSF A β 42 reduction.

The main limitation of this study is the relatively low sample size, but this was balanced by the long follow-up time and multiple samples from each subject. Our results may be biased by the selection of proteins studied, included mainly because of known associations with A β metabolism, microglia activity or synaptic function. It is possible that another set of proteins would have shown stronger predictive effects on longitudinal change of T-tau and P-tau. It is not known whether these proteins may help identify patients at risk for future cognitive decline or AD. When comparing protein levels between the 41 cognitively stable controls and the 5 controls who progressed to MCI, the only protein that was differently expressed between the groups was MMP2, which was reduced in participants who developed MCI. This is in agreement with previous studies, showing reduced CSF MMP2 levels in AD.³⁵ However, the small number of participants who developed MCI in this study makes the negative results for the other proteins difficult to interpret.

In sum, we found that baseline CSF proteins involved in the A β metabolism, microglial regulation and synaptic function predicted longitudinal change in CSF A β 42 in cognitively healthy people. Some of the proteins were only predictive in people with normal CSF A β 42, suggesting that they may be used as biomarkers predicting future development of A β pathology in persons without A β pathology at testing. No proteins predicted increases in CSF T-tau or P-tau, consistent with the view that these protein changes occur prior to widespread plaque accumulation and downstream axonal loss and tangle pathology. The results provide *in vivo* evidence for pathological mechanisms related to early A β 42 accumulation, and especially point to a role for early alterations of the A β metabolism and microglia activity. These proteins may be useful tools to identify cognitively normal subjects at risk for future A β 42 pathology, and subsequently AD.

CONFLICT OF INTEREST

JQT may accrue revenue in the future on patents submitted by the University of Pennsylvania, wherein he is the co-inventor and he received revenue from the sale of Avid to Eli Lilly as the co-inventor on imaging-related patents submitted by the University of Pennsylvania; and is the William Maul Measey-Truman G Schnabel, Jr, MD Professor of Geriatric Medicine and Gerontology. MW has been on scientific advisory boards for Pfizer and BOLT International; has been a consultant for Pfizer,

Janssen, KJ Associates, Easton Associates, Harvard Associates, Harvard University, inThought, INC Research, University of California, Los Angeles, Alzheimer's Drug Discovery Foundation and Sanofi-Aventis Groupe; has received funding for travel from Pfizer, AD PD meeting, Paul Sabatier University, Novartis, Tohoku University, MCI Group, France, Travel eDreams, Neuroscience School of Advanced Studies (NSAS), Danone Trading, BV, CTAD ANT Congres; serves as an associate editor of Alzheimer's & Dementia; has received honoraria from Pfizer, Tohoku University and Danone Trading, BV; has research support from Merck, Avid, DOD and VA; and has stock options in Synarc and Elan. The remaining authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

Data used in preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.ucla.edu). As such, the investigators within the ADNI contributed to the design and implementation of ADNI and/or provided data but did not participate in analysis or writing of this report. A complete listing of ADNI investigators can be found at http://adni.loni.ucla.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf.

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