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Plasma Transthyretin as a Candidate Marker for Alzheimer's Disease

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Abstract. Diagnosis of the progressive neurodegenerative disorder Alzheimer's disease (AD) can only definitively be made postmortem. The most promising AD biomarkers identified to date are found in cerebrospinal fluid (CSF). Among these, one of the most interesting candidates is transthyretin (TTR), the carrier of thyroxine and retinol, which also binds with amyloid-β (Aβ), and it has been suggested that it protects against Aβ deposition. A biomarker detectable in plasma would have great diagnostic value and could be of use for determining disease progression and the monitoring of therapeutic efficacy due to its greater accessibility over CSF-based markers. We aimed to validate TTR as a prognostic marker in AD and to determine its relation with cognitive measures. We examined the plasma protein levels of TTR in 90 people with late-onset AD and 50 age-matched non-demented controls (NDC) by immunoblotting and found lower plasma TTR levels in AD compared to NDC ($p = 0.004$). We then quantified plasma TTR by enzyme-linked immunosorbent assays in a larger independent cohort ($n = 270$) including subjects with mild to severe AD. Plasma TTR levels were significantly lower in AD cases with rapid cognitive decline and with severe cognitive impairment. Regression analyses showed plasma TTR levels also predicted cognitive decline over the ensuing 6 months. These data indicate that plasma TTR is a strong candidate AD biomarker that should be included in the development of blood based biomarker panels for disease diagnosis and also suggests that plasma TTR is a marker of disease severity and progression.

Keywords: Alzheimer's disease, cognitive impairment, plasma proteins, transthyretin

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INTRODUCTION

With the rapidly aging global population, the number of people with dementia is estimated to quadruple worldwide in the next 20 years [1]. Alzheimer's disease (AD) is by far the most common dementia and is progressive in nature. A biomarker to aid the early diagnosis of AD, allowing the use of disease modifying therapies before overt dementia manifests or in the monitoring of disease progression would therefore be of great clinical value.

Considerable progress in the search for biomarkers has been made with markers derived from amyloid

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plaques (amyloid-β (Aβ)) [2] and neurofibrillary tangles (tau and phospho-tau) [3]. The most promising sources for biomarkers in AD are cerebrospinal fluid (CSF) and blood plasma, because compared to brain tissue, these fluids are more easily accessible and, in the CSF, which is in close contact with the central nervous system, where key biochemical changes take place. However, while CSF is a good resource for the study of biomarkers in AD, its clinical application is limited by the relatively invasive nature of the procedure. Blood-based biomarkers have an advantage in that they are suitable for large scale studies, in community settings, with the ease of venepuncture allowing for repeatability in old and frail people and applicable to clinical settings.

Many approaches to identifying factors associated with disease characteristics such as speed of progression, have been employed. These include clinical factors, neuroimaging, genetics and various approaches to discover biomarkers in body fluids. Proteomic studies using CSF and blood have identified potential AD diagnostic markers, distinguishing AD patients from healthy elderly controls and other neurodegenerative disorders [4–6], and other studies have used protein-based studies to identify potential predictive markers in mild cognitive impairment (MCI) [7]. However, few studies have yet sought to go beyond diagnostic markers to identify potential prognostic markers in AD. Here we report the validation of one of the key proteins, transthyretin (TTR), identified from a mass spectrometry-gel based proteomics study in plasma, evaluating them further in larger independent cohorts using immunoblotting and quantitative enzyme-linked immunosorbent assays (ELISA). We investigated whether TTR distinguished AD from healthy controls and also its correlations with the rate of cognitive decline and severity in AD.

MATERIAL AND METHODS

Subjects and samples

The samples used in these analyses came from two studies: AddNeuroMed studies and the Alzheimer's Research Trust cohort, Kings College London (KCL-ART). As a part of the KCL-ART study, people with AD, MCI, and non-demented controls (NDC) have been recruited and sampled from 2001 onwards [8]. All subjects were white Europeans with grandparents born in the UK and underwent assessments annually. The AddNeuroMed project, a European Union study, recruited subjects with AD, MCI, and NDC from 6 centers in the UK, France, Italy, Finland, Poland, and Greece [9]. All subjects were assessed at 3-monthly intervals over a year. Assessments in both the studies included a semi-structured interview for demographics, case history, family history, medical history, and standardized tools used to assess cognition, function, behavior, global severity [8]. Patients with probable AD (National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association [NINCDS-ADRDA] criteria) in both the studies were identified as previously described [8] and evaluated with a standardized assessment shown to have high diagnostic validity [11]. Age-matched NDC, defined as having no evidence of cognitive impairment (with a MMSE greater than 28), were recruited systematically from primary care patient lists (KCL-ART study) [8]. The full standardized assessments in both of the studies are similar and included demographic and medical information, scales to assess function, behavior, and global levels of severity including the Cambridge Examination for Mental Disorders of Older People (CAMDEX) [12]; and cognitive assessment including Mini Mental State Examination (MMSE) [13] (both studies; all subjects) and Alzheimer disease assessment scale-Cognitive (ADAS-cog) [14] (AddNeuroMed only) [15, 16]. Peripheral venous blood was collected at baseline (initial assessment) and at subsequent time points, including plasma samples collected in 9 ml EDTA tubes and stored at -80°C according to rigorous standard operating procedures. In total, we studied 50 NDC and 90 AD subjects for immunoblotting (KCL-ART cohort) and 270 AD subjects for ELISA (AddNeuroMed cohort), with an additional 40 subjects (AddNeuroMed cohort) for determining correlation between the two techniques. Ethical approval was obtained from local ethic committees.

Criteria for cognitive decline and severity in AD patients

Cognitive decline was defined using MMSE scores, as this was available for all the subjects and previously described [15, 16]. Briefly, annualized fall in MMSE was calculated from the duration of disease and MMSE at the point of blood sampling and rapid cognitive decliners were defined as subjects with a drop of 2 or more points over a period of one year [15]. We further defined mild AD as those subjects with probable AD with MMSE scores of 20 points and above. Moderate to severe AD (Mod-severe AD) was defined as AD in those subjects with MMSE scores between 0–19.

MMSE score change over a period of 6 months post-venepuncture was calculated for prospective cognitive decline.

Validation of TTR using western blotting and enzyme-linked immunoassay

The discovery phase (mass spectrometry-gel based proteomics) for this study has been previously reported [15]. Briefly, plasma samples from AD subjects (AddNeuroMed cohort) characterized as rapid ($n = 22$) and non-rapid progressors ($n = 29$) were subjected to two-dimensional difference-in-gel electrophoresis (2DGE). PLS-DA model discriminating the fast from slow progressing AD groups was constituted by the integrated optical densities of silver-stained 2DGE spots. Transthyretin was identified as one of the proteins from these well-defined, discrete spots, present in all 51 gels by mass spectrometry LC-MS/MS [15].

Western blot analysis was carried out to measure TTR levels in a sample set of 90 AD subjects and 50 healthy controls (KCL-ART cohort). Plasma samples were diluted (4 μl raw plasma plus 96 μl of PBS containing protease inhibitor cocktail (Complete®, 1836145, Roche Applied Science, Penzberg, Germany) and mixed with 100 μl of 2 × reducing Laemmli sample buffer (S3401, Sigma). Samples were then boiled at 100°C for 5 min, centrifuged at 15,500 g and separated on NuPAGE® (24 well), 4–12% Bis-Tris SDS-polyacrylamide gels (Invitrogen, Paisley, UK). Proteins were electroblotted onto 0.2 μm nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany), blocked in 5% dried skimmed milk in PBS + 0.1% Tween (PBST) and probed with a rabbit anti-human TTR antibody (Dako, Ely, UK) for 2 h at room temperature. Primary antibody immunoreactivity was detected with an anti-rabbit antibody conjugated to a 680 nm fluorophor (Alexis, Invitrogen, Paisley) and visualized on an Odyssey near infrared scanner (LI-COR Biosystems, Nebraska, USA). Densitometric analysis was performed using the Odyssey software v 2.1. All samples were run in duplicate and intensities were normalized to a reference plasma sample run on each gel (also loaded in duplicate) to allow inter-gel comparisons. The densitometric values obtained for each duplicate run were averaged post normalization to the in gel control sample.

To validate the novel finding that transthyretin levels correlated with cognitive decline, the protein was assayed by a commercial ELISA kit (Assaypro-AssayMax Human prealbumin ELISA Kit). The assay was carried out as per the manufacturer's instruction. Baseline plasma samples from an independent cohort of AD subjects ($n = 270$) from both AddNeuroMed and KCL-ART were run in duplicate.

Genotyping

Venous blood was obtained for DNA extraction and genotyping for the apolipoprotein (APOE) alleles using standard methods [17]. The APOE haplotype was determined using two allelic discrimination assays (rs7412 and rs429358) based on fluorogenic 5′ nuclease activity: TaqMan single nucleotide polymorphism Genotyping Assays (Applied Biosystems).

Statistical analysis

Protein data was analyzed using SPSS version 17 (for Windows). Chi-square, student t-test, correlation analysis (Spearman non-parametric test) and non-parametric Mann-Whitney-Wilcoxon test were used to compare the sociodemographics, MMSE test scores and TTR protein levels between groups: rapid and non-rapid cognitive decliners; and mild and mod-severe AD subjects. Linear regression was performed with the loss of MMSE scores over 6 months follow up as the dependent variable and plasma transthyretin levels, age, baseline MMSE scores, duration of illness, gender, and APOE4 as predictive variables within the whole AD sample.

RESULTS

Discovery phase

We have previously reported the discovery phase experiments; comparing fast to slow progressors, using two-dimension gel electrophoresis (2DGE) and tandem mass spectrometry (LC/MS/MS) [15]. Proteins differing in plasma between fast and slow progressors included those previously identified by us, and by other groups, as potential markers for AD, including complement proteins and apolipoprotein A1. We have previously reported the validation studies for clusterin, a protein which is also altered in relation to degree of entorhinal cortex atrophy. One novel protein was identified in this discovery program, transthyretin (TTR), also known as pre-albumin.

Transthyretin levels lower in AD subjects compared to NDC

In order to validate this finding, we first compared the plasma TTR levels between AD subjects and age

Table 1
Comparison of plasma transthyretin level and socio-demographic-clinical parameters between the groups: a) AD subjects and non-demented controls; b) Within independent cohort of AD subjects: rapid cognitive decliners and non-rapid cognitive decliners and mild AD and moderate-severe AD

a) AD subjects and non-demented controls				b) Comparisons within an independent cohort of AD subjects ($n = 270$)					
Variables	AD ($n = 90$)	NDC ($n = 50$)	p-value	Rapid decliners ($n = 180$)	Non-rapid decliners ($n = 86$)	p-value	Mild AD ($n = 128$)	Mod-severe AD ($n = 142$)	p-value
Female/male	70/20	38/12	N.S[a]	120/60	57/29	N.S[a]	79/49	99/43	N.S[a]
Age, years	81.4 (6.5)	80.8 (7.2)	N.S[b]	77.5 (6.5)	77.1 (6.2)	N.S[b]	76.6 (5.9)	78 (6.6)	N.S[b]
TTR levels	63.9 (0.1)	82.3 (0.1)	0.01*¶	130.3 (64.6)	146.4 (62.9)	0.04*§	144.2 (61.2)	128.1 (65.8)	<0.01*§
APOE4	53 (60.2%)	9 (20%)	<0.001[a]	106 (60.6%)	46 (54.8%)	N.S[a]	71 (57.3%)	82 (60.5%)	N.S[a]
MMSE score, baseline	15.8 (8.1)	28.7 (1.1)	<0.001*	14.8 (7.6)	22.8 (4.7)	<0.001*	23.8 (2.6)	11.9 (6.5)	<0.001*
Duration of illness, months	n/a	n/a	n/a	3.9 (2.6)	6.3 (3.6)	<0.001[b]	4 (2.7)	5.2 (3.4)	<0.01[b]

Values are mean (SD) or n (%); [a]calculated using the χ^2 test, [b]calculated using the student t-test.
Wilcoxon paired test, ¶Western blotting experiments, §Enzyme linked immunoassay (ELISA).
AD, Alzheimer's disease; NDC, non-demented controls; MMSE, Mini Mental State Examination; TTR, transthyretin; APOE4, presence of one E4 allele; n/a, not applicable.

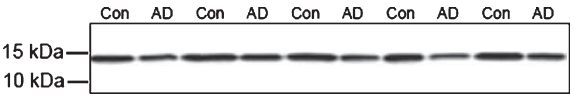

Fig. 1. Representative blot from immunoblotting experiment for plasma transthyretin levels in Alzheimer's disease patients and non-demented controls.

matched non-demented controls. Equal volumes of plasma from AD ($n = 90$) and controls ($n = 50$) (ART-KCL) were immunoblotted for TTR, in duplicate, as described above. A standard pooled sample was loaded in duplicate on each gel, to which each test sample was normalized, and which allowed inter-gel comparisons to be made. When assessing the reproducibility of the duplicate gels, a large positive correlation of 0.84 was obtained (Pearson correlation test). We found that TTR levels were significantly ($p = 0.004$) reduced in AD compared to NDC (Table 1a, Figs. 1 and 2). There was no association between APOE4 carrier status and TTR concentration ($p = 0.47$) as tested by analysis of covariance.

Transthyretin levels and cognition within AD subjects

We then used samples from an independent sample set of 270 AD subjects (AddNeuroMed = 177 and ART-KCL = 93). These included 178 females (66%), with a mean age of 77.4 years (±6.3) and mean MMSE score 17.49 (±7.8). APOE genotyping was available for 247 AD subjects, with 145 subjects having at least one E4 allele. The mean TTR level for the whole cohort was 135.5 μg/ml (±64). Information on anticholinesterase inhibitors treatment was available from the AddNeuroMed cohort, with $n = 123$ having

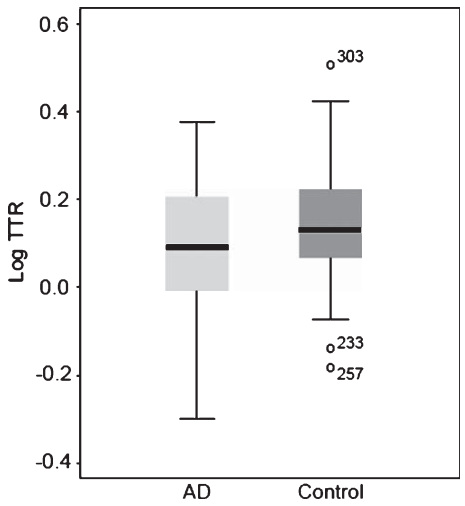

Fig. 2. Box plot showing lower plasma transthyretin levels in Alzheimer's disease subjects compared to non-demented controls.

ongoing treatment. We found no difference in the plasma TTR levels with and without treatment. There was also no difference in TTR levels between APOE4 carrier and non-carriers. There was no significant difference between depressed and non-depressed AD subjects as assessed by the neuropsychiatry inventory. There were 15 subjects with known thyroid dysfunction, however, there was no difference in TTR levels between subjects who had thyroid dysfunction ($n = 15$) and those without. There were no subjects with known liver dysfunction.

When comparing AD subjects by speed of decline, TTR levels were significantly lower in subjects with more rapid cognitive decline ($p = 0.036$), and also in subjects with moderate-severe AD ($p < 0.01$) (Table 1b) (Mann-Whitney U test).

Table 2
Linear regression analysis with the loss of MMSE scores over 6 months follow up as the dependent variable and plasma transthyretin levels, age, baseline MMSE scores, duration of illness, gender and APOE4 alternatively (Model 1) or simultaneously (Model 2) entered as predictive variables within the whole Alzheimer's disease sample

	R^2 (%)		Beta	T-value	P value
Model 1					
Plasma transthyretin	3.6		0.012	2.32	0.022*
Age in years	0.6		−0.039	−1.072	0.285
Duration of illness	0.4		−0.074	−0.924	0.356
MMSE baseline	1.8		0.092	1.903	0.058
Gender	0.2		−0.295	−0.592	0.555
APOE4	0.2		0.294	0.609	0.543
Model 2					
Plasma transthyretin + MMSE baseline	5.7	TTR	0.011	2.168	0.032*
		MMSE	0.100	1.779	0.077

R^2 (%) = R^2 value in percent for the overall model; *$p < 0.05$; MMSE, Mini Mental State Examination; TTR, Transthyretin; APOE4, presence of one E4 allele.

The change in MMSE scores from baseline over the following six months was then calculated. Linear regression analysis showed TTR levels as a better predictor factor for MMSE score change in the six months following venepuncture ($p = 0.029$), in both adjusted and unadjusted models with variables such as age, gender, duration of illness, baseline MMSE, and APOE4 carrier status (Table 2). Correlation analysis showed positive association of TTR levels with baseline MMSE scores; decreasing plasma TTR levels with lower MMSE scores ($p = 0.006$, $r^2 = 0.2$).

To determine the degree of correlation between the two techniques (ELISA and immunoblotting) used to measure TTR plasma levels, we performed both techniques on 40 new plasma samples, each run in duplicate in both assays. The samples were from 40 AD subjects (22 women) (AddneuroMed cohort), with a mean age 77.5 years (±6.6) and mean MMSE scores, 20.9 (±4.9). We found a good positive correlation between the two techniques ($p < 0.001$, $r^2 = 0.65$) (supplementary Figure 1; available online: http://www.j-alz.com/issues/28/vol28-2.html#supplementarydata06).

DISCUSSION

Previously we reported, in a discovery study, that TTR was one of the proteins in plasma discriminating between fast and slow progressing AD [15]. All other proteins from this discovery had been previously identified in biomarker studies [15]. Here we set out to determine whether this novel observation could be replicated in an independent sample set. By immunoblotting we found that TTR levels are significantly lower in AD subjects compared to the NDC. Measuring TTR by ELISA in an independent cohort of AD subjects, we found decreased TTR levels in moderate-severe stages of AD and in subjects presenting with rapid cognitive decline. We also found that plasma TTR level predicted subsequent decrease in MMSE score over the ensuing 6 months. The absolute concentration of TTR using TTR ELISA and immunoblotting correlated positively on a common set of plasma samples.

Previous studies have reported decreased TTR levels in CSF of patients with AD [18–23]. Low levels of TTR in CSF have been reported to be AD-specific compared with other dementia types, i.e., fronto-temporal dementia and Lewy body dementia [23, 24]. Lower TTR levels in CSF have been reported in severe AD [19, 22]. In a recent report, TTR is one of the six CSF biomarkers for AD describing six clinicopathological stages from cognitive normalcy to mild dementia, including stages defined by increased risk of cognitive decline [25]. Our findings are consistent with a recent report demonstrating lower serum TTR levels in AD subjects compared to NDC, although the study used a different detection method [26].

TTR, a 55-kDa homotetramer, is an abundant protein in CSF and human plasma, serving as the main transporter of thyroid hormones from the blood stream into CSF and in plasma, and is associated with retinol-binding protein [27]. It has been proposed that TTR acts as a scaffold protein, binding to Aβ and in so doing protects against Aβ deposition and the formation of senile plaques [28–30]. TTR seems to play an important role in keeping intracerebral proteins such as amyloid in a soluble form and helps prevent further aggregation [31]. In a recent study in mice, we found that deletion of insulin receptor substrate 2 (Irs2) resulting in insulin resistance increased tau pathology as expected but paradoxically decreased amyloid

pathology. We showed that this unexpected protection against plaque pathology was due to an increase in TTR expression [32], in line with a previous genome-wide expression study which found that increased TTR was one of the protective factors preventing transgenic mice with plaque pathology progressing to other pathological features of AD [33].

An alternative mechanism to explain the observation of lower TTR in more severe and more rapidly progressing AD is that TTR functions as a rate-limiting factor for the plasma transport of retinol [34]. Depletion of retinoic acid derivatives has been associated with deposition of Aβ peptides [35]. Whatever the mechanism, TTR is a prime candidate to influence Aβ pathology both directly and indirectly. The reasons for decreased plasma TTR levels in AD subjects could be from altered morphology of the choroid plexus in AD with possible change of expression profile including TTR production and its transport into blood [36]. Another possible explanation could be the down regulation of TTR expression in choroid plexus caused by activated β-secretase activity in AD with decreased sAβPPα [37]. Decreased hepatic TTR expression is another possible cause of reduced TTR in AD but none of our AD subjects had recorded liver dysfunction and additionally we did not find any differences in TTR levels of AD subjects with and without thyroid dysfunction.

In conclusion, significantly lower level of plasma TTR were found in AD subjects compared to non-demented controls and within AD subjects, TTR plasma levels were lower in subjects with rapid cognitive decline and severe cognitive impairment. In addition, TTR level predicted subsequent cognitive decline. These data suggest that plasma TTR is a strong candidate AD-specific biomarker that should be included in the development of blood-based biomarker panels for disease diagnosis and also suggests that plasma TTR may act as a marker for disease severity and progression.

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