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Research Article

Identification of preclinical Alzheimer's disease by a profile of pathogenic proteins in neurally derived blood exosomes: A case-control study

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Abstract

Background: Proteins pathogenic in Alzheimer's disease (AD) were extracted from neurally derived blood exosomes and quantified to develop biomarkers for the staging of sporadic AD.

Methods: Blood exosomes obtained at one time-point from patients with AD (n = 57) or frontotemporal dementia (FTD) (n = 16), and at two time-points from others (n = 24) when cognitively normal and 1 to 10 years later when diagnosed with AD were enriched for neural sources by immunoabsorption. AD-pathogenic exosomal proteins were extracted and quantified by enzyme-linked immunosorbent assays.

Results: Mean exosomal levels of total tau, P-T181-tau, P-S396-tau, and amyloid β 1–42 (Aβ1–42) for AD and levels of P-T181-tau and Aβ1–42 for FTD were significantly higher than for case-controls. Step-wise discriminant modeling incorporated P-T181-tau, P-S396-tau, and Aβ1–42 in AD, but only P-T181-tau in FTD. Classification of 96.4% of AD patients and 87.5% of FTD patients was correct. In 24 AD patients, exosomal levels of P-S396-tau, P-T181-tau, and Aβ1–42 were significantly higher than for controls both 1 to 10 years before and when diagnosed with AD.

Conclusions: Levels of P-S396-tau, P-T181-tau, and Aβ1–42 in extracts of neurally derived blood exosomes predict the development of AD up to 10 years before clinical onset.

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Keywords: Preclinical AD; Neural exosomes; P-Tau; Aβ1–42; Biomarkers

1. Introduction

Roles in the pathogenesis of Alzheimer's disease (AD) have been attributed to altered proteins accumulating inside and on the surface of neurons [1,2]. Increases in brain tissue oligomeric amyloid β (Aβ) peptides and phosphorylated tau (P-tau) detected by central nervous system (CNS) imaging and in cerebrospinal fluid (CSF) levels of soluble Aβ1–42 and P-tau have been documented years before the signs of AD [3–6]. Times for progression from preclinical stages to clinically apparent AD with threshold detectable amyloid deposition and abnormal elevation of CSF P-tau proteins

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are estimated to be up to 17 years [3,5]. The potential prognostic sensitivity of protein biomarkers is supported by the timing of induction of AD-like disease in rodent models after the transgenic overexpression of putatively neuropathogenic proteins [7–9].

In recent studies, low CSF levels of Aβ1–42 and high CSF levels of P-tau, and positive CNS images of amyloid deposits accurately predicted the development of mild cognitive impairment (MCI) and probable AD [10,11]. However, there was substantial overlap in these biomarkers between patients who subsequently developed AD and those who later manifested other forms of dementia or no signs of dementia, even when concentrations of these CSF proteins were considered together or as ratios. The overlap was even greater when plasma levels of these proteins were used for diagnosis or prediction [12–15]. This high level of prognostic uncertainty combined with the morbidity and the expense of repeated CSF sampling and of neuroimaging procedures emphasizes the importance of developing accurate blood-based tests that predict high risk for AD and distinguish AD from other forms of dementia.

Exosomes are one class of endosome-derived membrane vesicles shed by neural cells, that contain proteins and other constituents of their cellular origin [16]. Exosomes accept amyloid precursor protein from early endosomes, after its cleavage by β-secretase, and the Aβ peptide fragments subsequently generated by γ-secretase are secreted in exosomes [17]. Although this exosome pathway accounts for only a small portion of the total Aβ peptides in neural plaques, it constitutes a prionoid-like mechanism for CNS spread of proteinopathies [18]. The detection of exosome signature proteins in neural amyloid plaques supports the possibility of their role in the generation of AD-associated lesions [17]. Here we use a combination of chemical and immunochemical methods to harvest and enrich neurally derived exosomes from small volumes of plasma or serum in quantities that provide readily detectable amounts of proteins implicated in the pathogenesis of AD.

2. Materials and methods

2.1. Study design, subject characterization, and blood collection

Fifty-seven patients with amnestic MCI (aMCI) or dementia attributable to AD, who had donated blood at one time-point, were identified retrospectively at the Clinical Research Unit of the National Institute on Aging (CRUNIA) in Harbor Hospital, Baltimore, MD, at the Jewish Home of San Francisco (JHSF), San Francisco, CA, and in the neurology clinical services of the University of Rochester (UR), Rochester, NY, the University of California Irvine (UCI), Irvine, CA, and Georgetown University Medical Center, Washington, DC (GUMC) (Table 1). Twenty-four additional patients with AD had provided blood at two time-points in studies at the Mayo Clinic and the University of Kentucky, first when cognitively intact and later when diagnosed with AD. For both groups, the diagnosis of AD had been established according to the revised National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association (NINDS-ADRDA) criteria [19]. The patients classified as having aMCI had a Clinical Dementia Rating (CDR) global score of 0.5 [20]. Those with AD and mild to moderate dementia had a CDR global score of 1.0. Twenty-eight of the 57 single-time sample AD patients were taking an acetylcholinesterase inhibitor and/or memantine, and 12 were on antidepressant medications; blood was drawn at least 8 hours after their last medication.

Sixteen patients with behavioral variant frontotemporal dementia (bv-FTD) had been evaluated and selected for study at the Memory and Aging Center of the Department of Neurology of the University of California, San Francisco (Table 1). Their diagnosis and assignment to mild dementia or moderate dementia groups (Table 1) was based on standard clinical, mental status, and psychiatric criteria, including discriminant analyses of neuropsychiatric elements, phonological performance, and object understanding that distinguish FTD from AD [21,22]. Seven

Table 1
Characteristics of patients and control subjects

	Total			MCI		Dementia	
Diagnosis	Number	Male/female	Ages, mean ± SD (range)	Number	MMSE scores, mean ± SEM	Number	MMSE scores, mean ± SEM
AD	57	30/27	79.5 ± 6.05 (64–90)	29	27.6 ± 0.30	28	22.9 ± 1.02**
AC	57	30/27	79.6 ± 6.03 (64–90)	0		0	
				Mild dementia		Moderate dementia	
FTD	16	12/4	63.1 ± 8.79 (48–79)	9	26.7 ± 0.73	7	15.0 ± 3.65*
FTC	16	12/4	63.7 ± 7.43 (48–79)	0		0	

Abbreviations: MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; AD, Alzheimer's disease; AC, AD case-controls; FTD, frontotemporal dementia; FTC, FTD case-controls.

NOTE. The significance of differences in values between the MCI/mild dementia and dementia/moderate dementia groups were calculated by an unpaired t test; *$P < .01$ and **$P < .001$.

of the FTD patients were receiving an antidepressant, two were taking an acetyl-cholinesterase inhibitor and one was on memantine. Ninety-two cognitively normal subjects were recruited at the JHSF, CRU-NIA, and GUMC to be age- and gender-matching controls for the several groups of AD and FTD patients (five served as controls for two clinical groups). Each subject studied and some patient-designates signed a consent form approved with the study protocol at each institution. All plasma and serum were stored at −80°C.

2.2. Isolation of exosomes from plasma or serum for ELISA quantification of exosome proteins

One-half milliliter of plasma was incubated with 0.15 ml of thromboplastin-D (Fisher Scientific, Inc., Hanover Park, IL) at room temperature for 60 minutes, followed by the addition of 0.35 ml of calcium- and magnesium-free Dulbecco's balanced salt solution (DBS^{-2}) with protease inhibitor cocktail (Roche Applied Sciences, Inc., Indianapolis, IN) and phosphatase inhibitor cocktail (Pierce Halt, Thermo Scientific, Inc., Rockford, IL). For serum, 0.5 ml was mixed with 0.5 ml of DBS^{-2} containing the inhibitor cocktails. After centrifugation at 1500 × g for 20 minutes, supernates were mixed with 252 μl of ExoQuick exosome precipitation solution (EXOQ; System Biosciences, Inc., Mountainview, CA), and incubated for 1 hour at 4°C. Resultant exosome suspensions were centrifuged at 1500 × g for 30 minutes at 4°C and each pellet was resuspended in 250 μl of DBS^{-2} with inhibitor cocktails before the immunochemical enrichment of exosomes from a neural source, as described for immune cell exosomes [23].

Each sample received 100 μl of 3% bovine serum albumin (BSA; 1:3.33 dilution of Blocker BSA 10% solution in DBS^{-2} [Thermo Scientific, Inc.]) and was incubated for 1 hour at 4°C each with 2 μg of mouse anti-human neural cell adhesion molecule (NCAM) antibody (ERIC 1, sc-106, Santa Cruz Biotechnology, Santa Cruz, CA) that had been biotinylated with the EZ-Link sulfo-NHS-biotin system (Thermo Scientific, Inc.) or for some preparations with 1 μg of mouse anti-human CD171 (L1 cell adhesion molecule [L1CAM]) biotinylated antibody (clone 5G3, eBioscience, San Diego, CA) and then 25 μl of streptavidin-agarose resin (Thermo Scientific, Inc.) plus 50 μl of 3% BSA. After centrifugation at 200 × g for 10 minutes at 4°C and removal of the supernate, each pellet was suspended in 50 μl of 0.05 M glycine-HCl (pH 3.0) by vortexing for 10 seconds. Each suspension then received 0.45 ml of DBS^{-2} with 2 g/100 ml of BSA, 0.10% Tween 20 and the inhibitor cocktails followed by incubation for 10 minutes at 37°C with vortex-mixing and was stored at −80°C before enzyme-linked immnuosorbent assays (ELISAs). Relative yields of exosomes from plasma and serum at this stage were compared using both sources from six patients with AD. The respective mean levels of P-T181-tau and CD81 extracted from serum-derived exosomes were 58% and 56% of that from plasma-derived exosomes. Although the yield from serum was lower, it was correctible by normalization for the exosome marker CD81 as shown [23]. To recover exosomes for counting, immunoprecipitated pellets were resuspended in 0.25 ml of 0.05 M glycine-HCl (pH = 3.0) at 4°C, centrifuged at 200 × g for 15 minutes and supernate pH adjusted to 7.0 with 1 M Tris-HCl (pH 8.6). Exosome suspensions were diluted 1:200 to permit counting in the range of 3–15 × 10^8/ml, with an NS500 nanoparticle tracking system (NanoSight, Amesbury, UK), as described [23].

Exosome proteins were quantified by ELISA kits for human Aβ1–42, human total tau, and human P-S396-tau (Life Technologies/Invitrogen, Camarillo, CA), human P-T181-tau (Innogenetics Division of Fujirebio US, Inc., Alpharetta, GA) and human CD81 (Hölzel Diagnostika-Cusabio, Cologne, Germany) with the verification of the CD81 antigen standard curve using human purified recombinant CD81 antigen (Origene Technologies, Inc., Rockville, MD), according to suppliers' directions. The mean value for all determinations of CD81 in each assay group was set at 1.00 and the relative values for each sample used to normalize their recovery. The minor constituent of secreted neural exosomes P-S396-tau and the usually examined major neural exosome component P-T181-tau were both quantified to provide more complete information about the possible relationship between neurally secreted and plasma neurally derived exosome constituents in AD and FTD [24].

2.3. Statistical analyses

The statistical significance of differences between group means for patients with AD or FTD and their respective normal controls was determined with an unpaired t test including a Bonferroni correction in the interpretation (GraphPad Prism 6, La Jolla, CA). The significance of differences between serial values for AD patients taken before and after the onset of aMCI or dementia was calculated with a paired t test (GraphPad). Separate discriminant classifier analyses were conducted to define the best simple linear models for comparing AD with AC and FTD with FTC. Two discriminant analyses considered all variables and were performed step-wise. Final models retained only variables with a minimum partial F of 3.84 to enter and 2.71 to remove. Prior probabilities were considered equal for all groups. Fisher function coefficients and within group covariances were computed. Receiver operating characteristics (ROC) analyses were conducted under the nonparametric distribution assumption for standard error of area to determine the performance of the models for discriminating AD from AC and FTD from FTC. Discriminant and ROC analyses were conducted with SPSS v21.0 (IBM).

3. Results

3.1. Patient characteristics

The 57 patients with AD consisted nearly equally of those with aMCI or dementia, with the latter group having significantly lower MMSE scores ($P < .001$) (Table 1). The 16 patients with FTD had nearly equal numbers with mild or moderate dementia, with greater severity for the latter group documented by the significantly lower MMSE scores ($P < .01$). As cognitively normal control subjects were matched individually with patients, group male/female ratios, and mean (±SD) ages were expectedly nearly equal.

3.2. Exosomal protein levels

Cross-sectional comparisons of results of one-time studies of 57 AD patients and 57 matched case-controls (AC) revealed that AD exosomal concentrations of total tau (191 ± 12.3 pg/ml, mean ± SEM, $P = .0005$), P-T181-tau (106 ± 6.10 pg/ml, $P < .0001$), P-S396-tau (25.4 ± 2.25 pg/ml, $P < .0001$), and Aβ1–42 (18.5 ± 2.97 pg/ml, $P < .0001$) were significantly higher than for AC (130 ± 11.9 pg/ml, 16.9 ± 1.89 pg/ml, 3.88 ± 0.26 pg/ml, and 0.83 ± 0.13 pg/ml, respectively) (Fig. 1). P-S396-tau levels showed the least overlap with only five AD values in the AC range, of which two had P-T181-tau levels, two others had Aβ1–42 levels, and one had both P-T181-tau and Aβ1–42 levels above the AC range. Thus the AD profile of these three exosomal proteins together was completely distinct from that of AC. Step-wise discriminant analyses resulted in a model progressively incorporating P-T181-tau, P-S396-tau, and Aβ1–42, but not total tau, which produced a Wilk's lambda of 0.229 and an exact F of 119 ($P < .001$). The final model correctly classified 96.4% of MCI/AD patients contrasted with AC subjects (93% of MCI/AD and 100% of AC). The area under the curve (AUC) for the final model from the ROC analysis was 0.999 and individual AUC values for the individual proteins were 0.991, 0.988, 0.987, and 0.731, respectively, for P-T181-tau, P-S396-tau, Aβ1–42, and total tau (Fig. 1S).

Cross-sectional comparisons of the results of one-time studies of 16 FTD patients and 16 matched case-controls (FTC), showed that FTD exosomal concentrations of P-T181-tau (82.6 ± 9.20 pg/ml, mean ± SEM) and Aβ1–42 (7.54 ± 1.01 pg/ml) were significantly higher than for the FTC group (9.32 ± 2.86 pg/ml and 0.76 ± 0.35 pg/ml, respectively; both $P < .0001$), whereas those of total tau and P-S396-tau did not differ significantly between the FTD (135 ± 15.8 pg/ml and 2.13 ± 0.33 pg/ml, respectively) and FTC (148 ± 30.1 pg/ml and 3.13 ± 0.46 pg/ml) groups (Fig. 1). Fourteen of the 16 levels of P-T181-tau for FTD patients were higher than the upper end of the range for FTC subjects and the Aβ1–42 levels for the two FTD patients with P-T181-tau levels in the FTC range both were above the FTC Aβ1–42 range. In a step-wise discriminant analysis, only P-T181-tau entered the model, and attained a Wilk's Lambda value of 0.324 and an exact F of 62.5 ($P < .001$). In the final model, exosomal P-T181-tau correctly classified 87.5% of FTD patients contrasted with FTC subjects (75% of FTD and 100% of FTC). For the final model from the ROC analysis, AUC

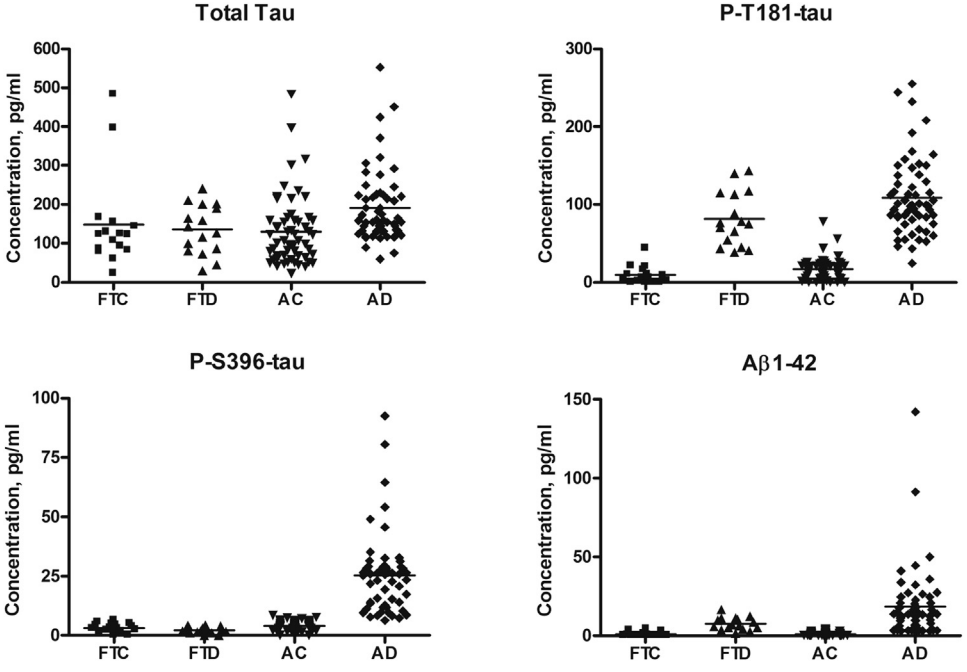

Fig. 1. Levels of proteins in blood exosomes of patients with Alzheimer's disease (AD), frontotemporal dementia (FTD), and cognitively normal matched case-controls (AC, FTC). The horizontal line in each cluster here and in Fig. 2 depicts the mean for that set.

for P-T181-tau was 0.992 and for Aβ1–42 was 0.969. Most remarkably and in contrast to the AD group, none of the concentrations of P-S396-tau for the FTD group was higher than the upper end of the range for the FTC subjects.

Resuspended initial precipitates from control subjects (n = 3) and AD patients with dementia (n = 3), respectively, contained 3.49 ± 0.90 × 10^9 exosomes/ml of plasma (mean ± SEM) and 2.78 ± 0.26 × 10^9 exosomes/ml of plasma as determined by the Nanosight system. Suspensions of immunoabsorbed exosomes from the same initial suspensions contained 0.417 ± 0.023 × 10^9 exosomes/ml of plasma and 0.472 ± 0.090 × 10^9 exosomes/ml of plasma. The range of yields of immunoabsorbed neurally derived exosomes for both AD and AC groups was 12% to 17% of the initial precipitates. Diameters of total plasma exosomes and immunoabsorbed putatively neural plasma exosomes ranged from 78 nm to 126 nm, which encompasses the expected exosomal size. No difference between exosomes of AD patients and control subjects was statistically significant, so that differences in the levels of pathogenic proteins are not due to divergent yields of exosomes and have been corrected by normalization with the exosomal marker CD81.

To support the capacity of neural adhesive protein immunoabsorption to enrich plasma exosomes from a neural source, immunoabsorption was carried out in parallel both with anti-NCAM-1 antibody and anti-L1CAM antibody for six plasmas of AD patients and six plasmas of matched controls (Table S1). Unlike the anti-NCAM-1 antibody, the anti-L1CAM antibody does not bind to NK and NKT cells of the immune system and is differently distributed in the nervous system. Extracted exosomal levels of CD81, P-T181-tau, P-S396-tau, total tau, and Aβ1–42 were statistically indistinguishable whether enriched with anti-NCAM-1 antibody or anti-L1CAM antibody.

3.3. Relationship of exosomal protein levels to severity and stage of AD

Comparing the 29 AD patients with aMCI to the 28 AD patients with dementia showed no differences in the exosomal levels of P-S396-tau, P-T181-tau, total tau, or Aβ1–42 (Table 2). This suggested that increased exosomal levels of these pathogenic proteins might be detectable early in the preclinical course. Blood exosomal proteins therefore were measured for an additional group of 24 AD patients at two time-points, the first at 1 to 10 years before their diagnosis and the second at the time of initial diagnosis of AD. This group consisted of 12 men and 12 women with a mean age (±SD) of 71.8 ± 7.30 years at the time of the first blood sample. The later diagnosis was aMCI for 13 and dementia for 11. Intervals between the two blood samples (number of patients) were: 1 year (one), 2 years (one), 3 years (four), 4 years (two), 5 years (two), 6 years (two), 7 years (three), 8 years (three), 9 years (three), and 10 years (three).

Table 2
Levels of serum exosome proteins in relation to severity of dementia in AD

Patient group	P-S396-tau	P-T181-tau	Total tau	Aβ1–42
AD, MCI	23.8 ± 3.27	114 ± 10.6	201 ± 20.9	23.0 ± 4.57
AD, dementia	27.0 ± 3.12	102 ± 7.08	181 ± 12.8	12.8 ± 1.60

Abbreviations: Aβ1–42, amyloid β1-42; AD, Alzheimer's disease; MCI, mild cognitive impairment.

NOTE. All values are mean ± SEM, pg/ml; none of the differences between values for the MCI and dementia groups were significant.

As for the single time-point values (Fig. 1), the mean levels (±SEM) of P-S396-tau (25.2 ± 1.85 pg/ml), P-T181-tau (91.1 ± 4.42 pg/ml), and Aβ1–42 (14.5 ± 1.41 pg/ml) at the time of diagnosis of AD were significantly higher than those of their case-controls (AC) (4.72 ± 0.64 pg/ml, 35.6 ± 3.49 pg/ml and 1.51 ± 0.52 pg/ml, respectively; all $P < .0001$) (Fig. 2). The mean level of total tau for the AD patients (165 ± 15.8 pg/ml) was no different from that of their AC group (148 ± 16.5 pg/ml). Furthermore, the mean preclinical (AP) level of total tau (154 ± 13.6 pg/ml) also was no different from that of AC. For P-S396-tau and P-T181-tau, the AP (19.2 ± 2.00 pg/ml and 85.7 ± 3.75 pg/ml, respectively) and AD values were significantly higher than those of the corresponding AC sets (both $P < .0001$). The mean P-S396-tau and P-T181-tau values of the AD group were no different from those of the corresponding AP group. Elevated exosomal levels of P-S396-tau and P-T181-tau thus were clearly detectable in a high-risk but cognitively normal AP group and had attained a plateau as early as 10 years before the clinical diagnosis of AD. For Aβ1–42, the mean levels for the AD and AP (6.64 ± 0.58 pg/ml) sets both were significantly higher than that of the AC set, but the mean AD level also was significantly higher than that of AP. Therefore Aβ1–42 may represent a biomarker for progression and early detection. A comparison of all AP and AD exosomal protein levels of patients converting to aMCI with those converting to AD did not show any significant differences. Furthermore, a comparison of all AP and AD exosomal protein levels of patients converting to aMCI or AD after 1 to 5 years with those converting after 6 to 10 years also did not show any significant differences.

4. Discussion

Levels of total tau, P-T181-tau, P-S396-tau, and Aβ1–42 previously quantified in plasma, serum or CSF have represented those in the fluid-phases. In contrast, the levels we now report are for proteins extracted predominantly from cellular structures consisting of neurally derived blood exosomes. When contrasted with fluid-phase levels, exosomal levels are nearly two orders of magnitude higher for total tau and P-T181-tau, and similar in magnitude for Aβ1–42 [15,25–27], and all were significantly greater than those in case-control exosomes (Fig. 1). When blood exosomal levels of P-S396-tau, P-T181-tau, and Aβ1–42 were considered together, the sensitivity of distinguishing AD from AC was 96% (Figs. 1 and 2, FigS1). In contrast to levels in AD, no

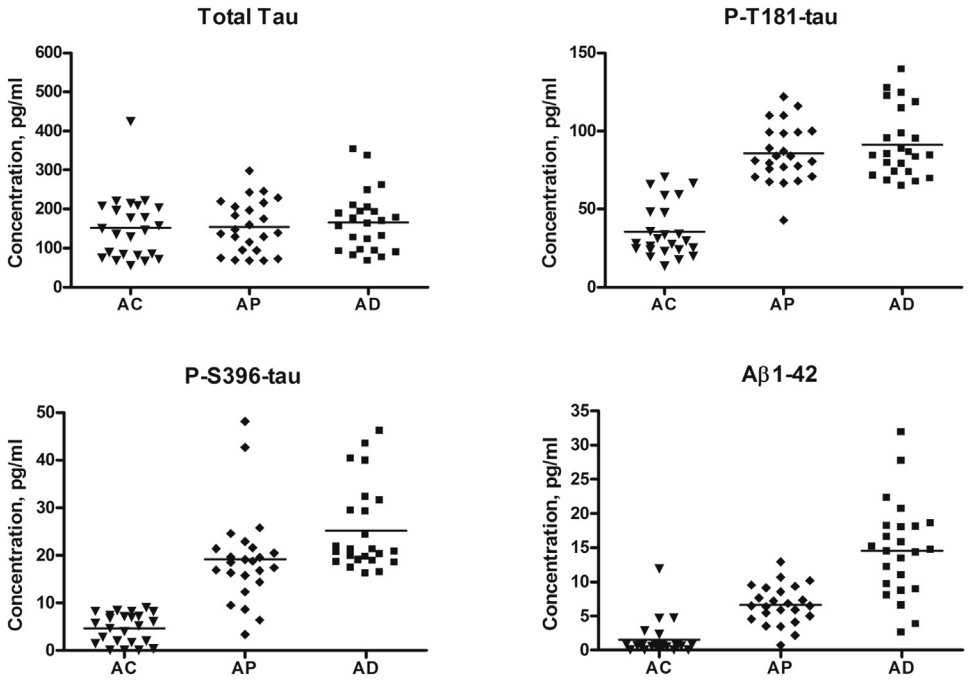

Fig. 2. Sequential levels of proteins in blood exosomes of patients with Alzheimer's disease (AD) measured first at a time of normal cognition (preclinical, AP) and later at the time of development of amnestic mild cognitive impairment (aMCI) or dementia (AD).

concentration of P-S396-tau for the FTD group was higher than the upper end of the range for the FTC controls. Thus blood exosomal P-S396-tau alone separates FTD from AD with a high specificity. Levels of P-S396-tau, P-T181-tau, and Aβ1–42 together also distinguished patients in the AP preclinical set from AC subjects with a sensitivity of 96% (Fig. 2). Most importantly, significantly elevated exosomal levels of these proteins were detected in high-risk, but cognitively normal AP subjects up to 10 years before clinical diagnosis. Furthermore, blood exosomal levels of Aβ1–42 continued to increase progressively from preclinical AP levels to significantly higher levels at the time of diagnosis of AD, implying value for exosomal Aβ1–42 as a progression biomarker (Fig. 2).

A recent fascinating report of higher or lower than normal plasma levels of multiple lipids and other cellular constituents during an average 2.1 year preclinical phase of AD provided a method with 90% accuracy in predicting progression to MCI or mild AD [28]. The broad spectrum of structures, organ distribution, functions, biosynthetic pathways, and biodegradative mechanisms of these molecules suggest that the disturbed pattern observed reflects major systemic perturbations in the early stages of AD. The two sets of results differ in three major respects. First, here we are assessing neural cell exosomal proteins implicated in the pathogenesis of AD, whereas they quantified plasma fluid-phase levels of amino acids and fats that are not characteristically altered in neural lesions of AD. Second, our present approach identified preclinical AD up to 10 years before clinical onset, as contrasted with the detection of plasma abnormalities to date only up to three years before diagnosis of dementia clinically. Third, the accuracy of classification of preclinical AD with our protein assays exceeded 96% as contrasted with 90% for the analyses of plasma lipids and amino acids. In further collaborative studies, we quantified neural exosomal P-tau and Aβ1-42 proteins in pairs of plasma samples from the 28 AD converters studied earlier by Dr. Federoff's group [28]. Our results showed significant elevations compared with cognitively normal matched controls in 100% of the preclinical samples, as contrasted with 89% preclinical identification by the profile of plasma tests of the Georgetown University Medical Center.

At this point in the evolving understanding of the clinical significance of our findings, it appears that detection in individuals of elevations of blood exosomal P-T181-tau and Aβ1–42 support the identification of present or future susceptibility to proteinopathic neurodegenerative disease, including AD and at least one form of FTD. Concurrent or subsequent recognition of elevated exosomal P-S396-tau suggests the presence or future likelihood of development of AD. At least two more points of information would strengthen the clinical usefulness of this blood exosomal profile of neuropathogenic proteins. The first would be quantification of levels of other relevant neurally derived exosomal proteins, such as TAR DNA-binding protein 43 (TDP-43), fused in sarcoma RNA-binding protein (FUS), and additional isoforms of P-tau, in relation to the type and severity of neurodegenerative disease. The second would be results of prospective longitudinal studies designed to delineate the neurological course of cognitively normal subjects with an abnormal

blood exosomal profile of neuropathogenic proteins as contrasted with the course of those having a normal profile of these proteins. With this knowledge, it may be possible to identify high-risk subjects early in their preclinical stage, define their point in the preclinical trajectory, and guide early applications of novel treatments.

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Author contributions: analysis of data, writing and/or editing of manuscript (MSF); evaluation of patients, analysis of data, writing and/or editing of manuscript (DK); evaluation of patients, analysis of data, writing and/or editing of manuscript (MM); evaluation of patients, analysis of data, writing and/or editing of manuscript (AB); laboratory benchwork, analysis of data (EE); evaluation of patients, analysis of data, writing and/or editing of manuscript (JBS); evaluation of patients, analysis of data, writing and/or editing of manuscript (ELA); evaluation of patients, analysis of data, writing and/or editing of manuscript (RCP); analysis of data, writing and/or editing of manuscript (HJF); evaluation of patients, analysis of data, writing and/or editing of manuscript (BLM); development of analytical methodology, laboratory benchwork, analysis of data, writing and/or editing of manuscript (EJG).

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RESEARCH IN CONTEXT

1. Case-control study: Abnormal cerebrospinal fluid (CSF) levels of amyloid β (Aβ1–42) and phosphorylation tau (P-tau), and positive central nervous system (CNS) images of amyloid deposits have diagnostic and predictive value for mild cognitive impairment and probable Alzheimer's disease (AD). However, there is substantial overlap in these preclinical biomarkers between patients who subsequently develop AD and those who later manifest other forms of dementia or no signs of dementia, even when concentrations of these CSF proteins are considered together or as ratios. This overlap of biomarkers is even greater when plasma levels of these proteins are used for diagnosis or prediction. We thus developed a method for quantifying AD-relevant pathogenic proteins in neurally derived blood exosomes.
2. Interpretation of results: Neurally derived blood exosomal levels of P-T181-tau, P-S396-tau, and Aβ1–42 were significantly higher for AD patients than case-controls, correctly classified 96.4% of AD patients and were significantly higher for AD patients than for controls one to ten years before diagnosis of AD. This high level of prognostic certainty for preclinical AD combined with the lower morbidity and expense compared with repeated CSF sampling and neuroimaging procedures emphasizes the potential importance of this novel blood-based approach to biomarker testing.
3. Future directions: The clinical usefulness of this blood exosomal profile of neuropathogenic proteins will be enhanced by quantification of levels of other relevant neurally derived exosomal proteins and by prospective longitudinal studies designed to delineate the neurological course of cognitively normal subjects with an abnormal blood exosomal profile of neuropathogenic proteins as contrasted with the course of those having a normal profile of these proteins.

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Table S1
Anti-NCAM-1 vs. anti-L1CAM antibodies for enrichment of neurally derived exosomes

AD (n = 6)									
CD81		Total tau		PS-396 tau		PT-181 tau		Aβ1–42	
NCAM-1	L1CAM	NCAM-1	L1CAM	NCAM-1	L1CAM	NCAM-1	L1CAM	NCAM-1	L1CAM
5.79 ± 0.79	5.93 ± 0.75	171 ± 24.4	167 ± 19.4	27.1 ± 3.29	26.1 ± 3.00	90.5 ± 10.1	92.6 ± 12.3	14.9 ± 3.66	18.0 ± 3.14
Controls (n = 6)									
CD81		Total tau		PS-396 tau		PT-181 tau		Aβ1–42	
NCAM-1	L1CAM	NCAM-1	L1CAM	NCAM-1	L1CAM	NCAM-1	L1CAM	NCAM-1	L1CAM
5.90 ± 0.69	5.95 ± 0.67	158 ± 18.8	154 ± 18.6	7.38 ± 0.46	7.32 ± 0.49	37.4 ± 6.73	37.7 ± 6.24	4.18 ± 1.70	4.07 ± 1.45

Abbreviations: NCAM-1, type 1 neural cell adhesion molecule; L1CAM, L1 cell adhesion molecule; AD, Alzheimer's disease; Aβ1–42, amyloid β 1–42.
NOTE. Each value is the mean ± SEM (pg/ml; ng/ml for CD81) for six determinations of proteins isolated from neurally derived exosomes.

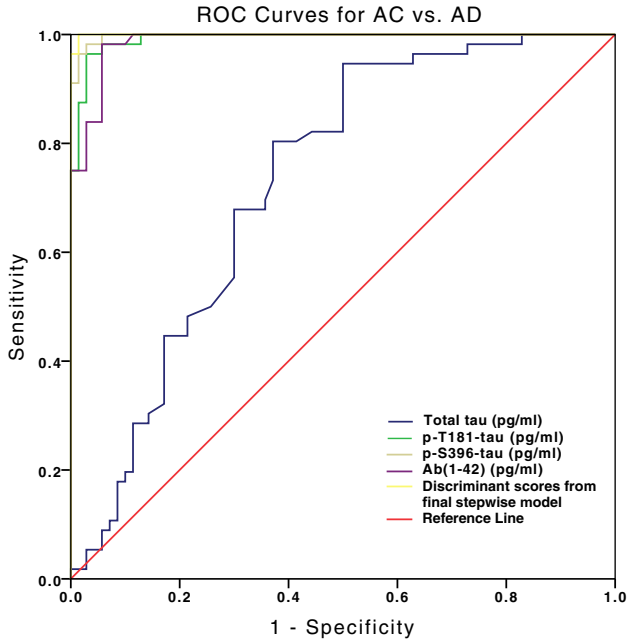

Fig. S1. Receiver operating characteristic (ROC) curves for the classification of patients with Alzheimer's disease (AD) vs. controls (AC) based on exosomal levels of total tau, P-181-tau, P-S396-tau, amyloid β (Aβ1–42), and using a discriminant model sequentially incorporating exosomal P-181-tau, P-S396-tau, and Aβ1–42 values.