BRIEF COMMUNICATION

Plasma neuronal exosomal levels of Alzheimer's disease biomarkers in normal aging

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Abstract

Plasma neuronal exosomal levels of pathogenic Alzheimer's disease (AD) proteins, cellular survival factors, and lysosomal proteins distinguish AD patients from control subjects, but changes in these exosomal proteins associated with normal aging have not been described for cognitively intact subjects. Plasma neuronal exosomal levels of P-T181-tau, P-S396-tau, $A\beta_{1-42}$, cathepsin D, repressor element 1-silencing transcription factor, and neurogranin were quantified longitudinally in cognitively intact older adults using two samples collected at 3- to 11-year intervals. Except for P-S396-tau, exosomal protein levels changed significantly with aging, but were largely outside the range observed in AD patients.

Background

Blood-based biomarkers of Alzheimer's disease (AD) are needed given high costs and invasiveness of most available biomarker technologies, including neuroimaging and quantification of analytes in cerebrospinal fluid (CSF).[1] Exosomes are released into the blood from a variety of cells, including neurons.[2] Exosomes have been shown to play a role in neuronal development and regeneration,[3] and they have been implicated in the transport of pathogenic proteins in the brain and in the progression of neurodegenerative diseases.[4]

Recent studies showed that neuronally derived plasma exosomal protein levels of β-amyloid$_{1-42}$ ($A\beta_{1-42}$) and tau proteins (P-T181-tau and P-S396-tau) were increased both in AD patients and in cognitively intact subjects (CIS) who transitioned to AD 2–10 years later compared to controls.[5] Similarly, neuronally protective repressor element 1-silencing transcription factor (REST) was decreased in both AD patients and in CIS who eventually transitioned to AD relative to controls.[6] Autolysosomal proteins, including cathepsin D, were higher both in AD patients and in CIS who eventually transitioned to AD relative to controls.[7]

Longitudinal studies of these neuronally derived plasma exosome proteins in CIS are needed as an appropriate framework for interpretation of elevated values. Additionally, neurogranin, a postsynaptic protein associated with

synapse loss, is reduced in the brains of AD patients,[8] but is increased in CSF[9,10] of AD patients compared to controls. Here, we examine levels of $A\beta_{1-42}$, P-T181-tau, P-S396-tau, REST, cathepsin D, and, for the first time, neurogranin, in CIS followed at the University of Kentucky Alzheimer's Disease Center (UK-ADC) in order to assess aging effects on the levels of these neuronally derived exosomal proteins.

Methods

Participant characterization, study design, and blood sampling technique

We studied plasma and CSF samples from 20 volunteers from the UK-ADC longitudinal cohort.[11] All volunteers were at least age 60 at UK-ADC enrollment. Volunteers were followed annually with in-person examinations, including neurocognitive assessment, physical exam, medical history, and neurological exam.

CIS provided blood at annual visits. Blood was centrifuged to yield platelet-free plasma and stored in 2.0-mL self-standing polypropylene tubes at −80°C. Plasma samples were collected from November 2011 to August 2013 ("second draw") from participants in a biomarker study, and each was paired with a sample collected from the same participant at least 3 years earlier ("first draw") in order to assess longitudinal protein changes in CIS. The CIS in this study also provided CSF at the second draw. Two participants had no available earlier first draw. The UK Institutional Review Board approved all research procedures; participants provided written informed consent.

CSF collection and measurement

Lumbar CSF was drawn using a 20-gauge needle the morning after fasting since midnight and was maintained in single use 0.5-mL aliquots in polypropylene storage tubes in a −80°C freezer. Briefly, CSF was collected into 15-mL sterile polypropylene collection tubes and transferred into storage tubes without any centrifugation step. CSF was batch shipped overnight to the Biomarker Research laboratory at the University of Pennsylvania Medical Center on dry ice. Upon receipt the samples were transferred to a −80°C freezer and maintained at this temperature until the day of analysis. $A\beta_{1-42}$, total tau, and P-T181-tau were measured using the multiplex xMAP Luminex platform (Luminex Corp, Austin, TX) with Fujirebio (INNO-BIA AlzBio3; Ghent, Belgium; for research use–only reagents) immunoassay kit–based reagents. The capture and detection antibodies for $A\beta_{1-42}$ were 4D7A3 and 3D6, respectively.[12] CSF analytes were assayed without dilution.

Isolation of exosomes from plasma for extraction and ELISA quantification of exosome proteins

Samples were relabeled prior to analysis to blind the laboratory. One-fourth ml of plasma was incubated with thromboplastin-D (Fisher Scientific, Inc., Hanover Park, IL) followed by addition of calcium- and magnesium-free Dulbecco's balanced salt solution with protease inhibitor cocktail (Roche Applied Sciences, Inc., Indianapolis, IN) and phosphatase inhibitor cocktail (Pierce Halt, Thermo Scientific, Inc., Rockford, IL) as described earlier.[5,6] After centrifugation at 3000*g* for 20 min at room temperature, supernatants were incubated with ExoQuick exosome precipitation solution (System Biosciences, Inc., Mountainview, CA), and the resultant suspensions were centrifuged for 30 min at 1500*g* at 4°C. Each pellet was resuspended in 300 μL of distilled water with inhibitor cocktails for immunochemical enrichment of exosomes from neuronal sources.[7]

Exosome suspensions were incubated with 2 μg of mouse anti-human CD171 (L1CAM neural adhesion protein) biotinylated antibody (clone 5G3, eBioscience, San Diego, CA) in 50 μL of 3% BSA for 60 min at 20°C followed by addition of 10 μL of Streptavidin-Plus UltraLink resin (Pierce-Thermo Scientific, Inc.) in 40 μL of 3% BSA.[7] After incubation for 30 min at 20°C, centrifugation at 400*g* for 5 min at 4°C, and removal of supernatants, pellets were resuspended in 50 μL of 0.05 mol/L glycine-HCl (pH 3.0), incubated 10 min at 4°C, and recentrifuged for 10 min at 4000*g* at 4°C. Each supernatant in a new Eppendorf tube received 5 μL of 1 mol/L Tris-HCl (pH 8.0) and 45 μL of 3% BSA followed by 0.40 mL of mammalian protein extraction reagent (Thermo Scientific, Inc.) containing protease and phosphatase inhibitors, mixed and stored at−80°C.

Exosome proteins were quantified by human-specific ELISAs for $A\beta_{1-42}$, P-S396-tau (Life Technologies/Invitrogen, Camarillo, CA), P-T181-tau (Innogenetics Division of Fujirebio US, Inc., Alpharetta, GA), REST (Cusabio, American Research Products, Inc., Waltham, MA), neurogranin (Cloud-Clone, Inc., American Research Products, Inc.), cathepsin D (EMD Millipore Corp., Billerica, MA), and tetraspanin exosome marker human CD81 (American Research Products-Cusabio), according to the suppliers' directions. One in ten representative preparations of exosomes was counted and CD63 levels were determined along with CD81. 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. Units for all analytes are pg/neuronal-derived exosomes in 1 mL of plasma (pg/ml). $A\beta_{1-42}$ and P-S396-tau in exosome extracts were quantified at a 1:2 (v:v)

dilution, whereas CD81, P-T181-tau, REST, and cathepsin D were quantified at a 1:4 (v:v) dilution. Evidence for enrichment of exosomes from neuronal sources has been provided previously.[7,13]

Statistical analyses

We used paired *t* tests to estimate mean change in protein levels over time, and we used linear regression to estimate mean P-T181-tau, P-S-396-tau, $A\beta_{1-42}$, cathepsin D, REST, and neurogranin levels at the first draw using age at draw as the predictor. Next, we regressed the change score on age at first draw and time between draws (years). Last, we compared protein levels at the second draw to distributions obtained from 10 age- and sex-matched AD patients for overlap. Plasma from AD patients was obtained from the National Institute on Aging plasma bank; AD diagnosis was made by neurological exam, psychometric testing, CSF protein analytes, and MRI using standard criteria.[13] All analyses were conducted with SAS 9.4® (SAS Institute, Inc.; Cary, NC).

Results

Median time between draws was 8.7 years (range 3.3–11.4), and all but four pairs occurred at least 7 years apart. Mini-Mental State Examination[14] scores decreased slightly over the interval ($P = 0.049$; Table 1). Paired *t* tests showed that P-T181-tau ($P = 0.0047$), $A\beta_{1-42}$ ($P = 0.014$), cathepsin D ($P = 0.0022$), and REST ($P = 0.0078$) increased over time (Table 2). Neurogranin decreased ($P < 0.0001$) and P-S396-tau ($P = 0.57$) did not change. Regression revealed significant association between P-S396-tau change and age at first draw ($P = 0.024$), with older participants experiencing larger increases. However, significance did not persist ($P = 0.31$) after excluding the oldest participant (age 92), who also had the largest increase in P-S396-tau level. This participant was diagnosed with probable AD 2 years following the second blood draw. No other change scores were associated significantly with age at first draw or time between draws. Analyses were repeated excluding participants with draws less than 7 years apart. Results were similar and conclusions did not change.

Cathepsin D, REST, and neurogranin levels in CIS were distinct from those of AD patients (Fig. 1). There was frequent overlap between CIS and AD patients for $A\beta_{1-42}$ levels (6/20) and less frequent for P-T181-tau (1/20) and P-S396-tau (2/20) (Fig. 1). In comparison, some participants also had CSF protein levels in the AD range[15]: CSF $A\beta_{1-42}$ (2/20; <192 pg/mL), total tau (1/20; >93 pg/mL), and P-T181-tau (10/20; >23 pg/mL).

Table 1. Sample characteristics of cognitively intact University of Kentucky Alzheimer's Disease Center research volunteers[1].

Characteristic	First draw ($n = 18$)	Second draw ($n = 20$)
Age, y	69.3 (5.6)	77.6 (6.1)[2]
Education, y	16.3 (2.5)	16.3 (2.5)
Female (%)	55.6	50.0
White race (%)	100	100
Mini-mental state examination	29.7 (0.5)	29.4 (0.8)[2]
APOE-ε4 carriers (n)	3	3
CSF $A\beta_{1-42}$ (pg/mL)	n/a	300.1 (76.9)
CSF P-T181-tau (pg/mL)	n/a	23.9 (8.4)
CSF total tau (pg/mL)	n/a	57.3 (17.3)

CSF, cerebrospinal fluid.
[1]Results presented are mean (SD).
[2]Significant change from first draw by paired *t* test ($P < 0.05$).

Table 2. Mean normalized protein levels (pg/mL) in plasma neuronal-derived exosomes.[1]

	First draw ($n = 18$)	Second draw ($n = 20$)
P-T181 tau	73.4 (25.3)	88.2 (22.7)[3]
P-S396 tau	10.9 (5.1)[2]	11.4 (4.3)
$A\beta$1-42	2.9 (1.3)	3.8 (1.9)[3]
Cathepsin D	1972.4 (1382.0)	4074.6 (2117.4)[3]
REST	906.4 (503.0)	1437.0 (720.0)[3]
Neurogranin	285.1 (76.8)	224.8 (61.0)[3]

[1]Results presented are mean (SD).
[2]Protein level at first draw was significantly associated with age at first draw. Significance did not persist after excluding the oldest participant.
[3]Significant change from first draw by paired *t* test ($P < 0.05$).

Discussion

This study provides evidence that neuronally derived exosome proteins $A\beta_{1-42}$, P-T181-tau, REST, and cathepsin D in older CIS increase over 3–11 years, whereas neurogranin decreases and P-S396-tau changes little over the same interval. Despite these changes, levels of REST, cathepsin D, and neurogranin were distinct from the ranges associated with AD. Importantly, we showed that levels of the neuroprotection factor REST, measured in plasma neuronal exosomes, increased with normal aging, similar to what has been shown in human brain tissue.[16] We also reported results of the first analyses of neurogranin in plasma neuronal exosomes; results were similar to the decreased levels seen in human brain tissue[7] in contrast to increases in CSF concentrations with aging and dementia.[9,10] This study further establishes the distinctive natures of the CSF and exosomal pathways for
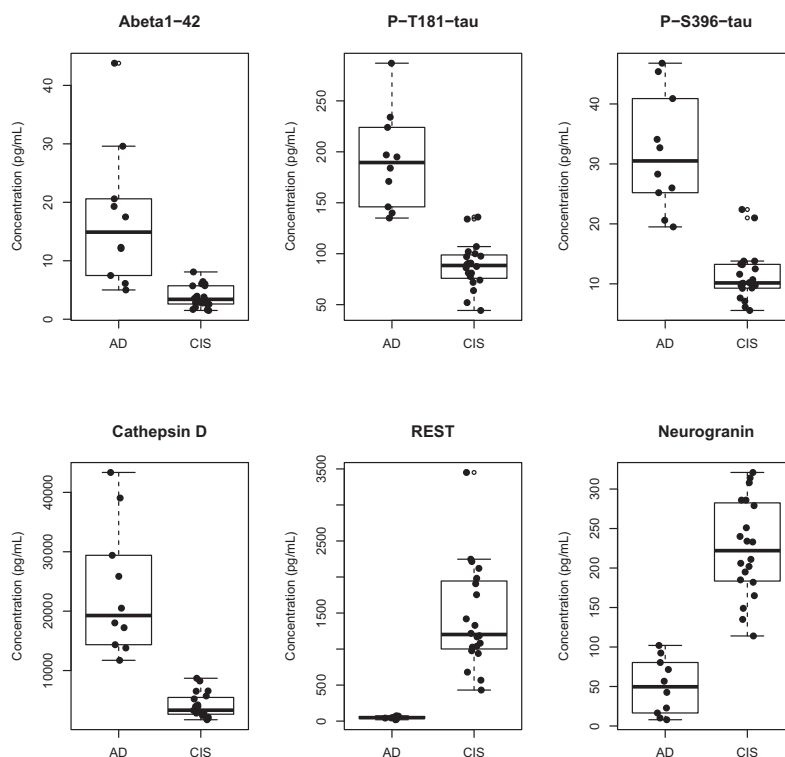
Figure 1. Levels of plasma exosomal proteins in age- and sex-matched cognitively intact subjects (CIS) and patients with Alzheimer's disease(AD).

exportation from CNS neurons of proteins relevant to the pathogenesis of dementias.

Changes in established AD biomarkers, $A\beta_{1-42}$, P-T181-tau, and P-S396-tau, moved some levels for CIS into the range of measurements observed in AD patients, as has been observed with CSF analytes. Overlap may represent variability in the distribution of values but may also identify participants at increased risk for future AD diagnosis.[17] Indeed, for the CIS who transitioned to Probable AD 2 years after the second draw, protein levels were in the AD range for both $A\beta_{1-42}$ (6.4 pg/mL) and P-S396-tau (22.4 pg/mL). Basic conclusions, except for the significance of P-S396-tau age associations, were not altered as a result of repeating statistical analyses excluding this participant.

Our data demonstrated that normal aging is associated with increases in certain AD biomarkers. Based on our prior work, preclinical AD cases may show mean protein levels similar to CIS on measures like $A\beta_{1-42}$, P-S396-tau, and P-T181-tau, although the upper range of measurement tends to be lower in CIS.[5] In comparison, REST and cathepsin D levels may be completely distinct in preclinical AD and CIS.[6,7] Future determination of thresholds for transition from normal aging to AD is critically important for interpretation of such biomarkers including $A\beta_{1-42}$, P-T181-tau, REST, cathepsin D, and neurogranin.

In contrast, P-S396-tau appears to exhibit distinctive specificity for the diagnosis of imminent or fulminant AD. This is consistent with our previous work demonstrating a lack of P-S396-tau elevations in FTD, and nonoverlapping P-S396-tau levels in controls who did not progress to AD over a 10-year period compared to those with AD and those who eventually developed AD.[5] The specificity of the antemortem CSF level of P-S396-tau epitope for fulminant AD or AD in situ with imminent clinical presentation has been published previously and parallels our findings in exosomes,[18] which represent a less invasive, peripheral blood-derived biomarker. While the utility of exosomal P-S396-tau for detection of fulminant AD appears consistent across studies, we note that this biomarker may lack diagnostic utility prior to the imminent development of symptomatic AD. Further research should address the temporal course of exosomal epitope changes heralding the onset of AD and the establishment of diagnostic thresholds for exosomal markers of preclinical AD.

Our findings add important information to existing literature on neuronally derived exosome-based biomarkers, but there are limitations in interpretation. Although the participants are well-characterized clinically, sample size is limited and may not be representative of the larger population of CIS. Additional participants may transition to

AD in the future. Replication studies are needed to confirm our results.

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Author contributions

E. L. A. performed the statistical analysis and drafted and revised the manuscript; G. A. J. evaluated participants and revised the manuscript; L. M. S. performed CSF analyses and revised the manuscript; J. Q. T. performed CSF analyses and revised the manuscript; E. J. G. developed the plasma analytical methodology, performed laboratory bench work, and drafted and revised the manuscript.

Conflict of Interest

EJG reports grants from NanoSomix, Inc., during the conduct of the study and grants from NanoSomix, Inc., outside the submitted work. In addition, Dr. Goetzl has a patent USA provisional regarding assay methods pending. LMS reports consulting fees from Eli Lilly and Janssen Research & Development regarding biomarker analyses and interpretation, and personal fees from Novartis, outside the submitted work. ELA, GAJ, and JQT report no conflicts of interest.

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