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SHORT REPORT

CSF alpha-synuclein aggregates by seed amplification and clinical presentation of AD

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Abstract

INTRODUCTION: Accumulating evidence suggests that α-synuclein (αSyn) can modulate Alzheimer's disease (AD) pathology. The aim of this study was to evaluate the prevalence and clinical features associated with cerebrospinal fluid (CSF) αSyn detected by seed amplification assay (SAA) in AD.

METHODS: Eighty AD patients with CSF AT(N) biomarker positivity (mean age 70.3 ± 7.3 years) and 28 non-AD age-matched controls were included. All subjects underwent standardized clinical assessment; CSF αSyn aggregates were detected by SAA.

RESULTS: CSF was αSyn-SAA positive (αSyn+) in 36/80 AD patients (45%) and in 2/28 controls (7.1%). AD αSyn+ and αSyn− patients were comparable for age, disease severity, comorbidity profile, and CSF core biomarkers. AD αSyn+ presented a higher prevalence of atypical phenotypes and symptoms.

CONCLUSIONS: Our findings demonstrate that concomitant CSF αSyn pathology is present in a significant proportion of AD patients starting in the early stages and can affect clinical presentation. Longitudinal studies are warranted to evaluate the significance for the disease course.

KEYWORDS
Alzheimer's disease, biomarkers, cerebrospinal fluid, copathology, phosphorylated tau, seed amplification assay, tau protein, α-synuclein

1 | INTRODUCTION

The presence of concomitant brain pathologies is very common in an aging population and represents an important modulating factor of Alzheimer's disease (AD) pathology.[1] Within the wide spectrum of known proteinopathies, α-synuclein (αSyn) pathology appears to be far the most common copathology in AD, ranging from 30% to 60% in different autopsy series.[1–3] In vivo, however, data on αSyn copathology are sparse, as the standard quantification methods for cerebrospinal fluid (CSF) and plasma are still inconclusive.

Recent evidence indicates that seed amplification assay (SAA) detects misfolded αSyn in the CSF of subjects with Parkinson's disease (PD) or dementia with Lewy bodies (DLB) with a high diagnostic accuracy.[4–8]

The detection of αSyn copathology in AD patients represents an important issue for both clinical and research scenarios, as it might impact disease presentation and progression and might explain different trends in response to either symptomatic strategies or to disease-modifying treatments.[9,10] Moreover, the presence of αSyn aggregates in the CSF of AD patients might be a biomarker for an underlying

αSyn-related pathology. In fact, DLB is the second most common neurodegenerative condition in the aging population, and its overlap with AD has been consistently supported by clinical and neuropathology series.[7,11] Despite the growing literature in the field, no large studies have evaluated the clinical characteristics and progression patterns of AD patients according to αSyn status.

In this study, we selected clinically defined AD patients with a biological confirmation of disease based on CSF core biomarkers, and we tested the presence of CSF αSyn aggregates using SAA. Patients underwent a standard protocol that included the evaluation of DLB core criteria and symptoms potentially related to αSyn pathology.[12]

The aim of the study was to investigate in AD patients the extent of αSyn-SAA positivity in the CSF and to assess its association with clinical characteristics.

2 | METHODS

2.1 | Study population

The study included patients with a clinical diagnosis of AD who underwent CSF assessment at the Neurology Unit of Brescia. All patients underwent routine blood analyses, magnetic resonance imaging (MRI), a standardized cognitive and behavioral assessment, including the Mini-Mental State Examination (MMSE), the Clinical Dementia Rating (CDR), Geriatric Depression Scale (GDS), and the Neuropsychiatric Inventory (NPI). The clinical diagnosis of AD was based on NIA-AA criteria confirmed on the basis of a CSF biomarker profile (see next section). The following exclusion criteria were applied: (1) prominent cortical or subcortical infarcts or brain/iron accumulation at imaging; (2) other neurological disorders or medical conditions potentially associated with cognitive deficits; (3) bipolar disorder, schizophrenia, history of drug or alcohol abuse, or impulse control disorder; (4) recent traumatic events or acute fever/inflammation potentially influencing CSF and plasma biomarkers; and (5) a possible or probable diagnosis of DLB.[11] DLB core criteria, namely, REM sleep behavioral disorder (RBD), hallucinations, and cognitive fluctuations, were specifically evaluated at baseline and follow-up according to the definitions and guidelines provided by the DLB Consortium.[11] Non-motor symptoms suggestive of a potential underlying α-synucleinopathy[12] were additionally evaluated. This study was approved by the local ethics committee (NP 1471, DMA, Brescia, approved in its last version on April 26, 2022) and was in conformity with the Helsinki Declaration; informed consent was obtained from all participants.

2.2 | Cerebrospinal fluid biomarkers

Lumbar puncture was performed in fasting conditions according to the standardized protocol of the outpatient clinic, from 9:00 to 11:00 a.m., after clinical informed written consent was obtained. CSF was collected in sterile polypropylene tubes and gently mixed to avoid gradient effects. The CSF was centrifuged and first processed for standard bio-

RESEARCH IN CONTEXT

1. **Systematic Review**: The authors reviewed the literature using conventional (e.g., PubMed) sources and meeting abstracts and presentations. While clinical characteristics and progression patterns of AD patients according to α-synuclein (αSyn) status have not yet been evaluated, recent evidence suggests that αSyn accumulates in AD brain and influences clinical course.

2. **Interpretation**: Our findings demonstrate that concomitant CSF αSyn pathology is present in a significant proportion of AD patients starting in the early stages and can affect clinical presentation.

3. **Future Directions**: This study proposes a framework for the generation of new hypotheses and for conducting additional studies. Further longitudinal studies are warranted to evaluate the impact of copathologies on clinical progression and course.

chemical analyses, and two milliliters of CSF were stored in cryotubes at $-80°$C before biomarker testing. Only patients with normal CSF standard biochemical measures were included in further analyses. CSF total and phosphorylated tau (t-tau and p-tau) and Aβ_{42} concentrations were measured by Lumipulse (Fujirebio, Ghent, Belgium). Standard cut-off values for AD used by our laboratory are Aβ_{42} < 650 ng/L, p-tau > 60 pg/ml, t-tau > 400 pg/ml, and p-tau/ Aβ_{42} ratio > 0.9.[13] Patients with a clinical diagnosis of AD but no A+T+N+ positivity were excluded from the study ($n = 9$). We analyzed 28 controls recruited at the University of Verona with a clinical diagnosis of non-αSyn and non-AD neurodegenerative diseases (i.e., A-T-N- standard markers). The group included patients with progressive supranuclear palsy ($n = 8$), corticobasal syndrome ($n = 1$), frontotemporal dementia ($n = 3$), other non-neurodegenerative neurological disorders ($n = 6$), and ten Creutzfeldt–Jakob cases neuropathologically confirmed (Figure S1 and Table S1).

2.3 | Expression and purification of recombinant human α-synuclein

Recombinant αSyn was expressed and purified from the periplasmic fraction as reported.[14] Briefly, wild-type human αSyn cDNA was cloned in the pET-28a plasmid (Novagen) and transformed into *Escherichia coli* BL21 (DE3). Cell cultures (1 L) were grown at 37°C to an optical density of 600 nm of 0.3 to 0.4, and the expression was induced with 0.1 mM isopropyl b-D-1-thiogalactopyranoside (IPTG) for 5 h. Cells were collected by centrifugation, resuspended in 100 ml of osmotic shock buffer (30 mM Tris–HCl pH 7.2, 40% sucrose, 2 mM EDTA) and incubated for 10 min at room temperature. The cells were centrifuged at 12,000 rpm, resuspended in 90 ml of cold water with 37.5 ml of

saturated MgCl2 solution, and, after 5 min incubation on ice, centrifuged again. The supernatant containing the periplasm proteins was boiled for 15 min and cleared by centrifugation. The soluble fraction, enriched in α-syn, was subjected to ammonium sulfate precipitation followed by extensive dialysis against 20 mM Tris–HCl, pH 8.0. Further purification of α-syn was performed by anion exchange chromatography loading the sample on a Q-Sepharose column (GE Healthcare) equilibrated with the same buffer and eluted with a 0 to 500 mM linear gradient of NaCl. The purity of αSyn was checked by SDS-PAGE. The protein was then dialyzed against 10 mM sodium phosphate buffer pH 7.4 and stored at $-80C$ until use.

2.4 | αSYN-SAA analysis of cerebrospinal fluid

15 μl of undiluted CSF was added to 85 μl of reaction mix composed of 100 mmol/L phosphate buffer (pH 8.2), 10 lmol/L ThT, 0.05 mg/ml human recombinant full-length (1 to 140 aa) αSyn, 0.0075% sodium dodecyl sulphate (SDS), and 37 $\pm$ 3 mg of 0.5-mm glass beads (Sigma). The reaction plates were incubated at 30°C in a BMG FLUOstar Omega plate reader with cycles of 1 min shaking (200 rpm double orbital) and 14 min rest. ThT fluorescence measurements (450 $\pm$ 10 nm excitation and 480 $\pm$ 10 nm emission; bottom read) were taken every 45 min.

The criteria for discriminating positive versus negative SAA tests of CSF are similar to those previously described for prion SAA analyses.[5,15] Briefly, a ThT fluorescence threshold was calculated as the average fluorescence for all samples during the initial 10 h of incubation plus 10 standard deviations (SD). Cut-off time was assessed at 80 h for both NS and CSF samples, based on the results from certain cases, in order to obtain the best specificity and sensitivity.
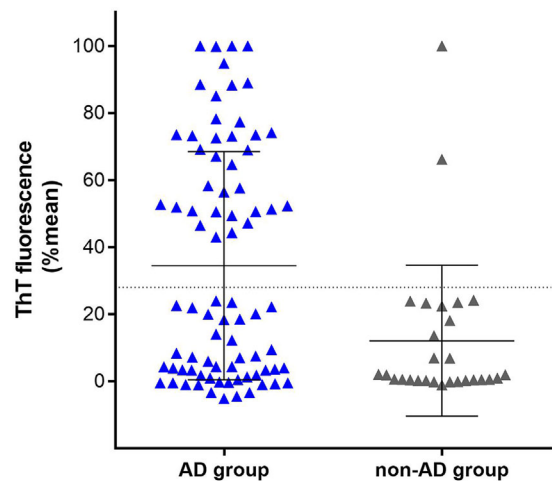
A sample was considered positive overall when at least two of four replicate wells crossed this calculated threshold. When only one of the quadruplicates crossed the threshold, the analysis was repeated, and if the data were confirmed, the sample was considered an undetermined negative. All the data were normalized as previously described.[8]

2.5 | Statistical analysis

Data are presented as mean + SD for continuous variables and number (%) for categorical variables. Group comparisons between AD patients with and without αSynuclein pathology were evaluated with Mann-Whitney and Fisher's tests for continuous and dichotomic variables, respectively.

3 | RESULTS

Eighty consecutive AD patients underwent CSF core analyses and enrolled in the study, including 40 subjects with mild cognitive impairment (MCI) and 40 with mild dementia (DEM). The mean age at CSF analyses was 70.3 $\pm$ 7.3 years and the mean disease duration 2.8 $\pm$ 1.7 years from the onset of cognitive symptoms. As controls, 28 non-

FIGURE 1 αSyn-SAA seeding activity in CSF of AD group and non-AD group. CSF sample was considered positive when at least two of four replicate wells crossed the calculated threshold (see methods in supplementary materials). Final average relative ThT fluorescence from four replicate readings obtained from CSF of AD group of patients (blue triangles) and for each control (non-AD group) (gray triangles) at 80 h. Bars show average $\pm$ SD for type of case.

AD subjects were enrolled (see methods in supplementary materials for further details). Sixty-eight out of 80 patients presented a typical AD phenotype characterized by prominent early memory impairment, whereas 12 presented atypical forms, namely, logopenic variant of primary progressive aphasia ($n = 5$), AD frontal variant ($n = 4$), and posterior cortical atrophy (PCA, $n = 3$). Thirty-six out of 80 CSF samples from AD patients (45.5%) and two out of 28 (7.1%) controls were αSyn-SAA positive ($p = 0.001$) (Figure 1). AD patients who tested positive in the αSyn-SAA assay (AD-αSyn+) were comparable for age, sex distribution, educational levels, and number and severity of comorbidities assessed on the cumulative illness rating scale (CIRS) and vascular risk factors compared to patients who tested negative in the assay (AD-αSyn−). No differences in treatment distribution at the time of CSF analyses were detected for anticholinesterase inhibitors (11.9% vs. 8.6%, $p = 0.46$), antidepressant (23.8% vs. 22.9%, $p = 0.57$), and antipsychotic (2.4% vs. 5.7%, $p = 0.43$). The subgroups of patients showed a similar distribution in MCI (44%) and dementia (47.5%) and did not differ either for disease severity (assessed with MMSE and CDR) or for neuropsychiatric symptoms (expressed by GDS and NPI). CSF core AD biomarker mean levels did not differ according to αSyn status (Table 1).

Compared to AD-αSyn−, AD-αSyn+ exhibited a higher prevalence of atypical phenotype (11.6% vs. 19.4%). Three AD-αSyn+ patients presented a frontal variant of AD, three presented a posterior cortical atrophy (PCA), whereas lvPPA was more commonly associated with AD-αSyn- ($n = 4$ out of 5 cases). The age- and sex-adjusted analyses revealed a slightly higher prevalence of isolated core DLB criteria or symptoms suggestive of an underlying α-synucleinopathy in AD-αSyn+, namely, hallucinations ($n = 3$ vs. 1, 7.7% vs. 2.3%, $p = 0.6$), parkinsonism ($n = 3$ vs. 0, 7.7% vs. 0%, $p = 0.04$), and symptomatic orthostatic hypotension ($n = 3$ vs. 0, 7.7% vs. 0%, $p = 0.04$), with no

TABLE 1 Demographics and clinical characteristics according to the aSyn CSF positivity

	AD αSyn+ (n = 36)	AD αSyn− (n = 44)	p-value
Age, years	70.70 ± 6.18	69.99 ± 8.22	0.97
Sex female, n (%)	19 (48.7%)	23 (53.5%)	0.66
Education, years	9.0 ± 4.3	9.4 ± 3.8	0.64
Comorbidity total score	17.7 ± 4.3	18.4 ± 3.4	0.66
Diabetes, n (%)	6 (16.6%)	6 (13.6%)	0.50
Hypertension, n (%)	15 (41.7%)	17 (38.6%)	0.53
Dyslipidemia, n (%)	9 (25%)	7 (15.9%)	0.25
Disease duration, years	2.87 ± 2.17	2.84 ± 1.48	0.49
MMSE, total score	20.4 ± 5.3	20.4 ± 5.4	0.38
CDR score	0.87 ± 0.54	0.93 ± 0.60	0.88
GDS, total score	2.00 ± 2.70	2.68 ± 2.73	0.51
NPI, total score	11.1 ± 9.2	9.5 ± 7.4	0.67
CSF core AD biomarkers			
T-Tau, pg/ml	749.4 ± 450.7	924.3 ± 628.4	0.48
P-Tau, pg/ml	99.9 ± 54.7	108.78 ± 67.3	0.47
$A\beta_{42}$, pg/ml	477.8 ± 118.9	504.6 ± 142.9	0.24

Abbreviations: $A\beta_{42}$, amyloid 1-42; CDR, Clinical Dementia Rating; GDS, Geriatric Depression Scale; MMSE, Mini-Mental State Examination; ml, milliliters; NPI, neuropsychiatric inventory; pg, picograms; P-Tau, phosphorylated tau protein; T-tau, total tau level.

differences for REM sleep Behavior Disorder (n = 3 for each group) or subthreshold parkinsonism evaluated by Movement Disorder Societ-y Unified Parkinson's disease Rating Scale part III (4.4 ± 6.8 vs. 2.5 ± 4.5, p = 0.07).

4 | DISCUSSION

The study highlighted that αSyn positivity by SAA is relatively common in AD patients and associated with a higher risk of DLB-like features or atypical presentations despite similar disease severity and core AD biomarker CSF levels.

This study evaluated a large series of clinical and ATN-positive AD patients who underwent a standard clinical evaluation and SAA for αSyn. We found that the CSF of AD patients tested positive for αSyn in 45% of patients, a rate in line with several neuropathological surveys conducted in tertiary dementia centers.[3,16,17] In one of the largest cohorts described to date, 177 of 626 (28%) participants with clinical AD collected at the Mayo Clinic Brain Bank from 2007 to 2016 showed significant Lewy bodies copathology, compatible with a secondary diagnosis of DLB.[18] In a different work, Robinson and coauthors also confirmed α-synucleinopathies as by far the most common neurodegenerative copathologies in AD, increasing with age in pathologically proven series.[1] At variance with previous studies, we observed no correlation between age and CSF αSyn aggregate detection. We might speculate, however, that, although αSyn copathology increases with age, it could also be impacted by age at presentation in some early-onset cases, thereby minimizing the role of age. In this context, Chung and coauthors argued for the need for a more complex model able to disentangle the real impact of overall copathologies in AD pathology at different age and disease stages. This issue might also be applied to core AD biomarkers, as they were similar in groups with and without αSyn-SAA assessment. A previous study by Vergallo and coauthors in a French cohort of at-risk subjects for AD found an interesting association between αSyn levels and amyloid burden and p-tau/t-tau levels.[19] This might suggest that further studies in patients with prodromal AD, negative for amyloid/tau or p-tau markers, will be pivotal to challenge the role of αSyn as modulator of AD pathology at this stage.

The higher rate of CSF samples that tested positive for αSyn compared to previous studies also needs to be discussed. First, other studies on the diagnostic accuracy of αSyn-SAA selected younger patients, mainly at the MCI stage and with fewer comorbidities, who might present a lower prevalence of neurodegenerative copathologies. Second, αSyn-SAA analysis is a standardized procedure, but it is still dependent on specific laboratory standards and procedures and needs to be implemented in interlaboratory validation studies.[20] However, the diagnostic accuracy of αSyn-SAA is very high when samples are obtained from neuropathologically proven DLB or PD, supporting the reliability of αSyn-SAA in detecting αSyn pathology.[4,7]

The clinical findings associated with α-synuclein positivity supports the claim of a genuine subcortical and cortical Lewy body copathologies. At the time of CSF assessment, we observed a higher prevalence of αSyn+ in AD patients with parkinsonism and orthostatic hypotension, features strongly associated with prodromal phases of both DLB and PD.[11,12] Furthermore, PCA and frontal variant of cognitive impairment, which are more frequent in patients with positive αSyn in CSF, might also indicate a true Lewy body pathology. PCA cases can indeed present AD or Lewy body pathologies, even in a pure form.[21] Behavioral changes associated with the frontal variant of AD are the most consistent clinical features associated with cortical Lewy body pathology in AD patients.[2]

Several limitations of this study should be acknowledged. First, the AD sample was clinically characterized at the time of assessment, but a longitudinal follow-up needs to be done to evaluate the impact of copathologies on progression and eventually on conversion to clinical DLB in some patients. Second, the study included patients at different stages of the disease, but all satisfied A+T+N+ core criteria. Thus, replication in prodromal AD phases, even in subjects with positive amyloid markers but no cognitive impairment, is pivotal since these subjects are the target for the ongoing disease-modifying trials. Third, the lack of advanced imaging assessment did not allow for a proper evaluation of αSyn positivity impact on brain structure or functional connectivity. Finally, the SAA detection and the control recruitment were performed at a different center, and interlaboratory validation studies are needed to test the reliability and reproducibility of results using different SAA protocols in different laboratories.[16]

Understanding the interaction between different copathologies in the aging brain is an important challenge for the research

community with relevant consequences for early diagnosis but also for the design and outcomes of clinical trials. The development of highly sensitive markers enables the detection of αSyn pathology in other neurodegenerative conditions and even in at-risk subjects,[22,23] thereby opening a new window of opportunity for the early detection of α-synucleinopathies. The overlap between αSyn and AD-related pathologies is supported by extensive preclinical, neuropathological data and needs to be further addressed in clinical research as well in ongoing longitudinal studies on at-risk subjects. A deeper understanding of the relationship between copathologies might allow for a more precise prediction of different trajectories observed by clinicians in clinical practice and might represent a pivotal step toward a personalized medical approach to formulating pharmacological and non-pharmacological strategies.

CONFLICTS OF INTEREST STATEMENT

Andrea Pilotto is a consultant and has served on the scientific advisory board of Z-cube (Technology Division of Zambon Pharma) and received speaker honoraria from Abbvie, Biomarin, and Zambon Pharmaceuticals.

Matilde Bongianni, Clara Tirloni, and Alice Galli declare no competing interests.

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CONSENT STATEMENT

This study was approved by the local ethics committee (NP 1471, DMA, Brescia, approved in its last version on April 26, 2022) and was in conformity with the Helsinki Declaration; informed consent was obtained from all participants.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.