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RESEARCH ARTICLE

ATN cerebrospinal fluid biomarkers in dementia with Lewy bodies: Initial results from the United States Dementia with Lewy Bodies Consortium

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Abstract

INTRODUCTION: The National Institute on Aging – Alzheimer's Association (NIA-AA) ATN research framework proposes to use biomarkers for amyloid (A), tau (T), and neurodegeneration (N) to stage individuals with AD pathological features and track

changes longitudinally. The overall aim was to utilize this framework to characterize pre-mortem ATN status longitudinally in a clinically diagnosed cohort of dementia with Lewy bodies (DLB) and to correlate it with the *post mortem* diagnosis.

METHODS: The cohort was subtyped by cerebrospinal fluid (CSF) ATN category. A subcohort had longitudinal data, and a subgroup was neuropathologically evaluated.

RESULTS: We observed a significant difference in $A\beta_{42/40}$ after 12 months in the A+T− group. *Post mortem* neuropathologic analyses indicated that most of the p-Tau 181 positive (T+) cases also had a high Braak stage.

DISCUSSION: This suggests that DLB patients who are A+ but T− may need to be monitored to determine whether they remain A+ or ever progress to T positivity.

KEYWORDS

ATN longitudinal data, ATN research framework, cerebrospinal fluid biomarkers, dementia with Lewy bodies, neuropathology, pre-mortem ATN status

HIGHLIGHTS

- Some A+T- DLB subjects transition from A+ to negative after 12-months.
- Clinically diagnosed DLB with LBP-AD (A+T+) maintain their positivity.
- Clinically diagnosed DLB with LBP-AD (A+T+) maintain their positivity.
- Monitoring of the A+T- sub-type of DLB may be necessary.

1 | BACKGROUND

Many individuals with dementia with Lewy bodies (DLB) are found to have both Lewy body pathology (LBP) and Alzheimer's disease pathology (LBP-AD) at autopsy. The characteristic neuropathological features associated with LBP include neuronal Lewy body inclusions and neurites, primarily containing aggregated α-synuclein protein. LBP can be observed in the brainstem and limbic and neocortical regions of the brain and is staged based on distribution.[1] Individuals with DLB show a progressive cognitive decline with key additional "core" clinical features including fluctuations and motor parkinsonism.[2] Although amyloid and tau pathological features of AD have been described in DLB cases that have come to autopsy and via biomarkers in cerebrospinal fluid (CSF), less is known about progressive changes in CSF amyloid β (Aβ) and tau in DLB.[3–5] Recently, different profiles of CSF Aβ reduction have been reported on DLB compared to AD. Specifically, while AD has been characterized by an isolated drop in $A\beta_{42}$, DLB comes with reductions in $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$.[6] Chaudhry et al. in 2020 showed that levels of soluble amyloid precursor protein β (sAPPβ) are higher and $A\beta_{42/40}$ ratios are lower in AD compared to DLB.[7]

While pathologic amyloid is common in DLB at autopsy, it has been suggested that diffuse amyloid deposition, and not neuritic plaques typically seen in AD,[5] is more common in DLB pre-mortem. Therefore, since amyloid deposition may be different in DLB compared to AD, it is important to develop DLB-specific tools to monitor amyloid deposition. The ATN research framework, proposed in 2011 and updated in 2018 by the NIA-AA, proposes to use biomarkers (namely amyloid (A), tau (T), and neurodegeneration (N)) to categorize individuals with an AD diagnosis.[8] The framework was conceptualized for a biologi-

cal construct of AD, not clinical symptoms of AD pathology. This ATN research framework can utilize cerebrospinal biomarkers where the ratio of the two Aβ peptides (CSF $A\beta_{42/40}$) is a measure for A, tau phosphorylated at threonine 181 (p-Tau 181) is a measure for T, and total tau (t-Tau) is a measure for N. Currently, little is known about how well ATN performs over time in non-AD dementia with potential mixed pathologies.[9] It has been used to study trends specific to other conditions, such as Parkinson's disease (PD)[10] and subjective cognitive decline.[11] Emerging evidence suggests that pathologicchanges in amyloid and tau are likely present at the earliest stages of AD, including presymptomatic disease, and this can be reflected in CSF and imaging biomarkers.[12] Aβ positivity alone, with limited tau pathology, is a frequent pathological finding in some DLB patients at autopsy, while others have the full pathologic changes in both AD and DLB. In this context, CSF Aβ with p-Tau 181 biomarkers could assist with discriminating DLB patients with and without coexistent AD, with potential relevance to emerging amyloid disease-modifying therapies.[13–15] Other studies have utilized RT-QuIC in CSF to distinguish between probable DLB, possible DLB, and AD.[16] Although ATN has been cross-sectionally described in DLB,[3,10,17] little is known about the trajectory of CSF Aβ and tau in DLB over time. Since DLB presents with fluctuations in symptoms, knowledge about longitudinal patterns of these biomarkers will determine whether they will remain stable during the progression of the disease.[2]

Therefore, additional studies are needed to fully understand both the prevalence and trajectory of these AD-related biomarkers in DLB. The overall objective of this investigation was to utilize the ATN framework to longitudinally characterize pre-mortem AD-related biomarkers of AD pathology in a clinically diagnosed and

rigorously characterized DLB cohort from the Dementia with Lewy Bodies Consortium (DLBC) (https://pdbp.ninds.nih.gov/Dementia-with-Lewy-Bodies-Consortium).[18,19] Our hypothesis was that a subtype of clinically diagnosed DLB patients with CSF AD-related pathologic change (LBP-AD) remain positive for CSF AD-related pathologic change after 12 months. A subcohort of DLB participants ($N = 8$) who had progressed to autopsy were also evaluated for the relationship between AD-related neuropathology assessments and CSF ATN category.

2 | METHODS

2.1 | Participants

Participants were recruited by the Cleveland Alzheimer's Disease Research Center (CADRC), Cleveland Clinic Lou Ruvo Center for Brain Health Aging and Neurodegenerative Disease Biobank (LRCBH-Biobank) located at the Cleveland Clinic in Cleveland, Ohio, and the DLBC.[19–21] Recruitment, patient consent, and sample collection for research studies were approved by each individual DLBC site and the Cleveland Clinic Institutional Review Board under the LRCBH-Biobank, CADRC, and DLBC protocols.

Participants had CSF collected between 2014 and 2021. A total of 354 individuals underwent a formal clinical consensus diagnosis utilizing the structured National Alzheimer's Disease Coordinating Center (NACC) D1 form (https://naccdata.org/data-collection/forms-documentation/uds-3). Clinical diagnoses of AD or DLB were made according to published criteria.[2] In particular, the DLB patients satisfied the diagnostic criteria for probable DLB.[2] As part of the DLBC protocol, DLB participants underwent dopamine transporter imaging (DAT scan) as previously described.[22] All participants underwent a baseline visit consisting of comprehensive neurological evaluation and neuropsychological assessment. Patients with DLB completed the Montreal Cognitive Assessment (MoCA)[23] and the Mini-Mental State Examination (MMSE)[24] as measures of global cognitive functioning. In addition to MoCA and MMSE, DLB patients completed an extensive neuropsychological battery composed of two tests within each of the five domains (attention and working memory, executive, language, memory, and visuospatial), according to the Movement Disorder Society (MDS) recommendations criteria.[25] Only individuals above 45 years of age with CSF available for ATN analysis were selected for this study and included 112 cognitively normal (CN) (65 from the CBH-Biobank, 47 from the CADRC), 133 AD (123 from CBH-Biobank, 10 from CADRC), and 109 DLB (28 from CBH-Biobank, 5 from CADRC, 76 from DLBC) subjects (Table 1).

2.2 | Sample processing

The procedures involved in collecting, processing, and storing biofluid samples were as recommended by NCRAD (https://ncrad.iu.edu/). Briefly, to avoid preanalytical variations, a lumbar puncture occurs in the morning after fasting. The CSF was aliquoted into 500-μL

RESEARCH IN CONTEXT

1. **Systematic Review**: Neuropathological hallmarks of AD, amyloid plaques and neurofibrillary tangles, have been described in dementia with Lewy bodies (DLB). CSF biomarkers for amyloid (A), tau (T), and neurodegeneration (N) can be utilized to define pre-mortem pathological status in DLB. Little is known about change in ATN status overtime in DLB.

2. **Interpretation**: Some DLB patients positive for CSF Aβ42/40 (A+), but negative for p-Tau 181 (T-) (A+T-N-, A+T-N+), changed from CSF Aβ42/40 positive to negative at 12 months (pvalue = .0153). This was not observed in the LBP-AD (A+T+) group. Findings from a small DLB subgroup with post-mortem neuropathologic analyses indicated that A+T+ cases had the highest CERAD scores.

3. **Future Directions**: There is a critical unmet medical need for more DLB longitudinal and neuropathological studies to comprehensively characterize biomarkers of AD-related pathology during the life of the patient

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amber tubes, immediately frozen, and stored at $-80°$C as previously described.[26] All CSF Aβ_{40}, Aβ_{42}, p-Tau 181, and t-Tau analyses were performed on the first freeze thaw.

2.3 | ATN classification

CSF Aβ_{40}, Aβ_{42}, p-Tau 181, and t-Tau were measured according to manufacturer specifications (Luminex xMAP technology; EMD Millipore, Chicago, IL, USA: HNABTMAG-68K),[27] modified by a 1:10 dilution of CSF. Each Aβ and tau kit comes with an Aβ and tau standard, as well as Aβ and tau quality controls. The kit provides the expected concentrations of each working standard as well as each of the quality controls. The standards, controls, and cohort samples were all run in duplicate. If the coefficient of variation for any of the replicate wells was greater than 25%, or if both replicate wells had a bead count of less than 35 beads for a given analyte, the assay for that sample was repeated. Patient groups with outliers were compared for age range, average age, sex, APOE ε4, and race. APOE ε4 status was obtained from NINDS BioSEND, generated using Fluidigm Juno and BiomarkHD platforms, from SNPs rs7412 and rs429358, and was performed in-house using TaqMan genotyping as previously described.[28] To identify outliers for each ATN category and each disease group, ROUT analysis (Q = 1%) was used[29] in the GraphPad Prism version 8.3.1 for Windows (GraphPad Prism, San Diego, CA, USA, www.graphpad.com). Outliers were identified for Aβ_{40}, Aβ_{42}, A$\beta_{42/40}$, p-Tau 181, and t-Tau using GraphPad Prism. Outliers were excluded out of an abundance of caution for assay technical error. There was no evidence of biological differences based on regression models that included age, sex, or

TABLE 1 Cohort description

Cohort characteristics	CN	AD	DLB	CN versus AD	CN versus DLB	AD versus DLB
n =	112	133	109			
Disease Duration (years)	–	6.0	2.5			
Age range (years)	48-81	52-86	57-87			
Average age (years)	69.2	66.6	69.8	0.0036	.4720	0.0009
Percentage male	47.7	51.9	85.2	0.6012	<0.0001	<0.0001
Percentage *APOE ε*4+ *	40.5	66.4	32.4	<0.0001	0.5348	<0.0001
Percentage White	91.9	89.5	98.1	0.2974	0.0100	0.0030
AD-related biomarkers (ATN)						
CSF $A\beta_{40}$ (pg/mL) mean (SD)	4266 (1518)	4428 (1650)	2242.22 (1494.50)	0.4297	<0.0001	<0.0001
CSF $A\beta_{42}$ (pg/mL) mean (SD)	735.34 (314.18)	489.18 (252.45)	317.37 (266.12)	<0.0001	<0.0001	<0.0001
CSF $A\beta_{42/40}$ (A) mean (SD)	0.17 (0.04)	0.11 (0.04)	0.13 (0.06)	<0.0001	<0.0001	0.0045
CSF p-Tau 181 (pg/mL) (T) mean (SD)	68.33 (38.66)	176.23 (85.83)	102.75 (70.02)	<0.0001	<0.0001	<0.0001
CSF t-Tau (pg/mL) (N) mean (SD)	343.91 (209.82)	686.53 (366.79)	474.33 (349.92)	<0.0001	0.0030	<0.0001

Note: *APOE ε*4 status is missing for five AD subjects, one CN subject, and 10 DLB subjects.
Abbreviations: AD, Alzheimer's disease; *APOE*, apolipoprotein E; CSF, cerebrospinal fluid; CN, cognitively normal; DLB, dementia with Lewy bodies; SD, standard deviation.

*APOE ε*4 status upon outlier removal (Supplemental File). The outliers included three samples removed for the $A\beta_{42/40}$ ratio (3 AD); eight removed for $A\beta_{42}$ (6 AD, 2 DLB); 12 removed for p-Tau 181 (3 CN, 2 AD, 7 DLB); and 12 removed for t-Tau (4 CN, 2 AD, 6 DLB). Upon identification of outliers (Table 2), the dataset without outliers was used for all subsequent analyses. Receiver operator characteristic (ROC) analyses were performed with and without outliers to determine sensitivity and specificity for each analyte. The Youden index was calculated to demonstrate the effectiveness of the biomarker and to select cut points for each biomarker.[30] ATN pie charts were created to demonstrate the distribution of ATN according to AD-related pathological category using the Matplotlib (3.4.3) library in Python (3.8.0) (Python Software Foundation. Python Language Reference, version 3.8. Available at http://www.python.org).[31]

2.4 | Statistical analysis

All statistical analyses were conducted in RStudio, using psych,[32] partial ROC (pROC),[33] ROCit,[34] verification,[35] cutpointr,[36] OptimalCutpoints,[37] readxl,[38] dplyr,[39] ggplot2,[40] ggpubr,[41] tidyverse,[42] and stats[43] packages. The optimal cut points for each biomarker were determined in RStudio by the ROC analysis, using the "cutpointr" function from the cutpointr package with the Youden index specified as the metric function[36] (Table 2). The significance (*p* value) between groups was determined using the Fisher's exact test performed in GraphPad Prism.[44] Binomial logistic regression was performed in RStudio to identify the correlation between longitudinal data and different independent variables, namely, $A\beta_{42/40}$, p-Tau 181, t-Tau, age, race, sex, and *APOE ε*4. A Sankey diagram analysis was performed to exhibit individual conversion from one ATN category to another after 12 months (https://sankeymatic.com).

3 | NEUROPATHOLOGY

All tissues for neuropathologic assessment were fixed in formalin and embedded in paraffin. After processing, these were deparaffinized in xylene and rehydrated in an ethanol gradient to deionized water. Sections were stained with Mayer's hematoxylin solution (Sigma-Aldrich, St. Louis, MO, USA) and counterstained with Eosin Y solution, alcoholic (Sigma-Aldrich) for H&E staining. Antigen retrieval for p-Tau (pSer202/Thr205) AT8 (Invitrogen) A1:500 and phospho-TDP43 (Millipore) 1:2000 was heat activated in a sodium citrate buffer pH 6.0 by autoclave at 121°C for 20 min. Antigen retrieval for *α*-synuclein (LB509) 1:1000, Syn 303 1:30,000 (gifts of the Trojanowski lab, University of Pennsylvania), and A*β* (6E10) (Biolegend) 1:1000 involved 1-min treatment in 88% formic acid followed by rinsing in deionized water for 5 min. Tissues were treated for endogenous peroxidases with 3% peroxide solution for 30 min and blocked with 3% normal goat serum for 1 h. All primary antibody incubation was overnight at room temperature. Next, addition of a biotinylated anti-mouse or anti-rat secondary antibody (Vector Labs) was incubated for 1 h followed by 1 h avidin-biotin complex, Vectastain ABC solution (Vector Labs). Finally, antibodies were detected with diaminobenzidine chromagen substrate solution (Vector Labs). Positive control cases were used for each antibody assessed. Negative controls were used with the same sections in the absence of the primary antibody. Hematoxylin and eosin (H&E) staining was used on neocortical, limbic, and brain stem sections to evaluate for cerebrovascular disease including

TABLE 2 Optimal cut-point identification

(a)

| ROC characteristics | Without outliers | | | | |
	$A\beta_{40}$	$A\beta_{42}$	$A\beta_{42/40}$ (A)	p-Tau 181 (T)	t-Tau (N)
N (CN/AD)	237 (106/122)	231 (106/122)	230 (106/122)	230 (106/122)	230 (106/122)
AUC	0.4739	0.7861	0.9231	0.9386	0.8624
Optimal cut point	2492	601.11	0.1385	110.08	455.45
Youden	0.0326	0.4915	0.7482	0.7536	0.5786
Sensitivity	0.2213	0.8689	0.9180	0.8197	0.7295
Specificity	0.8113	0.6226	0.8302	0.9340	0.8491
P value	0.3904	<0.0001	<0.0001	<0.0001	<0.0001

(b)

| ROC characteristics | With outliers | | | | |
	$A\beta_{40}$	$A\beta_{42}$	$A\beta_{42/40}$ (A)	p-Tau 181 (T)	t-Tau (N)
N (CN/AD)	245 (112/133)	245 (112/133)	245 (112/133)	245 (112/133)	245 (112/133)
AUC	0.4758	0.7484	0.8603	0.9040	0.8200
Optimal cut point	2451	601.11	0.1385	110.08	401.43
Youden	0.0338	0.4370	0.6518	0.6913	0.5202
Sensitivity	0.1053	0.8120	0.8571	0.7895	0.7970
Specificity	0.9286	0.6250	0.7946	0.9018	0.7232
p value	0.4297	<0.0001	<0.0001	<0.0001	<0.0001

AD-related biomarker positivity and ATN categories were established using optimal cut points as defined by Youden index and receiver operator characteristic (ROC) analyses for CN and AD. The following are described: area under curve (AUC), sample size (N), optimal cut points, Youden index, sensitivity, specificity, and significance (p value). These were determined for ATN without (a) and with outliers (b) (cut points highlighted in gray).

TABLE 3 Neuropathological assessment

Case no.	Pre-mortem Dx	Post-mortem Dx	PMI	Time from baseline LP to death	Thal stage	Braak Stage	CERAD Score	ABC score	$A\beta40$	$A\beta42$	$A\beta42/40$	p-Tau 181	t-Tau	ATN status
1	DLB	(limbic)	42.85	569days	1	III	absent	A1B2C0	1105	155	0.140271493	36.52	420.93	A-T-N-
2	DLB	(neorcortical)	80.75	82days	1	II	absent	A1B1C0	293.92	52.85	0.179810833	37.51	153.24	A-T-N-
3	DLB	(limbic)	21.50	138days	2	II	absent	A1B1C0	764.81	107.9	0.141080791	37.61	279.27	A-T-N-
4	DLB	(limbic)	50.25	537days	0	III	absent	A0B3C0	663.05	77.94	0.117547696	28.49	214.43	A+T-N-
5	DLB	(limbic)	48.00	30 days	2	III	absent	A1B2C0	1340	144.38	0.107746269	49.97	558.76	A+T-N+
6	DLB	(neocortical)	21.18	1066days	2	II	sparse	A1B1C1	1050	117.93	0.112314286	113.8	351.06	A+T+N-
7	DLB	(Amygdala-Predominent)	40.95	825days	5	IV	frequent	A3B3C3	839.58	40.03	0.047678601	122.9	401.51	A+T+N-
8	DLB	(neocortical)	7.93	28 days	5	IV	sparse	A3B2C1	1758	191.82	0.109112628	157.9	613.27	A+T+N+

Note: Autopsy-related features and corresponding CSF ATN status at baseline clinical visit. *Post mortem* diagnosis for all cases was DLB.
Abbreviations: ABC, amyloid, Braak and CERAD; CERAD, Consortium to Establish a Registry for Alzheimer's Disease; DLB, dementia with Lewy bodies; Dx, diagnosis; LBD, Lewy body disease; LP, lumbar puncture; PMI, *post mortem* interval.

microinfarcts, neuronal loss, and gliosis. AD pathology was evaluated using $A\beta$ and p-Tau antibodies. Neuropathologic change of AD was evaluated by assessing for Thal amyloid stage,[45] Braak stage for neurofibrillary tangles,[46] and CERAD neuritic plaque frequency[47] to an ABC score that accounts for variability in the person evaluating the tissue.[1] Identification of Lewy body inclusions and Lewy neurites was evaluated in neocortical, limbic, and brain stem regions and was classified as none, brainstem-predominant, limbic transitional, neocortical diffuse, or amygdala predominant[1] (Table 3). Additionally, neocortical and limbic regions were evaluated for phospho-TDP43 neuropathologic change.[1]

4 | RESULTS

4.1 | Cohort description

Sample characteristics are shown in Table 1. Two-sample *t* test indicated significant differences for age between CN and AD (p = .0036) and AD and DLB (p = .0009). A chi-squared test indicated significant differences for sex distribution between DLB and the other study groups, that is, DLB and CN (p < .0001) and DLB and AD (p < .0001). For *APOE* ε4 carrier status, significant differences were seen between CN and AD (p value < .0001) and AD and DLB (p value < .0001). Overall,
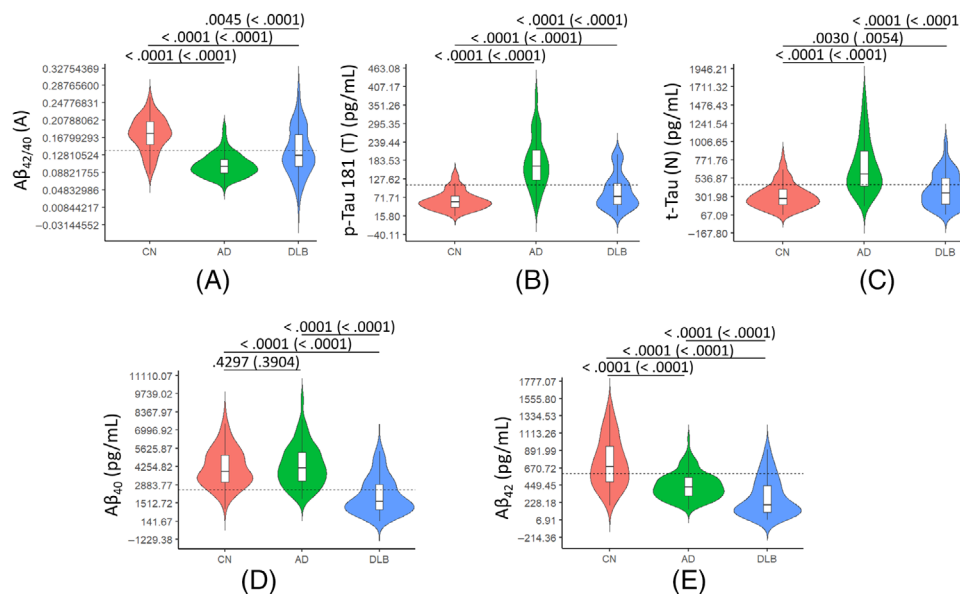
FIGURE 1 AD-related biomarker differences between clinically diagnosed groups. $A\beta_{42/40}$ is significantly lower than controls, for both AD and DLB, while DLB is significantly higher than AD (A). p-Tau 181 is significantly higher in both AD and DLB compared to CN, while DLB is significantly lower than AD (B). t-Tau is significantly higher in AD and somewhat significantly higher in DLB compared to CN, while DLB is significantly lower than AD (C). $A\beta_{40}$ in DLB is significantly lower than both CN and AD (D). $A\beta_{42}$ in DLB is significantly lower in both AD and DLB compared to CN, while DLB is significantly lower than AD (E). The p values for each pairwise comparison are shown at the top of each plot. This dataset was without outliers. *P* values for both with outliers (without parentheses on the left) and without outliers (in parentheses on the right) are shown.

the most significant differences between AD and DLB were observed for both gender and *APOE ε4* carrier status.

4.2 | Optimal cut point identification

ATN classification was determined for the entire dataset with and without outliers, and data without outliers were utilized to establish ATN categories (Table 2). The area under curve (AUC), optimal cut points, Youden index, and other factors calculated using the ROC analysis are shown in Table 2. For $A\beta_{40}$, the optimal cut point was 2451 pg/mL. For $A\beta_{42}$, the optimal cut point was 601.11 pg/mL. Individuals with $A\beta_{42/40}$ ratio < 0.1385 were classified as "A" positive, individuals with p-Tau 181 > 110.08 pg/mL were classified as "T" positive, and individuals with t-Tau > 445.45 pg/mL were classified as "N" positive (Table 2). These cut points were used to give each case an ATN status.

4.3 | AD-related biomarker differences between clinically diagnosed groups

CSF $A\beta_{42}$ ($p < .0001$), $A\beta_{42/40}$ ($p < .0001$), p-Tau 181 ($p < .0001$), and t-Tau ($p < .0001$) levels were significantly different between CN and AD. CSF $A\beta_{40}$ ($p < .0001$), $A\beta_{42}$ ($p < .0001$), $A\beta_{42/40}$ ($p < .0001$), and p-Tau 181 levels ($p < .0001$) were significantly different between CN and DLB. CSF $A\beta_{40}$ ($p < .0001$), $A\beta_{42}$ ($p < .0001$), p-Tau 181 levels ($p < .0001$) and t-Tau levels ($p < .0001$) were significantly different between AD and DLB (Figure 1). The only non-significant differences were for $A\beta_{40}$

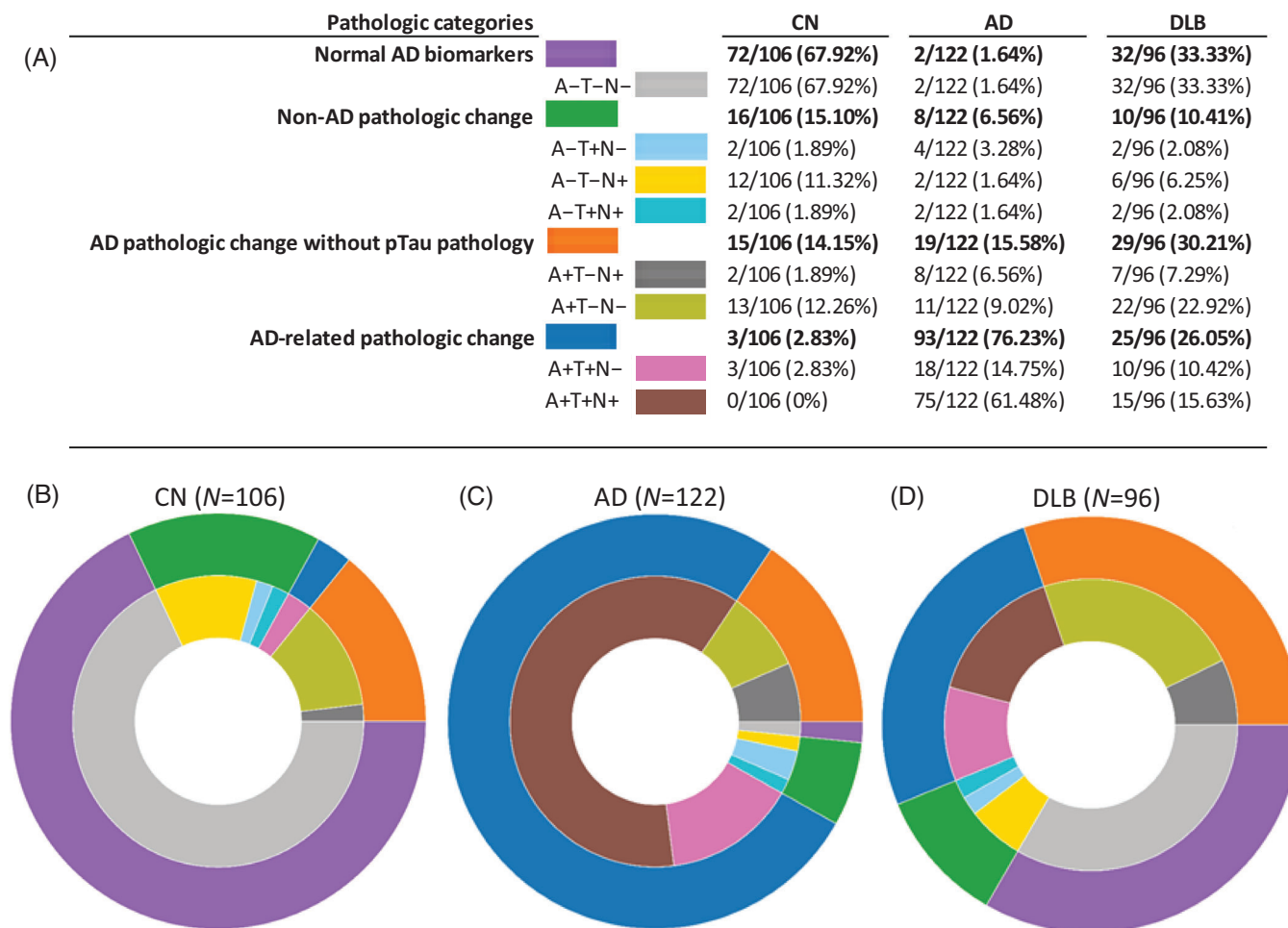between CN and AD, t-Tau between CN and DLB, and $A\beta_{42/40}$ between AD and DLB.

4.4 | CSF ATN biomarker status and pathological status of clinically diagnosed groups

Four broad pathologic categories were determined utilizing ATN cut points (Figure 2): (1) normal AD biomarkers (composed of A−T−N−) (Figure 2B-D); (2) non-AD pathologic change (composed of A−T+N−, A−T−N+ and A−T+N+) (Figure 2B-D); (3) AD pathologic change without p-Tau 181 pathology (composed of A+T−N+ and A+T−N−) (Figure 2B-D); and (4) AD-related pathologic change (composed of A+T+N− and A+T+N+) (Figure 2B-D). Specific ATN categories are represented by various colors in the inner ring, the color scheme is consistent through all the charts (Figure 2B-D). CN and DLB had a majority of normal AD biomarkers, whereas AD had a majority of AD-related pathologic change.

4.5 | Longitudinal DLB subcohort (*n* = 27) and AD-related biomarkers at baseline and 12 months

Twenty-seven DLB patients out of the original cohort of *N* = 96 had CSF collected at both baseline and 12 months. ATN status was determined using the baseline cut points. A non-significant increase in $A\beta_{42/40}$ ($p = .0589$), and no significant differences for both p-Tau 181 and t-Tau (Figure 3A-C) were observed between baseline and 12 months. When using the cut points for $A\beta_{40}$ and $A\beta_{42}$ alone, a significant change

(A)

Pathologic categories		CN	AD	DLB
Normal AD biomarkers	(purple)	**72/106 (67.92%)**	**2/122 (1.64%)**	**32/96 (33.33%)**
A−T−N−	(gray)	72/106 (67.92%)	2/122 (1.64%)	32/96 (33.33%)
Non-AD pathologic change	(green)	**16/106 (15.10%)**	**8/122 (6.56%)**	**10/96 (10.41%)**
A−T+N−	(light blue)	2/106 (1.89%)	4/122 (3.28%)	2/96 (2.08%)
A−T−N+	(yellow)	12/106 (11.32%)	2/122 (1.64%)	6/96 (6.25%)
A−T+N+	(cyan)	2/106 (1.89%)	2/122 (1.64%)	2/96 (2.08%)
AD pathologic change without pTau pathology	(orange)	**15/106 (14.15%)**	**19/122 (15.58%)**	**29/96 (30.21%)**
A+T−N+	(dark gray)	2/106 (1.89%)	8/122 (6.56%)	7/96 (7.29%)
A+T−N−	(olive)	13/106 (12.26%)	11/122 (9.02%)	22/96 (22.92%)
AD-related pathologic change	(blue)	**3/106 (2.83%)**	**93/122 (76.23%)**	**25/96 (26.05%)**
A+T+N−	(pink)	3/106 (2.83%)	18/122 (14.75%)	10/96 (10.42%)
A+T+N+	(brown)	0/106 (0%)	75/122 (61.48%)	15/96 (15.63%)

FIGURE 2 CSF ATN biomarker status and pathologic status of clinically diagnosed groups. ATN categories represent four pathological types (AD-related pathologic change, non-AD pathologic change, AD pathologic change without p-Tau pathology, normal AD biomarkers) (A). The distribution of these pathological categories is represented as the outer ring of the pie charts. The distribution of the ATN categories is represented by the inner ring of the pie charts (B–D). This dataset was without outliers.

over time was observed in the DLB group ($A\beta_{40}$ *p* value < .0001 and $A\beta_{42}$ *p* value < .0001) (Figure 3D-E). When these data were observed for longitudinal changes, 52% of the $A\beta_{40}$ values changed their status from positive to negative (Figure 3D), while no cases changed their status for $A\beta_{42}$ values. In other words, even though there was a significant difference between baseline and follow-up $A\beta_{42}$ values, none of the participants changed their status from positive to negative or vice versa (Figure 3E). Binomial logistic regression was performed to determine whether there was an association between the time duration of follow-up (time) and AD-related biomarkers while taking into account covariates: age, race, sex, and *APOE* ε4. The dependent variable was time (baseline and 12 months in the case of DLB and varying at second visit in the case of CN or AD/mild cognitive impairment (MCI) groups) and the independent variables were individual AD-related biomarkers, age, race, sex, *APOE* ε4 alleles, MoCA scores, and MDS-Unified Parkinson's Disease Rating Scale (MDS-UPDRS) Part III scores. Different models were used in different combinations of these variables with time, such as Time ~ $A\beta_{42/40}$ ratio, Time ~ $A\beta_{42/40}$ ratio + age, Time ~ $A\beta_{40}$ ratio + age + race, Time ~ $A\beta_{42/40}$ ratio + age + race + sex, and

so on. Some models that were significant (*p* < .05) for DLB cases were as follows: Time ~ $A\beta_{40}$ (*p* = .0003), Time ~ $A\beta_{40}$ + *APOE* ε4 ($A\beta_{40}$ *p* value = .0003, *APOE* ε4 *p* value = .2137), Time ~ $A\beta_{42}$ (*p* = .0002), Time ~ $A\beta_{42}$ + *APOE* ε4 ($A\beta_{42}$ *p* value = .0002, *APOE* ε4 *p* value = .6941), Time ~ $A\beta_{42/40}$ ratio + *APOE* ε4 ($A\beta_{42/40}$ ratio *p* value = .0384, *APOE* ε4 *p* value = .2763). All the significant models can be viewed in the Supplementary File. In summary, for DLB, time (baseline, 12 months) was found to be significantly associated with $A\beta_{40}$, $A\beta_{42}$, $A\beta_{42/40}$ ratio, and *APOE* ε4 in models with multiple covariates.

4.6 | Longitudinal CN subcohort (*n* = 28) and AD/MCI (*n* = 7) $A\beta_{40}$, $A\beta_{42}$, and AD-related biomarkers at baseline and second visit

Twenty-eight CN, five AD, and two MCI/AD patients had CSF collected at both baseline and follow-up visits of varying time points (6 to 60 months for CN and 12 to 54 months for AD/MCI). A significant change in $A\beta_{42}$ (*p* = .0042), $A\beta_{42/40}$ (*p* value = .0029), and p-Tau 181
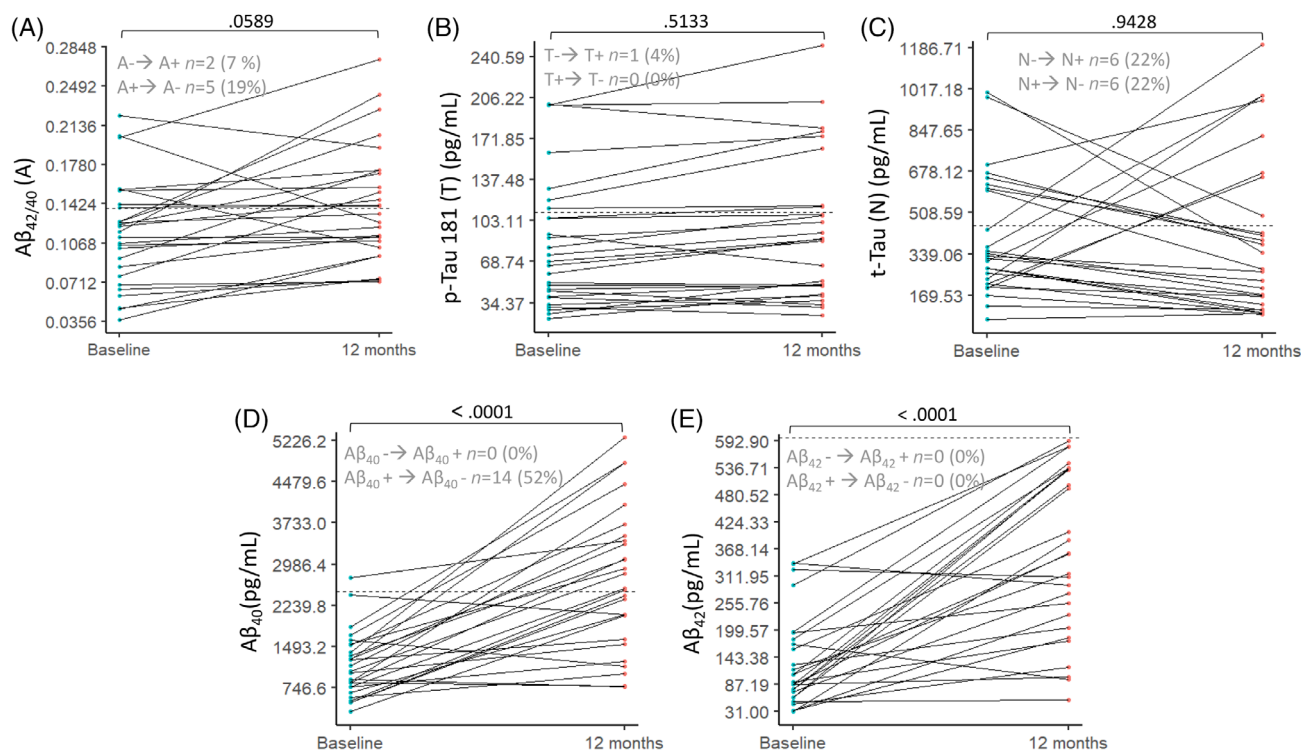
FIGURE 3 Longitudinal DLB subcohort ($n = 27$) and AD-related biomarkers at baseline and 12 months. There is a non-significant change in $A\beta_{42/40}$ levels from baseline to 12 month in DLB (p value = .0589); two changed from A− to A+, and five cases changed from A+ to A− (A). There was no significant difference from baseline to 12 months in p-Tau 181 levels (p value = .5133); one changed from T− to T+ (B) or t-Tau levels (p value = .9428); six changed from N− to N+ and six changed from N+ to N− (C). There was a significant increase in $A\beta_{40}$ levels (p value < .0001); 14 changed from positive to negative (D). There was a significant increase in $A\beta_{42}$ levels (p value < .0001); none changed from positive to negative (E). These data are without outliers. Analyte positivity was determined as above or below cut-point dotted line. Youden-derived cut-point values are shown in Table 2. P values were determined from linear regression.

($p = .0185$), but not $A\beta_{40}$ ($p = .1016$) or t-Tau ($p = .8956$), was observed between baseline visit and second visit for the CN cohort. The most notable change was observed for $A\beta_{42}$, where 43% of CN individuals changed their measures from positive to negative (Supplementary Figure 2). No significant changes were observed for AD and MCI/AD grouped together ($n = 7$) (Supplementary Figure 3). Individual ATN status change for CN and AD and MCI/AD cohorts are provided in Supplementary Figure 4. Binomial logistic regression analysis was done for these groups. Six models were significant ($p < .05$) for CN cases: Time $\sim A\beta_{42}$ ($p = .0076$), Time $\sim A\beta_{42}$ + age ($p = .0064$ for $A\beta_{40}$), Time $\sim A\beta_{42/40}$ ratio ($p = .0067$), Time $\sim A\beta_{42/40}$ ratio + age ($p = .0066$ for $A\beta_{42/40}$ ratio), Time $\sim$ p-Tau 181 ($p = .0267$), and Time $\sim$ p-Tau 181 + age ($p = .0223$ for p-Tau 181). There were no significant models for AD and MCI/AD cases. In summary, for CN, time, $A\beta_{42}$, $A\beta_{42/40}$ ratio, p-Tau 181, and age were significantly associated in different combinations.

4.7 | Longitudinal DLB cohort ($n = 27$) $A\beta_{40}$, $A\beta_{42}$, and AD-related biomarkers at baseline and 12 months stratified by ATN status

Upon stratification of the DLB cohort by the following ATN categories: (A−T−N+, A−T−N−); (A+T+N−, A+T+N+); (A+T−N−, A+T−N+)

(Figure 4), a significant increase in the $A\beta_{42/40}$ ratio was observed for the ATN categories positive for A but negative for T (A+T−N−, A+T−N+) ($p = .0128$) (Figure 4H). In addition, 50% in this group ($N = 6$) transitioned from A+ to A− (Figure 4H). Of these six participants, three were in the upper quartile near the cut point and three were in the upper mid-quartile further from the cut point. Other than this, $A\beta_{40}$ and $A\beta_{42}$ values also showed a significant change from baseline to 12 months for the ATN categories positive for A but negative for T (A+T−N−, A+T−N+) ($A\beta_{40}$ p value = .0007, $A\beta_{42}$ p value < .0001) and for the ATN categories positive for both A and T (A+T+N− and A+T+N+) ($A\beta_{40}$ p value < .0001, $A\beta_{42}$ p value < .0001). None of the other DLB ATN subgroups exhibited significant change after 12 months (Figure 4). In summary, the A+T− categories in DLB showed a significant $A\beta_{42/40}$ ratio increase over time.

4.8 | DLB AD-related biomarker fold-change between baseline and 12-month follow-up

Fold-change (follow-up measure divided by baseline measure) was calculated for A, T, and N. $A\beta_{42/40}$ (A) fold-change from baseline to 12 months was significantly lower in the DLB cases without AD biomarkers (A−T−N−, A−T−N+) as compared to those without
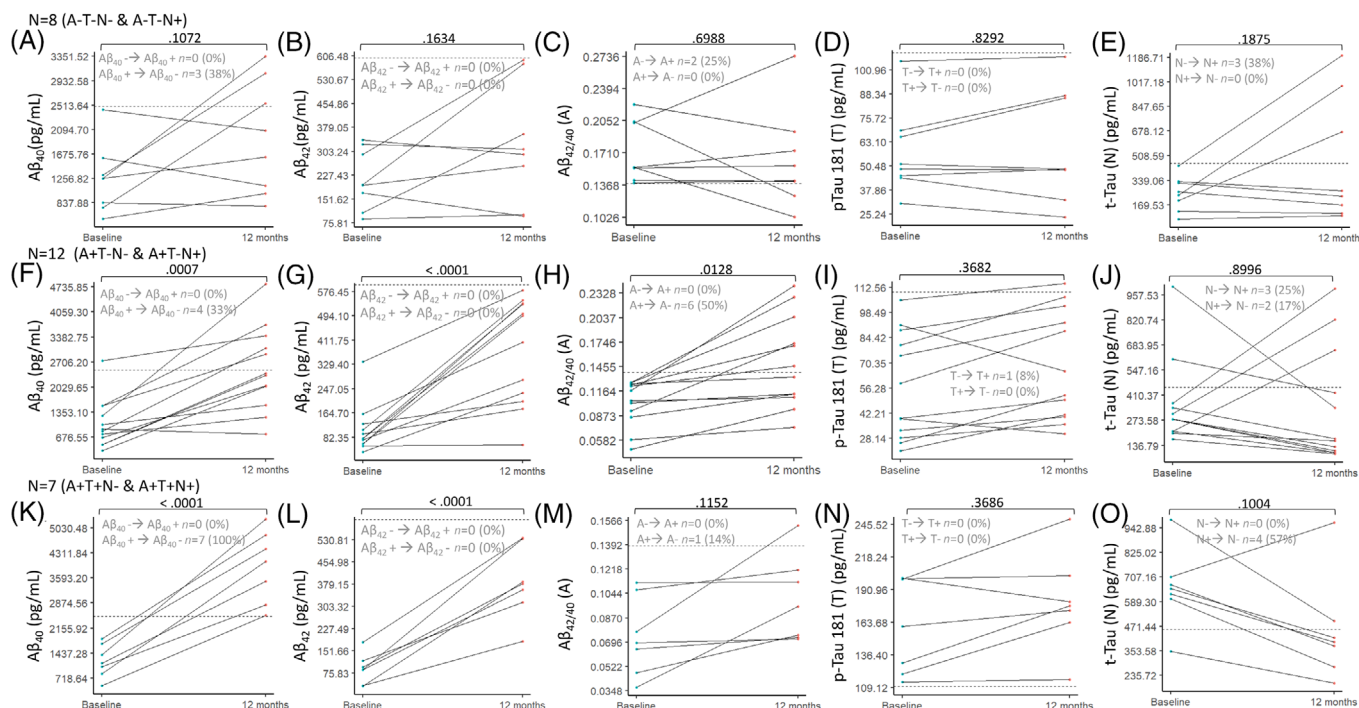
FIGURE 4 Longitudinal DLB cohort ($n = 27$) $A\beta_{40}$, $A\beta_{42}$, AD-related biomarkers at baseline and 12 months stratified by ATN status. There were no significance differences for the A−T−N−, A−T−N+ group (A−E). The A+T−N−, A+T−N+ group ($N = 12$) (F−J) had a significant difference from baseline to 12 months in $A\beta_{40}$ (p value = .0007) (F), $A\beta_{42}$ (p value < .0001) (G), and $A\beta_{42/40}$ levels (p value = .0128) (F). There was a significant difference from baseline to 12 months in $A\beta_{40}$ (p value < .0001) (K) and $A\beta_{42}$ levels (p value < .0001) (L) in the A+T+N−, A+T+N+ group ($N = 7$) (K−O). Analyte positivity was determined as above or below cut-point dotted line. Youden-derived cut-point values are shown in Table 2. P values were determined from linear regression.

evidence of p-Tau pathology (A+T−N−, A+T−N+, p value = .0269) and those with AD-related pathologic change (A+T+N− & A+T+N+, p value = .0490) (Figure 5A). There was a non-significant greater fold-change in p-Tau 181 (T) (Figure 5B) and non-significant lower fold-change in t-Tau (N) (Figure 5C). An approximately threefold change for $A\beta_{40}$ in the AD-related pathologic change group was non-significantly higher (A+T+N− & A+T+N+, p value = .0580) than the A− group (A−T−N+, A−T−N−) (Figure 5D), while an approximately fivefold change for $A\beta_{42}$ was significantly higher (A+T+N− & A+T+N+, p value = .0268) than the A− group (A−T−N−, A−T−N+). The $A\beta_{42/40}$ ratio fold-change was significantly lower for the A− group (A−T−N+, A−T−N−) as compared to those without evidence of p-Tau pathology (A+T−N−, A+T−N+, p value = .0485) and those with AD-related pathologic change (A+T+N− & A+T+N+, p value = .0268) (Figure 5E). Specifically, there were eight DLB patients that were A−T−N− at baseline; two out of these eight converted to A+T−N+ (25%), one converted to A−T−N+ (13%), and five remained the same (63%) after 12 months (Figure 5F). Two DLB patients were A+T−N+ at baseline; one reverted to A−T−N− (50%), one converted to A+T−N− (50%). There were 10 DLB patients that were A+T−N− at baseline; four out of these 10 reverted to A−T−N− (40%), one converted to A−T−N+ (10%), two converted to A+T−N+ (20%), one converted to A+T+N− (10%), and two remained the same (20%) after 12 months. One DLB patient was A+T+N− at baseline and remained the same after 12 months. Six DLB patients were A+T+N+ at baseline; one changed to A−T+N− (17%),

three reverted to A+T+N− (50%), and two remained the same (33%) after 12 months (Figure 5F). No significant changes over time were observed in MoCA and MDS-UPDRS scores for DLB (Supplementary Figure 1). In CN with longitudinal data ($n = 28$), there was a significant difference in CSF $A\beta_{42}$, $A\beta_{42/40}$ ratio, and p-Tau 181 at a follow-up visit (Supplementary Figure 2). In a small group of MCI and AD patients, no significant change was observed over time (Supplementary Figure 3). However, only one CN case changed their ATN status from A+ at baseline to A− at follow-up, and one other case changed from T− to T+ (Supplementary Figure 2-4). For a MCI and AD longitudinal cohort, the sample size was much smaller ($n = 7$), and there was no significant change observed, while two cases changed ATN status over time (Supplementary Figure 3-4). In summary, significant changes were observed within the fold-changes for DLB between A−T− and A+T− for $A\beta_{42}$ and $A\beta_{42/40}$ ratio and between A−T− and A+T+ for $A\beta_{40}$, $A\beta_{42}$, and $A\beta_{42/40}$ ratios.

4.9 | Post mortem neuropathology for eight individuals with DLB

Autopsy data were available for eight individuals with a clinical diagnosis of DLB and CSF biomarker data (Table 3). Three individuals out of this autopsy cohort had normal AD biomarkers (A−T−N−), two of which (cases 1 and 2) had Thal stage 1, Braak stage II and III, and
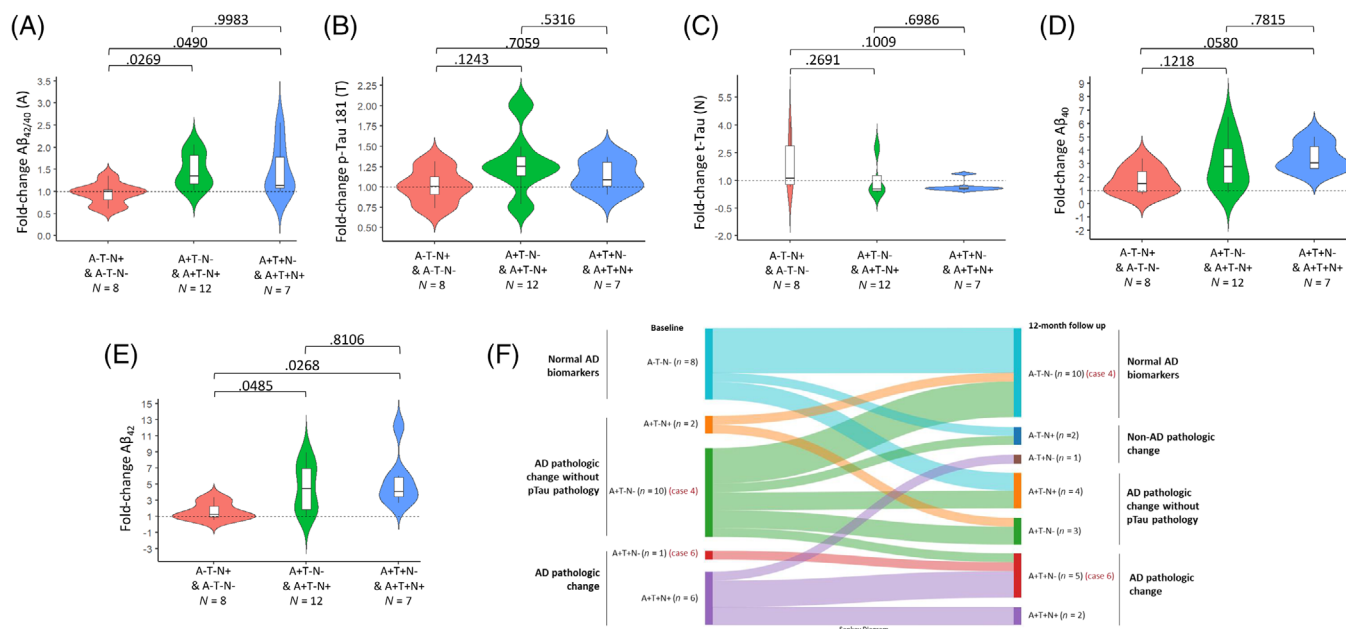
FIGURE 5 DLB AD-related biomarker fold-change between baseline and 12-month follow-up. Fold-change for Aβ42/40 for A$-$T$-$N+ and A$-$T$-$N$-$ was significantly different from A+T$-$N$-$ and A+T$-$N+ (p value = .0269) and from A+T+N$-$ and A+T+N+ (p value = .0490) (A). Ratio fold-change for p-Tau 181 for A+T$-$N$-$ and A+T$-$N+ was not significantly different from either group (B). Ratio fold-change for t-Tau for A$-$T$-$N+ and A$-$T$-$N$-$ was not significantly different from either group (C). Ratio fold-change for Aβ40 for A$-$T$-$N+ and A$-$T$-$N$-$ was not significantly different from either group (D). Ratio fold-change for Aβ42 for A$-$T$-$N+ and A$-$T$-$N$-$ was significantly different from A+T$-$N$-$ and A+T$-$N+ (p value = .0485) and from A+T+N$-$ and A+T+N+ (p value = .0268) (E). Individual ATN status changes after 12 months for some DLB patients where three changed A$-$T$-$N$-$ status, two changed A+T$-$N+, seven changed A+T$-$N$-$ status, and five changed A+T+N+ status. Out of eight cases that were pathologically examined (Table 3), only two had longitudinal data available (Cases 4 and 6). Case 4 was A+T$-$N$-$ at baseline and changed to A$-$T$-$N$-$ at follow-up, and Case 6 was A+T+N$-$ at baseline and remained the same at follow-up (F). These data were without outliers. Fold-change = measure at 12 months/measure at baseline. The dashed line at Y = 1 represents a ratio fold change of 1, which means the value remained the same. P values were determined from one-way ANOVA post hoc Tukey HSD test.

an "absent" CERAD score. Two individuals had AD pathologic change without p-Tau pathology (A+T$-$N$-$, A+T$-$N+). Three individuals had AD-related pathologic change (A+T+N$-$, A+T+N+), two of which had Thal stage 5 and Braak stage IV, with one having frequent CERAD score and the other having a sparse CERAD score. All of these had *post mortem* LBP changes consistent with the clinical diagnosis of DLB, of which four were limbic DLB, three were neocortical DLB, and one was amygdala-prominent. Only two individuals in this subcohort had longitudinal CSF data (cases 4 and 6). Case 4 changed CSF ATN status from A+T$-$N$-$ to A$-$T$-$N$-$, while case 6 did not change ATN status from baseline to 12 months (Figure 5F). In summary, an association between A+ state and amyloid deposition was found.

5 | DISCUSSION

The purpose of this study was to utilize the NIA-AA ATN research framework to characterize pre-mortem AD-related pathology longitudinally in a clinically diagnosed cohort of DLB. We observed lower levels of CSF A$\beta_{42/40}$ and higher levels of p-Tau 181 and t-Tau in AD and DLB, compared to CN (Figure 1), as previously described,[48] suggesting that at least some of the clinically diagnosed DLB cohort harbored significant coexistent AD pathology as described by others.[13-15] To

examine the distribution of AD-related pathologic change, CSF ATN categories were established (Figure 2). These categories were devised from the cut points calculated by the ROC analysis and were 0.1385 for A$\beta_{42/40}$ ratio, 110.08 pg/mL for p-Tau 181, and 445.45 pg/mL for t-Tau as opposed to 0.057 for A$\beta_{42/40}$ ratio,[49] 64.54 pg/ml for p-Tau,[50] and 508 pg/ml for t-Tau[51] in the literature. ATN categories were stratified by AD pathologic change without p-Tau pathology (A+T$-$N$-$, A+T$-$N+), AD pathologic change with p-Tau (A+T+N$-$, A+T+N+), non-AD pathologic change (A$-$T+N$-$, A$-$T$-$N+, A$-$T+N+), and normal AD biomarkers (A$-$T$-$N$-$). Interestingly, the same ATN distribution as in our study was previously reported, 30% A+T$-$ and 26% A+T+.[17] This suggests that not only is there a large proportion of A+ individuals in DLB as previously described,[3,10,17] but many of the A+ individuals do not meet criteria for p-Tau biomarker thresholds. The stratified longitudinal results (Figures 3 and 4) suggest that within the A+T$-$ DLB group, CSF Aβ biomarker positivity is not static and instead may fluctuate. Conversion from one ATN category to another was described previously in a CN elderly population.[52] However, to our knowledge, this has not been described in DLB until now. This may represent an early and plastic state of amyloid deposition in DLB, with diffuse, amyloid-only pathology, as suggested by others.[5] Collectively, the pathophysiological role of A+ or Aβ in DLB remains unclear and could be related to a complex interplay between Aβ and

other pathological proteins, such as interactions between Aβ, tau, and α-synuclein.[4] Further study of how pathological α-synuclein might influence ATN status during DLB progression is needed. In support of some of the longitudinal findings in this study, a recent longitudinal DLB study describes changes in cognitive scores in DLB patients that differ by CSF Aβ positivity.[17] Another study discussed the clinical differences between A−T−, A+T−, A−T+, and A+T+ groups,[3] suggesting that A+ and T+ have different clinical outcomes. Together, this emerging evidence suggests that some DLB patients may have fluctuating AD-related biomarker positivity within a 12-month period, which could have important implications for clinical outcomes, diagnosis, and potential for amyloid therapies in DLB. In addition, an association was observed in this study between APOE ε4 genotype and AD-related Aβ biomarkers in DLB, supported by previous reports of the relationship between CSF Aβ measures and APOE ε4 genotype in AD[53] and DLB.[6]

Through neuropathological assessment, there appeared to be a good relationship between A+ state and amyloid deposition as determined by Thal stage for diffuse plaques (except for case 4), neuritic plaque pathology (ie, CERAD positive), and A+T+ cases. Interestingly, case 4 (A+T−N− at baseline) changed ATN status during life, reverting to A−T−N− after 12 months. Others have also described an absence of neuritic plaques in DLB. A 2019 study reported that about 50% of the DLB patients had absent to sparse CERAD scores, most were neocortical and had Braak stages of I–VI, and about 40% had Thal phases of 0–3.[54] Recently 60% of the DLB patients in China were reported as amyloid PET positive.[55] Amyloid PET imaging of DLB patients indicates lower mean cortical Aβ ligand binding compared to AD.[56,57] Together, this suggests that CSF Aβ positivity in DLB could be a biomarker of a different type of amyloid pathology, likely diffuse, in the context of a normal pTau marker, where the pathobiologic pathway for amyloid pathology in A+T− DLB is somehow different than that observed in A+T+ AD or LBP-AD. It is possible that CSF amyloid is more sensitive to changes in amyloid status over time, particularly while in a pathologically diffuse form. Future longitudinal studies comparing the sensitivity and stability of CSF amyloid and amyloid imaging measurements over time may assist with the interpretation of results with these two measures. It is beyond the scope of this study to address the question of whether there is an association between 12-month change in ATN status and clinical measures or comorbidity to better understand the underlying pathophysiological mechanism or how AD pathologic biomarker indices predict clinical features and prognosis.

A few limitations are notable. Some of the CN individuals were collected as part of multiple studies of aging risk factors, such as APOE ε4. Indeed, the frequency of APOE ε4 was higher than previous reported in the general community[58,59] and therefore could impact the established cut points in our study by defining lower cut points for Aβ and higher cut points for tau. This may bias our findings toward the null hypothesis, suggesting that the differences identified may actually be larger than observed here. The high frequency of APOE ε4 carriers in the CN cohort may skew the CN versus AD comparison to favor fewer

A+. When the APOE ε4+ subjects are removed from the CNs, the cut point for A increases to 0.1415 and is 0.1313 when APOE ε4− subjects are removed. This is in contrast to the value of 0.1385 used for this study. Biomarker values at the borderline of a cut point may behave differently.[60,61] Therefore, it is critical to continue to study ATN in a variety of DLB cohorts to replicate these findings. ATN cut points vary across studies and can be influenced by the heterogeneity of the cohorts from which they are derived and may vary by sex, age, APOE ε4, analytes used (e.g., Aβ42 vs. Aβ42/40), and assay or platform variability across studies.[61–65] This cohort included only participants older than 45 years, and a majority were males and white, which prevented further analyses pertaining to sex or race differences. In the future, a study of more representative participants in terms of age, race, and sex would be important to allow conclusions to be generalized. In addition, the results from the longitudinal DLB cohort should be approached with caution, and replication in future studies is imperative, given the small sample size and limited available neuropathological data. No relationship was observed between time and longitudinal change in MoCA scores and a change in ATN status (data not shown). Future work will benefit from a larger sample size, a control group that better represents the population, and statistical models that take into account the heterogeneity of both the control and AD cohorts from which the cut point is established.[66,67] Furthermore, with more time points, it might be possible to tell whether ATN conversion is an indication of duration of disease. PET imaging biomarkers in AD longitudinal studies have found that amyloid deposition tends to plateau or increase over time in AD.[68,69] In DLB, an initial increase in amyloid deposition has been shown to reach a threshold and later decrease.[70] In addition, others have observed AD-related imaging biomarker reversal from positive to negative in a CN elderly cohort.[11] Together, this supports the idea that AD-related biomarkers may behave differently over time in CN controls or AD, compared to DLB. Unfortunately, our CN and AD cohorts were collected at different and limited time points and were not comparable to DLB here. Another limitation is that only one method was used to measure these AD-related biomarkers. Technical and handling errors can contribute to variability[71–73] and are a concern for the finding that some analyte values revert from positive to negative despite careful quality control. Fully automated methods can decrease technical error and may improve assessment in future studies. As suggested by other studies that observe differences in amyloid in DLB compared to AD,[74] further study of amyloid neuropathological status in DLB is warranted. Lastly, CSF t-Tau was utilized as the biomarker of neurodegeneration, while other biomarkers of neurodegeneration, (N) such as neurofilament light chain or neuroimaging, could offer better utility as N instead of t-Tau.[75]

In conclusion, some A+T− clinically diagnosed DLB patients transition from A+ to negative, while A+T+ DLB (LBP-AD) maintain their positivity. This change in biomarker levels after 12 months suggests a subtype of DLB (A+T−N−, A+T−N+) is distinct from LBP-AD cases (A+T+N, A+T+N). This DLB A+T− group warrants further characterization since monitoring over time may be necessary before considering amyloid-focused therapeutic strategies in DLB patients.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

CONSENT STATEMENT

All human subjects or their caregivers provided informed consent. Author disclosures are available in the supporting information.

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SUPPORTING INFORMATION

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

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