CSF Biomarkers of Alzheimer Disease in Patients With Concomitant α-Synuclein Pathology

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Running Head: CSF markers of AD are altered by concomitant LBD Study Type: Cross-sectional

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Abstract

Background and objectives: Cerebrospinal fluid (CSF) biomarkers amyloid-β42 (Aβ42), phosphorylated tau (p-tau181), total tau (t-tau) and neurogranin (Ng) can diagnose Alzheimer's disease (AD) in life. However, it is unknown if CSF concentrations, and thus their accuracies, are affected by concomitant pathologies common in AD, such as α-synuclein (αSyn). Our primary goal was to test if biomarkers in patients with AD are altered by concomitant αSyn. We compared CSF Aβ42, p-tau181, t-tau and Ng levels across autopsy-confirmed AD and concomitant AD and αSyn (AD+αSyn). Antemortem CSF levels were related to postmortem accumulations of αSyn. Finally, we tested how concommitant AD+αSyn affected diagnostic accuracy of two CSF-based strategies: the ATN framework and the t-tau/Aβ42 ratio.

Methods: Inclusion criteria were neuropathologic diagnoses of AD, mixed AD+αSyn, and αSyn. A convenience sample of non-impaired controls were selected with available CSF and a mini mental state exam (MMSE)≥27. αSyn without AD and controls were included as reference groups. Analyses of covariance (ANCOVAs) tested planned comparisons were CSF Aβ42, p-tau181, t-tau, and Ng differences across AD and AD+αSyn. Linear models tested how biomarkers were altered by αSyn accumulation in AD, accounting for pathologic amyloid-β and tau. Receiver operating characteristic and area under the curve (AUC), including 95% confidence intervals (CI), evaluated diagnostic accuracy.

Results: Participants were 61 AD, 39 mixed AD+αSyn, 20 αSyn, and 61 Controls. AD had similar median age (73 [IQR=12]), MMSE (23 [IQR=9]), and sex distribution (Male=49%) compared to AD+αSyn age (70 [IQR=13]; p=0.3), MMSE (25 [IQR=9.5]; p=0.19), and sex distribution (Male=69%; p=0.077). ANCOVAs showed AD+αSyn had lower p-tau181 ($F(1,94)=17$, p=0), t-tau ($F(1,93)=11$, p=0.0004), and Ng levels ($F(1,50)=12$, p=0.0004) than AD; there was no difference in Aβ42 (p=0.44). Models showed increasing αSyn related to lower p-tau181 (β=-0.26, SE=0.092, p=0.0065), t-tau (β=-0.19, SE=0.092, p=0.041), and Ng levels (β=-0.2, SE=0.066, p=0.0046); αSyn was not a significant factor for Aβ42 (p=1). T-tau/Aβ42 had the highest accuracy when detecting AD, including mixed AD+αSyn cases (AUC=0.95; CI=0.92 to 0.98).

Discussion: Findings demonstrate that concomitant αSyn pathology in AD is associated with lower CSF p-tau181, t-tau, and Ng levels, and can affect diagnositic accuracy in AD patients.

1. Introduction

Cerebrospinal fluid (CSF) signatures of Alzheimer's disease (AD) neuropathologic change (ADNC) include decreased amyloid-β peptide 1-42 ($A\beta_{42}$) related to accumulation of amyloid-β plaques,[1,2] increased tau phosphorylated at threonine 181 ($p\text{-}tau_{181}$) associated with tau neurofibrillary tangles,[2] increased total tau (t-tau) associated with neurofibrillary tangles and neurodegeneration,[3,4] and increased neurogranin (Ng) linked to synaptic degeneration.[5] Ratios, such as $p\text{-}tau_{181}/A\beta_{42}$[6,7] and $t\text{-}tau/A\beta_{42}$,[8,9] also indicate ADNC with high accuracy. Because of their sensitivity to AD neuropathologic processes, these CSF biomarkers can be used in life to stratify AD patients from individuals without AD, including suspected non-AD pathophysiology (SNAP).

However, binary stratification of AD from SNAP can obscure the presence of mixed pathology, common in AD.[10,11] In particular, α-synuclein positive Lewy body disease (LBD) is observed in an estimated 30-50% of AD cases.[10,12] Likewise, roughly 50% of LBD patients show significant ADNC.[3,13] Thus discrimination of AD from SNAP, a designation that includes LBD, becomes muddled when AD and SNAP are concomitant. The 2018 ATN framework[4] could potentially fill this gap. By combining amyloid-β (A), tau (T), and neurodegeneration (N) statuses using CSF $A\beta_{42}$, $p\text{-}tau_{181}$ and t-tau respectively, ATN provides a diagnosis along a continuum of AD: Normal (A-T-N-), early Alzheimer's pathologic change (A+T-N-), AD (A+T+N±), concomitant Alzheimer's pathologic change and SNAP (A+T-N+), and SNAP (A-T±N±). While more detailed than binary classification by CSF ratios, it is unclear if the ATN continuum is more accurate when diagnosing AD, or if it successfully detects SNAP.[14]

It is unknown how concomitant LBD affects CSF, an issue for any CSF-based strategy. Postmortem work suggests that CSF $A\beta_{42}$ in LBD correlates with α-synuclein, independent

of amyloid-β plaque burden.[8] Early Parkinson's disease (PD) patients have lower CSF p-tau$_{181}$, t-tau, and Ng than healthy controls,[15,16] and longitudinal CSF p-tau$_{181}$ and t-tau levels may decline in first three years in PD.[15] Thus, α-synuclein may influence CSF biomarkers in a manner distinct from ADNC, thereby affecting diagnostic accuracy and interpretation when AD and LBD are mixed. Since there are currently no biomarkers that can positively identify α-synuclein pathology, autopsy work is needed to determine if diagnostic accuracy is affected when AD and LBD pathologies cooccur.

To address this gap, this study compares CSF Aβ$_{42}$, p-tau$_{181}$, t-tau, and Ng levels in autopsy-confirmed AD patients and patients with mixed AD and LBD (AD+LBD); we include LBD without AD and non-impaired controls as reference groups. We first test if CSF biomarkers differ across AD and AD+LBD. Models also test if CSF levels are affected by the other common age-associated pathology in our sample, transactive response DNA-binding protein of 43 kDa (TDP-43). We next test the direct association of CSF with increasing postmortem α-synuclein burden in AD brain tissue. Finally, we evaluate the diagnostic accuracy of ATN and t-tau/Aβ$_{42}$ ratio, and test which strategy best detects ADNC in a mixed pathology sample. We also test biomarkers sensitive to LBD, including Ng.

2. Methods

2.1 Patient Criteria

Patients were autopsied at the University of Pennsylvania (Penn) Center for Neurodegenerative Disease Research (CNDR), and were retrospectively selected from the Penn Integrated Neurodegenerative Disease Biobank and Database (INDD).[17,18] Inclusion criteria were a primary pathological diagnosis of either LBD (n=32) or AD (n=88) and a clinical phenotype of either amnestic AD (n=86) or LBD spectrum, including Parkinson's

disease (PD; n=3), PD with dementia (n=16), or dementia with Lewy bodies (DLB; n=15). Primary pathologic diagnosis was performed by expert neuropathologists (EBL, JQT) using neuropathological criteria.[19,20] All patients were assessed for CSF $A\beta_{42}$, p-tau_{181}, and t-tau using the xMAP Luminex platform.[21] A subset of patients (32 AD, 24 AD+LBD, 14 and LBD patients) were assessed for Ng using an in-house enzyme-linked immunosorbent assay (ELISA), previously described.[5] Exclusion criteria were a neuropathologic diagnosis of frontotemporal lobar degeneration (FTLD)[22] or cerebrovascular disease.[19] One outlier for CSF t-tau was excluded for levels of 929 pg/mL (>9 standard deviations). A subset of this data (n=22) was included in a previous publication focused on LBD.[8] Non-impaired healthy controls (n=66) with a mini-mental state exam (MMSE)[23] of 28 or above and available CSF $A\beta_{42}$, p-tau_{181}, and t-tau were included as a comparison group; a subset of these controls had CSF Ng (n=14).

Demographics were recorded as age of onset (earliest reported symptom), age at CSF collection, interval from CSF-to-death, age at death, disease duration at CSF (time from symptom onset to CSF), global cognition (MMSE), and sex. Two participants in our sample self-reported as Black/African American, both AD+LBD; the rest self-reported as White. Consent was obtained according to the Declaration of Helsinki and approved by the Penn Institutional Review Board.

2.2 Neuropathological Assessment and Patient Groupings

Tissue samples were processed as previously described, with immunohistochemical staining for phosphorylated tau, amyloid-β, TDP-43, and α-synuclein using well-characterized antibodies.[18,24] Of the total sample, 100 patients met criteria for intermediate/high AD neuropathologic change (ADNC).[19] LBD was determined by α-synuclein positive Lewy bodies in the brainstem, limbic, or neocortical regions.[25] Of the

100 ADNC patients, 39 had both AD and LBD neuropathologic diagnoses (AD+LBD); because several patients had high levels of both pathologies, this grouping made no distinction between primary AD or primary LBD. "AD" patients had no or scant α-synuclein pathology (*i.e.* amygdala-only α-synuclein; n=61).[26] "LBD" patients had not/low ADNC (n=20). In addition to assessments for AD and LBD neuropathologic alterations,[18] 44 patients had co-occurring TDP-43 proteinopathy consistent with limbic-predominate age-related TDP-43 encephalopathy (LATE)[27] with or without hippocampal sclerosis (HS).[28] Group comparisons thus accounted for the presence of TDP-43.

2.2.1 Pathologic Burden

Pathologic burden for amyloid-β, tau, and α-synuclein was assessed prospectively in neocortical, limbic, and brainstem regions standardly sampled at autopsy according to criteria:[19] middle frontal, angular, superior/middle temporal, occipital, amygdala, cingulate, CA1/subiculum, entorhinal, pons and medulla regions. Sampling was randomized between left and right hemispheres. Each region was scored for pathological severity using semi-quantitative 5-point scale (*i.e.* 0=none, 0.5=rare, 1=low, 2=intermediate, 3=high). An average burden score for amyloid-β, tau, and α-synuclein pathology was calculated across all regions.[8]

2.3 Statistical Analyses

Shapiro-Wilks tests indicated non-normal distribution of demographic and CSF variables. Kruskal-Wallis tests performed group-wise comparisons for continuous variables. Mann–Whitney–Wilcoxon tests performed planned comparisons between AD and AD+LBD. Chi-square tests compared categorical variables. To perform non-parametric comparisons for analyses of covariance (ANCOVAs) and linear models, all continuous variables were rank-

transformed.[29] Permutation testing calculated *p*-values based on unique sum of squares (20,000 iterations). Statistical tests were performed with a significance threshold of α=0.05. ANCOVAs using type II sum of squares tested whether CSF concentrations differed between AD and AD+LBD patients; covariates included age at CSF, CSF-to-death interval, sex, and TDP-43 copathology. Effect size for ANCOVAs (partial η^2) was calculated using 20,000 iterations (>0.01 is considered small, >0.09 medium, and >0.25 large). To ensure CSF differences were not due to phenotype,[14] we repeated ANCOVAs in the subset of ADNC with an amnestic phenotype.

Linear models tested CSF levels as a function of α-synuclein burden, while accounting for amyloid-β and tau burden; CSF-to-death and sex were also included as covariates. Amyloid-β and tau accumulation were collinear, and therefore amyloid-β was not included in final models. To ensure that observed effects of α-synuclein on CSF were not due to lower AD pathology, models were repeated in a subset of ADNC patients with high ADNC, high amyloid-β (burden>2) and high tau (burden>2).

ROC analyses using bootstrapping (500 iterations) tested diagnostic accuracy of each analyte ($A\beta_{42}$, p-tau, t-tau, and Ng) and two ratios (p-$tau_{181}/A\beta_{42}$ and t-$tau/A\beta_{42}$) when discriminating ADNC patients (AD, AD+LBD) from LBD. AUC and 95% confidence interval (CI) were reported; Youden's index determined best threshold that maximized sensitivity and specificity. We tested classification of AD, AD+LBD, LBD and Controls using two diagnostic strategies: ATN and t-$tau/A\beta_{42}$. To not bias results to favor a strategy, thresholds were specific to this sample. Finally, ROC analyses tested diagnostic accuracy of each analyte and ratios when discriminating LBD positive patients (LBD, AD+LBD) from AD without LBD.

Analyses were conducted using R statistical software, using Companion to Applied Regression (car),[30] effectsize,[31] lmPerm,[32] and cutpointr[33] packages.

3. Results

3.1 Demographic Characteristics

Table 1 compares demographic characteristics across groups. Wilcoxon tests performed planned pairwise comparisons across AD and AD+LBD patients. AD and AD+LBD showed no difference for onset age (p=0.29), age at CSF collection (p=0.3), age at death (p=0.48), CSF-to-death interval (p=0.36), or MMSE scores (p=0.19). Chi-square tests showed no differences in sex ($\chi^2(1)=3.1$, p=0.077), APOE ε4 alleles (p=0.73), or presence of TDP-43 (p=0.73) across AD and AD+LBD.

	Control	AD	AD+LBD	LBD	p
n	61	61	39	20	
Age at Onset (years)	--	69.0 [59.8, 74.0]	65.0 [60.0, 72.0]	58.5 [54.8, 64.5]	0.002
Age at CSF (years)	67.0 [62.0, 71.0]	73.0 [65.0, 77.0]	70.0 [63.0, 76.0]	69.0 [64.8, 77.2]	0.060
CSF to Death (years)	7.5 [6.0, 11.2]	6.0 [5.0, 8.0]	7.0 [4.5, 9.0]	6.0 [2.0, 7.2]	0.118
Age at Death (years)	82.0 [77.0, 84.8]	79.0 [70.0, 85.0]	78.0 [68.5, 83.0]	76.5 [70.8, 80.2]	0.474
MMSE (max = 30)	30.0 [29.0, 30.0]	23.0 [17.0, 26.0]	25.0 [17.5, 27.0]	27.0 [25.0, 29.0]	<0.001
Sex = Male (%)	18 (29.5)	30 (49.2)	27 (69.2)	17 (85.0)	<0.001
TDP43+ (%)	0 (0.0)	22 (36.1)	18 (46.2)	4 (20.0)	0.212
APOE ε4 (%)					<0.001
0 alleles	43 (74.1)	16 (26.7)	9 (23.7)	13 (65.0)	
1 allele	14 (24.1)	30 (50.0)	22 (57.9)	7 (35.0)	
2 alleles	1 (1.7)	14 (23.3)	7 (18.4)	0 (0.0)	
Phenotype (%)					<0.001
Control	61 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	
AD	0 (0.0)	61 (100.0)	25 (64.1)	0 (0.0)	
DLB	0 (0.0)	0 (0.0)	9 (23.1)	6 (30.0)	
PD	0 (0.0)	0 (0.0)	0 (0.0)	3 (15.0)	
PDD	0 (0.0)	0 (0.0)	5 (12.8)	11 (55.0)	

Table 1: Patient characteristics. Demographic and pathological characteristics of patients and unimpaired controls. For continuous variables, descriptive statistics median and interquartile range (median [IQR]) are provided; Kruskal-Wallis tests performed group comparisons. For categorical variables, count (percentage [%]) are provided; chi-square tests performed frequency comparisons. *p*-values are reported for group comparisons.

3.2 CSF comparisons

Figure 1 illustrates CSF differences across groups. As expected, biomarkers reflected that LBD patients had clinically insignificant/absent AD pathology, with higher CSF $A\beta_{42}$, and lower CSF p-tau_{181} and t-tau than AD and AD+LBD; Ng was also lower in LBD than AD,

but levels were similar between LBD and AD+LBD. Controls had higher $A\beta_{42}$, and lower p-tau, t-tau, and Ng than AD; Controls had higher $A\beta_{42}$, and lower p-tau and t-tau than AD+LBD; Controls had higher $A\beta_{42}$, t-tau, and Ng than LBD.

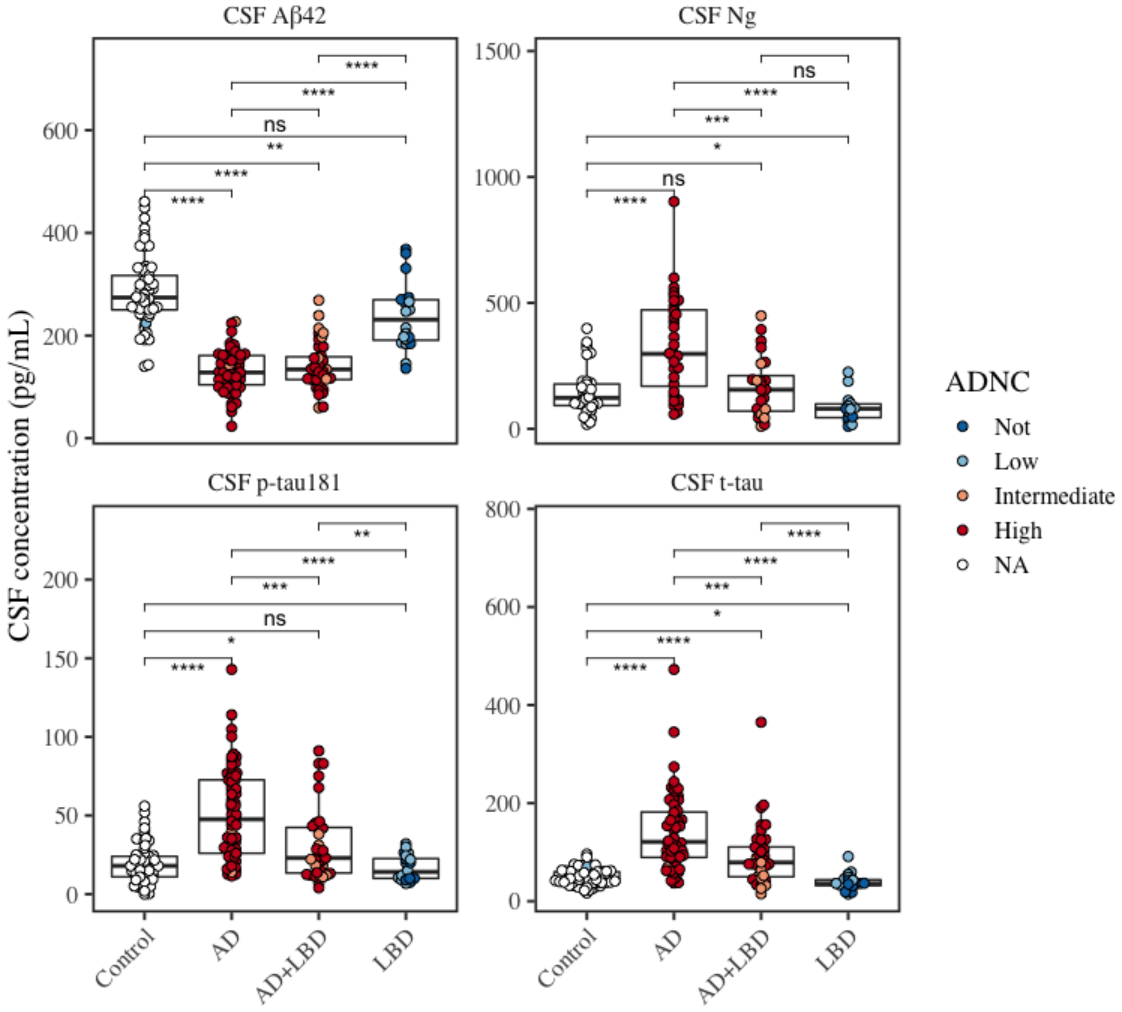

Figure 1. Comparisons of CSF concentrations. CSF levels of $A\beta_{42}$, p-tau_{181}, t-tau, and Ng across AD, AD+LBD, LBD, and Controls. Color indicates ADNC from not (dark blue) to high (dark red), or not assessed (white). Asterisks represent p-values from Wilcoxon pairwise comparisons (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, or not significant [ns]). Boxplots show median, interquartile range (IQR), and outliers.

ANCOVAs tested CSF differences across AD and AD+LBD, covarying for age, CSF-to-death interval, sex, and TDP-43 pathology. There was no difference in CSF $A\beta_{42}$ across

AD and AD+LBD (p=0.44), nor any other covariate (all p>0.1). CSF p-tau$_{181}$ was significantly lower for AD+LBD than AD (F(1,94)=17, p<2.0e^{-16}) with a medium effect size (η^2=0.152); CSF-to-death interval (F(1,94)=3.5, p=0.072) and all other factors were non-significant (all p>0.1). CSF t-tau was lower in AD+LBD compared with AD (F(1,93)=11, p=0.0004) with a medium effect size (η^2=0.106) and was lower in men than women (F(1,93)=9.2, p=0.0022; η^2=0.09); no other factors were significant (all p>0.1). CSF Ng was lower in AD+LBD than AD (F(1,50)=12, p=0.0004) with a medium effect size (η^2=0.19) and in men compared to women (F(1,50)=12, p=0.0014; η^2=0.197); all other factors were non-significant (all p>0.1).

We tested if observed differences could be in part due to lower ADNC in AD+LBD than AD patients. Wilcoxon test comparisons showed that AD+LBD patients indeed had lower amyloid-β (median=2.3; IQR=0.53) than AD (median=2.4; IQR=0.6; W=1568, p=0.0073). AD+LBD also had lower tau burden (median=2.1; IQR=0.95) than AD (median=2.5; IQR=0.35; W=1640, p=0.0015). In accordance with their pathological diagnosis, AD+LBD had greater α-synuclein burden (median=1.4; IQR=1.4) than AD (median=0; IQR=0.15; W=89, p=4.4e^{-15}). Thus, subsequent linear models accounted for differences in burden of amyloid-β and tau across ADNC. We also repeated models in ADNC patients with only high levels of amyloid-β and tau pathology.

3.2.1 CSF comparisons in amnestic ADNC

Because AD+LBD consisted of patients with heterogeneous phenotypes (Table 1), we repeated ANCOVAs within ADNC patients with an amnestic phenotype (Supplementary Section 5.1). Results were consistent; amnestic AD+LBD had significantly lower p-tau (p=0.0077), t-tau (p=0.046), and Ng (p=0.029) than amnestic AD; there was no difference between amnestic AD+LBD and AD in CSF Aβ_{42} (p=0.56). Moreover in amnestic ADNC,

we saw no differences in amyloid-β (p=0.17) or tau burden (p=0.2) according to Wilcoxon tests; as expected α-synuclein was higher in amnestic AD+LBD than AD (W=89, p=1.7e^{-11}).

3.3 CSF associations with α-synuclein burden in AD and AD+LBD

Linear models tested CSF p-tau$_{181}$, t-tau, and Ng as dependent variables of postmortem α-synuclein within ADNC patients (AD, AD+LBD) while accounting for differences in tau burden (Figure 2); CSF-to-death interval and sex were also included as covariates. CSF p-tau$_{181}$ was negatively associated with α-synuclein (β=-0.26, SE=0.092, p=0.0065) and positively associated with pathological tau (β=0.31, SE=0.093, p=0.0016); neither CSF-to-death (p=0.18) nor sex (p=0.21) were significant. For CSF t-tau, levels were negatively associated with α-synuclein (β=-0.19, SE=0.092, p=0.041), positively associated with tau burden (β=0.32, SE=0.092, p=0.00045), lower in men than women (β=-14, SE=5.3, p=0.0066), and had no association with CSF-to-death (p=0.86). For CSF Ng, levels were negatively associated with α-synuclein (β=-0.21, SE=0.071, p=0.0044) and were lower in men than women (β=-14, SE=3.7, p=0.0008); there was no association with tau burden (p=0.74) or with CSF-to-death (p=0.16).

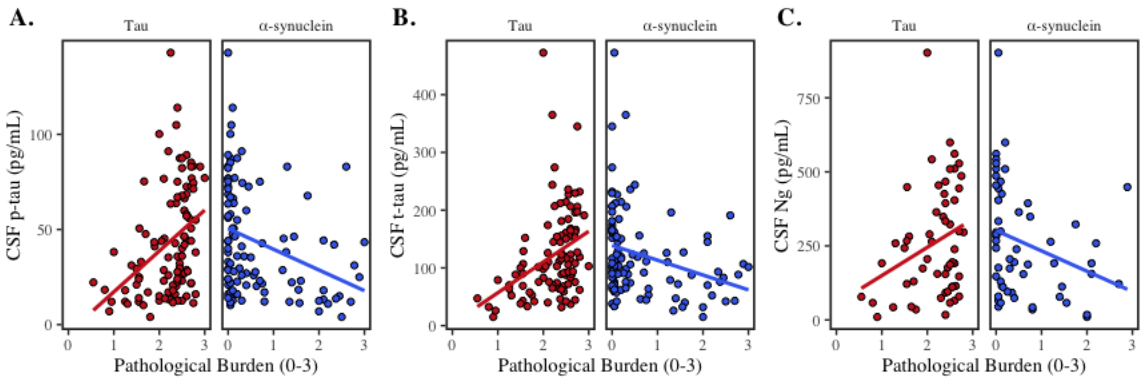

Figure 2. CSF and pathology burden in ADNC patients (AD, AD+LBD). CSF p-tau$_{181}$ (Panel A) and Ng (Panel B) levels relate to pathological tau (red) or amyloid-β (red) and α-synuclein burden (blue).

3.3.1 CSF associations with α-synuclein burden in high ADNC

To confirm CSF associations with α-synuclein even in high ADNC, linear models were repeated within the subset of patients with high ADNC, high amyloid-β (burden>2), and high tau (burden>2; Supplementary Section 5.2). Results were consistent; both CSF p-tau$_{181}$ (p=0.031) and Ng (p=0.0036) significantly declined with increasing accumulation of α-synuclein; t-tau was not significantly associated with α-synuclein burden in high ADNC (p=0.084).

3.4 Comparing diagnostic schemes

ROC analyses tested CSF biomarkers and established ratios when detecting ADNC in a mixed pathologic sample (Table 2). The t-tau/Aβ$_{42}$ ratio had the highest AUC when discriminating ADNC patients (AD and AD+LBD) from LBD; CSF p-tau had the worst performance.

Measure	AUC	AUC 5%CI	AUC 95%CI	Threshold	Sensitivity	Specificity
t-tau/Aβ42	0.95	0.91	0.98	0.32	0.84	0.95
p-tau/Aβ42	0.92	0.88	0.97	0.13	0.78	0.90
Aβ42	0.91	0.85	0.96	180.79	0.86	0.90
t-tau	0.90	0.85	0.95	59.05	0.80	0.86
Ng	0.82	0.73	0.90	127.76	0.70	0.86
p-tau	0.81	0.74	0.88	25.75	0.64	0.81

Table 2: Receiver Operating Characteristic (ROC) Analyses to detect ADNC. ROC metrics are calculated using bootstrapping with 500 iterations. CSF measures are listed in descending order of area under the curve (AUC) when discriminating ADNC (AD, AD+LBD) from non-AD (LBD) patients. Best threshold was calculated (Youden's index) for this sample, and sensitivity and specificity at that threshold are reported, and the 5%-95% CI for AUC.

Figure 3 evaluates two established diagnostic strategies using sample-specific thresholds (Table 2): the ATN framework and the t-tau/$A\beta_{42}$ ratio. While less detailed, t-tau/$A\beta_{42}$ made fewer obvious errors than ATN. Supplementary Section 5.3 discusses these classifications and misclassifications in pathologic detail.

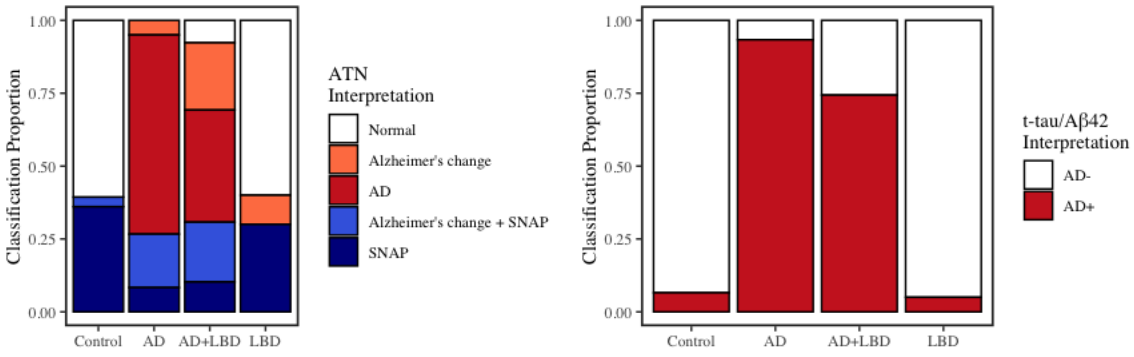

Figure 3. ATN and t-tau/$A\beta_{42}$ Classifications. Barplot of how each strategy classifies Controls, AD, AD+LBD, and LBD patients. ATN Classifications (Left Panel): Normal (A-T-N-), early Alzheimer's pathologic change (A+T-N-), AD (A+T+N±), concomitant Alzheimer's pathologic change and SNAP (A+T-N+), and SNAP (A-T±N±). t-tau/$A\beta_{42}$ Classifications (Right Panel): AD+ (≥0.32) and AD- (<0.32).

Under ATN, 12% of AD/AD+LBD patients were misclassified as “Normal” or “SNAP”; 12% of AD/AD+LBD patients were classified as “Alzheimer’s pathologic change” (A+T-N-) despite significant tau pathology at autopsy; 60% of LBD patients were misclassified as “Normal”; 39% of controls were classified as SNAP or Alzheimer’s and SNAP.

Under t-tau/$A\beta_{42}$, 14% of AD/AD+LBD patients were misclassified as negative for AD and 5% of LBD patients were misclassified as positive for AD. 7% of controls were classified as AD positive.

3.4.1 Posthoc analyses detecting mixed AD and LBD pathology with Ng

Follow-up ROC analyses investigated whether CSF analytes could detect LBD pathology in a mixed cohort (Supplementary Section 5.3.1). When discriminating LBD positive patients (LBD, AD+LBD) from LBD negative (AD), CSF Ng had the best accuracy (AUC=0.82; CI=0.74 to 0.9; Threshold=195.5).

4. Discussion

Increased CSF p-tau_{181}, t-tau, and Ng levels, as well as decreased CSF $A\beta_{42}$, are all established biomarkers of AD pathological processes.[4,34,35] In this study we show evidence that concomitant α-synuclein, common in AD,[10,12] is associated with lower CSF p-tau_{181} and Ng, even after accounting for amyloid-β and tau burden. Models indicated that CSF $A\beta_{42}$ levels were largely invariant to α-synuclein pathology. While CSF t-tau was negatively associated with α-synuclein and was lower in AD+LBD than AD, results were not robust in patients with high ADNC. High t-tau/$A\beta_{42}$ had the best performance (AUC=0.95) when determining AD positivity across all patients.

Our finding that α-synuclein was negatively associated with CSF p-tau_{181}, t-tau, and Ng has important implications for diagnostic strategy. Lower CSF levels may be characteristic

of LBD pathology.[5,16] Evidence shows that CSF $A\beta_{42}$, p-tau_{181}, and t-tau are lower in Parkinson's disease patients than healthy controls at baseline.[15] Likewise, we found that LBD patients had lower $A\beta_{42}$, t-tau and Ng than healthy controls. Lower CSF p-tau_{181} associated with α-synuclein has important implications for the ATN framework, which is increasingly applied in the AD field and has been recently applied to DLB [36]. Under ATN, p-tau_{181} is the established CSF biomarker of T status, and a patient does not meet the definition of AD unless they are both A+ and T+. Even using sample-specific thresholds to define ATN status, many AD+LBD and the majority of LBD patients were misclassified. Indeed, 22 (56%) of AD+LBD patients were classified as T- despite significant tau pathology at autopsy. The t-tau/$A\beta_{42}$ ratio had previously been identified by our group as the best metric in LBD spectrum patients to identify concomitant AD,[8] and it also had the best accuracy in this study. We found $A\beta_{42}$ was invariant to α-synuclein pathology, and in high ADNC, t-tau was not significantly associated with α-synuclein. This study provides a mechanism for why the t-tau/$A\beta_{42}$ ratio may best identify AD; it combines the two markers which may be less influenced by concomitant α-synuclein and thus may more reliably reflect ADNC.

We did not identify a diagnostic strategy that could reliably detect primary or mixed LBD in life. While ATN has a designation for concomitant pathologies, a similar percentage of AD+LBD and AD were classified as concomitant Alzheimer's and SNAP (21% *vs.* 18%) indicating that this designation does not reliably detect LBD using the current biomarkers. ATN also classified a minority of LBD patients as SNAP (30%). These results highlight the need for accurate biomarkers of SNAP, including LBD, which might greatly improve diagnostic accuracy and precision.[37,38] ROC results using Ng to identify LBD were

modest, but indicated that low Ng in AD might indicate mixed LBD (AUC=0.82). Ng is a post-synaptic protein, and while both AD and LBD are associated with synaptic dysfunction, their synaptic CSF profiles may differ.[39] Previous work has shown elevated levels of CSF Ng specific to AD patients,[5,40] while studies on patients with Parkinsonian disorders found decreased CSF Ng,[16] hypothetically due to reduced synaptic activity.[41] Special preparations of human brain tissue highlight a high burden of α-synuclein pathology at synapses.[42] Future, longitudinal work and the future development of *in vivo* α-synuclein markers might disentangle how CSF Ng levels change with accumulating AD and LBD pathology.

There are important caveats to consider when interpreting results. First, we focused on end-stage disease and models accounted for the time-interval from CSF to death, but it is possible that pathological findings at autopsy do not fully represent biological state at CSF collection. Still, we note that patients were symptomatic at CSF collection and we'd thus expect significant pathological accumulation.[43] Moreover, CSF p-tau and t-tau levels are relatively stable after dementia onset.[44] Second, CSF levels may also differ by race[45] and non-whites with AD may be more likely to have concomitant LBD.[12] However, with limited diversity in our sample we were not able to explore how CSF and pathology differed by race. Third, while we found no evidence that TDP-43 influenced CSF levels, there are different subtypes of TDP-43,[22,46] and aggregation across multiple subtypes might obscure underlying differences. Fourth, to reduce bias that might favor one diagnostic strategy, we used sample-specific thresholds to compare ATN and t-tau/$A\beta_{42}$ accuracy. Therefore, diagnostic performance must still be validated in an independent sample.

Finally, while this work used a well-established immuno-assays,[21] future work should explore these associations in newer, high-precision, second generation immunoassays.

In summary, this study finds α-synuclein accumulation is associated with lower CSF p-tau$_{181}$ and Ng in AD, and that this can inform biomarker interpretation to improve accuracy in stratifying patients with AD from SNAP, including LBD.

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Potential Conflicts of Interest

Competing interests: HZ has served at scientific advisory boards and/or as a consultant for Abbvie, Alector, Eisai, Denali, Roche Diagnostics, Wave, Samumed, Siemens Healthineers, Pinteon Therapeutics, Nervgen, AZTherapies, CogRx, and Red Abbey Labs, has given lectures in symposia sponsored by Cellectricon, Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work). KB has served as a consultant, at advisory boards, or at data monitoring committees for Abcam, Axon, Biogen, JOMDD/Shimadzu. Julius Clinical, Lilly, MagQu, Novartis, Prothena,

Roche Diagnostics, and Siemens Healthineers, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, all unrelated to the work presented in this manuscript. All other authors report no conflicts of interest relevant to this study.

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Figure Legends

Figure 1. Comparisons of CSF concentrations. CSF levels of $A\beta_{42}$, p-tau_{181}, t-tau, and Ng across AD, AD+LBD, LBD, and Controls. Color indicates ADNC from not (dark blue) to high (dark red), or not assessed (white). Asterisks represent p-values from Wilcoxon pairwise comparisons (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, or not significant [ns]). Boxplots show median, interquartile range (IQR), and outliers.

Figure 2. CSF and pathology burden in ADNC patients (AD, AD+LBD). CSF p-tau_{181} (Panel A) and Ng (Panel B) levels relate to pathological tau (red) or amyoloid-β (red) and α-synuclein burden (blue).

Figure 3. ATN and t-tau/$A\beta_{42}$ Classifications. Barplot of how each strategy classifies Controls, AD, AD+LBD, and LBD patients. ATN Classifications (Left Panel): Normal (A-T-N-), early Alzheimer's pathologic change (A+T-N-), AD (A+T+N±), concomitant Alzheimer's pathologic change and SNAP (A+T-N+), and SNAP (A-T±N±). t-tau/$A\beta_{42}$ Classifications (Right Panel): AD+ (≥0.32) and AD- (<0.32).

Tables

	Control	AD	AD+LBD	LBD	p
n	61	61	39	20	
Age at Onset (years)	NA [NA, NA]	69.0 [59.8, 74.0]	65.0 [60.0, 72.0]	58.5 [54.8, 64.5]	0.002
Age at CSF (years)	67.0 [62.0, 71.0]	73.0 [65.0, 77.0]	70.0 [63.0, 76.0]	69.0 [64.8, 77.2]	0.060
CSF to Death (years)	7.5 [6.0, 11.2]	6.0 [5.0, 8.0]	7.0 [4.5, 9.0]	6.0 [2.0, 7.2]	0.118
Age at Death (years)	82.0 [77.0, 84.8]	79.0 [70.0, 85.0]	78.0 [68.5, 83.0]	76.5 [70.8, 80.2]	0.474
MMSE (max = 30)	30.0 [29.0, 30.0]	23.0 [17.0, 26.0]	25.0 [17.5, 27.0]	27.0 [25.0, 29.0]	<0.001
Sex = Male (%)	18 (29.5)	30 (49.2)	27 (69.2)	17 (85.0)	<0.001
TDP43+ (%)	0 (0.0)	22 (36.1)	18 (46.2)	4 (20.0)	0.212
APOE ε4 (%)					<0.001
0 alleles	43 (74.1)	16 (26.7)	9 (23.7)	13 (65.0)	
1 allele	14 (24.1)	30 (50.0)	22 (57.9)	7 (35.0)	
2 alleles	1 (1.7)	14 (23.3)	7 (18.4)	0 (0.0)	
Phenotype (%)					<0.001
Control	61 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	
AD	0 (0.0)	61 (100.0)	25 (64.1)	0 (0.0)	
DLB	0 (0.0)	0 (0.0)	9 (23.1)	6 (30.0)	
PD	0 (0.0)	0 (0.0)	0 (0.0)	3 (15.0)	
PDD	0 (0.0)	0 (0.0)	5 (12.8)	11 (55.0)	

Table 1: Patient characteristics. Demographic and pathological characteristics of patients and unimpaired controls. For continuous variables, descriptive statistics median and interquartile range (median [IQR]) are provided; Kruskal-Wallis tests performed group comparisons. For categorical variables, count (percentage [%]) are provided; chi-square tests performed frequency comparisons. *p*-values are reported for group comparisons.

Measure	AUC	AUC 5%CI	AUC 95%CI	Threshold	Sensitivity	Specificity
t-tau/Aβ42	0.95	0.91	0.98	0.32	0.84	0.95
p-tau/Aβ42	0.92	0.88	0.97	0.13	0.78	0.90
Aβ42	0.91	0.85	0.96	180.79	0.86	0.90
t-tau	0.90	0.85	0.95	59.05	0.80	0.86
Ng	0.82	0.73	0.90	127.76	0.70	0.86
p-tau	0.81	0.74	0.88	25.75	0.64	0.81

Table 2: Receiver Operating Characteristic (ROC) Analyses to detect ADNC. ROC metrics are calculated using bootstrapping with 500 iterations. CSF measures are listed in descending order of area under the curve (AUC) when discriminating ADNC (AD, AD+LBD) from non-AD (LBD) patients. Best threshold was calculated (Youden's index) for this sample, and sensitivity and specificity at that threshold are reported, and the 5%-95% CI for AUC.

5. Supplemental Material

5.1 Comparisons in amnestic AD and AD+LBD

There were no demographic differences across AD and AD+LBD patients with an amnestic phenotype (Supplemental Table 1).

	AD	AD+LBD	p
n	61	27	
Age at Onset (years)	69.0 [59.8, 74.0]	65.0 [57.5, 72.5]	0.419
Age at CSF (years)	73.0 [65.0, 77.0]	69.0 [61.0, 76.0]	0.389
CSF to Death (years)	6.0 [5.0, 8.0]	8.0 [5.0, 10.5]	0.089
Age at Death (years)	79.0 [70.0, 85.0]	78.0 [67.0, 83.0]	0.690
MMSE (max = 30)	23.0 [17.0, 26.0]	25.0 [16.0, 27.0]	0.368
Sex = Male (%)	30 (49.2)	16 (59.3)	0.521
TDP43+ (%)	22 (36.1)	13 (48.1)	0.405
APOE ε4 (%)			0.987
0 alleles	16 (26.7)	7 (25.9)	
1 allele	30 (50.0)	14 (51.9)	
2 alleles	14 (23.3)	6 (22.2)	

Supplementary Table 1: Characteristics of amnestic ADNC patients. Demographic and pathological characteristics of amnestic AD and AD+LBD patients. For continuous variables, descriptive statistics median and interquartile range (median [IQR]) are provided; Kruskal-Wallis tests performed group comparisons. For categorical variables, count (percentage [%]) are provided; chi-square tests performed frequency comparisons. *p*-values are reported for group comparisons.

In amnestic ADNC patients, ANCOVAs investigated if CSF levels were influenced by concomitant LBD, covarying for age at CSF, CSF-to-death interval, sex, and TDP-43 pathology. There was no difference in CSF $A\beta_{42}$ across AD and AD+LBD (p=0.56), nor in any other covariate (all p>0.1). CSF p-tau_{181} was significantly lower for AD+LBD than AD ($F(1,82)=6.9$, p=0.0077); age at CSF (p=0.085), CSF-to-death interval (p=0.12), sex

(p=0.2), and TDP-43 pathology (p=0.27) were all not significant. CSF t-tau levels was significantly lower in amnestic AD+LBD than AD (F(1,81)=3.8, p=0.046) and lower in men compared to women (F(1,81)=5.3, p=0.027); age at CSF (p=0.65), CSF-to-death (p=0.57), and TDP-43 pathology (p=0.37) were not significant factors for t-tau levels. CSF Ng was significantly lower for AD+LBD than AD (F(1,41)=5, p=0.029). Men also had lower Ng levels compared to women (F(1,41)=8.4, p=0.0074); age at CSF (p=0.51), CSF-to-death (p=0.13), and TDP-43 pathology (p=0.71) were not significant factors for Ng levels.

5.2 CSF associations with α-synuclein burden in high ADNC +/- LBD

To confirm the association of postmortem α-synuclein pathologic burden with antemortem CSF p-tau$_{181}$ and Ng levels, linear models were repeated in the subset of patients with high levels of ADNC, high amyloid-β burden (amyloid-β > 2) and high tau burden (tau > 2). Results confirmed that CSF p-tau$_{181}$ levels were negatively associated with α-synuclein burden (Supplementary Table 2A), while adjusting for interval from CSF-to-death and sex. Likewise, CSF Ng levels were negatively associated with pathological α-synuclein (Supplementary Table 2C), while adjusting for interval from CSF-to-death and sex. CSF t-tau was not significantly associated with pathological α-synuclein (Supplementary Table 2B).

A. p-tau181	β	SE	t value	p
Tau Burden	0.13	0.12	1.13	0.2635
α-syn Burden	-0.26	0.12	-2.22	0.0293
CSF to Death	0.30	0.11	2.68	0.0090
Sex (Male)	-9.33	4.14	-2.25	0.0309

B. t-tau	β	SE	t value	p
Tau Burden	0.03	0.13	0.27	0.7732
α-syn Burden	-0.22	0.13	-1.71	0.0860
CSF to Death	0.11	0.12	0.87	0.3794
Sex (Male)	-9.00	4.53	-1.98	0.0511

C. Ng	β	SE	t value	p
Tau Burden	-0.09	0.10	-0.87	0.5014
α-syn Burden	-0.30	0.09	-3.21	0.0054
CSF to Death	0.15	0.09	1.74	0.0654
Sex (Male)	-6.54	3.03	-2.16	0.0440

Supplementary Table 2: Linear models in High ADNC patients (AD, AD+LBD). Effect of global tau and α-synuclein on CSF p-tau$_{181}$ (Panel A), CSF t-tau (Panel B) and CSF Ng levels (Panel C). Patients have high ADNC, tau burden > 2, and amyloid-β burden > 2. All models include CSF-to-death and sex as covariates. *p*-values are based on permutation testing with 20,000 iterations.

5.3 Comparing diagnostic schemes

Supplementary Table 3 compares classifications of all groups using the ATN framework and the t-tau/Aβ$_{42}$ ratio.

For ATN, Aβ$_{42}$≤181 was considered A+, p-tau$_{181}$≥26 was T+, and t-tau≥59 was N+. Despite sample-specific thresholds, Fisher's tests indicated that AD+LBD patients were less likely to be T+ (OR=0.26, CI=0.1 to 0.65, *p*=0.0027) or N+ than AD (OR=0.25, CI=0.07 to 0.83, *p*=0.015). There was no difference between ADNC groups in A status (*p*=0.21).

Under ATN, 12 (12%) AD/AD+LBD patients were misclassified as "Normal" or "SNAP"; of these misclassified, 6 had intermediate ADNC and 6 had high ADNC. 12 (12%) AD/AD+LBD patients were classified as "Alzheimer's pathologic change" (A+T-N-) despite significant tau pathology at autopsy (3 were Braak=2; 9 were Braak=3). 12 (60%)

LBD patients were misclassified as "Normal." Of the 2 LBD patients who were classified as "Alzheimer's pathologic change," one had negligible amyloid-β (Thal=0/CERAD=0) and the other had mild amyloid-β pathology (Thal=2/CERAD=1).

Next we evaluated the t-tau/$A\beta_{42}$ ratio, which classified patients simply as AD positive (≥0.32) or negative (<0.32; Table 4). Under t-tau/$A\beta_{42}$, 14 (14%) of AD/AD+LBD patients were misclassified as negative for AD; of these misclassified, 7 had intermediate ADNC and 7 had high ADNC. 1 (5%) LBD patient with low ADNC (Thal=2/Braak=1/CERAD=1) was misclassified as positive for AD by t-tau/$A\beta_{42}$.

A. ATN Classification	Control	AD	AD+LBD	LBD
A-T-N-	37 (61%)	0 (0%)	3 (8%)	12 (60%)
A+T-N-	0 (0%)	3 (5%)	9 (23%)	2 (10%)
A+T+N-	0 (0%)	1 (2%)	0 (0%)	0 (0%)
A+T+N+	0 (0%)	40 (67%)	15 (38%)	0 (0%)
A+T-N+	2 (3%)	11 (18%)	8 (21%)	0 (0%)
A-T+N-	5 (8%)	2 (3%)	0 (0%)	4 (20%)
A-T-N+	9 (15%)	0 (0%)	2 (5%)	2 (10%)
A-T+N+	8 (13%)	3 (5%)	2 (5%)	0 (0%)

B. t-tau/Aβ42 Classification	Control	AD	AD+LBD	LBD
AD-	57 (93%)	4 (7%)	10 (26%)	19 (95%)
AD+	4 (7%)	56 (93%)	29 (74%)	1 (5%)

Supplementary Table 3: ATN and t-tau/$A\beta_{42}$ Classifications. Summary of how each strategy classifies Controls, AD, AD+LBD, and LBD patients. Count and percentage (%) is provided for each diagnostic category. ATN Classifications (Panel A): Normal (A-T-N-), early Alzheimer's pathologic change (A+T-N-), AD (A+T+N±), concomitant Alzheimer's pathologic change and SNAP (A+T-N+), and SNAP (A-T±N±). t-tau/$A\beta_{42}$ Classifications (Panel B): AD+ (≥0.31724) and AD- (<0.31724).

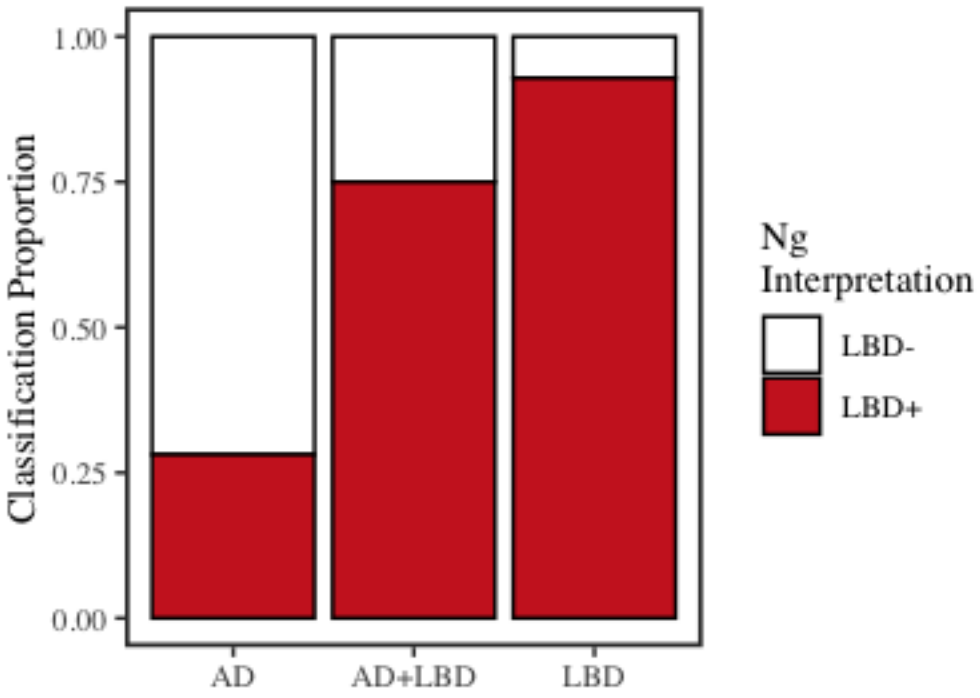
5.3.1 Posthoc analyses detecting mixed AD and LBD pathology with Ng

Our previous analyses showed that low Ng levels in ADNC may be indicative of concomitant α-synuclein pathology in AD patients. We performed follow-up tests to investigate if Ng or other analytes could detect LBD pathology in a mixed cohort. ROC analyses (Supplementary Table 4) showed that Ng had the best accuracy when discriminating LBD positive patients (LBD, AD+LBD) from patients negative for LBD (AD).

Measure	AUC	AUC 5%CI	AUC 95%CI	Threshold	Sensitivity	Specificity
Ng	0.82	0.74	0.90	195.49	0.82	0.72
p-tau/Aβ42	0.81	0.74	0.87	0.20	0.69	0.77
t-tau	0.80	0.73	0.86	80.69	0.66	0.78
t-tau/Aβ42	0.79	0.72	0.86	0.51	0.66	0.87
p-tau	0.78	0.71	0.85	33.46	0.78	0.64
Aβ42	0.68	0.60	0.75	179.57	0.42	0.92

Supplementary Table 4: Receiver Operating Characteristic Analyses to detect LBD. ROC metrics are calculated using bootstrapping with 500 iterations. CSF measures are listed in descending order of area under the curve (AUC) when discriminating LBD (LBD, AD+LBD) from non-LBD (AD) patients. Best threshold was calculated (Youdon's index) for this sample, and sensitivty and specificity at that threshold are reported.

Supplementary Fig 1 shows the classification of patients using Ng<195.5 to detect LBD in a mixed sample of LBD and AD. Results were fair, with 18 of 24 AD+LBD patients (75%) correctly identified as LBD positive. Conversely, 23 of 32 AD patients (72%) were correctly identified as negative for LBD. For LBD patients, 13 of 14 (93%) were correctly classified as LBD positive.



Supplementary Figure 1. Ng Classifications of LBD+/-. Barplot of how CSF Ng classifies AD, AD+LBD, and LBD patients. Ng Interpretation: LBD+ (<195.5) and LBD

We next tested how Ng predicted LBD using a logistic regression that also incorporated sex and disease duration at CSF; we substituted disease duration for CSF-to-death interval because interval to death is unknown at the time of CSF collection. Results showed that higher Ng was significantly associated with likelihood of LBD pathology (β=-0.0084, *p*=0.00073); neither sex (*p*=0.7) nor disease duration (*p*=0.077) were significantly associated with LBD pathology. Still, the AUC was somewhat higher with sex and disease duration included (0.86), than Ng alone (AUC=).

Extras

Figure 1. CSF levels across AD, AD+LBD, and LBD patients. Comparisons of CSF levels of $A\beta_{42}$, p-tau_{181}, t-tau, and Ng across AD, AD+LBD, and LBD patients. Color indicates ADNC from not (dark blue) to high (dark red). Dotted lines represent median level for controls. Asterisks represent p-values from Wilcoxon pairwise comparisons (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, or not significant [ns]). Boxplots show median, interquartile range (IQR), and outliers.

3.2.1 Correlation with clinical outcomes