An examination of a novel multipanel of CSF biomarkers in the Alzheimer’s disease clinical and pathological continuum

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Abstract

Introduction: This study examines the utility of a multipanel of cerebrospinal fluid (CSF) biomarkers complementing Alzheimer’s disease (AD) biomarkers in a clinical research sample. We compared biomarkers across groups defined by clinical diagnosis and pTau₁₈₁/Αβ₄₂ status (+/−) and explored their value in predicting cognition.

Methods: CSF biomarkers amyloid beta (Αβ)₄₂, p Tau₁₈₁, t Tau, Αβ₄₀, neurogranin, neurofilament light (NFL), α-synuclein, glial fibrillary acidic protein (GFAP), chitinase-3-like protein 1 (YKL-40), soluble triggering receptor expressed on myeloid cells 2 (sTREM2), S100 calcium binding protein B (S100B), and interleukin 6 (IL6), were measured with the NeuroToolKit (NTK) for 720 adults ages 40 to 93 years (mean age = 63.9 years, standard deviation [SD] = 9.0; 50 with dementia; 54 with mild cognitive impairment [MCI], 616 unimpaired).

Results: Neurodegeneration and glial activation biomarkers were elevated in pTau₁₈₁/Αβ₄₂+ MCI/dementia participants relative to all pTau₁₈₁/Αβ₄₂− participants. Neurodegeneration biomarkers increased with clinical severity among pTau₁₈₁/Αβ₄₂+ participants and predicted worse cognitive performance. Glial activation biomarkers were unrelated to cognitive performance.
1 | INTRODUCTION

Alzheimer’s disease (AD) is a progressive neurodegenerative disease with an extended preclinical phase wherein pathologic amyloid β (Aβ) and tau proteins aggregate before onset of cognitive impairment.1–3 Over the past two decades, tremendous progress has been made in detecting abnormal forms of Aβ and tau proteins in cerebrospinal fluid (CSF) and positron emission tomography (PET) imaging.4–7 In particular, CSF in vitro diagnostic (IVD) immunoassays for Aβ42, tau phosphorylated at serine-181 (pTau181), and total tau protein (tTau) concentrations have demonstrated excellent diagnostic precision in AD.1,8 However, there is still heterogeneity in progression to symptomatic AD that may be explained by other pathophysiologies. The NeuroToolKit (NTK) is a panel of automated CSF immunoassays developed to complement established core AD biomarkers Aβ42, pTau181, and tTau9–12 with markers for glial activation and inflammation, synaptic degeneration, and damage to long axons, to provide new tools to explore disease pathogenesis (see Wild et al. [2020], this issue).

Our objectives were to (1) confirm the utility of core AD biomarker positivity derived from CSF measured using automated Elecsys CSF NTK immunoassays in clinical research sample and (2) explore associations with biomarkers in the NTK that are not specific to AD, but may indicate the presence and severity of neurodegeneration and glial activation and thereby account for variability in clinical diagnosis and cognitive performance.

2 | METHODS

2.1 | Participants

Using a uniform preanalytic protocol across included longitudinal studies, CSF was obtained from N = 720 adults ages 45 to 93 years (M = 63.9, standard deviation [SD] = 9.0; 51.6% female) participating in the Wisconsin Registry for Alzheimer’s Prevention study (WRAP, n = 205),13 the Wisconsin Alzheimer’s Disease Research Center (WADRC, n = 411), or affiliated studies (Statins in Healthy, At-Risk Adults: Impact on Amyloid and Regional Perfusion [SHARP, n = 63; NCT00939822]; the Alzheimer’s Disease Connectome Project [ADCR n = 9]; Fitness Aging in the Brain, [FAB, n = 40]). Enrollment criteria varied across studies (see supporting information). The combined sample includes cognitively unimpaired (CU) individuals, participants with mild cognitive impairment (MCI) or dementia due to suspected AD, and is enriched for parental history of AD dementia (determined through review of parental medical records, autopsy reports, results of a dementia questionnaire, or participant self-report). All participants had decisional capacity and completed an informed consent process before undergoing study procedures. Lumbar punctures (LPs) were performed within 1 year of cognitive testing. If participants completed multiple LPs, their most recent LP was selected for analysis.

Discussion: The NTK contains promising markers that improve the pathophysiological characterization of AD. Neurodegeneration biomarkers beyond tTau improved statistical prediction of cognition and disease stages.

KEYWORDS

Alzheimer’s disease, amyloid positron emission tomography imaging, biomarker validation, cerebrospinal fluid biomarkers, glial activation, inflammation, neurodegeneration
(neurogranin, neurofilament light protein [NFL], and α-synuclein),
and markers of glial activation (glial fibrillary acidic protein [GFAP],
chitinase-3-like protein 1 [YKL40], and soluble triggering receptor
expressed on myeloid cells 2 [sTREM2]).

2.5 Amyloid PET imaging

A subset of 185 participants also underwent dynamic [C-11]Pittsburgh
compound B (PiB) PET imaging (0–70 minutes post-injection) within
2 years of their most recent LP (mean time between PiB and LP
was 0.35 ± 0.71 years). Imaging methods and PiB quantification have
been previously described.16 PiB(+/−) status was determined by visual
inspection inter-rater reliability = 0.95, intra-rater reliability = 0.96.26

2.6 Biomarker positivity

We used receiver operating characteristic (ROC) analyses to derive
positivity thresholds for AD biomarkers (ADβ) using PiB(+/−) as the
standard of comparison. ROC analyses were conducted using the Mat-
Lab perfcurve function (The Mathworks, Natick, Massachusetts, USA).
The optimal threshold for Aβ42/40 and pTau181/Aβ42 discrimination
was based on equally weighted cost functions for positive and nega-
tive agreement.17 Due to the greater availability of Elecsys® IVD immunoassays in clinical settings, pTau181/Aβ42 was used in analy-
ses requiring continuous measures of ADB, and pTau181/Aβ42 pos-
itivity status was used for analyses with dichotomous ADB status
(ADβ[+/−]).

Thresholds for pTau181, tTau, NFL, neurogranin, and α-synuclein sta-
tus were determined by establishing a reference group of 223 CSF
amyloid (Aβ42/40) negative, cognitively unimpaired younger partici-
pants (ages 40–60 years). Biomarker positivity thresholds for these
analytes were set at +2SD above the mean of this reference group.18

2.7 Cognitive outcomes

The primary cognitive outcome was clinical diagnosis. As a sec-
ondary cognitive outcome, we examined the cross-sectional relation-
ship between biomarkers and cognitive performance, using a three-
best Preclinical Alzheimer Cognitive Composite (PACC3) described by
Jonaitis et al.19 and based on the work of Donohue et al.20 Due to
variations in cognitive batteries across cohorts, Trail-Making Test B
replaced Digit Symbol as the executive function measure and Craft
Story Delayed Recall was used to impute Logical Memory II-A based
on a published crosswalk.21 Continuous cognitive outcomes were
matched to the nearest LP visit. Only matches less than a year apart
were included, and no cognitive visit was matched more than once.

2.8 Statistical analysis

Statistical analyses were conducted in R.22 Sample characteristics
were compared across clinical diagnosis using analysis of variance
for continuous measures and chi-square for categorical measures.

| HIGHLIGHTS |
|--------------------------------------------------|
| • Alzheimer’s disease (AD) biomarker positive (pTau/Aβ42) participants had higher levels of neurodegeneration biomarkers across levels of clinical severity. |
| • Biomarkers for glial activation were differentiated in cognitively impaired, but not cognitively unimpaired, participants. |
| • Biomarkers of neurodegeneration beyond tau accounted for additional variation in cognitive performance over time. |
| • An expanded panel of cerebrospinal fluid biomarkers that include neurodegeneration and neuroinflammatory markers represents an important array of tools that may play a role in staging AD and other neurodegenerative diseases. |

RESEARCH IN CONTEXT

1. Systematic review: The authors reviewed the extant liter-
ature using PubMed and Google Scholar. A small num-
ber of studies have been published using the same Neuro-
ToolKit (NTK) automated assay for core Alzheimer’s dis-
eease (AD) biomarkers. However, this study examines the
extended NTK assay, which includes additional markers
for neurodegeneration and glial activation.

2. Interpretation: Our results indicate that the NTK panel of
neurodegeneration and neuroinflammatory markers rep-
resents an important array of tools that may play a role in
staging AD and confer new insights into the pathogenesis
of AD and its clinical manifestation.

3. Future directions: A number of hypotheses are gener-
ated from these results. For example, focusing on devel-
oping meaningful thresholds for neurofilament light may
enhance detection of subjects with neurodegeneration
(N+). Also, studies with a more clinically diverse sample
are required to establish the contexts under which glial
markers signify or contribute to risk for AD.

Associations between CSF values and clinical severity were tested
with linear regressions. The R package emmeans 1.4.3.01
was used to compare mean differences among groups defined by ADB status and clinical diagnosis.

We evaluated the potential added explanatory value of exploratory
NTK biomarkers when modeling cognitive outcomes using categori-
ical (clinical diagnosis) and continuous (PACC3) measures of cogni-
tion. Observations were excluded if they were missing ADB or NTK
biomarkers, or any covariates (n = 47). SHARP participants (n = 66)
received a different cognitive battery and were excluded from analy-
zes of continuous cognitive performance. Logistic regression was used
### TABLE 1  Sample demographics at most recent LP by clinical diagnosis

|                          | Total          | Dementia       | MCI            | CU            | P       |
|--------------------------|----------------|---------------|----------------|---------------|---------|
| n (% Female)             | 720 (63.3%)    | 50 (36.0%)    | 54 (42.6%)     | 616 (67.4%)   | <.001   |
| non-Hispanic, White, n (%) | 676 (93.9%)    | 49 (100%)     | 44 (93.6%)     | 576 (94.3%)   | .17     |
| Age, m (SD)              | 63.9 (± 9.0)   | 72.6 (± 8.5)  | 72.4 (± 8.4)   | 62.4 (± 8.1)  | <.001   |
| APOE4+, n (%)            | 251 (34.8%)    | 33 (67.3%)    | 27 (50.0%)     | 220 (35.7%)   | .02     |
| Parental AD+, n (%)      | 501 (69.6%)    | 31 (63.3%)    | 29 (53.7%)     | 441 (71.6%)   | .02     |
| Education, m (SD)        | 16 (± 2.6)     | 14.4 (± 2.6)  | 16.1 (± 2.6)   | 16.2 (± 2.4)  | <.001   |
| MMSE, m (SD)             | 28.5 (± 2.5)   | 21.6 (± 3.7)  | 27.4 (± 2.0)   | 29.4 (± 0.9)  | <.001   |
| CDR Sum of Boxes, m (SD) | 0.67 (± 1.5)   | 4.5 (± 1.6)   | 1.7 (± 1.3)    | 0.08 (± 0.27) | <.001   |
| ASCVD ≥ 7.5, n %         | 323 (55.4%)    | 33 (89.2%)    | 29 (85.2%)     | 261 (50.9%)   | <.001   |
| Hypertension, n (%)      | 162 (25.2%)    | 28 (56.0%)    | 21 (42%)       | 113 (20.8%)   | <.001   |
| Diabetes, n %            | 44 (6.7%)      | 6 (12%)       | 5 (9.8%)       | 33 (6.1%)     | .19     |
| MDD, n %                 | 202 (31.2%)    | 19 (38.0%)    | 19 (38.0%)     | 164 (31.0%)   | .39     |
| Number LPs, 1/2/3/4+     | –              | 48/2/0/0      | 47/6/0/1       | 362/81/115/58 | –       |
| LP interval in years, m (SD) | –             | 3.7 (2.8)    | 2.0 (1.5)      | –             | –       |
| PIB PET, n               | 185            | 2             | 16             | 167           |         |
| PIB(+), n (%)            | 47 (25%)       | 2 (100%)      | 10 (63%)       | 35 (21%)      | <.001   |
| Age at PIB, m (SD)       | 67.0 (± 7.6)   | 71.0 (± 4.5)  | 71.9 (± 8.4)   | 66.5 (± 7.5)  | .02     |
| Years at(PIB – LP), n (SD)| 0.4 (± 0.7)   | 0.3 (± 0.5)   | 0.2 (± 0.6)    | 0.4 (± 0.7)   | .17     |

Abbreviations: AD, Alzheimer’s disease; APOE4+, apolipoprotein E4 carrier; ASCVD, atherosclerotic cardiovascular disease 10 year risk percent (≥ 7.5 is high risk); CDR, Clinical Dementia Rating; CU, cognitively unimpaired; Dementia, dementia due to suspected AD or other causes; LP, lumbar puncture; MCI, mild cognitive impairment due to suspected AD or other causes; MDD, major depressive disorder; MMSE, Mini-Mental State Examination; PIB PET, [C-11] Pittsburgh compound B positron emission tomography; SD, standard deviation.

Notes: Clinical status (MCI/Dementia) was determined based on National Institute on Aging-Alzheimer’s Association (NIA-AA) criteria, without reference to biomarkers. Each LP visit was matched to the participant’s nearest consensus conference (average time [age Diagnosis-age LP] = .25 ± .30 years). Six participants were missing MMSE (5 CU, 1 MCI); n = 79 CU participants were missing CDR Sum of Boxes due to variations in cognitive testing across cohorts (see supporting information). Parental history of AD was determined through parent medical records, autopsy reports, results of a dementia questionnaire, or participant self-report. ASCVD 10-year risk was calculated using the 2013 American College of Cardiology/American Heart Association algorithm (n = 145 CU participants were missing data). Diagnosis of hypertension, diabetes, and MDD was obtained at study entry (3 MCI and 72-87 CU participants were missing data).

### 3 RESULTS

#### 3.1 Sample characteristics and CSF analytes

Sample characteristics are shown by clinical diagnosis in Table 1. Participants were aged 40 to 93 years (M = 63.9, SD = 9.0), mostly white, and highly educated. Cognitively unimpaired participants were younger, more educated, and less likely to carry the APOE4 risk allele compared to impaired groups. Performance on the Mini-Mental State Examination (MMSE) and the Clinical Dementia Rating Scale Sum of Boxes (CDR-SB) tracked with diagnostic category, as expected (Table S3 in supporting information).

#### 3.2 Biomarker positivity thresholds

##### 3.2.1 CSF amyloid and ADB ratios

ROC analyses indicated high diagnostic consistency between PiB visual positivity and Aβ42/40 and pTau181/Aβ42. Area under the curve was 97% for both ratios. ROC derived thresholds for biomarker positivity were 0.046 for Aβ42/40 (96% negative agreement, 92% positive agreement) and 0.038 for pTau181/Aβ42 (98% negative agreement, 83% positive agreement). Applying these thresholds to...
the full study sample resulted in 46/50 (92%) dementia, 31/54 (61%) MCI, and 98/604 (16%) of CU participants identified as Aβ42/40(+), and 46/50 (92%) dementia, 31/54 (61%) MCI, and 66/606 (11%) CU participants identified as pTau/Aβ42 positive (i.e., ADB(+)). Aβ42/40, and pTau181/Aβ42, positivity agreed in 669/708 (94%) of cases with disagreement observed for 36 cases classified as Aβ42/40(+)/pTau/Aβ42(−), and 3 cases classified as Aβ42/40(−)/pTau/Aβ42(−).

### 3.2.2 | Tau and neurodegeneration positivity

The average pTau181 concentration among the reference group of cognitively unimpaired, amyloid negative adults aged 40 to 60 years was 15.1 (SD = 4.8) pg/ml resulting in a pTau181 + threshold of 24.8 pg/ml. Applying this threshold to the non-reference sample indicated 38/49 (78%) dementia, 25/47 (53%) MCI, 68/385 (18%) of the CU participants were pTau181 positive. Similarly derived positivity thresholds for other CSF neurodegeneration analytes are reported in Table S2a in supporting information. Of these neurodegeneration markers (NfL, neurogranin, and α-synuclein), neurogranin was the only analyte that did not indicate stepwise increases in the proportion of positive cases with increasing clinical severity. The proportion of NfL and α-synuclein positivity within each diagnostic group was highest in dementia cases (20/50 [40%] NfL(+); 18/50 [36%] α-synuclein(+]), followed by MCI cases (12/54 [22%] NfL(+); 11/54 [20%] α-synuclein(+)), and then CU cases (15/401 [4%] NfL(+); 35/401 [9%] α-synuclein(+)). However, biomarkers varied in agreement for neurodegeneration positivity (Cohen’s kappa ranged 0.36–0.51; Table S2b in supporting information).

### 3.3 | CSF analyte observations by ADB status

Scatterplots and correlations between CSF analytes for biomarker groups (AD, neurodegeneration, and glial activation) are shown by ADB status in Figure 1A-C (See Figure S1 in supporting information for correlations between all CSF analytes). Correlations between Aβ42, Aβ40, pTau181, and tTau were typical of those observed in AD (Figure 1A). Due to the high correlation between pTau181, and tTau (r = .98), tTau was excluded from subsequent regression analyses with clinical diagnosis and cognition.

Correlation patterns for CSF analytes related to neurodegeneration and glial activation were consistent across ADB status for all NTK analytes. All neurodegeneration markers (Figure 1B) correlated highly with tTau (range r = .62–.87). Neurogranin was highly correlated with α-synuclein (r = .81), while NfL was only moderately correlated with α-synuclein (r = .50) and neurogranin (r = .38). Glial activation biomarkers (Figure 2C) were all modestly inter-correlated (r = .22–.62) with S100B showing the lowest correlation with other glial activation markers. IL6 values were unrelated to the remaining analytes (Figure S1).

### 3.4 | CSF analyte observations by clinical diagnosis and ADB status

Descriptive statistics for all CSF analytes and derived ratios stratified by clinical diagnosis and ADB status are shown in Table 2. Distributions of analytes are shown in Figure 2A-D. Aβ40, Aβ42, and pTau181 (Figure 2, panel A) exhibited the expected distributions for combinations of clinical and ADB status. Aβ40 did not differ across clinical or ADB groups. Aβ42 was lower for all ADB+ and did not differ between clinical groups. Phospho-Tau181 was low in all ADB-, was higher in unimpaired ADB+, and was highest in impaired (MCI and dementia) ADB+.

Neurodegeneration analytes (Figure 2, panel B) showed similar patterns between ADB and clinical status groups for neurogranin and α-synuclein. These markers did not differ across clinical groups in ADB- and were higher in ADB+ compared to ADB- both within and across clinical groups (not enough ADB- dementia cases for comparison). NfL indicated stepwise increases in ADB+ with increasing clinical severity.

CSF analytes of glial activation YKL-40, S100B, GFAP, and sTREM2 (Figure 2, panel C) exhibited similar patterns in ADB+ wherein impaired ADB+ had higher values compared to unimpaired ADB+. In general, these analyte distributions had considerable overlap between ADB+ and ADB- in the unimpaired group. YKL-40 and GFAP were higher for ADB+ compared to ADB- in the MCI group. IL6 (Figure 2, panel D) was unrelated to ADB status or clinical group.

### 3.5 | Relationships between cognitive outcomes and extended NTK analytes

Continuous ADB significantly predicted clinical diagnosis and cognitive performance (Ps < .001). Adding gliosis biomarkers did not improve model fit for either clinical status or PACC3 (clinical status: χ²[4] = 4.0, P = .41; PACC3: χ²[4] = 6.7, P = 0.15). For both clinical status (Table 3) and PACC3 (Table 4), adding neurodegeneration biomarkers improved the overall model fit when compared to a model that included continuous ADB and covariates (clinical status: χ²[2] = 17.3, P = .0006; PACC3: χ²[3] = 23.5, P = .00032). The regression coefficient for neurogranin was opposite in sign to our expectation. Secondary analyses of individual neurodegeneration biomarkers suggested that this was an artifact of statistical suppression. As individual biomarkers, NfL best predicted clinical status and PACC3 over and above continuous ADB. To visualize the latter findings, we plotted a loess curve of PACC3 against NfL grouping by ADB status (Figure S2 in supporting information).

### 4 | DISCUSSION

The development of CSF assays for Aβ and tau proteins launched a rapid expansion of biomarker research. Nevertheless, questions surrounding heterogeneity in the clinical manifestation of AD, and the contribution of co-occurring pathology to clinical symptoms.
onset,30 and progression30,31 require an expanded set of biomarkers reflecting neurodegeneration and neuroinflammatory processes. Recent studies have investigated the NTK core AD biomarkers,10,24 and exploratory NTK biomarkers in cognitively unimpaired adults.12 We examined established and novel biomarkers in the NTK in subjects that span clinical severity to explore their characteristics in the context of AD biomarker status and clinical diagnosis and their added value in predicting cognition.

### 4.1 Biomarker positivity

#### 4.1.1 Concordance of CSF ratios with amyloid PET

CSF and PET biomarkers of AD provide overlapping, but not completely redundant, information given that their targets differ (eg, the Aβ1-42 protein fragment vs fibrillar amyloid with PET) as do...
their sensitivity to pathology. Amyloid PET positivity may repre-
sent a slightly more mature phase of the disease\textsuperscript{32} and has been
used as a standard to which AD CSF biomarkers can be compared.
For this study, having an empirically derived threshold for CSF Aβ
positivity based on maximizing agreement with amyloid PET was
an important strength. The derived thresholds for Aβ$_{42}$/Aβ$_{40}$ (0.046)
and pTau/Aβ$_{42}$ (0.038) conferred an area under the curve of .97 in
classifying participants with known amyloid PET status. While this
agreement is excellent, it is important to note the differences in
physiologic meaning of the signal—lower CSF levels of Aβ$_{42}$ likely
reflect impaired clearance, whereas PET signal likely reflects years of
accumulated fibrillar amyloid deposition. CSF is likely to begin reflect-
ing AD pathology earlier than PET imaging, thus some individuals with
early amyloid pathology that has not yet shown up on PET imag-
ing may have been misidentified as ADB–. The alternative to using
PET amyloid as the standard would be to use autopsy cases (which
were not available), distribution-based cutpoints (which we resorted
to for other analytes), or published CSF amyloid cut points (assum-
ing site-specific differences in pre-analytic procedures have no effect,
which is unlikely). Relying on currently published thresholds\textsuperscript{10,24} would
have led to overestimating the number of biomarker positive CU
participants.
4.1.2 Concordance of the AD ratios

Aβ42/40 and ptau/Aβ42 exhibited 95% agreement in this mixed sample of dementia, MCI, and CU participants. This is a high degree of concordance and suggests near equivalence between these ratios for identifying biomarker positive cases defined by PET visual ratings. Because the ptau/Aβ42 ratio simultaneously comprises both proteinopathies, concords well with amyloid PET studies, and may be more available to the research and clinical community than Aβ42/40, we used ptau/Aβ42 as the primary AD biomarker grouping variable. Nevertheless, ptau/Aβ42 has the potential to misidentify individuals as ADB—very early in the disease process.

4.2 Interrelationship between neurodegenerative analytes and clinical diagnosis/ADB status

As noted in the NIA-AA research framework, neurodegeneration is a non-specific feature of several neurodegenerative diseases. Because we and others have observed remarkably high agreement between...
**TABLE 2**  Descriptive statistics and mean differences within clinical diagnosis and Alzheimer’s disease biomarker (ADB) status

| Measure | Dementia | ADB+ | ADB− | ADB+ | ADB− | CU | ADB+ | ADB− |
|---------|---------|------|------|------|------|----|------|------|
| N       | 46      | 4    | 33   | 21   | 70   | 536|      |      |
| Age, M (SD) | 72.3 (8.0) | 76.5 (14.0) | 74.1 (7.6) | 69.8 (9.2) | 69.1 (6.6) | 61.6 (8.0) |
| Female, n (%) | 18 (39%) | 0 (0.0%) | 13 (39%) | 10 (48%) | 45 (64%) | 362 (68%) |
| APOE4+, n (%) | 32 (70%) | 1 (25%) | 21 (63%) | 6 (29%) | 41 (59%) | 176 (33%) |
| Alzheimer’s biomarkers |  |  |  |  |  |  |  |
| Aβ42 pg/mL, m (SD) | 425 (229) | 1152 (369) | 464 (161) | 1061 (394) | 463 (152) | 991 (366) |
| Aβ40 pg/mL, m (SD) | 14002 (5874) | 15682 (4296) | 15360 (5288) | 14986 (4553) | 14477 (4439) | 14444 (4675) |
| Aβ42/40, m (SD) | 0.031 (0.007) | 0.074 (0.008) | 0.031 (0.006) | 0.071 (0.009) | 0.034 (0.009) | 0.069 (0.013) |
| pTau181 pg/mL, m (SD) | 39.7 (19.6) | 19.1 (5.28) | 34.4 (17.6) | 17.8 (6.36) | 27.4 (10.27) | 16.4 (5.46) |
| Neurodegeneration biomarkers |  |  |  |  |  |  |  |
| tTau pg/mL, m (SD) | 390 (182) | 286 (131) | 347 (148) | 217 (77.7) | 284 (99.6) | 189 (63.0) |
| NfL pg/mL, m (SD) | 225 (112) | 279 (277) | 199 (130) | 149 (123) | 129 (80.2) | 89.9 (55.6) |
| Neurogranin pg/mL, m (SD) | 1116 (583) | 805 (238) | 1067 (481) | 795 (320) | 1040 (414) | 753 (289) |
| α-Synuclein pg/mL, m (SD) | 240 (118) | 246 (116) | 231 (101) | 177 (94.7) | 195 (78.3) | 156 (63.4) |
| Gliosis biomarkers |  |  |  |  |  |  |  |
| YKL-40 ng/mL, m (SD) | 238 (96.7) | 239 (132) | 226 (87.2) | 176 (68.1) | 179 (61.2) | 144 (53.7) |
| GFAP pg/mL, m (SD) | 15.2 (6.72) | 10.1 (4.00) | 15.2 (5.89) | 11.4 (5.26) | 11.2 (3.14) | 9.13 (3.27) |
| S100B ng/mL, m (SD) | 1.25 (0.331) | 1.03 (0.214) | 1.31 (0.303) | 1.11 (0.381) | 1.15 (0.248) | 1.14 (0.249) |
| sTREM2 ng/mL, m (SD) | 9.95 (3.57) | 8.93 (1.61) | 9.75 (3.68) | 8.90 (2.56) | 8.51 (2.43) | 7.94 (2.43) |
| Inflammation biomarkers |  |  |  |  |  |  |  |
| IL6 pg/mL, m (SD) | 5.47 (5.00) | 4.38 (0.724) | 3.88 (1.84) | 5.25 (5.97) | 4.01 (1.99) | 4.68 (3.16) |

Abbreviations: Aβ, amyloid beta; ADB, Alzheimer’s disease biomarker status; APOE4+, apolipoprotein E4 carrier; CU, cognitively unimpaired; Dementia, dementia due to suspected AD or other causes; GFAP, glial fibrillary acidic protein; MCI, Mild cognitive impairment due to suspected AD or other causes; NfL, neurofilament light protein; SD, standard deviation; sTREM2, soluble triggering receptor expressed on myeloid cells 2; YKL-40, chitinase-3-like protein 1.

Notes: Clinical status (MCI/dementia) was determined based on National Institute on Aging-Alzheimer’s Association (NIA-AA) criteria without reference to dementia due to suspected AD or other causes; GFAP, glial fibrillary acidic protein; MCI, Mild cognitive impairment due to suspected AD or other causes; Nfl, neurofilament light protein; SD, standard deviation; sTREM2, soluble triggering receptor expressed on myeloid cells 2; YKL-40, chitinase-3-like protein 1.

TABLE S2 in which a steeper relationship between lower cognitive scores and NfL was observed among ADB+ than ADB− participants. In

**tTau and pTau181, CSF tTau does not appear to be a fully independent measure of neurodegeneration in AD. The other NTK markers may serve an important need in this regard. Indeed, Nfl, an indicator of axonal degradation, has been used as a useful neurodegeneration marker in multiple sclerosis, non-AD tauopathies, synucleinopathies, and traumatic brain injury as well as AD. Nfl is also in agreement with magnetic resonance imaging metrics in this population and correlates with pre-dementia disease progression. In the present analyses, Nfl, neurogranin, and α-synuclein exhibited moderate to strong agreement with tTau and at least moderate agreement with each other, suggesting these markers of neurodegeneration are reflecting common aspects of neurodegeneration. Further, they exhibited elevation within diagnostic stage by ADB status or significant elevation differences across diagnoses (as shown in Figure 2). Nfl exhibited the most characteristic stepwise increase across clinical diagnosis in AD biomarker positive subjects and this was further evident in Figure S2 in which a steeper relationship between lower cognitive scores and Nfl was observed among ADB+ than ADB− participants.**
contrast, neurogranin, a post-synaptic protein marker, was significantly elevated in the cognitively unimpaired group who were ADB+ and remained elevated across clinical diagnoses consistent with our prior observations. Total α-synuclein, a presynaptic marker, exhibited a similar pattern and was also strongly correlated with neurogranin ($r = .80$). CSF α-synuclein was initially found to be slightly decreased in Parkinson’s disease and Lewy body dementia, but subsequent studies showed a pronounced increase in CSF α-synuclein in neurodegenerative disorders with marked neurodegeneration, including Creutzfeldt-Jakob disease and AD. Elevation of this protein in our sample likely
FIGURE 2  Continued

† p<0.05 compared with ADB-/CU
* p<0.001 compared with ADB-/CU
reflected synaptic degeneration rather than deposition of α-synuclein in
Levy bodies. Its utility as a novel marker of neurodegeneration continues
to undergo study.

Although promising as continuous markers of neurodegeneration (N), among AD and CU participants a lower proportion were identified as positive when defined by NFL, neurogranin, or α-synuclein compared to tTau. Agreement across neurodegeneration biomarkers was moderate. The method for choosing thresholds for these analytes (2 SD above the mean of a CU Aβ42/40 negative group) is a reasonable approach but assumes a monotonic relationship between age and biomarker concentration. Ongoing work in the field will lead to more precise methods for defining a meaningful threshold for N+/−.

### 4.3 | Interrelationship between inflammation and gliosis analytes and clinical diagnosis/ADB status

Activation of microglia in response to amyloid plaques is a well-known feature of AD, and inflammatory pathways have been shown to play a role in AD pathogenesis.40,41 Despite the involvement of inflammatory processes in AD pathophysiology, IL6 was unrelated to either markers of glial activation or clinical diagnosis, and may be more relevant at a more advanced disease state.42 Markers of glial activation exhibited low to moderate intercorrelation, indicating potentially unique physiologic meaning of each analyte. YKL-40, a glycoprotein expressed by microglia and astrocytes, and GFAP, an indicator of reactive astrocytes,43 were both elevated in ADB+ cognitively impaired subjects compared to their biomarker negative peers. The YKL-40 finding replicates previous observations.44 From Figure 2, the effect sizes of glial and microglial markers observed here appear lower than for the neurodegeneration markers. Nevertheless, these results are promising and warrant further study, particularly in the context of co-occurring diseases.

### 4.4 | Core AD biomarkers and cognition

Before examining the effect of the NTK panel, we first confirmed that AD biomarkers were related to cognition defined by clinical diagnosis and global cognitive performance. Aβ42 alone predicted impairment, but did not distinguish between MCI and AD, perhaps due to the well-known observation that levels of this protein plateau by the dementia stage.45,46 Normalizing against total amyloid production (ie, by the Aβ42/40 ratio), led to clear differentiation by clinical diagnosis, as did pTau181 and pTau181/Aβ42.

### 4.5 | NTK biomarkers and cognition

Results from hierarchical regression analyses suggest that as a group, neurodegeneration biomarkers add value in predicting both clinical impairment (MCI/dementia vs CU) and global cognitive performance. The information in these markers is overlapping, as can be
TABLE 4  Results of linear mixed model predicting continuous cognitive performance on the preclinical Alzheimer’s cognitive composite (PACC3; \( n = 617 \)) from NTK biomarkers

| Term                        | Neurodegeneration (\( \chi^2(3) = 23.5, P = .000032 \)) | Gliosis (\( \chi^2(4) = 6.7, P = .15 \)) |
|-----------------------------|----------------------------------------------------------|------------------------------------------|
|                             | \( \beta \) (SE)                                       | \( P \)                                   | \( \beta \) (SE)                                       | \( P \)                                   |
| Intercept                   | -1.88 (0.22)                                            | <.001                                    | -1.82 (0.22)                                            | <.001                                    |
| Sex, male                   | -0.5 (0.068)                                            | <.001                                    | -0.58 (0.068)                                            | <.001                                    |
| Parental AD +               | 0.012 (0.07)                                            | .86                                      | -0.0013 (0.071)                                          | .99                                      |
| APOE4+                      | -0.0054 (0.067)                                         | .94                                      | -0.018 (0.068)                                          | .79                                      |
| Education, years            | 0.11 (0.013)                                            | <.001                                    | 0.11 (0.013)                                            | <.001                                    |
| Prior exposure to cognitive tests |                                              |                                          |                                                      |                                          |
| 1 exposure                  | 0.18 (0.056)                                            | <.001                                    | 0.21 (0.056)                                            | <.001                                    |
| 2 exposures                 | 0.28 (0.059)                                            | <.001                                    | 0.31 (0.059)                                            | <.001                                    |
| 3 exposures                 | 0.54 (0.065)                                            | <.001                                    | 0.56 (0.066)                                            | <.001                                    |
| 4 exposures                 | 0.6 (0.077)                                             | <.001                                    | 0.63 (0.077)                                            | <.001                                    |
| 5 exposures                 | 0.54 (0.13)                                             | <.001                                    | 0.56 (0.13)                                             | <.001                                    |
| 6 exposures                 | 0.58 (0.25)                                             | .023                                    | 0.64 (0.25)                                             | .012                                    |
| 8 exposures                 | -0.88 (0.53)                                            | .1                                      | -0.98 (0.53)                                            | .066                                    |
| Age at cognitive testing    |                                                         |                                          |                                                      |                                          |
| Linear term                 | -0.041 (0.0044)                                         | <.001                                    | -0.04 (0.0049)                                          | <.001                                    |
| Quadratic term              | -0.0012 (0.00031)                                       | <.001                                    | -0.0012 (0.00031)                                       | <.001                                    |
| p\( \beta \)42              | -0.42 (0.034)                                           | <.001                                    | -0.38 (0.033)                                           | <.001                                    |
| NfL, sd                     | -0.1 (0.033)                                            | .002                                    | -                                     |                                          |
| Neurogranin, sd             | 0.17 (0.045)                                            | <.001                                    | -                                     |                                          |
| &-\text{Synuclein, sd}      | -0.059 (0.042)                                          | .16                                     | -                                     |                                          |
| YKL-40, sd                  | -                                                      | -                                      | -0.044 (0.044)                                          | .31                                      |
| S100B, sd                   | -                                                      | -                                      | 0.059 (0.028)                                           | .035                                    |
| GFAP, sd                    | -                                                      | -                                      | -0.058 (0.042)                                          | .17                                      |
| sTREM2, sd                  | -                                                      | -                                      | 0.018 (0.037)                                           | .63                                      |

Abbreviations: APOE4+, apolipoprotein E4 carrier; GFAP, glial fibrillary acidic protein; NfL, neurofilament light protein; NTK, NeuroToolKit; PACC, preclinical Alzheimer’s cognitive composite; PACC3, Preclinical Alzheimer Cognitive Composite; sTREM2, soluble triggering receptor expressed on myeloid cells 2; YKL-40, chitinase-3-like protein 1.

Notes: NTK biomarkers were standardized prior to analysis, and age is mean-centered on baseline age; PACC3 is comprised of Rey AVLT-total over five trials, Logical Memory IIA (Story Recall Delayed or cross-walked Craft Story), and Trail-Making Test Part B. \( n = 111 \) participants were excluded due to missing covariates, cerebrospinal fluid values, or cognitive testing (1 dementia, 5 mild cognitive impairment, 105 cognitively unimpaired). Excluded participants were younger (\( t[158.5] = 6.9 \), \( P < .001 \)) and less likely to be biomarker positive (\( \chi^2[1] = 16.7, P < .001 \)) than participants included in the analyses. No other demographic differences were found.

seen by the suppression effects observed in the full model; however, their relatively moderate concordance with one another indicates that the overlap is only partial. Of the three neurodegeneration markers, NfL appears to have the best concordance with clinical diagnosis. In contrast, adding gliosis markers to the regression did not improve model fit for either clinical status or global cognition, which suggests these markers do not explain additional variance in cognition beyond core AD markers. This conclusion must be tempered by the constraint of the study design. It is possible that glial markers may exhibit effects in certain contexts, such as the presence of vascular disease, or their effects are non-linear across disease state.

4.6 Limitations

Although the development of immunoassays has the potential to greatly reduce assay variability, raw values, particularly for A\( \beta \), may still be affected by preanalytic fluid collection protocols, which vary across studies. As such, the values and cut points described here may not generalize to studies in which preanalytic protocols differ. Our cohort is typical of dementia research (white, educated, relatively high functioning) but may not represent the typical AD patient, or individuals from higher risk populations like African Americans and Latinxs. Results need to be interpreted in light of this limitation. Although the time interval between LP and PET imaging was up to 2 years, given the stability of
amloid in the brain we do not anticipate a smaller time interval would change our findings.

5 | CONCLUSION

The NTK panel of neurodegeneration and neuroinflammatory markers represents an important array of tools that may play a role in staging AD, provide complementary outcomes for clinical trials, and confer new insights into the pathogenesis of AD and its clinical manifestation. In this sample, which spanned the spectrum of AD clinical stages, we observed informative interrelationships among the analytes and found that the neurodegeneration markers, but not glial activation, improved prediction of cognitive performance.

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CONFLICTS OF INTEREST

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