Original Investigation

Neurogranin as a Cerebrospinal Fluid Biomarker for Synaptic Loss in Symptomatic Alzheimer Disease

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IMPORTANCE Neurogranin (NGRN) seems to be a promising novel cerebrospinal fluid (CSF) biomarker for synaptic loss; however, clinical, and especially longitudinal, data are sparse.

OBJECTIVE To examine the utility of NGRN, with repeated CSF sampling, for diagnosis, prognosis, and monitoring of Alzheimer disease (AD).

DESIGN, SETTING, AND PARTICIPANTS Longitudinal study of consecutive patients who underwent 2 lumbar punctures between the beginning of 1995 and the end of 2010 within the memory clinic–based Amsterdam Dementia Cohort. The study included 163 patients: 37 cognitively normal participants (mean [SE] age, 64 [2] years; 38% female; and mean [SE] Mini-Mental State Examination [MMSE] score, 28 [0.3]), 61 patients with mild cognitive impairment (MCI) (mean [SE] age, 68 [1] years; 38% female; and mean [SE] MMSE score, 27 [0.3]), and 65 patients with AD (mean [SE] age, 65 [1] years; 45% female; and mean [SE] MMSE score, 22 [0.7]). The mean (SE) interval between lumbar punctures was 2.0 (0.1) years, and the mean (SE) duration of cognitive follow-up was 3.8 (0.2) years. Measurements of CSF NGRN levels were obtained in January and February 2014.

MAIN OUTCOME AND MEASURE Levels of NGRN in CSF samples.

RESULTS Baseline CSF levels of NGRN in patients with AD (median level, 2381 pg/mL [interquartile range, 1651-3416 pg/mL]) were higher than in cognitively normal participants (median level, 1712 pg/mL [interquartile range, 1206-2724 pg/mL]) (P = .04). Baseline NGRN levels were highly correlated with total tau and tau phosphorylated at threonine 181 in all patient groups (all P < .001), but not with Aβ42. Baseline CSF levels of NGRN were also higher in patients with MCI who progressed to AD (median level, 2842 pg/mL [interquartile range, 1882-3950 pg/mL]) compared with those with stable MCI (median level, 1752 pg/mL [interquartile range, 1024-2438 pg/mL]) (P = .004), and they were predictive of progression from MCI to AD (hazard ratio, 1.8 [95% CI, 1.1-2.9]; stratified by tertiles). Linear mixed-model analyses demonstrated that within-person levels of NGRN increased over time in cognitively normal participants (mean [SE] level, 90 [45] pg/mL per year; P < .05) but not in patients with MCI or AD.

CONCLUSIONS AND RELEVANCE Neurogranin is a promising biomarker for AD because levels were elevated in patients with AD compared with cognitively normal participants and predicted progression from MCI to AD. Within-person levels of NGRN increased in cognitively normal participants but not in patients with later stage MCI or AD, which suggests that NGRN may reflect presymptomatic synaptic dysfunction or loss.

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The core cerebrospinal fluid (CSF) biomarkers Aβ42, total tau, and tau phosphorylated at threonine 181 (P-tau$_{181}$) reflect the neuropathological hallmarks of Alzheimer disease (AD), amyloid plaques, and neurofibrillary tangles.[1] Clinically, AD is characterized by cognitive decline, but once a patient has AD pathology, these core CSF biomarkers appear not to reflect further functional decline owing to their relative stability during clinical AD.[2-4]

The synapse plays a central and essential role in cognitive function because it subserves neuronal transmission. Synaptic loss is an early event in the pathogenesis of AD[5] and has been shown to correlate with cognitive decline.[6] Biomarkers that reflect synaptic integrity could therefore be useful for both an accurate, early diagnosis and disease prognosis. A promising biomarker candidate is the postsynaptic protein neurogranin (NGRN),[7] which is expressed exclusively in the brain, particularly in dendritic spines.[7] Neurogranin binds to calmodulin in the absence of calcium and is involved in synaptic plasticity and long-term potentiation, processes essential for learning.[8] Decreased levels of NGRN have been reported in AD brain tissue samples compared with control samples,[9,10] and recent studies have reported increases in CSF NGRN levels in patients with AD compared with controls.[11,12] We aimed to evaluate the diagnostic and prognostic utility of NGRN as a CSF biomarker in a cohort of patients with AD or mild cognitive impairment (MCI) and cognitively normal participants, and to assess its dynamics during disease progression in longitudinal CSF samples obtained from participants over 2 years.

Methods

Participants

From the Amsterdam Dementia Cohort, we included 65 patients with AD, 61 patients with MCI, and 37 cognitively normal participants, all of whom had CSF samples obtained at 2 time points.[2] At baseline, all patients underwent standard dementia screening, including physical and neurological examinations, laboratory tests, electroencephalography, and magnetic resonance imaging. Cognitive screening included a Mini-Mental State Examination but usually also involved comprehensive neuropsychological testing. The diagnosis of probable AD was made according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association.[13] The diagnosis of MCI was made according to the criteria of Petersen et al.[14] All of the patients with probable AD or MCI experienced subjective cognitive decline, and, in addition, they scored in a cognitive domain below expected (<1 SD), and yet they did not have dementia. When the results of all examinations were normal, the patients were considered to have subjective memory complaints. The cognitively normal group consisted of 31 participants with subjective memory complaints, 2 participants with a psychiatric disorder (eg, depression), 2 participants with temporal epilepsy (treated with medication), and 2 healthy volunteers. Diagnoses were made by consensus of a multidisciplinary team. Our study was approved by the ethical review board of the VU University Medi-

cal Center in Amsterdam, the Netherlands, and all participants provided written informed consent.

Follow-up

Patients were followed up clinically on an annual basis. Of the 61 patients with MCI (with a mean [SE] follow-up of 2.7 [0.3] years), 17 remained stable, 36 progressed to AD,[13] and 8 progressed to other types of dementia (2 patients progressed to frontotemporal lobar degeneration,[15] 3 patients to vascular dementia,[16] 1 patient to dementia with Lewy bodies, 1 patients to progressive supranuclear palsy,[17] and 1 patient to normal-pressure hydrocephalus). Of the 37 cognitively normal participants (with a mean [SE] follow-up of 4.0 [0.5] years for 31 cognitively normal participants), 6 with subjective memory complaints progressed to MCI, 3 progressed to AD, 1 progressed to vascular dementia, and 27 remained stable. During follow-up, patients were asked to undergo a second lumbar puncture (minimum interval, 6 months). Owing to technical reasons (inadequate amount of CSF aliquoted in selected vials), NGRN was unavailable for 1 patient at baseline and 3 patients at follow-up.

Analyses of CSF Samples

Samples of CSF were obtained by standard lumbar puncture, using a 25-gauge Quincke needle, and collected in 10-mL polypropylene tubes. Within 2 hours, CSF samples were centrifuged at 1800g for 10 minutes at 4°C, aliquoted in polypropylene tubes of 0.5 or 1 mL, and stored at −80°C until further analysis of NRGN. Baseline CSF Aβ42, total tau, and P-tau$_{181}$ were measured with an enzyme-linked immunosorbent assay (INNOTEST ELISA; Fujirebio [formerly Innogenetics]) at the VU University Medical Center.[18] Because the manufacturer does not supply controls, the consistency of the assay's performance was monitored using pools of surplus CSF samples. The mean (SD) intra-assay coefficient of variation was 2.0% (0.5%) for Aβ42, 3.2% (1.3%) for total tau, and 2.9% (0.8%) for P-tau$_{181}$ as calculated from averaging the coefficient of variation of duplicates from 5 runs (with 36 samples each) randomly selected over 2 years. The mean (SD) interassay coefficient of variation was 10.9% (1.8%) for Aβ42, 9.9% (2.1%) for total tau, and 9.1% (1.8%) for P-tau$_{181}$, as analyzed in a high and low pool from 13 consecutive pool preparations used in total in 189 to 231 runs. Samples of CSF were analyzed for NGRN using a sandwich immunoassay developed on a Singulex Erenna system at Washington University in St Louis, Missouri.

NGRN Assay

Recombinant glutathione S-transferase (GST)–NGRN fusion protein was produced in pGEX-4T-1 (GE Healthcare Biosciences), expressed in *Escherichia coli*, and then purified according to the manufacturer. Rabbits were immunized with the GST-NGRN fusion protein at Harlan Bioproducts for Science (Madison, Wisconsin). Antiserum samples were first passed over a glutathione-GST column to remove anti-GST antibodies and then a glutathione-GST-NGRN column to obtain affinity-purified anti-NGRN antibodies. This material was epitope-mapped using spot-peptide membrane arrays, where spot 1 comprised residues 1 to 10, spot 2 residues 2 to 11, spot 3 residues 3 to 12, and so on, until the entire sequence of 78 residues was covered. Two peptides were synthesized as a result of this mapping experiment. Epitope affinity columns were prepared with N-terminal

Table 1. Baseline Characteristics of Patients in the Separate Clinical Diagnostic Categories

Characteristic	Cognitively Normal Participants (n = 37)	Patients With MCI (n = 61)	Patients With AD (n = 65)
Age, mean (SE), y	64 (2)	68 (1)[a]	65 (1)
Female sex, No. (%)	14 (38)	23 (38)	29 (45)
Mean (SE) baseline MMSE score[b]	28 (0.3)	27 (0.3)[a]	22 (0.7)[c,d]
APOE genotype, No./Total No. (%) of ε4 carriers[e]	15/36 (42)	33/58 (57)	45/64 (70)[a]
Follow-up time, mean (SE), y	2.4 (0.2)	2.0 (0.1)	1.9 (0.1)
Core CSF biomarkers, median (IQR), pg/mL			
Aβ42	704 (518-1010)	481 (369-651)[c]	407 (327-489)[c,d]
Total tau	304 (188-387)	527 (278-846)[c]	613 (422-878)[c]
P-tau$_{181}$	48 (36-72)	70 (45-102)[c]	80 (61-105)[c]
NGRN, median (IQR), pg/mL			
Baseline	1712 (1206-2724)	2391 (1418-3326)	2381 (1651-3416)[a]
Follow-up	2015 (1522-2997)	2909 (1686-3489)	2451 (1669-3124)

Abbreviations: AD, Alzheimer disease; CSF, cerebrospinal fluid; IQR, interquartile range; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; NGRN, neurogranin; P-tau$_{181}$, tau phosphorylated at threonine 181.

[a] $P < .05$ vs cognitively normal participants.

[b] Baseline scores (range, 0-30 [with 30 indicative of perfect performance]) were available for 160 patients, and follow-up scores were available for 148 patients; the mean (SE) cognitive follow-up period was 3.8 (0.2) years.

[c] $P < .01$ vs cognitively normal participants.

[d] $P < .01$ vs patients with MCI.

[e] The Fisher exact test or analysis of variance with post hoc Bonferroni corrections was used when applicable. The CSF biomarkers were log-transformed for analyses of variance, which were adjusted for sex and age.

peptide S10-D23 and C-terminal peptide G49-G60, each with a nonnative N-terminal cysteine for conjugation to the column. Synthetic 78-mer human NGRN was prepared and characterized by AAPPTec using C18-reversed phase-high performance liquid chromatography and electrospray ionization-mass spectrometry, and this material was used as the immunoassay standard. The stock mean (SD) concentration, prepared in phosphate-buffered saline with azide, was determined by amino acid analysis in triplicate (at AAA Service Laboratory, Inc, in Damascus, Oregon) and found to be 1.12 (0.03) mg/mL.

A sandwich immunoassay was developed for CSF on a Singulex Erenna system using the 2 epitope-specific rabbit antibodies (recognizing N-terminal epitope S11-D23 and C-terminal epitope G49-G60 antibodies). The C-terminal–specific antibody (P-4793) was coupled to magnetic beads and used as the capture antibody, and the N-terminal–specific antibody (P-4794) was labeled with a fluorescent dye and used as the capping/detection antibody. Three control CSF pools were prepared at concentrations corresponding to low (approximately 400 pg/mL), medium (approximately 1500 pg/mL), and high (approximately 2900 pg/mL) levels of NGRN. These were aliquoted and stored at −80°C for one-time use and included in every assay. For these 3 control

Table 2. Correlations of CSF Neurogranin With Core CSF Biomarkers

| Biomarker | Pearson Correlation Coefficient[a] | | |
	Cognitively Normal Participants (n = 37)	Patients With MCI (n = 61)	Patients With AD (n = 65)
Aβ42	−0.07	−0.18	0.23
Total tau	0.79[b]	0.89[b]	0.87[b]
P-tau$_{181}$	0.80[b]	0.88[b]	0.89[b]

Abbreviations: AD, Alzheimer disease; CSF, cerebrospinal fluid; MCI, mild cognitive impairment; P-tau$_{181}$, tau phosphorylated at threonine 181.

[a] Assessed with log-transformed baseline measures of the CSF biomarkers.

[b] $P < .001$.

CSF pools, the intra-assay precision (coefficient of variation) for 15 replicates was 6.6%, 7.1%, and 5.8%, respectively, and the interassay coefficient of variation for 23 assays was 5.7%, 6.2%, and 5.4%, respectively. The mean (SD) dilutional linearity recovery for 5 samples was 98.2% (9.45%), the mean (SD) spike recovery for 5 different samples was 104.8% (2.5%), and the lower limit of quantitation was approximately 2 pg/mL. To circumvent interassay variability, baseline and follow-up samples were analyzed on the same assay plate.[19,20] The teams involved in the CSF analysis were not aware of the clinical diagnoses.

Statistical Analysis

Cross-sectional differences among groups were assessed using analysis of variance, with post hoc Bonferroni corrections, or the Fisher exact test when applicable. The CSF biomarkers were log-transformed to fit the assumptions needed for analysis of variance and were adjusted for sex and age. Pearson correlations were assessed per diagnostic group using baseline log-transformed CSF biomarker levels. Cox proportional hazards models, adjusted for sex and age, were performed to analyze the predictive value of the CSF biomarkers for progression of MCI to AD and for progression of cognitively normal to either MCI or AD. For the Cox analyses, NGRN was evaluated as tertiles (<1666 pg/mL at tertile 1, 1666-2734 pg/mL at tertile 2, and >2734 pg/mL at tertile 3). Hazard ratios are presented with 95% CIs. Kaplan-Meier curves were created for illustrative purposes. Finally, age- and sex-adjusted linear mixed models were applied to assess within-person annual changes over time in CSF biomarker levels by diagnosis. The CSF NGRN level was the dependent variable, while diagnosis (treated as a categorical variable), time (ie, the interval between lumbar punctures in years; treated as a continuous variable), and the interaction between diagnosis and time were the independent variables. Diagnostic categories were recoded as dummy variables in order to estimate the mean (SE) values for each category. All linear mixed models were specified with a random intercept and/ or slope based on −2LL (minus twice the log likelihood) criteria.[21] For statistical analyses, we used SPSS Statistics 21.0 (for Windows; IBM). Statistical significance was set at $P < .05$.

Results

Baseline Characteristics

The baseline characteristics of the patients are shown in **Table 1**. The baseline levels of NGRN in the patients with AD were higher than those in the cognitively normal participants ($P = .04$).

Table 3. Progression From MCI to AD

Variable	Patients With Stable MCI (n = 17)	Patients With MCI Progressing to AD (n = 36)	Risk of Progression to AD, HR (95% CI)
Age, mean (SE), y	64 (2)	70 (1)[a]	
Female sex, No. (%)	6 (35)	13 (36)	
Mean (SE) baseline MMSE score[b]	28 (0.6)	26 (0.4)[a]	
CSF biomarkers,[c] median (IQR), pg/mL			
Aβ42	579 (493-814)	410 (322-507)[d]	1.6 (1.0-2.6)
Total tau	274 (212-418)	739 (463-950)[d]	2.3 (1.4-3.7)
P-tau$_{181}$	47 (40-79)	90 (65-124)[d]	2.1 (1.3-3.5)
Neurogranin	1752 (1024-2438)	2842 (1882-3950)[d]	1.8 (1.1-2.9)

Abbreviations: AD, Alzheimer disease; CSF, cerebrospinal fluid; HR, hazard ratio; IQR, interquartile range; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination.

[a] $P < .05$ vs patients with stable MCI.

[b] Baseline scores (range, 0-30 [with 30 indicative of perfect performance]) were available for 52 patients.

[c] Cox analyses were used with CSF biomarkers in tertiles (binning was performed for all included patients). The Fisher exact test or analysis of variance was used when applicable. The CSF biomarkers were log-transformed for analyses of variance, which were adjusted for sex and age. For Aβ42, the tertiles were inverted.

[d] $P < .01$ vs patients with stable MCI.

Figure 1. Kaplan-Meier Curve for Progression From Mild Cognitive Impairment (MCI) to Alzheimer Disease (AD), Stratified by Tertiles of Neurogranin Levels

No. at risk
Patients with MCI 53   46   28   17
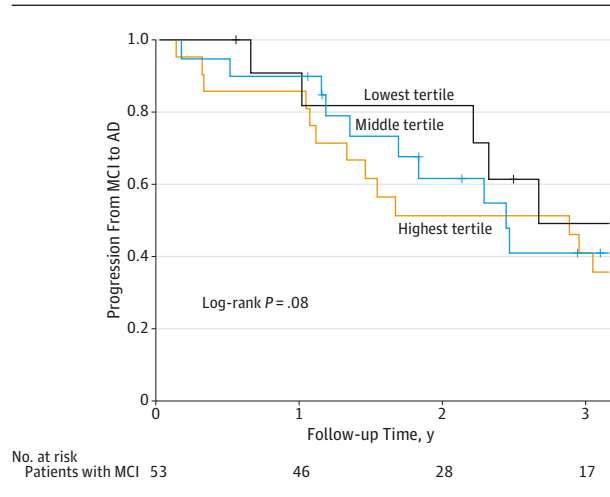
Neurogranin levels were evaluated at the lowest tertile (<1666 pg/mL), the middle tertile (1666-2734 pg/mL), and the highest tertile (>2734 pg/mL). The lowest tertile was used as reference.

Figure 2. Annual Change in Neurogranin (NGRN) Levels
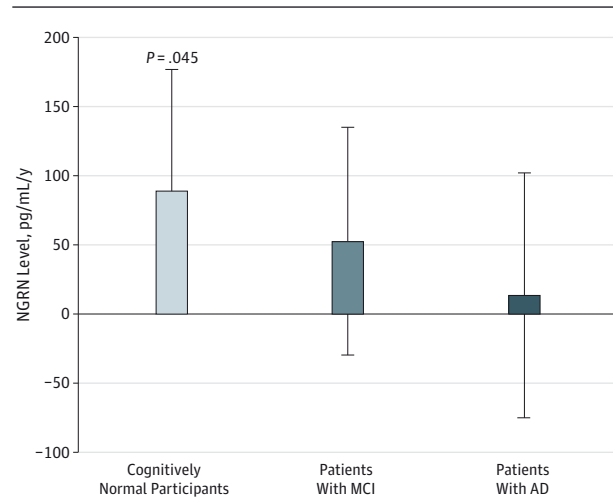
The annual change in NGRN levels obtained from samples of cerebrospinal fluid were assessed using age- and sex-adjusted linear mixed models. The NGRN level was the dependent variable, and clinical diagnosis (treated as a categorical variable), time (ie, the interval between lumbar punctures in years; treated as a continuous variable), and the interaction between diagnosis and time were the independent variables. The reported β represents the estimated change in NGRN levels per year, and the error bars represent the 95% CIs of the reported effects.

There were no differences in NGRN levels between the patients with MCI and the patients with AD or between the patients with MCI and the cognitively normal participants ($P = .25$ and $P = .31$). Baseline NGRN levels were strongly positively correlated with total tau and P-tau$_{181}$ levels in all clinical groups but were not correlated with Aβ42 level, as shown in Table 2.

Predictive Value of Baseline Levels of Novel Biomarkers for Progression

Further analyses with analysis of variance showed that baseline levels of NGRN were higher in patients with MCI who progressed to AD than in patients with MCI who remained stable. Cox regression analyses revealed that baseline NGRN levels were predictive of progression from MCI to AD in the same order of magnitude as the core biomarkers Aβ42, total tau, and P-tau$_{181}$, as shown in Table 3 and illustrated by Figure 1. In the

cognitively normal group, there was a trend, albeit nonsignificant, of NGRN levels predicting progression within the AD continuum (hazard ratio, 1.6 [95% CI, 0.64-4.0]).

Longitudinal Changes in Novel Biomarkers

Linear mixed-model analyses showed that levels of NGRN increased within cognitively normal participants (mean [SE] level, 90 [45] pg/mL per year; $P < .05$) but not in patients with MCI (mean [SE] level, 53 [42] pg/mL per year; $P = .22$) or AD (mean [SE] level, 14 [45] pg/mL per year; $P = .75$), as shown in Figure 2. This indicates that NGRN levels increase in a very early (asymptomatic) stage but not in later stages of the AD continuum.

Discussion

In our longitudinal study with repeated CSF sampling, we showed that NGRN has potential as a novel biomarker for synaptic dysfunction in AD. Baseline NGRN levels are higher in patients with MCI who progressed to AD than in patients with MCI who were clinically stable, and these higher levels predicted progression to AD. In addition, levels of NGRN were higher in patients with AD than in cognitively normal participants. Longitudinally, we showed that levels of NGRN increased over time in participants who were cognitively normal but not in patients with MCI or AD who were already cognitively impaired.

Our study confirms that CSF levels of NGRN are increased in patients with AD compared with cognitively normal participants.[11,12] This is consistent with postmortem studies in both mouse models[22] and human tissue.[10] The measurement of CSF NGRN level could possibly aid in the early diagnosis of AD. In addition, we show that NGRN levels are higher in patients with MCI who progress to AD than in patients with MCI who remain stable and that levels are predictive of progression from MCI to AD, as has recently also been reported by another group in a similar cohort using a different assay.[12] Thus, the CSF NGRN level appears to have a prognostic as well as a diagnostic value.

Levels of NGRN in CSF were much higher in the present study than in the study by Kvartsberg et al,[12] which also used an enzyme-linked immunosorbent assay. Differences in absolute values may be a result of the different antibodies used in the 2 assays. Further studies comparing both enzyme-linked immunosorbent assays are needed to clarify this matter. However, the direction and order of magnitude of the variance between patient groups were the same. It is possible that differences in the calibration and/or antibodies used contributed to the absolute differences. Among the limitations, the cognitively normal group was a mixed group of participants that also included patients with psychiatric disorders and temporal lobe epilepsy, which may hamper generalizability. In addition, our cognitively normal group was biased toward participants who showed decline (6 progressed to MCI, and 4 to dementia), which could have even diluted the baseline effect. This bias could be due to the fact that progressors are more likely to return to our clinic for a second lumbar puncture.[2] However,

thanks to this follow-up, we were able to evaluate change over time in NGRN levels for all stages of the AD continuum. Another limitation was that the size of the group of cognitively normal participants was too small to reliably analyze for risk of progression with Cox proportional hazards models. However, the trend was in the same order of magnitude as shown for the group of patients with MCI. This supports the notion that synapse loss is a very early process during AD pathogenesis.

With our longitudinal analyses, we found that NGRN levels increased in time in cognitively normal participants but not in patients with MCI or AD. This pattern is also consistent with the view that synaptic changes mainly occur in the earliest phase of the AD continuum, even before the stage of MCI.[23] Within the AD continuum (as proposed by Jack et al[24]), it could be hypothesized that CSF NGRN levels increase very early. Further studies are needed to confirm our findings. Additional cross-sectional studies are needed to clarify whether NGRN is specific for AD, or whether it also reflects synaptic changes in other neurodegenerative diseases.[25]

We observed high positive correlations between CSF NGRN levels and both total tau and P-tau$_{181}$ levels, but not with Aβ42 levels. The lack of correlation between NGRN level and Aβ42 level is in line with studies showing that both synapse loss and clinical stage are unrelated to the amount of amyloid plaques.[26-28] The CSF total tau level, on the other hand, is related to cognitive deterioration, likely reflecting neuronal cell death.[29-31] The NGRN level could be a more specific marker for pathological changes that lead to cognitive deterioration because it represents the more specific, and potentially earlier, process of synapse loss. Importantly, it reflects a mechanism that could be useful in treatment trials to monitor the effects of drugs on synaptic integrity.

Conclusions

In summary, NGRN levels are lower in the cognitively normal participants than in patients with AD, and then they increase over time. Furthermore, increased levels of NGRN are associated with progression to AD in patients with MCI. In addition to the "core CSF biomarkers" Aβ42, total tau, and P-tau$_{181}$, the NGRN level could have added value because it is a reflection of a pathophysiological process that is directly related to cognitive changes (ie, synapse function).

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