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RESEARCH ARTICLE

Thimet oligopeptidase as a potential CSF biomarker for Alzheimer's disease: A cross-platform validation study

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Abstract

INTRODUCTION: Our previous antibody-based cerebrospinal fluid (CSF) proteomics study showed that Thimet oligopeptidase (THOP1), an amyloid beta (Aβ) neuropeptidase, was increased in mild cognitive impairment with amyloid pathology (MCI-Aβ+) and Alzheimer's disease (AD) dementia compared with controls and dementia with Lewy bodies (DLB), highlighting the potential of CSF THOP1 as an early specific biomarker for AD. We aimed to develop THOP1 immunoassays for large-scale analysis and validate our proteomics findings in two independent cohorts.

METHODS: We developed in-house CSF THOP1 immunoassays on automated Ella and Simoa platforms. The performance of the different assays were compared using Passing–Bablok regression analysis in a subset of CSF samples from the discovery cohort ($n = 72$). Clinical validation was performed in two independent cohorts (cohort 1: $n = 200$; cohort 2: $n = 165$) using the Ella platform.

RESULTS: THOP1 concentrations moderately correlated between proteomics analysis and our novel assays ($Rho > 0.580$). In both validation cohorts, CSF THOP1 was increased in MCI-Aβ+ (>1.3-fold) and AD (>1.2-fold) compared with controls; and between MCI-Aβ+ and DLB (>1.2-fold). Higher THOP1 concentrations were detected in AD compared with DLB only when both cohorts were analyzed together. In both cohorts, THOP1 correlated with CSF total tau (t-tau), phosphorylated tau (p-tau), and Aβ40 ($Rho > 0.540$) but not Aβ42.

DISCUSSION: Validation of our proteomics findings underpins the potential of CSF THOP1 as an early specific biomarker associated with AD pathology. The use of antibody-based platforms in both the discovery and validation phases facilitated the translation of proteomics findings, providing an additional workflow that may accelerate the development of biofluid-based biomarkers.

KEYWORDS
Alzheimer's disease, biomarkers, CSF, THOP1

1 | BACKGROUND

Alzheimer's disease (AD) is the most common form of dementia worldwide, affecting over 50 million people, with numbers rising every year.[1] The underlying neuropathology of AD, the accumulation of amyloid beta (Aβ) plaques, neurofibrillary tangles (NFTs), and overall neurodegeneration can be measured in the cerebrospinal fluid (CSF) to support AD diagnosis.[2] However, other dementia types such as dementia with Lewy bodies (DLB) can have AD co-pathology and overlapping clinical features, which lead to up to 15% of AD misdiagnosis.[3,4] Furthermore, biological therapies targeting distinct pathological mechanisms of AD have recently become available in the United States.[5,6] Therefore, several biomarkers will likely be required to cover different contexts of use in clinical settings and trials (e.g., differential diagnosis, prognosis, disease stage and monitoring, patient selection and stratification, monitoring of treatment effects).

Unraveling novel fluid biomarkers is a long and multidisciplinary process including many phases, starting from biomarker discovery, followed by analytical (including immunoassay development) and clinical validation, and finally, by implementation in clinical settings.[7–10] To date, unbiased mass spectrometry (MS) proteomics discovery studies have uncovered numerous CSF biomarker candidates.[11–13] However, only a few of these biomarker candidates have made it through the thorough analytical and clinical validation phases (or so-called qualification and verification).[14,15] This might be explained by the use of different technological platforms between the biomarker discovery (e.g., MS) and validation phases (e.g., antibody-based immunoassays). Although MS identifies peptides from trypsinized proteins, antibody-based technologies detect proteins in their native conformation, which may explain to some extent the cross-technology translational gap often encountered in biomarker studies.[8] The emergence of antibody-based proteomics platforms (e.g., proximity extension assays; PEA technology) together with the use of more sensitive and automated immunoassay-based technologies may smoothen the development of optimal immunoassays for high-throughput screening and facilitate the validation and ultimately clinical implementation of fluid biomarkers.[7,8,16–19]

We previously used antibody-based multiplex arrays to map the CSF proteome of AD and a group of non-AD dementias, which revealed that Thimet oligopeptidase 1 (THOP1) is increased in patients with mild cognitive impairment (MCI) and AD dementia compared with other dementia groups (e.g., DLB and frontotemporal dementia [FTD]) and controls. In addition, a data-driven modeling approach selected THOP1 within a CSF biomarker panel that discriminated AD from a group of non-AD dementias.[18] THOP1 is a neuropeptidase able to cleave Aβ peptides among others.[20,21] It co-localizes with Aβ plaques and NFTs in the brain from AD-disease models and patients with AD.[22,23] Moreover, THOP1 concentrations were increased in temporal cortex tissue with AD pathology compared to controls, suggesting that protein concentrations in CSF could reflect brain-specific changes.[22] The increased concentrations in MCI and AD stages, and the brain-specific changes observed previously, underpin the potential of THOP1 as a novel and specific CSF biomarker increasing along the symptomatic continuum of AD.

RESEARCH IN CONTEXT

1. **Systematic review**: We have previously performed an large antibody-based CSF proteomics study, which showed increased concentrations of the amyloid-beta neuropeptidase THOP1, in MCI-Aβ+ and AD compared with controls and patients with DLB. Increased THOP1 levels were also detected in AD post-mortem brain tissue, highlighting the potential use of CSF THOP1 as an early specific biomarker for AD.
2. **Interpretation**: Our findings show that THOP1 could be a useful biomarker to detect an early neuroprotective response against AD pathology or as part of a CSF protein panel to discriminate AD from other neurodegenerative dementia. The use of antibody-based platforms in both the discovery and validation phases facilitated the translation of proteomics findings, providing an additional workflow that may accelerate the development of fluid biomarkers.
3. **Future directions**: The clinical context of use for THOP1 should be explored in future studies.

In this study, we aimed to develop specific CSF THOP1 immunoassays and validate our discovery findings using two independent clinical cohorts. This approach allowed us to additionally evaluate whether the use of immunobased platforms for both discovery and validation can accelerate the biomarker development workflow using CSF THOP1 as an example.

2 | METHODS

2.1 | Patient cohorts

To evaluate the analytical performance of our in-house immunoassays, we selected patients from the Amsterdam Dementia Cohort (ADC) also included in our original discovery study; 24 AD, 24 DLB, and 24 controls (Additional File 1; Table S1).[18,24] For clinical validation of CSF THOP1 changes, we selected two independent clinical cohorts: the Sant Pau Initiative on Neurodegeneration cohort (SPIN; total = 200, including 53 controls, 50 MCI-Aβ+, 47 AD, and 50 DLB) and the ADC (total = 165, including 33 controls, 44 MCI-Aβ+, 34 AD, and 54 DLB. A subset of the DLB cases (n = 27) were selected from the DEmEntia with LEwy bOdies Project (DEvELOP).[24–27]

All participants in each center, including patients with MCI, AD, and DLB were diagnosed according to consensus guidelines as described previously.[28–30] Concentrations of Aβ42, Aβ40, phosphorylated tau

TABLE 1 Patient characteristics validation cohorts.

	Validation cohort 1			
	Control	**MCI-Aβ+**	**AD**	**DLB**
N	53	50	47	50
Sex = F (%)	32 (60.4)	24 (48.0)	30 (63.8)	18 (36.0)
Age (years)	61 (58–66)[a-c]	72 (68–75)[c,d]	73 (68–76)[c,d]	78 (74–80)[a,b,d]
MMSE	29 (29–30)[a-c]	26 (25–28)[b,c]	23 (19–25)[a,d]	23 (19–24)[a,d]
Aβ42 (pg/mL)	1194 (903–1397)[a-c]	518 (425–648)[c,d]	521 (413–621)[c,d]	677 (497–943)[a,b,d]
Aβ40 (pg/mL)	12346 (9800–15103)	12290 (10348–14734)	13116 (10332–14494)	10452 (9063–14799)
t-tau (pg/mL)	257 (194–309)[a-c]	610 (489–862)[c,d]	762 (556–966)[c,d]	409 (283–761)[a,b,d]
p-tau (pg/mL)	36 (27–48)[a-c]	98 (82–135)[c,d]	125 (82–158)[c,d]	64 (41–115)[a,b,d]
APOE ε4 carrier (%)	13 (24.5)	25 (50.0)	22 (46.8)	17 (34.0)
	Validation cohort 2			
	Control	**MCI-Aβ+**	**AD**	**DLB**
N	33	44	34	54
Sex = F (%)	11 (33.3)	19 (43.2)	14 (41.2)	9 (16.7)
Age (years)	58 (54–61)[a-c]	67 (64–71)[d]	66 (62–68)[c,d]	69 (66–73][b,d]
MMSE	28 (27–29)[a-c]	26 (25–27)[b-d]	20 (18–23)[a,c,d]	24 (22–26)[a,b,d]
Aβ42 (pg/mL)	1491 (1292–1700)[a-c]	835 (672–976)[d]	649 (543–786)[c,d]	841 (659–1140)[b,d]
t-tau (pg/mL)	154 (134–182)[a-c]	288 (241–358)[c,d]	327 (253–470)[c,d]	187 (145–248)[a,b,d]
p-tau (pg/mL)	16 (12–64)[a,b]	86 (80–93)[c,d]	86 (76–99)[c,d]	29 (16–71)[a,b]
APOE ε4 carrier (%)	7 (21.2)	33 (75.0)	24 (70.6)	28 (51.9)

Note: Continuous data are represented as median ± interquartile range and dichotomous data as the number of cases with a percentage of the total (%). Differences between groups were determined using the Kruskal–Wallis test with Bonferroni correction or the chi-square test.
Abbreviations: AD, Alzheimer's disease; Aβ40, amyloid beta 1-40; Aβ42, amyloid beta 1-42; DLB, dementia with Lewy bodies; F, female; MCI-Aβ+, mild cognitive impairment with amyloid pathology; MMSE, Mini-Mental State Examination; p-tau, phosphorylated tau; t-tau, total tau.
[a] $p < 0.05$ compared to MCI.
[b] $p < 0.05$ compared to AD.
[c] $p < 0.05$ compared to DLB.
[d] $p < 0.05$ compared to controls.

(p-tau), and total tau (t-tau) (i.e., core AD CSF biomarkers) were used to support AD diagnosis and measured in each center using commercially available kits (SPIN: Lumipulse G β-Amyloid 1-42, β-Amyloid 1-40, Total Tau and pTau 181 on Lumipulse G600 automated platform, Fujirebio Ghent, Belgium; and ADC: Innotest ELISA INNOTEST Aβ(1-42), hTAUAg, pTau (181P), or Elecsys Aβ42, t-tau and p-tau (181P) CSF assays [Roche Diagnostics], and Lumipulse G Amyloid 1-40). In the ADC, enzyme-linked immunosorbent assay (ELISA) CSF concentrations were corrected for the drift in biomarker concentrations that occurred over the years.[31] Patients with AD dementia were included if all the classical CSF biomarkers were positive (SPIN: 0.062 < Aβ42/Aβ40 ratio, tTau > 456 pg/mL and pTau > 63 pg/mL; ADC ELISA: Aβ42 < 813 pg/mL, pTau > 52 pg/mL, and tTau > 375 pg/mL; ADC Elecsys: Aβ42 < 1000 pg/mL, pTau > 19 pg/mL, and tTau > 235 pg/mL).[32,33] Controls with subjective cognitive decline were included if no signs of dementia were present during the diagnostic work-up. All the controls included in the study were negative for any of the classical AD CSF biomarkers.[32,33] Informed consent was obtained from all participants or their authorized representatives, following the ethical consent by the VU University Medical Center Amsterdam and with the Helsinki Declaration of 1975. Patient characteristics of the validation cohorts are summarized in Table 1.

CSF was collected and biobanked according to established protocols,[34,35] CSF samples from the discovery cohort had two freeze and thaw (f/t) cycles prior to analysis.

2.2 | In-house immunoassay

We sought to determine whether we were able to set up a THOP1 immunoassay for measurements in CSF. Therefore, we first developed two immunoassays on different analytical platforms (i.e., Ella and Simoa); both assays have the same antibody setup. The capture antibody was a polyclonal anti-human THOP1 antibody (1 mg/mL, R&D systems, AF3439), and the biotinylated polyclonal anti-human THOP1 antibody (0.2 mg/mL, R&D systems, BAF3439) was used as a detection antibody. For both assays, the calibration curve was prepared with THOP1 recombinant human protein (aa2-aa689, 0.4mg/mL, R&D systems, 3439-ZN-010). To continue clinical validation, the platform with the lowest coefficients of variation (CV)%, higher sensitivity, and

stronger correlation with PEA-based proteomics results was considered to perform best.

2.3 | THOP1 Ella assay

An automated Ella immunoassay for the detection of THOP1 concentrations in CSF was developed following manufacturer's instructions (ProteinSimple, Bio-Techne, San Jose, California, USA).[36] First, THOP1 capture antibody (1 mg/mL, R&D systems) was conjugated to digoxigenin-*N*-hydroxysuccinimide ([NHS] 0.67 mg/mL, Sigma-Aldrich, Saint-Louis, USA) using sodium bicarbonate (75 mg/mL, Sigma-Aldrich), which will bind the anti-digoxigenin antibodies in the 48-Digoxigenin cartridges (ProteinSimple) by incubating reagents for 1 hour in the dark at room temperature. Thereafter, unreacted digoxigenin-NHS molecules were removed by purification across the Zeba Spin Desalting Columns (40K MWCO, Thermo Fisher Scientific, Waltham, USA). The concentration from conjugated THOP1 antibodies was measured by Nanodrop Spectrophotometer (Isogen Lifescience, Utrecht, The Netherlands) and stored at 4°C until further use.

The final THOP1 Ella assay included THOP1 digoxigenin(dig)-conjugated capture antibody (3.5 μg/mL), THOP1 biotinylated detection antibody (5.0 μg/mL), and calibration curve with THOP1 recombinant protein ranging from 1000 pg/mL to 156 pg/mL. All reagents were diluted in 1% casein-PBS (phosphate-buffered saline; Thermo Fisher Scientific). CSF samples were diluted four times in sample diluent (1% casein-PBS) and measured in triplicate, automatically performed by the technology, on the customizable 48-Digoxigenin cartridges (ProteinSimple). Concentrations of THOP1 were calculated from a five-parameter logistic (5-PL) calibration curve using the Simple Plex Explorer Software (ProteinSimple, version 3.7.1.12). The assay was validated analytically in-house for parallelism, dilutional linearity, recovery, protein stability, and lower limit of detection (LLOD) parameters, following international guidelines for immunoassay validation (Additional File 1).[37]

2.4 | THOP1 Simoa assay

In parallel, a THOP1 immunoassay was developed on the Simoa HD-X automated immunoassay platform (Quanterix, Billerica, Massachusetts, USA) following the manufacturer's protocol (Quanterix). First, the THOP1 capture antibody (1 mg/mL, R&D systems) was coupled to carboxylated paramagnetic beads (Quanterix), which were activated with 0.3 mg/mL EDC (Thermo Fisher Scientific). Capture antibody was diluted in assay buffer, containing 0.5% casein-PBS (Thermo Fisher Scientific) with 0.1% Tween20 (Merck Millipore, Burlington, Massachusetts, USA) to 0.2 mg/mL. Our in-house assay followed an automated two-step procedure. In step one, non-reactive paramagnetic beads (Helper beads, Quanterix) at a ratio of 250K:250K (assay bead to helper bead ratio), diluted in Bead Diluent (Quanterix), were incubated with 250 μL sample (CSF diluted eight times or calibration curve from 500 to 7.8 pg/mL with THOP1 recombinant protein) and THOP1 detection antibody (1 μg/mL, R&D systems) for 60 min, all diluted in assay buffer (0.5% casein-PBS, 0.1% Tween20). Followed by a washing procedure, step two included incubation with streptavidin-β-galactosidase (SβG; 50 pM, Quanterix) for 5 min and 15 s. After a second washing procedure, resorufin-β-d-galactopyranoside (RGP, Quanterix) was added and fluorescent imaging in Average Enzymes per Bead (AEB) was performed. CSF samples were measured in duplicate, per manufacturer's instructions, and concentrations were calculated from a four-parameter logistic (4-PL) fit calibration curve using the Simoa HD-X Analyzer Software (v.3.0.2003.4001). This assay was analytically validated following international guidelines for immunoassay validation (Additional File 1).[37]

2.5 | Statistical analysis

Data analysis was performed with R version 4.0.3 (packages emmeans, mcr, and pROC).[38–40] To compare THOP1 between our in-house assays and previous PEA-based proteomics results, Spearman *Rho* correlations analysis was performed.[18] Passing–Bablok regression analysis and Bland–Altman plots were carried out to compare THOP1 concentrations between the Ella and Simoa platforms.

Normal data distribution was assessed by Shapiro–Wilk test. THOP1 concentrations were log-transformed and the influence of covariates (age and sex) was determined by linear regression analysis. To determine THOP1 differences between clinical groups, analysis of covariance (ANCOVA) adjusted for age was performed using log-transformed THOP1 values followed by Bonferroni post-hoc correction. Post-hoc correction was based on the number of groups in each comparison (i.e., discovery cohort corrected for three comparisons and validation cohorts for six comparisons).[41] To determine whether there are THOP1 differences between AD and DLB without AD co-pathology, subsequent analysis was performed by stratifying the DLB group for amyloid status (negative amyloid status: cohort 1: CSF Aβ42/Aβ40 ratio > 0.062; cohort 2: CSF Aβ42 > 813 pg/mL). Independent clinical cohorts were analyzed separately. As a sensitivity analysis, ANCOVA analysis was repeated using both cohorts together, including the center as an additional confounder.

The associations between untransformed THOP1 concentrations with CSF biomarkers concentrations and Mini-Mental State Examination (MMSE) scores were determined. The CSF biomarker concentrations on the Elecsys were first transformed to the predicted ELISA concentrations using formulas determined previously.[42] Thereafter, associations were tested with Spearman *Rho* correlations (<0.3 = weak, 0.3–0.5 = moderate, >0.5 = strong correlation).[43] *p*-values <0.05 were considered statistically significant.

3 | RESULTS

3.1 | THOP1 immunoassays show good analytical performance and correlated with the proteomics platform

THOP1-specific immunoassays were developed on the Ella and Simoa automated platforms. Our THOP1 assay on the Ella platform was

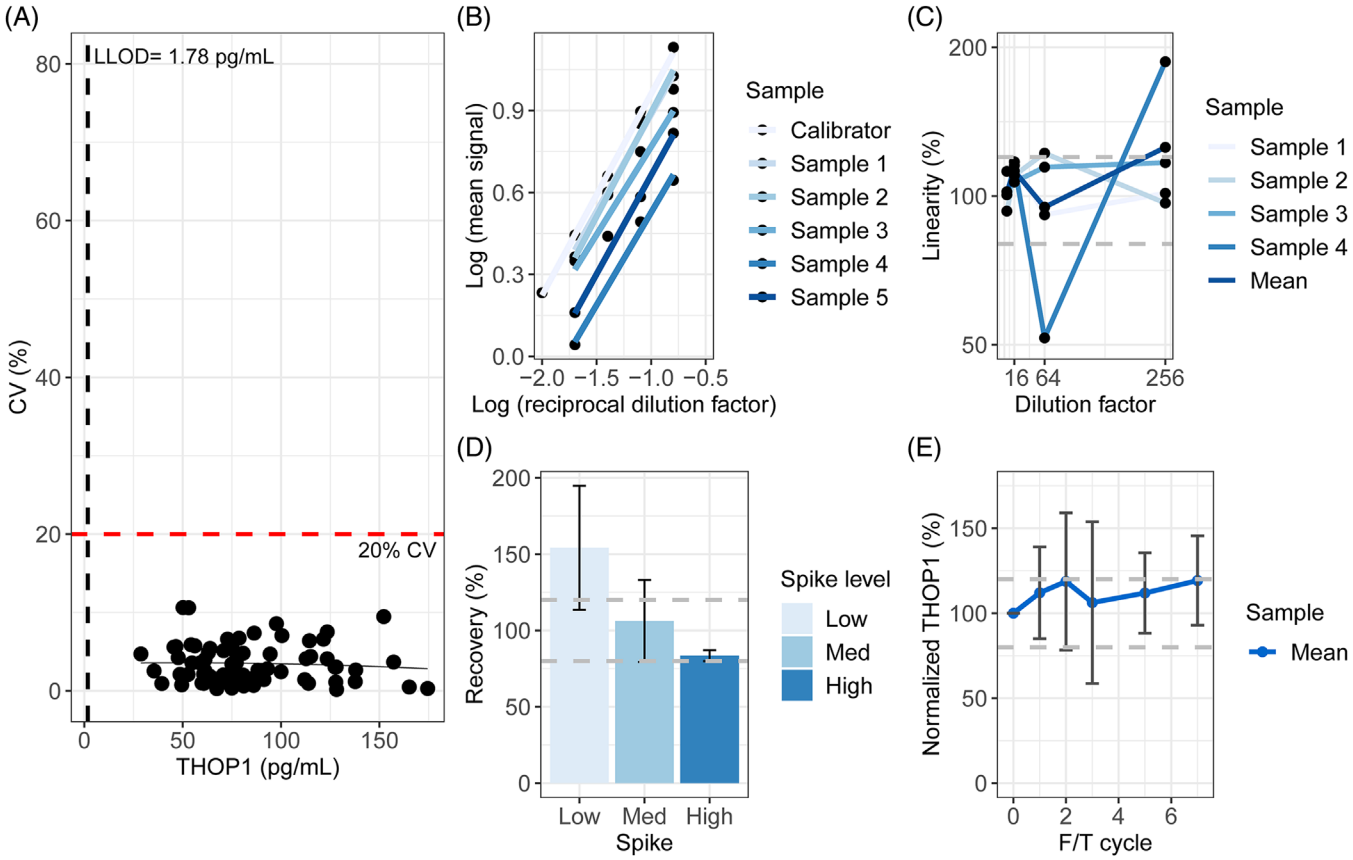

FIGURE 1 THOP1 assay was developed and analytically validated on the Ella platform. (A) The CV% of CSF samples with triplicate or duplicate measurements is plotted against the average THOP1 concentration. Precision plots show that all samples had a CV% <20 and all samples were above the LLOD of 1.78 pg/mL. (B) THOP1 Ella assay showed the THOP1 signal in CSF samples (log-transformed relative fluorescence unit; RFU signal) following a two-fold serial dilution (reciprocal relative dilution, log-transformed) was parallel to the signal obtained from the standard curve. (C) RFU signal of CSF samples measured in a serial dilution shows that the mean %linearity of the assay is within range (85%–115%). Graph is plotted with a log-transformed y-axis. (D) % Recovery of low, medium, and high spiked CSF samples measured on the Ella platform detects that only the medium and high spiked CSF samples were within range. (E) THOP1 concentrations in CSF samples were normalized to reference condition with zero freeze and thaw (f/t) cycles and presented in % as the mean of three samples, which showed stable protein concentrations up to seven f/t cycles. Dashed lines show the acceptance range of 85% to 115%. Error bars in D and E represent the standard deviation of the four and three CSF samples measured, respectively. Abbrevations: CSF, cerebrospinal fluid; CV, coefficient of variation; LLOD, lower limit of detection; THOP1, thimet oligopeptidase.

developed within 4 weeks, full-time, by one experienced technician (Figure S1A) and showed optimal parallelism and dilutional linearity, both between the acceptance criteria of 85% and 115% (Figure 1B–C). The mean recovery of low-spiked samples was not within the acceptable criteria; however, samples with medium or high spikes showed acceptable recovery (Figure 1D). THOP1 concentrations were not influenced by different f/t cycles (Figure 1E). The LLOD of this THOP1 assay was 1.78 pg/mL and precision plots show that THOP1 concentrations were detectable in 100% of the CSF samples ($n = 72$), all with CVs below 20% (Figure 1A). Intra- and inter-assay CVs were 5.9% and 14.2%, respectively.

The THOP1 in-house Simoa assay was developed within 6 weeks, full-time, by one experienced technician (Figure S1B) and showed optimal parallelism and recovery performance (between 85% and 115%, Figure 2B–C). Results for dilutional linearity within the lower dilutions (1:4 and 1:16) were not within the acceptance criteria, whereas in the higher dilutions (dilution 1:64 to 1:256; the range of unspiked CSF samples) good analytical performance was observed (Figure 2D). THOP1 concentrations on the Simoa platform were increased after two f/t cycles but such an effect was driven by one sample outlier and is thus likely not reflecting changes in protein measurements (Figure 2E). The LLOD was determined as 5.2 pg/mL and precision plots show that THOP1 concentrations were detectable in 98.6% of the CSF samples ($n = 71$), but three samples had a CV above 20% (Figure 2A). Intra- and inter-assay CVs were determined as 10.2% and 19.3%, respectively.

Next, we measured a subset of CSF samples from our discovery cohort to verify our in-house immunoassays. We observed that THOP1 concentrations measured on the Ella platform showed a stronger correlation to the PEA-proteomic platform compared to the Simoa assay (Ella: $Rho = 0.720$, Simoa: $Rho = 0.584$, both $p < 0.001$, Figure 3A–B). In addition, a strong correlation was observed between our novel

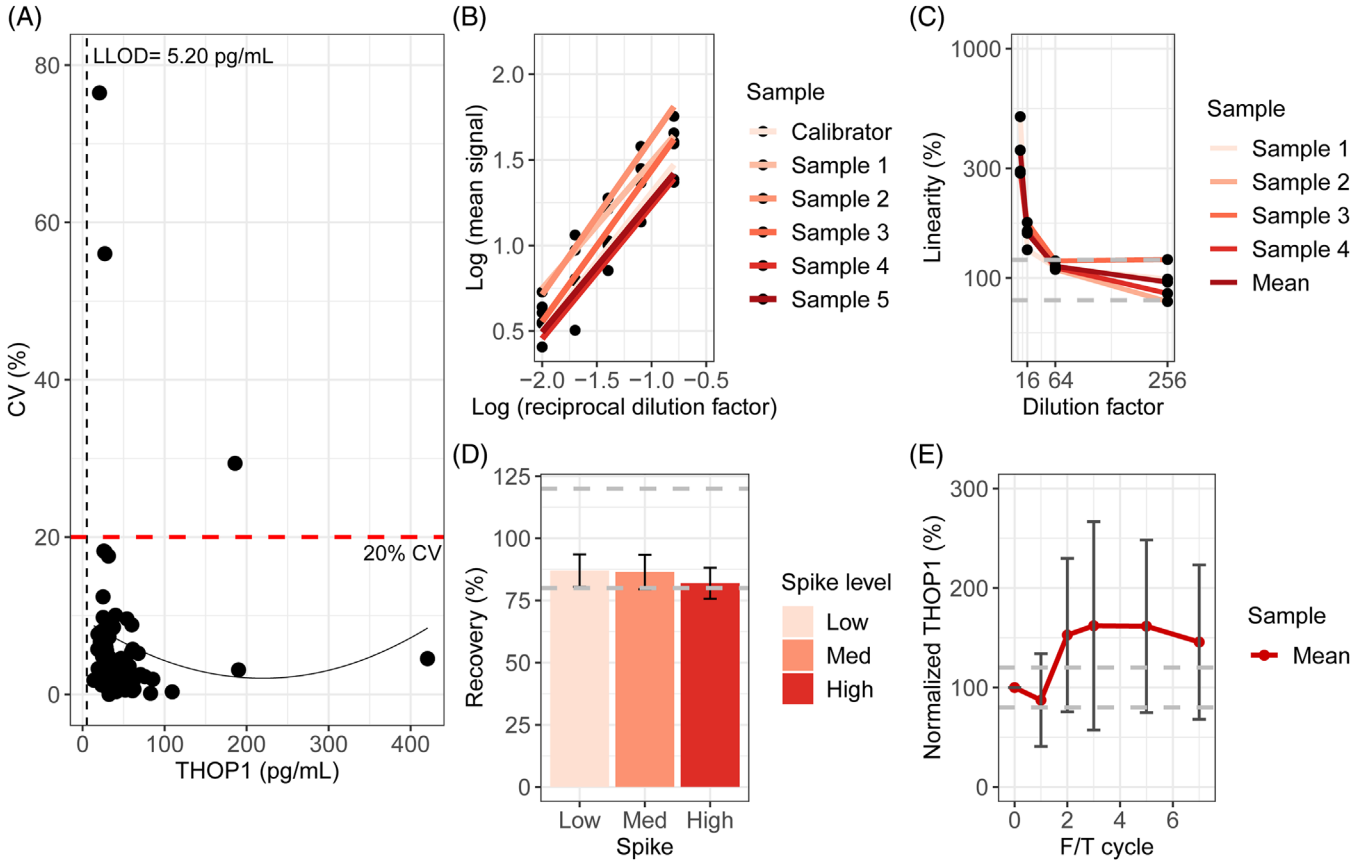

FIGURE 2 THOP1 assay was developed and analytically validated on the Simoa platform. (A) The CV% of CSF samples with duplicate measurements are plotted against the average THOP1 concentration. Precision plots show that three samples had a CV% >20 and all samples were above the LLOD of 5.2 pg/mL. (B) THOP1 concentrations on the Simoa platform show that levels in CSF samples following a two-fold serial dilution are parallel to the signal obtained from the standard curve. (C) AEB signals of CSF samples measured in a serial dilution show a matrix effect in dilutions 1–4. Upon further diluting, the % linearity is within the acceptable range. Graph is plotted with a log-transformed y-axis. (D) % Recovery of low, medium, and high spiked CSF samples measured on the Simoa platform show that all samples are within range. (E) THOP1 concentrations are stable up to two freeze and thaw (f/t) cycles. Dashed lines show the acceptance range of 85% to 115%. Error bars in D and E represent the standard deviation of the four or three CSF samples measured, respectively. Abbrevations: AEB, average enzymes per bead; CSF, cerebrospinal fluid; CV, coefficient of variation; LLOD, lower limit of detection; THOP1, thimet oligopeptidase.

in-house assays (*Rho* = 0.713, $p < 0.001$, Figure 3C). This was further confirmed by Passing–Bablok regression analysis, which showed platform agreement between the THOP1 Ella and Simoa assays (Figure S2A). The Bland–Altman plot showed acceptable variation between immunoassays (Figure S2B). Similar to our proteomics findings, we observed that THOP1 was increased in patients with AD compared to DLB (Ella: 1.6-fold, Simoa: 1.7-fold; both $p < 0.001$) and controls (Ella: 1.8-fold, $p < 0.001$; Simoa: 1.7-fold, $p = 0.07$; Figure 3D–F). Furthermore, both THOP1 assays discriminated AD from controls and DLB patients with high accuracy (AD vs controls: Ella area under the curve [AUC] = 0.947, 95% confidence interval [CI]: 0.892–1 and Simoa AUC = 0.840, 95% CI: 0.703–0.977; AD vs DLB: Ella AUC = 0.927, 95% CI: 0.86–0.994, and Simoa AUC = 0.809, 95% CI: 0.668–0.950; Figure S3). Despite both platforms showing comparable results, the THOP1 Ella assay showed slightly lower CVs% and higher sensitivity, therefore, we continued the clinical validation in two independent cohorts on the Ella platform.

3.2 | CSF THOP1 concentrations were highest in MCI-Aβ+ and AD groups in both validation cohorts

Subsequently, we measured CSF THOP1 concentrations using the Ella platform in two validation cohorts. In validation cohort 1, we observed an increase in THOP1 concentrations in MCI-Aβ+ (1.4-fold, $p < 0.001$) and AD patients (1.3-fold, $p < 0.001$) compared to controls. CSF THOP1 concentrations were higher in MCI-Aβ+ compared to DLB (1.2-fold, $p < 0.05$, Figure 4A) but there were no statistically significant differences between AD and DLB ($p > 0.05$, Figure 4A). In addition, THOP1 discriminated AD and MCI-Aβ+ from controls with good accuracy (MCI-Aβ+ vs controls: AUC = 0.800, 95% CI: 0.713–0.886 and AD vs controls: AUC = 0.797, 95% CI: 0.711–0.884; Figure S4A–B) but not between AD and DLB patients (AUC = 0.620, 95% CI: 0.507–0.7334; Figure S4C). The increased CSF THOP1 concentrations observed in MCI-Aβ+ and AD patients were also replicated in validation cohort 2 (MCI-Aβ+ vs controls: 1.3-fold, $p < 0.001$, AUC = 0.867,

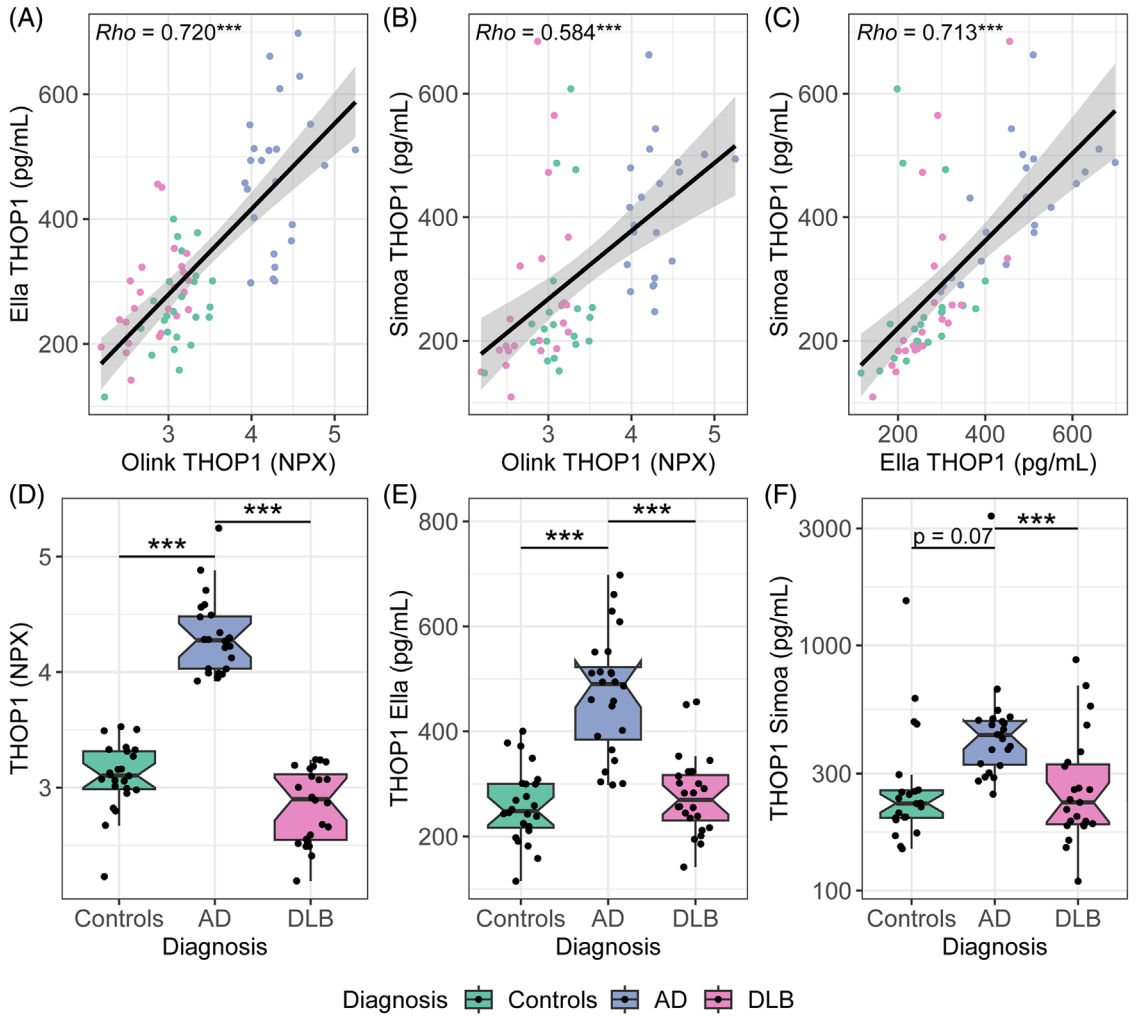

FIGURE 3 THOP1 concentrations are translatable across different platforms. THOP1 concentrations in CSF are increased in patients with AD compared to controls and patients with DLB on three different platforms; antibody-based proteomics (A), Ella (B), and Simoa (C). THOP1 concentrations strongly correlated between Ella and antibody-based proteomics (D) and moderately between Simoa and antibody-based proteomics (E). The novel THOP1 assays correlated strongly between the automated Ella and Simoa platforms (F). Abbrevations: AD, Alzheimer's disease; CSF, cerebrospinal fluid; DLB, dementia with Lewy bodies; THOP1, thimet oligopeptidase. ***indicates $p < 0.001$.

95% CI: 0.789–0.945; AD vs controls: 1.2-fold, $p < 0.05$, AUC = 0.667, 95% CI: 0.531–0.803; MCI-Aβ+ vs DLB: 1.2-fold, $p < 0.001$, AD vs DLB: $p > 0.05$, AUC = 0.609, 95% CI: 0.484–0.733; Figure 4C and Figure S4). When analyzing the THOP1 concentrations in both cohorts together, similar results were observed with an additional significant difference between AD and DLB (Figure S5A).

Considering the presence of AD copathology in DLB cases, we next investigated whether CSF THOP1 concentrations could be influenced by the development of amyloid pathology in this group. In validation cohort 1, we observed that CSF THOP1 concentrations were higher in DLB-Aβ+ compared with DLB-Aβ- groups (1.3-fold, $p < 0.05$). Furthermore, CSF THOP1 concentrations were higher in AD compared to the DLB-Aβ- but not between AD and DLB-Aβ+ (Figure S6A). These changes were not detected in validation cohort 2 (Figure S6B). However, when both cohorts were analyzed together, increased THOP1 concentrations in MCI-Aβ+ and AD groups compared with DLB-Aβ+ and DLB-Aβ- groups were detected (Figure S5B).

3.3 | CSF THOP1 concentrations correlate with CSF markers reflecting total amyloid load and tau pathology

To understand the relationship of CSF THOP1 with the AD pathological hallmarks and cognitive scores, we next performed correlation analysis with these markers. In the complete validation cohort 1, we observed strong positive correlations of THOP1 with CSF concentrations of Aβ40 (*Rho* = 0.625, $p < 0.001$), p-tau (*Rho* = 0.712, $p < 0.001$), and t-tau (*Rho* = 0.721, $p < 0.001$) but not with Aβ42 (*Rho* = −0.09, $p > 0.05$, Figure 4B). A weak negative correlation between THOP1 and MMSE scores was observed (*Rho* = −0.239, $p < 0.001$). These findings were similar in validation cohort 2 (Aβ40: *Rho* = 0.647, $p < 0.001$; p-tau: *Rho* = 0.540, $p < 0.001$; t-tau: *Rho* = 0.656, $p < 0.001$; Aβ42: *Rho* = −0.132, $p > 0.05$), although no significant correlation between THOP1 and MMSE scores was observed (*Rho* = −0.122, $p > 0.05$, Figure 4D). When we stratified for clinical diagnosis, similar correlations for Aβ40,

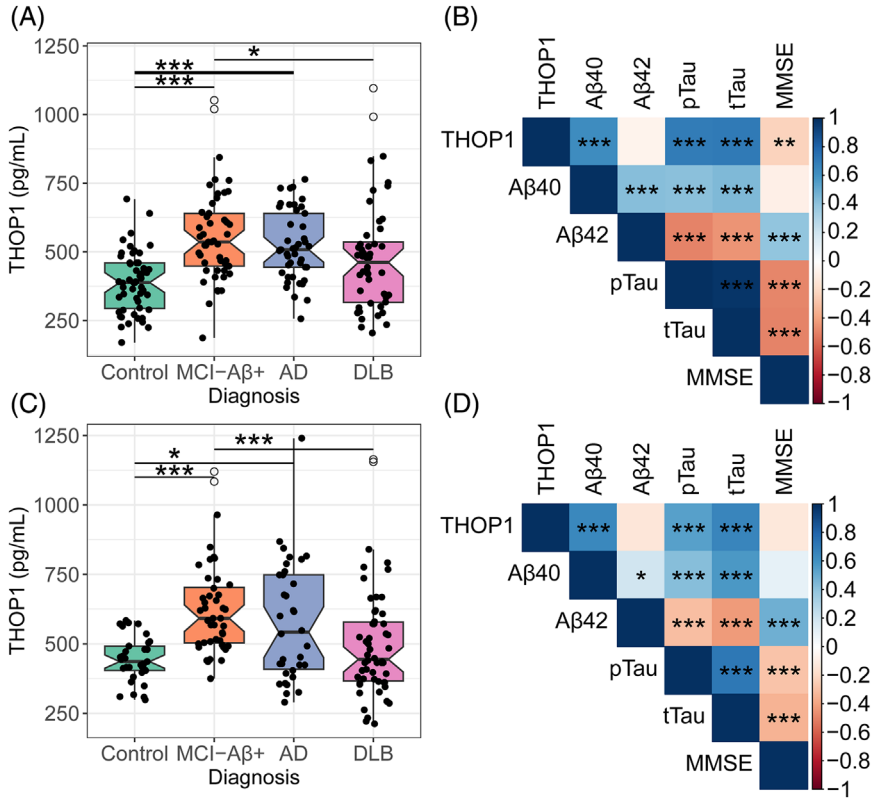

FIGURE 4 THOP1 concentrations are increased in MCI-Aβ+ and AD and associated with Aβ40, p-tau, and t-tau in both validation cohorts. THOP1 concentrations were measured on the Ella platform, which showed increased THOP1 concentrations in AD compared to MCI-Aβ+ compared to controls and patients with DLB in validation cohort 1 (A) and validation cohort 2 (C). The correlation matrix heatmap represents Spearman's correlation coefficient of THOP1 with the classical AD CSF biomarkers and MMSE scores. The blue color depicts a positive correlation coefficient, whereas red depicts a negative correlation coefficient. Significant correlations between THOP1 and Aβ40, p-tau, and t-tau in validation cohort 1 (B) and validation cohort 2 (D) were observed. Abbrevations: AD, Alzheimer's disease; Aβ40, amyloid beta 1-40; Aβ42, amyloid beta 1-42; CSF, cerebrospinal fluid; DLB, dementia with Lewy bodies; MCI-Aβ+, mild cognitive impairment with amyloid pathology; MMSE, Mini-Mental State Examination; p-tau, phosphorylated tau; THOP1, thimet oligopeptidase; t-tau, total tau. Statistical significance is indicated as: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

p-tau, and t-tau were shown as observed in the total cohort. However, we detected a moderate correlation between THOP1 and Aβ42 concentrations in controls (cohort 1: $Rho = 0.536$, $p < 0.001$, cohort 2: $Rho = 0.346$, $p < 0.05$, Figure S7), but not in other diagnostic groups.

4 | DISCUSSION

In this study, two novel THOP1 immunoassays were developed and validated analytically on different automated platforms. We observed increased CSF THOP1 concentrations in MCI-Aβ+ and AD stages compared to controls, and between MCI-Aβ+ and DLB in two independent cohorts, validating our proteomics discovery findings. This highlights the potential of the neuropeptidase THOP1 as a specific AD CSF biomarker in early disease stages. Furthermore, we show that the use of immunoassay-based platforms for both discovery and validation phases may accelerate the development of novel body-fluid–based biomarkers.

THOP1 is a neuropeptidase able to cleave Aβ peptides, among others.[44] Neuronal cell culture models have shown that although THOP1 overexpression increased Aβ degradation, downregulation of THOP1 made neurons more vulnerable to amyloid toxicity, suggesting a neuroprotective role of THOP1 against Aβ plaque toxicity.[22] In our previous discovery study, we found increased CSF THOP1 concentrations in MCI and AD stages compared to controls and a group of non-AD dementias. THOP1 was also selected in a CSF protein panel that could discriminate AD from a group of non-AD dementias with high accuracy (AUC > 0.87), further supporting its potential as a differential diagnostic biomarker.[18] Here, we have developed and technically validated immunoassays that can be used in two different automated and sensitive platforms (i.e., Ella and Simoa) allowing high-throughput measurements of CSF THOP1 for further validation in independent cohorts. We observed that CSF THOP1 was increased in both MCI and AD patients compared to controls in two independent clinical cohorts and when both cohorts were analyzed together, thereby validating our previous discovery findings. Our CSF findings also concur with previous studies that show increased THOP1 levels in AD temporal cortex tissue compared to controls, and in early affected brain areas (Braak III–IV).[22] CSF THOP1 concentrations may thus reflect early brain-specific changes. These changes might be the result of a neuroprotective mechanism to cope with the increased Aβ load.[21] However, it is important to note that the increased CSF THOP1 concentrations detected in this study do not necessarily imply an increased neuropeptidase activity within the brain. Understanding the enzyme activity of THOP1 could provide additional insights into the pathophysiological role that this protein may play in AD.

We did not observe any differences between AD and DLB patients in both independent cohorts, which is in contrast with our previous discovery findings.[18] The overlapping clinical and pathological features between AD and DLB may partly explain the similar CSF THOP1 concentrations between these dementias. Indeed, in the first validation cohort, we observed that the CSF THOP1 concentrations in AD were similar to those observed in amyloid-positive DLB cases and higher than those detected in amyloid-negative DLB cases. However, these differences were not detected in the second validation cohort. Although when both datasets were merged, significant differences

in CSF THOP1 concentrations between AD and DLB were observed independently of amyloid pathology. This suggests that the lack of significance observed in the independent cohorts is probably due to the lower sample sizes and not to the presence of amyloid comorbid pathology in the DLB group.

Strong correlations between THOP1 with t-tau, p-tau, and Aβ40 concentrations in the total cohort were observed. The strong relationship with these biomarkers suggests that THOP1 changes are associated with upstream and downstream mechanisms in AD such as tau pathology, neurodegeneration, and total Aβ load.[45,46] Despite the THOP1 increases in response to Aβ neurotoxicity,[22] the association between Aβ42 and THOP1 only in the control group suggests that THOP1 may not respond to Aβ fibril and plaque formation, further supporting a relationship of THOP1 with normal Aβ physiological functioning.[20,21] Altogether, the increased THOP1 concentrations detected in the MCI stage and the significant correlations with AD CSF biomarkers may suggest that this protein could be a useful biomarker for detecting an early neuroprotective response against AD pathology. CSF THOP1 could also be used in a CSF protein panel that together reflects the multifactorial nature of AD and can accurately discriminate AD from other non-AD dementias, as we showed previously.[18] The immunoassays developed in this study could thus be considered the first step in developing such a CSF protein panel using accessible immunoassays. These assays might also be useful for measuring protein concentrations in other matrices and animal or cellular models aiming to better understand the role of these proteins in AD pathogenesis.

Biomarker development is often hampered by the cross-technology translation gap between the discovery and validation phases, thereby limiting the successful clinical implementation of novel fluid biomarkers.[8] Our study allowed us to explore if the use of immunobased technologies during the discovery phase facilitates the development of the corresponding analytical assays for large-scale validations in subsequent steps,[18,47] providing an additional pipeline that may smoothen the development and validation of novel biomarker candidates. Both THOP1-specific immunoassays were developed within 4–6 weeks. Based on our previous experience, the period for developing and validating other in-house immunoassays for biomarker candidates coming from MS proteomics studies (e.g., CSF APOL1) was, over 6 months.[48] We observed that THOP1 concentrations strongly correlated across the different THOP1 immunoassays, suggesting that the different antibody-based platforms are likely detecting similar protein isoforms. Noteworthy, the comparison between our in-house immunoassays showed some outliers on the Simoa platform, which could indicate that the assay still requires additional optimization (e.g., higher sample dilution, or longer antibody incubation times). Considering that our Simoa assay showed slightly higher variability and was less sensitive, we thus continued our clinical validation on the Ella platform. Overall, these data suggest that the use of technologies based on the same principle (i.e., antibody-based) may have a great translational advantage resulting in faster immunoassay development and showing reproducible findings in subsequent validation studies. This is further supported by the successful results we obtained in the ongoing follow-up work from the discovery study (e.g., development of different immunoassays to validate our AD-differential diagnostic protein panel, development and validation of other biomarker candidates including inflammatory markers related to AD as well as specific biomarkers for DLB; unpublished data Y.S Hok-A-Hin & K. Bolsewig).[18,49] Other different factors (i.e., type of biomarker; standard ELISA vs more-sensitive and automated Ella and Simoa platforms) may also contribute to the efficient translation of our discovery findings. Although the workflow described in this study is designed for the analysis of novel CSF biomarkers in AD, it is likely also applicable for the development of biomarkers in other human biological fluids and other biomedical fields.

Among the limitations of this study is that we cannot exclude that some of the MCI cases included in this study may ultimately progress to another type of dementia. Still, MCI cases were selected based on amyloid positivity and thus they are at high risk of progressing to AD. Considering the recent advances in blood-based biomarkers within the AD field[10] it would be of interest to investigate whether this CSF biomarker candidate could be measured in blood. However, gene expression levels of THOP1 were also associated with rheumatoid arthritis,[50] possibly affecting THOP1 concentrations in blood. Thus, to what extent THOP1 changes in blood would reflect brain-specific changes should be investigated further.

In conclusion, considering the increased THOP1 concentrations in MCI and AD stages, CSF THOP1 might be useful as part of a CSF protein panel to discriminate AD from other neurodegenerative dementia in very early stages of the disease. Furthermore, our data suggest that the strategy followed, using antibodies in both discovery and validation studies, may facilitate the translation of proteomics findings and accelerate the development of body-fluid–based biomarkers.

AUTHOR CONTRIBUTIONS

Yanaika S. Hok-A-Hin contributed to the study design and was responsible for acquisition, experiments, statistical analysis, interpretation of the data, and drafting of the manuscript. Daimy N. Ruiters developed and analytically validated the THOP1 immunoassays. Katharina Bolsewig helped with the measurements of clinical samples. Afina W. Lemstra, Wiesje M. van der Flier, Alberto Lleó, and Daniel Alcolea provided patient material. All authors participated in data interpretation and critically revised the manuscript. Marta del Campo and Charlotte E. Teunissen conducted the study concept and design, data acquisition and interpretation, critically revised the manuscript, and supervised the study. All authors read and approved the final manuscript.

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CONFLICTS OF INTEREST STATEMENT

Y.S.H., K.B., D.R., A.W.L., and M.C. report no conflicts of interest. D.A. participated in advisory boards from Fujirebio-Europe and Roche Diagnostics and received speaker honoraria from Fujirebio-Europe, Roche Diagnostics, Nutricia, Krka Farmacéutica S.L., Zambon S.A.U., and Esteve Pharmaceuticals S.A., D.A. declares a filed patent application (WO2019175379 A1 Markers of synaptopathy in neurodegenerative disease). A.L. participated in advisory boards from Fujirebio-Europe, Grifols, Eisai, Novartis, Roche Diagnostics, Otsuka Pharmaceutical, Nutricia, Zambón S.A.U., and Biogen, and received speaker honoraria from Eli Lilly, Biogen, KRKA, and Zambon. A.L. declares a filed patent application (WO2019175379 A1 Markers of synaptopathy in neurodegenerative disease). Research programs of W.F. have been funded by ZonMW, NWO, EU-FP7, EU-JPND, Alzheimer Nederland, Hersenstichting CardioVascular Onderzoek Nederland, Health~Holland, Topsector Life Sciences & Health, stichting Dioraphte, Gieskes-Strijbis fonds, stichting Equilibrio, Edwin Bouw fonds, Pasman stichting, stichting Alzheimer & Neuropsychiatrie Foundation, Philips, Biogen MA Inc, Novartis-NL, Life-MI, AVID, Roche BV, Fujifilm, Eisai, and Combinostics. W.F. holds the Pasman chair. W.F. is recipient of ABOARD, which is a public-private partnership receiving funding from ZonMW (#73305095007) and Health~Holland, Topsector Life Sciences & Health (PPP-allowance; #LSHM20106). W.F. has been an invited speaker at Biogen MA Inc, Danone, Eisai, WebMD Neurology (Medscape), NovoNordisk, Springer Healthcare, NovoNordisk, and European Brain Council. W.F. is a consultant to Oxford Health Policy Forum CIC, Roche, and Biogen MA Inc. W.F. participated in advisory boards of Biogen MA Inc, Roche, and Eli Lilly. All funding is paid to her institution. W.F. is member of the steering committee of PAVE and Think Brain Health. W.F. was associate editor of *Alzheimer's Research & Therapy* in 2020/2021. W.F. is associate editor at *Brain*. C.T. has a collaboration contract with ADx Neurosciences, Quanterix, and Eli Lilly, and performed contract research or received grants from AC-Immune, Axon Neurosciences, Bioconnect, Bioorchestra, Brainstorm Therapeutics, Celgene, EIP Pharma, Eisai, Grifols, Novo Nordisk, PeopleBio, Roche, Toyama, and Vivoryon. C.T. serves on editorial boards of *Medidact Neurologie*/Springer, *Alzheimer's Research & Therapy*, *and Neurology: Neuroimmunology & Neuroinflammation* and is editor of a Neuromethods book (Springer). She had speaker contracts for Roche, Grifols, and Novo Nordisk. Author disclosures are available in the supporting information.

DATA AVAILABILITY STATEMENT

The data sets used and or analyzed in the current study are available from the corresponding author upon reasonable request.

CONSENT STATEMENT

Informed consent was obtained from all participants or their authorized representatives, following the ethical consent by the VU University Medical Center Amsterdam and with the Helsinki Declaration of 1975.

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SUPPORTING INFORMATION

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

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