Bayoumy *et al.*
Alzheimer's Research & Therapy (2025) 17:81
https://doi.org/10.1186/s13195-025-01716-8

Alzheimer's
Research & Therapy

RESEARCH

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Novel CSF β-synuclein-specific assays signal early synaptic degeneration in Alzheimer's disease

Sherif Bayoumy[1*], Julie Goossens[2], Charlotte De Rocker[2], Senna Y. Sie[1], Nolan J. Barrett[1], Wiesje M. van der Flier[3,4], Charlotte E. Teunissen[1], Eugeen Vanmechelen[3†] and Inge M. W. Verberk[1†]

Abstract

Background Beta-synuclein (β-syn), measured at N-terminal epitopes, is an emerging cerebrospinal fluid (CSF) biomarker for synaptic degeneration in Alzheimer's disease (AD). Targeting the mid-region or C-terminus of β-syn may enhance analytical specificity due to the distinct structures of these regions across the synuclein protein family, unlike targeting the N-terminus, which is conserved across the family. This study aimed to confirm that β-syn is a promising CSF biomarker in AD, using novel assays designed to target different regions of β-syn, to investigate whether these regions are differentially affected in AD.

Methods We developed two novel CSF β-syn-specific ELISAs targeting mid-region and C-terminus epitopes and assessed their analytical performance. Using these novel assays in combination with the established N-terminus ELISA, we analyzed a proof-of-concept cohort comprising biomarker-confirmed AD ($n=25$) and non-AD subjects ($n=25$) and a larger clinical cohort ($n=160$) from the Amsterdam Dementia Cohort, wich included 41 individuals with subjective cognitive decline (SCD, controls; AD biomarker negative; 64.3 ± 3.3 years, 23 females), 39 with SCD (AD biomarker positive; 65.7 ± 3.1 years, 17 females), 40 with mild cognitive impairment due to AD (MCI-AD; 66.2 ± 2.9 years, 20 females), and 40 with AD dementia (AD-dem; 65.3 ± 3.4 years, 20 females).

Results Both the mid-region and C-terminus assays demonstrated reliable analytical performance. All assays consistently detected β-syn in all clinical samples above their limits of detection, with a good average intra-assay coefficient of variation (range of the three assays: 2.7–6.5%CV) in the proof-of-concept cohort and clinical cohort (range of the three assays: 3.9–7.5%CV). CSF β-syn levels, with all the assays, were significantly elevated in all the AD groups compared with the controls in both cohorts. The diagnostic performance of the assays for distinguishing AD patients from controls was comparable (Delong's $p>0.05$, AUC 0.71–0.80). Notably, mid-region β-syn significantly differentiated SCD-AD patients from AD-dem patients ($p=0.035$) and MCI-AD patients at a trend level. Only mid-region and C-terminal levels correlated with MMSE scores (mid-region rho = -0.22, $p=0.006$; C-terminal rho = -0.19, $p=0.016$; N-terminus rho = -0.14, $p=0.069$).

Conclusion Our novel assays demonstrated good analytical and clinical performance. CSF β-syn reliably indicates early synaptic degeneration in AD. The mid-region assay uniquely differentiated SCD-AD from AD-dem, showing promise for early disease detection.

†Eugeen Vanmechelen and Inge M. W. Verberk Shared senior authorship

*Correspondence:
Sherif Bayoumy
S.S.A.Bayoumy@amsterdamumc.nl
Full list of author information is available at the end of the article

Keywords Alzheimer´s disease, Preclinical alzheimer's disease, CSF biomarker, β-Synuclein, Synaptic degeneration, Novel ELISAs

Background

Alzheimer's disease (AD) is the most prevalent form of dementia [1] and is characterized by the deposition of cerebral amyloid-β (Aβ) plaques in the brain's extracellular space and tangles of phosphorylated tau in neurons. These pathological hallmarks are accompanied by progressive neuronal and synaptic loss [2–6]. Synaptic loss is strongly correlated with cognitive decline [7–9] and is suggested to be among the earliest pathophysiological changes in AD [10, 11]. Fluid biomarkers that reflect synaptic loss in AD patients are highly desirable, as they might have potential use in diagnosis, prognosis, and disease or treatment efficacy monitoring.

Among the synaptic biomarkers, the presynaptic protein beta-synuclein (β-syn), which is expressed predominantly in presynaptic terminals of the central nervous system (CNS), is emerging as an early and specific biomarker for AD [12, 13]. β-syn levels were found to be significantly elevated in the cerebrospinal fluid (CSF) and serum of patients with AD, but were not elevated in patients with other types of dementia [14–16]. Notably, compared with core fluid AD biomarkers, which change with AD-related pathophysiological changes, β-syn elevations are among the earliest detected changes [14–20].

CSF β-syn results have thus far been generated using either a direct mass-spectrometry assay [15] or an immunoassay [16]. Immunoassays are more accessible and scalable methods with clinical feasibility for measuring β-syn in bodily fluids. The published immunoassay [16] employs an antibody that targets the N-terminus epitopes of β-syn. The N-terminus is, however, conserved across the synuclein family, and targeting β-syn-specific regions such as the mid-region or C-terminus may enhance specificity due to their distinct structures [21], which in turn could enhance diagnostic accuracy of β-syn detection. In addition, α-syn copathology is observed in 30%–45% of AD patients [18, 22, 23], potentially interfering with the specificity of the β-syn measurements in this subset of patients with AD and α-syn copathology. In contrast, the epitopes in the mid-region and C-terminus are more unique for β-syn [15]. Therefore, targeting these mid-region or C-terminus epitopes has the potential to increase the reliability and specificity of β-syn measurements as a biomarker in AD. Whether this specificity when measuring β-syn at the mid-region and C-terminus epitopes improves its use as biomarkers in AD remains unexplored.

This study aimed to confirm the diagnostic potential of CSF β-syn and assess whether targeting β-syn-specific regions could enhance the diagnostic accuracy of detecting synaptic degeneration across the AD continuum. To achieve this goal, we established novel β-syn ELISAs targeting mid-region and C-terminus epitopes and investigated the analytical and clinical performance of β-syn detected at these epitopes in comparison to an in-house assay targeting N-terminus epitopes.

Methods

Clinical samples

We included two cohorts: a proof-of-concept cohort and a clinical validation cohort. The proof-of-concept cohort was a convenience sample of remnant CSF materials from routine diagnostics performed by the Department of Laboratory Medicine of Amsterdam UMC from biochemically defined AD patients (CSF core AD biomarker profile positive: Roche Elecsys CSF amyloid β_{1-42} (Aβ42) < 1000 pg/mL, CSF phosphorylated tau (pTau181)> 19.0 pg/mL, and CSF total tau (Tau) > 235.0 pg/mL; $n = 25$) and controls with a negative CSF AD biomarker profile ($n = 25$). No additional clinical information about these remnant samples was available. The clinical validation cohort was a selection of patients from the Amsterdam Dementia Cohort [24, 25] and included patients with subjective cognitive decline (SCD), mild cognitive impairment (MCI), and AD dementia who visited the Memory Clinic of the Alzheimer Center Amsterdam between 2003 and 2021. All individuals in the Amsterdam Dementia Cohort underwent a standardized dementia diagnostic work-up, which included physical, neurological, and neuropsychological evaluation, brain MRI, Mini-Mental State Examination (MMSE) scores, which are used as a measure of global cognition, apolipoprotein E (APOE) genotyping (APOE ε4 carriage defined as having at least one ε4 allele), trail-making test A (TMTA) scores as a measure of attention, and trail-making test B (TMTB) scores as a measure of executive function, CSF AD biomarker analysis, and/or an amyloid PET scan. The clinical diagnoses were based on multidisciplinary consensus according to applicable criteria for SCD, MCI, and AD-dementia [25–29].

CSF levels of Aβ42, pTau181, and Tau were measured using ELISA INNOTEST Aβ42, hTAUAg and phospho-Tau(181P) kits (Fujirebio Europe, Ghent, Belgium) or Aβ42 t-TAUAg, and phospho-Tau181 Elecsys biomarker

assays (Roche Diagnostics GmbH, Basel, Switzerland). A positive CSF AD biomarker profile was defined on the basis of an increased CSF (pTau181; T)/(Aβ42; A) ratio > 0.02 for the values measured with Elecsys and T/A > 0.054 for those measured with Innotest [30]. To ensure consistency, the Innotest values were transformed into their Elecsys equivalents using previously described Eqs. [31]. Our clinical groups consisted of 41 A- individuals with SCD (controls), 39 A + individuals with SCD (SCD-AD), 40 A + individuals with MCI (MCI-AD), and 40 A + individuals with dementia (AD-dem). The CSF samples were collected and stored [32, 33] at −80 °C until use following established guidelines. The Medical Ethical Committee of the Amsterdam UMC approved the study. All participants of the Amsterdam Dementia Cohort provided informed consent, and the study was performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments.

β-syn assays

Specificity of the utilized antibodies

Linear epitope mapping of two rabbit monoclonal antibodies, EP1537Y (Abcam, ab221908) and EP1646Y (Abcam, ab189217), and two mouse monoclonal antibodies, ADx β-syn1 and ADx β-syn2, was conducted using three libraries of overlapping synthetic peptides (BioSynth, Lelystadt, NL) [34]. One array consisted of 10 amino acid-long peptides that covered the full sequence of human β-syn, and each peptide had a 9 amino acid overlap with the former peptide. The second array was based on 15 amino acid-long peptides that overlapped with 14 amino acids of the former peptide covering the complete β-syn protein. The third array was based on 20 amino acid-long peptides that overlap with 19 amino acids of the former peptide, also covering the whole protein (see also Fig. S2-S5 and Table S1 in the supplementary material). The binding capacity of the antibodies to each of the generated peptides was determined with a BioSynth-based ELISA at two different antibody concentrations and with a non-β-syn isotype antibody, with ADx252 for the rabbit antibodies and ADx202 for the mouse immunoglobulin g1 (IgG1) antibody. We conducted preliminary cross-reactivity experiments to assess the specificity of three antibodies (EP1646Y: N-terminus, EP1537Y: mid-region, and ADx β-syn2: C-terminus) towards α-syn. Using plates coated with 0.2 µg/mL α-syn, each antibody was tested in a serial dilution (1,000,000 to 2 pg/mL) (Fig. S1). We used a recombinant human α-syn protein encoding (the 1–140) bp sequence.

β-syn measurements

To measure β-syn levels, we developed three ELISAs to specifically target the N-terminus, mid-region, or C-terminus of the β-syn protein (Fig. 1). The N-terminus assay was performed according to a protocol developed elsewhere [16] with some changes, whereas the mid-region and C-terminus assays were developed in-house. In each of the assays a different capture antibody (N-terminus:

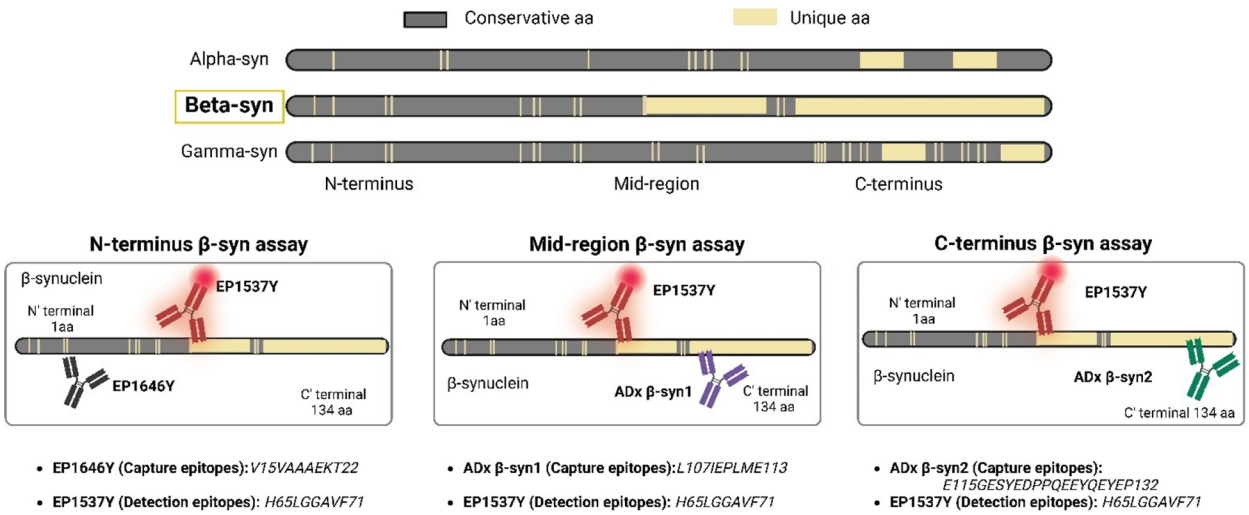

Fig. 1 Schematic representation of synuclein proteins (α, β, and γ-synuclein) illustrating their conserved and unique epitopes. The lower panel shows the results of the β-syn-targeting ELISAs: N-terminus assay (using an EP1537Y antibody for detection and N-terminus capture), mid-region assay (utilizing EP1537Y and ADx β-syn1 antibodies), and C-terminus assay (using EP1537Y for detection of ADx β-syn2 for capture). The placement of antibodies reflects their approximate binding sites on the β-syn protein. These in-house β-syn-specific assays offer targeted epitope recognition, enhancing the specificity of synaptic degeneration biomarker detection in AD. The exact binding epitopes of the antibodies are listed in the figure, starting with the first amino acid binding position and ending with the last amino acid

EP1646Y, mid-region: ADx β-syn1, and C-terminus: ADx β-syn2) while utilizing the same β-syn-specific biotinylated detection antibody (EP1537Y, Abcam, ab221908). We purchased a recombinant truncated human β-syn (residues 1–122, UniProt: P37840) expressed in E. coli without a tag from rPeptide and used it as a calibrator for all three assays. The amino acid sequence is as follows: MDVFMKGLSM AKEGVVAAAE KTKQGVTEAA EKTKEGVLYV GSKTREGVVQ GVASVAEKTK EQASHLGGAV FSGAGNIAAA TGLVKREEFP TDLK-PEEVAQ EAAEEPLIEP LMEPEGESYE. The recombinant protein was diluted in the same diluent as the CSF samples, that is, composed of PBS + 0.1% casein + 0.1% Tween + 0.05% ProClin300 for the N-terminus and C-terminus assays and composed of Dulbecco PBS (DPBS; with Ca2 + Mg2) + 0.1% casein + 0.1% Tween + 0.05% ProClin300 for the mid-region assay. The detailed protocols of the ELISAs are described in Table 1 and the supplementary material.

All three assays followed similar protocols: 96-well plates were coated overnight with 2 µg/mL of the corresponding capture antibody in PBS. The wells were washed with PBS containing Tween 20 and ProClin™300, blocked, and then incubated with 150 ng/mL detection antibody and 1:4 diluted CSF samples or calibrator materials in the assay diluent. The mid-region assay differed slightly from the other two assays in that it used Dulbecco's PBS as the sample diluent instead of regular PBS and had a shorter incubation time (1 h (hr) vs. 2 h). All three assays employed a streptavidin poly-horseradish peroxidase (polyHRP) conjugate and TMB substrate for detection, with consistent shaking and incubation conditions, and measurements were conducted in duplicate.

Analytical validation of the β-syn assays

We assessed the sensitivity, precision, parallelism, dilution linearity, recovery, specificity, and stability of the novel mid-region and C-terminus ELISAs following the protocol developed by the BIOMARKAPD consortium [35]. The analytical performance of the N-terminus assay has been described elsewhere [16]. For the analytical validation of our in-house assays, the acceptance criteria were set at < 20% CV for precision and between 80 and 120% for parallelism, recovery, and linearity calculations. Sensitivity was also assessed for the N-terminus ELISA. For sensitivity, the functional lowest limit of detection (LLoD) was interpolated from the standard curve from the mean signal of 16 blanks plus 10 × the standard deviation, which was subsequently multiplied by the assay's sample dilution factor. The intra-assay precision was calculated as the percentage of coefficient of variation (%CV) of duplicate measurements of the 160 clinical samples. For inter-assay precision, we measured a panel of three quality control (QC) CSF samples with high, medium, and low β-syn levels over three independent days and calculated the %CV. Parallelism was calculated by comparing the average slopes of four CSF samples that were serially diluted 4 times to the slope of the calibrators, starting with a twofold dilution factor. Dilution linearity was assessed by spiking three CSF samples with 1000 pg/mL or 5000 pg/mL β-syn for the mid-region or the C-terminus assays, respectively, and performing 4-fold serial dilution of these samples to levels below the LLoD. The recovery response was assessed using five CSF samples spiked with high (500 pg/mL), medium (mid-region: 125 pg/mL or C-terminus: 250 pg/mL), or low (mid-region: 32 pg/mL or C-terminus: 125 pg/mL) levels of β-synuclein. The recovery percentage for each sample was calculated relative to unspiked samples. Further details are provided in Table 1.

Statistical analysis

All the statistical analyses were performed using IBM SPSS Statistics (v.28.0.1.1) or RStudio (v.4.0.3). We conducted descriptive statistics to summarize demographic characteristics, using the chi-square test and the Kruskal–Wallis test. The β-syn levels were nonnormally distributed, so we applied the Mann–Whitney U test for the proof-of-concept cohort and the Kruskal–Wallis test followed by the Dunn post hoc test with Bonferroni correction for the clinical validation cohort. We calculated the fold changes between the control and AD groups using the medians of each of the groups. The diagnostic accuracy of β-syn for the detection of SCD-AD, MCI-AD, or AD-dem patients compared with that of controls was determined using receiver operating characteristic (ROC) curve analyses. The Delong test was used to compare the generated areas under the curves (AUCs) of each β-syn measurement [36]. We calculated cutoffs of β-syn levels in the clinical cohort between controls and patients at each AD stage by maximizing the Youden index [37]. Spearman's correlation analyses were applied to assess the correlation between the β-syn levels obtained with the three assays, as well as to assess associations of β-syn levels with the core AD biomarkers, MMSE scores and TMT B/A ratios. In addition, the ratios of β-syn to the core AD biomarkers were calculated to further assess diagnostic accuracy in the clinical cohort, in line with earlier research [16]. We considered p values < 0.05 as statistically significant and p *values* < 0.10 as indicative of a statistical trend.

Results

Antibody specificity and epitope mapping

Epitope mapping using three peptide libraries (10-mers, 15-mers, and 20-mers) covering the full length of the 134

Table 1 Analytical characteristics of the β-syn ELISAs

		N-terminus β-syn	Mid-region β-syn	C-terminus β-syn
Assay	Platform	ELISA	ELISA	ELISA
	Status	Prototype	Prototype	Prototype
	Developers	Ulm University Hospital	Amsterdam UMC / ADx NeuroSciences	Amsterdam UMC / ADx NeuroSciences
	Biofluid	CSF	CSF	CSF
	Fold sample dilution	4	4	4
Calibration curve	Type	Recombinant	Recombinant	Recombinant
	No. of calibrator points	10	10	10
	Range, pg/mL	4–1000	4–1000	4–1000
	Curve fit	$1/y^2$-weighted 5PL	$1/y^2$-weighted 5PL	$1/y^2$-weighted 5PL
Antibodies	Capture	EP1646Y	ADx- β-syn1	ADx- β-syn2
	Detector	EP1537Y	EP1537Y	EP1537Y
Clinical samples measurements (Amsterdam Dementia Cohort)	Number	160	160	160
	Range concentration, pg/mL	60.0–976.0	13.2–252.8	22.7–636.9
	Range, CV%	0–33.0%	0–26.0%	0–32.6%
	Average CV%	4.9%	3.9%	7.5%
	n measured < LLOQ	0	0	0
	n measured > 20%CV	2	1	11
Analytical validation results				
Sensitivity	Functional LLoD, pg/mL	2.15	0.74	2.42
Concentrations of QC panels	QC1: high, pg/mL	NA	57.8	23.8
	QC2: intermediate, pg/mL		90.3	179.8
	QC3: low, pg/mL		154.0	278.6
Precision of QCs	Average Intra-assay %CV	NA	7.2	16.7
	Average Inter-assay %CV		31.8	17.4
Parallelism	Average slope of samples	NA	0.78	0.70
	Range of slopes of samples		0.87–70	0.64–0.76
	Average slope of calibrator		0.82	0.67
	Parallelism, %		94%	105%

Table 1 (continued)

		N-terminus β-syn			Mid-region β-syn			C-terminus β-syn	
Dilution linearity	Spiked concentration, pg/ml				1000			1000	
		Df (x)	Mean %L		Mean %L			Mean %L	
	Dilution linearity factor with mean %Linearity	1	NA		-			-	
		4			144			58	
		16			103			99	
		64			139			96	
		256			87			73	
Recovery	Spiked concentration (pg/mL) With mean %Recovery	Spike	Mean %R		Spike (pg/mL)	Mean %R		Spike (pg/mL)	Mean %R
		NA			500	67		500	63
					125	64		250	64
					32	67		125	57
Stability	Freeze/ thaw cycles	Average response (%)		Standard deviation	Average normalized response (%)		Standard deviation	Average response (%)	Standard deviation
	0 F/T	NA			100.0		2	100.0	15
	1 F/T	NA			100.0		2	100.0	10
	2 F/T	NA			100.0		4	105.2	15
	3 F/T	NA			100.0		2	100.0	11

The analytical performance of the ELISAs was assessed based on sensitivity, precision, parallelism, dilution linearity and recovery. The sensitivity was determined by calculating the detection limits of each assay. We utilized a commercially available recombinant truncated human β-syn (residues 1–122, UniProt: P37840), lacking the 12 C-terminal amino acids of the full-length 134-residue protein. The recombinant protein was untagged. The functional LLoD was calculated as the mean signal of 16 blanks plus 10 times the SD multiplied by the dilution factor of four times. Quality control (QC) samples were made of remnant CSF samples. The mean intra- and inter-assay variation was determined by measuring the QC panels over three independent runs on three days. The 160 clinical samples were measured in duplicate. Precision was evaluated by calculating the intra-assay and inter-assay coefficients of variation (CV%) of these measurements. Parallelism was evaluated to ensure the consistency of assay performance across different sample dilutions, and dilution linearity confirmed the linear response of the assays to diluted samples. For parallelism, four samples were measured after being serially diluted four times 2-fold, starting with a 2-fold dilution that reached 32a-fold). For dilution linearity, three samples were spiked with 1000 pg/mL recombinant β-syn and subsequently measured undiluted and diluted 256-fold. Recovery tests were used to assess the accuracy of the assays in quantifying known amounts of β-syn. PL: polynomial; LLoD: lowest limit of detection; QC: quality control; CV: coefficient of variation; %L: % linearity; %R: % recovery, F/T: freeze / thaw cycles

amino acid human β-syn protein revealed that the rabbit monoclonal antibody EP1646Y, used in the N-terminus assay, primarily binds to the N-terminus repeat structure of synucleins, with its main epitope on $V_{15}VAAAEKT_{22}$ of β-syn. EP1646Y showed cross-reactivity with human α-syn in our experiment. ADx β-syn1 targets $L_{107}IEPLME_{113}$ (according to the results of the mid-region ELISA), and ADx β-syn2 binds the extreme C-terminus $E_{115}GESYEDPPQEEYQEYEP_{132}$ (Table S1, Fig. S2-S5). Neither antibodies showed cross-reactivity with human α-syn in our experiments. The antibody EP1537Y, the detector in all three assays, targets the $H_{65}LGGAVF_{71}$ region of the β-syn protein and shows some reactivity with the N-terminus repeat structure of synucleins.

Analytical performance of the β-syn ELISAs

The developed mid-region and C-terminus ELISAs demonstrated robust analytical performance (Table 1). The Sensitivity analysis revealed that the mid-region and C-terminus ELISAs were able to detect low levels of CSF β-syn, with a functional LLoD of 0.7 pg/mL for the mid-region assay and 2.4 pg/mL for the C-terminus assay. The parallelism analysis revealed a high degree of agreement between the calibrator protein and endogenous CSF β-syn levels upon serial dilution for the mid-region assay (mean parallelism of 105%) and the C-terminus assay (94%; Fig. S6, supplementary material). The dilution linearity responses of the spiked samples were within the accepted limits for the linear range of the mid-region and C-terminus assays (mean of 118% for the mid-region assay and 98% for the C-terminus assay) (Table 1). No hook effect was observed for either assay. The average recovery response of low, intermediate, and high spikes deviated from the acceptance criteria for both the mid-region and C-terminus assays (average of 66% for the mid-region assay and 61% for the C-terminus assay).

Freeze–thaw stability experiments revealed that β-syn levels remained stable throughout three freeze–thaw cycles (mid-region assay, average normalized stability: 100% ± 2%; C-terminus assay, average normalized stability: 101% ± 13%) (Table 1).

Demographic details and biomarker values of the two cohorts

The demographic and clinical parameters of the patients in the cohorts are summarized in Table 2. In the proof-of-concept cohort (controls and AD-dem), the individuals in the control group were significantly younger than the individuals in the AD-dem group were ($p=0.02$); consequently, there was a significant and moderate associations between age and N-terminal β-syn ($rho=0.45$, $p<0.001$), mid-region β-syn ($rho=0.35$, $p=0.017$, $N=47$) and C-terminal β-syn levels ($rho=0.421$, $p=0.002$, $N=50$) across the cohort. Sex did not differ between the controls and the AD-dem group, and there was no association between sex and β-syn. In the clinical cohort (controls, SCD-AD, MCI-AD, AD-dem), the controls were significantly younger than those with MCI-AD were ($p=0.018$). There were no differences in age between the other groups. There was no significant association between age and β-syn levels measured with any of the three ELISAs. The sex distribution did not differ among the four groups, and there was no association between sex and β-syn levels, as measured with any of the three ELISAs.

Clinical performance of the β-syn ELISAs

All clinical samples from both the proof-of-concept and clinical cohorts were measured above the functional LLoDs with all three β-syn assays (Fig. 2). In the proof-of-concept cohort

Table 2 Descriptive statistics of the proof-of-concept cohort and the clinical cohort

	Proof of concept cohort				
Clinical groups	**Control**		**AD-dem**		***p*. value**
Count, N (50 total)	25		25		-
Sex F/M	10/15		16/9		0.157
Age (years), mean (SD)	63 (9)		68 (8)		0.047
CSF Ab42 (pg/mL), median (IQR)	1213 (1062—1585)		491 (433—623)		< 0.001
CSF Tau (pg/mL), median (IQR)	208 (166—234)		449 (340—527)		< 0.001
CSF pTau181 (pg/mL), median (IQR)	17.5 (13.8 – 19.6)		41.9 (33.5 – 53.4)		< 0.001
CSF N-terminus β-syn (pg/mL), median (IQR)	174 (138–217)		317 (262–389)		< 0.001
CSF mid-region β-syn (pg/mL), median (IQR)	36 (28–47)		73 (56–86)		< 0.001
CSF C-terminus β-syn (pg/mL), median (IQR)	79 (56–106)		177 (148–243)		< 0.001
Clinical cohort					
Clinical groups	**Control**	**SCD-AD**	**MCI-AD**	**AD-dem**	***p*. value**
Count, N (160 total)	41	39	40	40	-
Sex F/M	23/18	17/22	20/20	20/20	0.741
Age (years), mean (SD)	64 (3)	66 (3)	66 (3)	65 (3)	0.021
MMSE-score, mean (SD)	27.9 (1.7)	28.1 (1.4)	26.6 (2.2)	19.9 (5.2)	< 0.001
TMT A, median (IQR)	37.0 (33.5 – 41.0)	38.0 (29.5 – 44.0)	42.5 (30.8 – 49.0)	55.5 (37.5 – 87.8)	0.001
TMT B, median (IQR)	90.5 (69.5 – 123.0)	90.0 (76.5 – 132.0)	107.0 (88.0 – 144.0)	208.0 (99.0 – 252.0)	< 0.001
TMT B / TMT A, median (IQR)	2.5 (2.1 – 3.0)	2.6 (2.2 – 3.2)	2.6 (2.2 – 3.4)	4.8 (2.9 – 6.1)	< 0.001
TMT B – TMT B, median (IQR)	57.0 (39.5 78.8)	53.0 (42.0 – 79.0)	72.0 (43.5 – 102.0)	167.0 (63.0 – 220)	< 0.001
APOE e4 carriers, %	27%	69%	77.5%	65%	< 0.001
CSF Aβ42 (pg/mL), median (IQR)	1679 (1256–1700)	815 (545–926)	786 (575–916)	581 (472–717)	< 0.001
CSF Tau (pg/mL), median (IQR)	165 (134–235)	248 (186–313)	287 (224–361)	356 (280—539)	< 0.001
CSF pTau181 (pg/mL), median (IQR)	15.7 (11.0–20.1)	24.7 (18.6—33.9)	29.9 (22.0 – 39.4)	39.3 (26.5 – 53.8)	< 0.001
CSF N-terminus β-syn (pg/mL), median (IQR)	162 (109—223)	249 (179—356)	254 (170—330)	268 (158—456)	< 0.001
CSF mid-region β-syn (pg/mL), median (IQR)	36 (27—49)	52 (44—56)	58 (47—86)	62 (46—81)	< 0.001
CSF C-terminus β-syn (pg/mL), median (IQR)	83 (53—129)	152 (79—207)	153 (110—225)	152 (99—233)	< 0.001

The data are presented as the mean, standard deviation (SD), median, interquartile range (IQR), and percentage (%). AD status was determined according to the CSF levels of phosphorylated tau181 (pTau181)/Aβ42 ratio. The pTau181: tau phosphorylated at threonine 181, Aβ42 amyloid beta$_{1\text{-}42}$, *β-syn* beta-synuclein, *SCD* subjective cognitive decline, *MCI* mild cognitive impairment, *AD* Alzheimer's disease, *MMSE* Mini-Mental State Examination, *TMT* trail making test A and B, TMT B / TMT A: ratio of trail making test B and A, TMT B – TMT B: trail making test A score subtracted from B. CSF biomarker levels were measured using either the Innotest or Elecsys platforms, with Innotest values converted to their Elecsys equivalents using the equations described earlier [31], resulting in a single CSF variable per analyte for consistency. We applied the Kruskal–Wallis's test to determine the differences between the groups and the results are reported as *p* values. The results were rounded

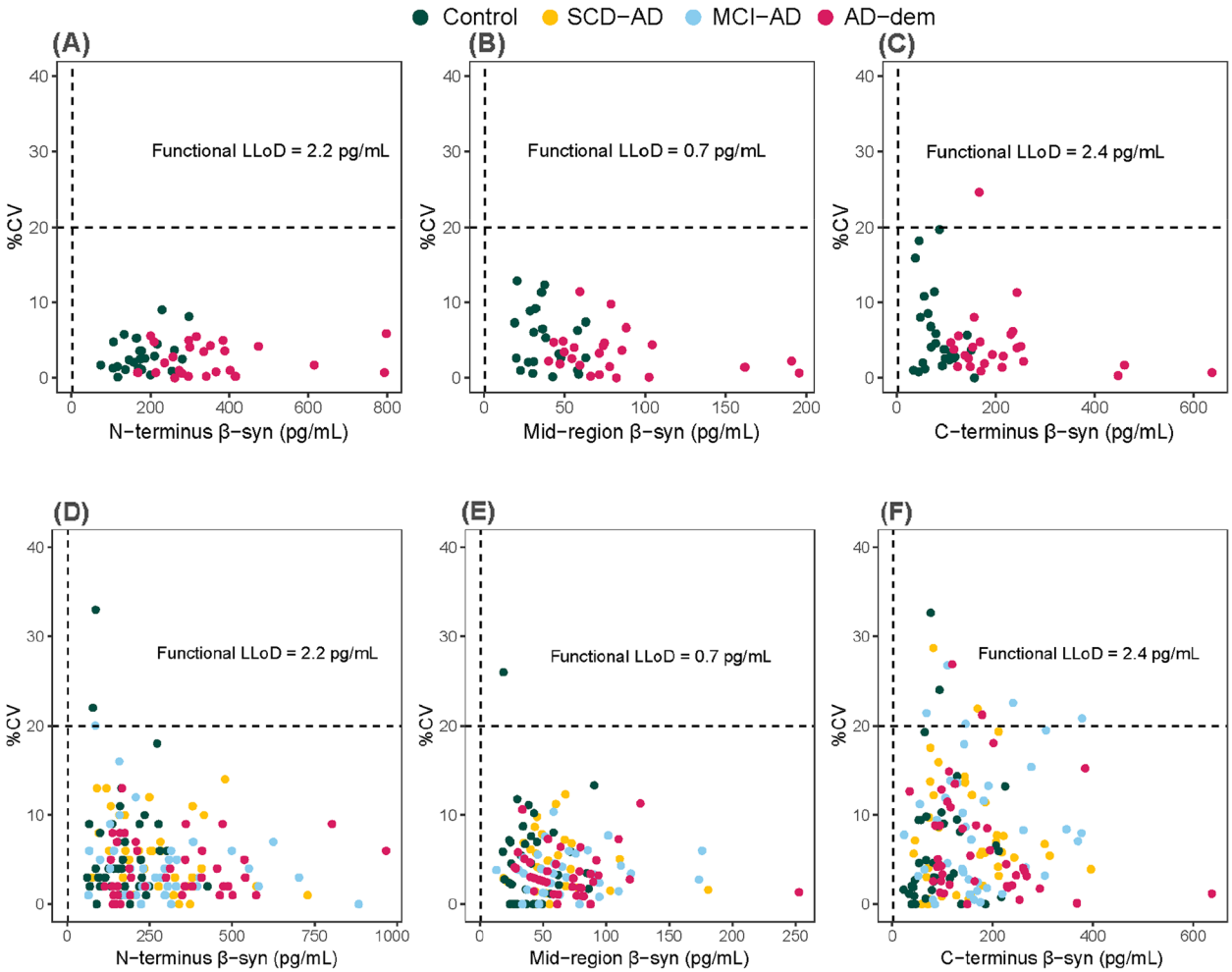

Fig. 2 Precision profiles of the CSF β-syn measurements using the three ELISAs. Performance in the proof-of-concept cohort (**A-C**) and the clinical cohort (**D-F**). In the figures, the levels of β-syn measured with each assay are plotted on the x-axis against the percentage coefficient of variation of the duplicate measurements (%CV) on the y-axis. The results were color-coded per group as follows: control, SCD-AD, MCI-AD, and AD-dem. For all the figures, the vertical dashed line represents the functional lowest limit of detection (LLoD) (i.e., mean of 16 blanks*10 × standard deviation of the blanks*assay's CSF dilution factor). The horizontal line shows the limit of accepted precision (20% CV). CSF: cerebrospinal fluid, β-syn: beta-synuclein, SCD: subjective cognitive decline, AD: Alzheimer's disease, MCI: mild cognitive impairment, AD-dem: dementia due to AD

(Fig. 2A-C), the average intra-assay CVs were 2.7% (with no sample > 20%CV) for the N-terminus assay, 5.6% (with no sample > 20%CV) for the mid-region assay and 6.5% (with 1 sample > 20%CV) for the C-terminus assay. In the clinical cohort (Fig. 2D-F), the mid-region assay demonstrated the highest precision in detecting β-syn in the CSF samples, with an average intra-assay CV of 3.9% (with 1 sample > 20%CV) compared with the N-terminus assay with a 4.9% CV (with 3 samples > 20% CV) and the C-terminus assay with a 7.5%CV (with 11 samples > 20%CV) for the C-terminus assay.

Diagnostic performance of CSF β-syn across AD stages

In the proof-of-concept cohort, β-syn levels were significantly elevated in the AD-dem group compared with those in the control group with all three assays (Fig. 3). The C-terminus of CSF β-syn exhibited greater difference in median levels in AD-dem patients than in controls (2.3-fold) and had the highest AUC among the three assays (AUC = 0.96, 95% CI = 0.91–1.00, Delong $P < 0.001$; compared with the other two assays). Compared with that of the controls, the N-terminus of β-syn had a median fold change of 1.8 in the AD-dem group, with an AUC of 0.91 (95% CI: 0.82–0.99) (Fig. 3, Table 2). Mid-region β-syn showed a median fold change of 2.0 in the AD-dem group, with an AUC of 0.92 (95% CI: 0.85–0.99).

In the clinical cohort, CSF β-syn levels were significantly elevated in individuals with AD compared with controls, independent of their syndromal disease stage,

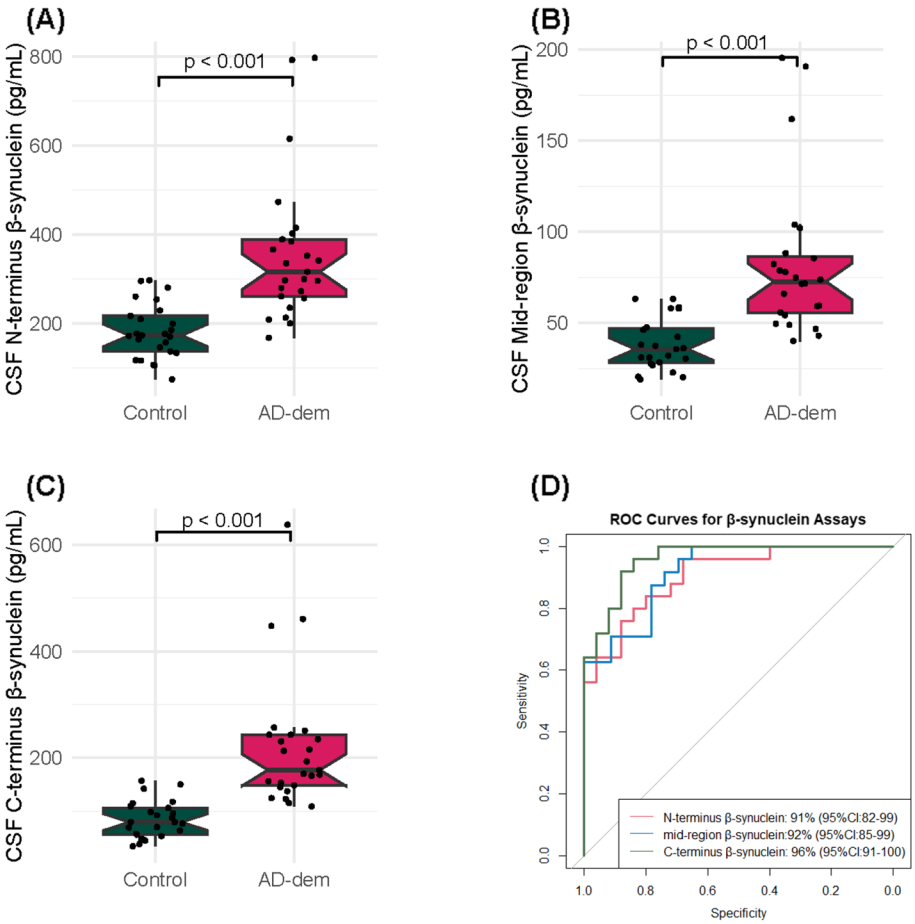

Fig. 3 Comparative analysis of CSF β-syn measurements using N-terminus, mid-region, and C-terminus ELISAs in the proof-of-concept cohort. (**A-C**) Box plots illustrating the β-syn levels measured at different epitopes (N-terminus, mid-region, and C-terminus) in the control and AD dementia groups. (**D**) ROC curves for β-syn measured at different epitopes: N-terminus β-syn: AUC = 0.91 (95% CI: 0.82–0.99), Mid-region β-syn: AUC = 0.92 (95% CI: 0.85–0.99), and C-terminus β-syn: AUC = 0.96 (95% CI: 0.91–1.00). The *p values* represent the results of the Wilcoxon test for comparisons of group levels. β-syn: beta-synuclein, ROC: receiver operating characteristic, AUC: area under the curve, AD: Alzheimer's disease

according to all three assays (all: $p < 0.001$) (Fig. 4). No differences were detected in CSF β-syn levels across the AD groups (SCD-AD, MCI-AD, and AD-dem), except between SCD-AD and AD-dem, as measured with the mid-region β-syn assay ($p = 0.035$). At the trend level, the mid-region assay also showed promise in discriminating between SCD-AD patients and MCI-AD patients ($p = 0.083$). Overall, the three CSF β-syn measurements exhibited comparable diagnostic accuracy for the AD groups versus the control group (Delong $P > 0.05$). For the controls vs SCD-AD comparison, the optimal cutoff values were 168.5 pg/mL for N-terminus β-syn (sensitivity: 82%, specificity: 58.5%), 43.25 pg/mL for mid-region β-syn (sensitivity: 77%, specificity: 66%), and 141.02 pg/mL for C-terminus β-syn (sensitivity: 56%, specificity: 83%) (elaborated results of all comparisons are shown in Table S2). The median fold-change in the N-terminus of β-syn was 1.5 to 1.7 in the control group versus the AD groups, with moderate diagnostic accuracy and AUCs ranging from 0.73 to 0.74 (Fig. 5). Compared with those in the controls, mid-region β-syn exhibited fold changes between 1.4 and 1.7 across the AD groups and moderate diagnostic accuracy, with AUCs ranging from 0.71 to 0.80 (Fig. 5). The C-terminus CSF β-syn levels exhibited consistent fold changes (1.8) in the medians of the control group versus the AD groups with moderate diagnostic accuracy and AUCs ranging from 0.71 to 0.78 (Fig. 5, Table S2).

We evaluated whether the ratios of β-syn to the core AD biomarkers, or among different β-syn measurements, could enhance diagnostic sensitivity and differentiate between AD patients and controls. Among these measurements, the β-syn/Aβ42 ratio demonstrated superior diagnostic performance compared with individual measurements of β-syn or Aβ42 alone in terms of better discrimination between the clinical groups

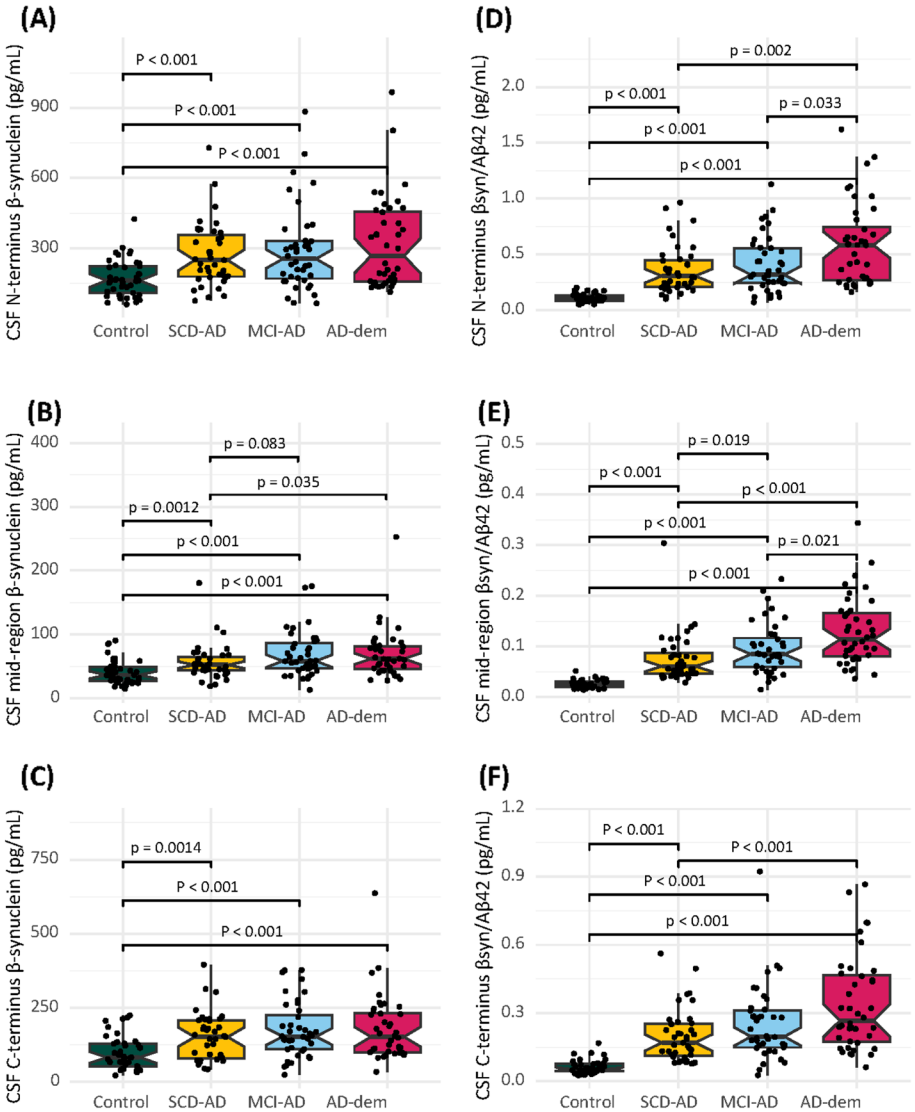

Fig. 4 Comparative analysis of CSF β-syn measurements using N-terminus, mid-region, and C-terminus ELISAs in the clinical cohort. (**A**-**C**) Box plots illustrating the levels of β-syn epitopes (N-terminus, mid-region, and C-terminus) across four clinical groups: control, SCD-AD, MCI-AD, and AD-dem. (**D**-**F**) Box plots illustrating the ratio of β-syn epitopes (N-terminus, mid-region, and C-terminus) to CSF amyloid beta 42 (Aβ42) across four clinical groups: control, SCD-AD, MCI-AD, and AD-dem. β-syn: beta-synuclein, AD: Alzheimer's disease

(Fig. 4. D-F, Fig. S7) and in terms of AUCs (Fig. 5). Specifically, the mid-region β-syn/Aβ42 ratio was the only measure that significantly distinguished between SCD-AD and MCI-AD, outperforming ratios involving the N- or C-terminus β-syn assays (Fig. 4, Fig. S7).

Correlations between CSF β-syn levels and AD biomarkers, cognitive scores, and executive functions

The different CSF β-syn measurements correlated strongly with the different CSF β-syn measurements in both the proof-of-concept cohort (Fig. S8; Spearman's rho: 0.96, $p < 0.001$) and the clinical cohort (Fig. 6; range Spearman's rho: 0.69–82, $p < 0.001$). In the proof-of-concept cohort, all β-syn levels showed a strong positive correlation with CSF tau and pTau181 (Fig. S7; Spearman's rho range: 0.80–0.88, $p < 0.001$), and a moderate negative correlation with CSF Aβ42 (Fig. S7; range Spearman's rho: −0.54- −0.62, $p < 0.001$). Similarly, in the clinical cohort, all β-syn levels were strongly positively correlated with CSF tau and pTau181 (Fig. 6; Spearman's rho range: 0.77–0.88, $p < 0.001$). However, β-syn levels in the clinical cohort showed no overall correlation with CSF Aβ42, except for a moderate association observed only in the control group (Fig. 6).

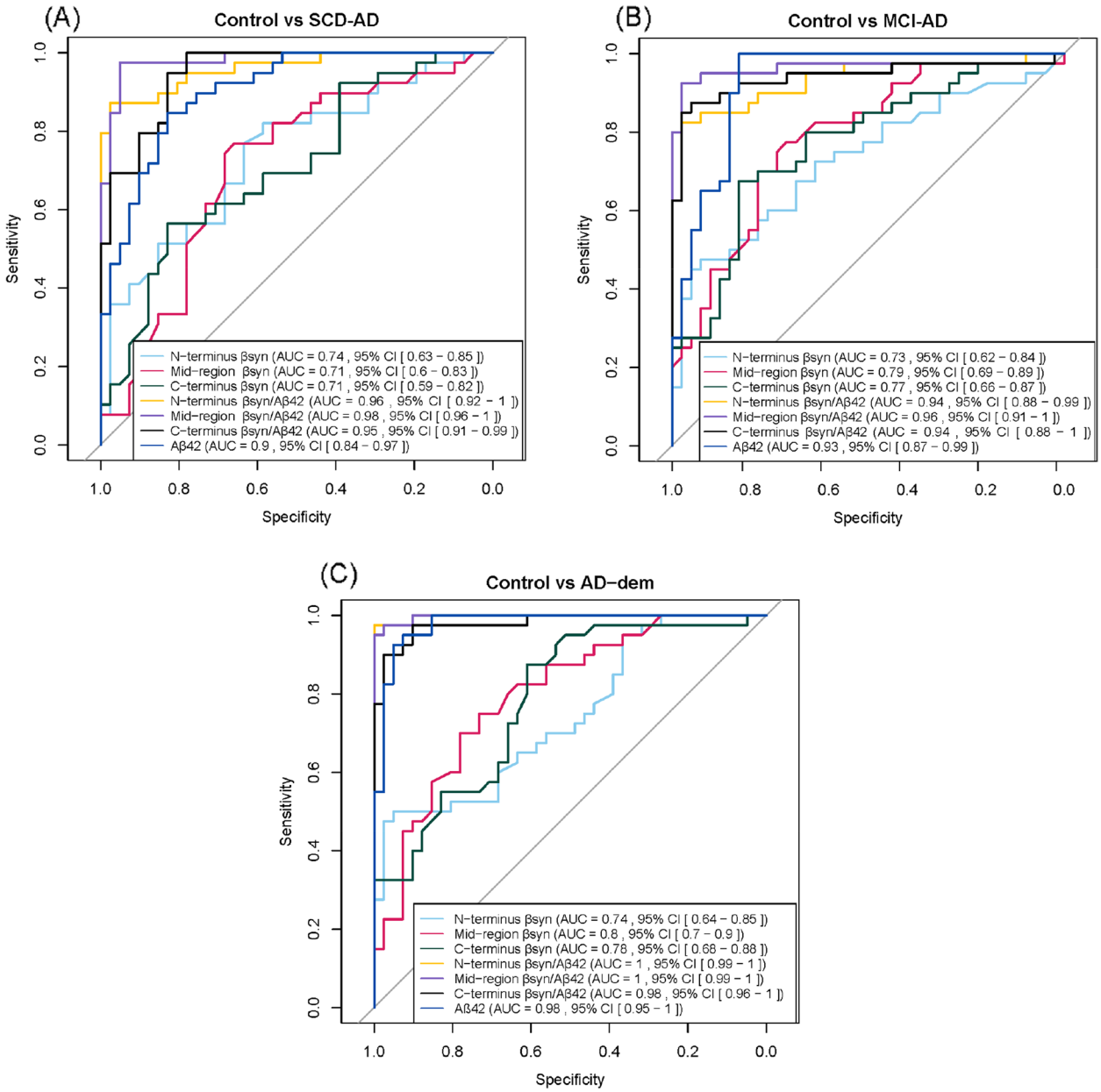

Fig. 5 ROC curves for β-syn (N-terminus, mid-region, and C-terminus) and Aβ42 levels, and β-syn/Aβ42 ratios. The figure depicts their diagnostic performance in distinguishing between SCD controls and SCD-AD (A), MCI-AD (B) and AD-dem (C). SCD-AD: subjective cognitive decline due to AD, MCI-AD: mild cognitive impairment due to AD, AD-dem: dementia due to AD, β-syn: beta-synuclein, Aβ42: amyloid beta $_{(1\text{-}42)}$, ROC: receiver operating characteristic, AUC: area under the curve

Additionally, the levels measured with the mid-region and C-terminus β-syn assays, but not those measured with the N-terminus β-syn assay, showed modest negative but significant correlations with MMSE scores across the clinical cohort (rho = −0.19 and −0.22, respectively; Fig. 6), although not within the diagnostic groups. None of the β-syn measurements correlated with psychomotor speed or executive functions (measured by the TMTA and TMTB).

Discussion

We introduce two novel in-house CSF ELISAs for detecting mid-region and C-terminus β-syn in CSF and present them in accordance with an in-house version of the existing N-terminus β-syn ELISA [16, 18]. Our novel assays demonstrated good analytical performance in terms of sensitivity, precision, and parallelism. We hypothesized that targeting β-syn-specific epitopes such as those in the mid-region and C-terminus regions, with highly sensitive

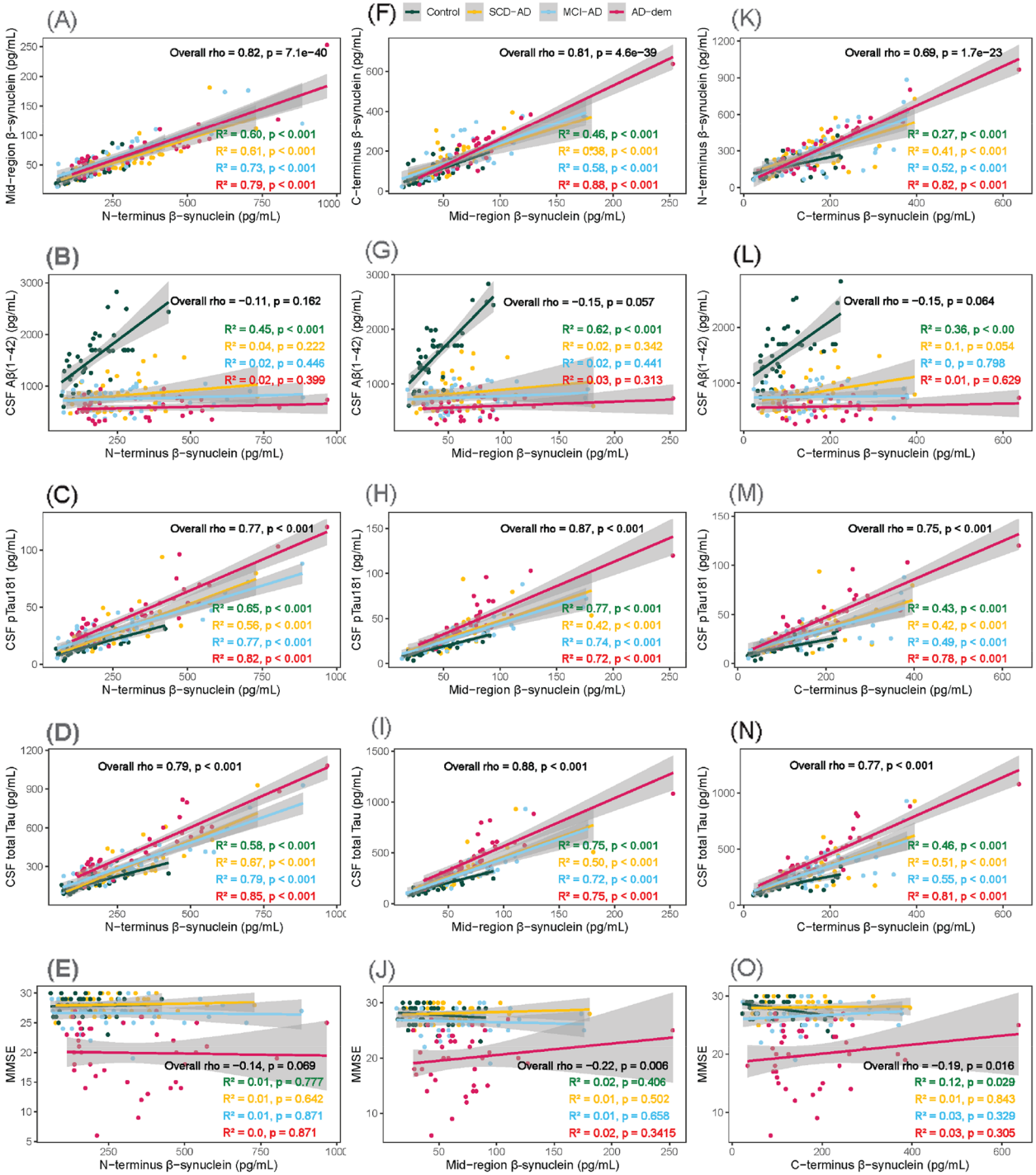

Fig. 6 Correlation plots between CSF β-syn levels measured with the N-terminus, mid-region, and C-terminus ELISAs and between CSF β-syn levels and the core AD biomarkers, Mini-Mental State Examination (MMSE) scores, and trail making test B/A ratio scores in the clinical cohort. **A**-**C** Scatter plots illustrating the correlations between the N-terminus, mid-region, and C-terminus β-syn levels in CSF. **D**-**F** Correlations between CSF Aβ42 and β-syn levels. **G**-**I** Correlations between pTau181 and β-syn levels. **J**-**L** Correlations between CSF Tau and β-syn levels. **M**–**O** Correlations between MMSE scores and β-syn levels. **P**-**R** Correlations between the TMT B/A ratio and β-syn levels. The colors represent different clinical groups: control (dark green), SCD-AD (yellow), MCI-AD (light blue), and AD-dem (magenta). Each plot includes a Spearman correlation coefficient in R-squared for each of the clinical groups and in (overall rho) for the overall correlation of all clinical groups along with the associated *p* value. The shaded gray areas represent the 95% confidence intervals for the linear regression lines. CSF: cerebrospinal fluid, β-syn: beta-synuclein, Aβ42: amyloid beta$_{1-42}$; pTau181: phosphorylated tau at threonine 181, TMT B/A: trail-making test B score divided by the A test score

and specific assays could enhance the detection of synaptic degeneration across the AD continuum. Therefore, we targeted β-syn-specific epitopes that are not conserved across the synuclein protein family with our mid-region and C-terminus assays. Epitope mapping and specificity experiments confirmed the specificity of the utilized antibodies for β-syn in our novel mid-region and C-terminus assays. We showed that CSF β-syn levels were already altered in the early stages of AD, which confirms earlier findings that β-syn is a promising early biomarker for AD. Notably, all three assays showed comparable diagnostic potential for differentiating AD patients from controls with SCD, suggesting the clinical utility of β-syn measured at various regions as a synaptic biomarker in AD, although only our mid-region β-syn assay seems to have some value in discriminating between the AD syndromal stages.

The availability of multiple analytically validated β-syn assays that target different epitopes while all showing similar clinical performance support the use of β-syn as a synaptic biomarker for AD. We observed that the levels measured by the mid-region β-syn assay were consistently lower than those measured by the N- and C-terminal assays, despite the use of similar assay components. However, this does not indicate differential performance; the mid-region assay showed strong analytical reliability. The lower levels detected with the mid-region assay might reflect unique conformational changes or interactions with proteolytic peptides around the mid-region epitopes. Our findings highlight the need for further studies to explore whether and how conformational changes and proteolytic interactions impact disease pathology as well as β-syn detection. Overall, our novel assays showed good analytical performance, and have potential for further improvement. Such improvements may further increase measurement accuracy and facilitate scalable assay production to make the assays available to the wide research community for further validation.

Our findings confirm the diagnostic potential of β-syn as a biomarker for early synaptic dysfunction in AD patients, which aligns with the findings of previous studies [14, 16–18, 22]. Across the AD continuum, we observed that CSF β-syn levels were consistently elevated in AD patients using all three assays. Notably, the mid-region β-syn assay uniquely differentiated between SCD-AD patients and AD-dem patients, as well as between SCD-AD patients and MCI-AD patients, at a trend level. Furthermore, the β-syn/Aβ42 ratio showed enhanced diagnostic accuracy across AD stages, with the mid-region β-syn/Aβ42 ratio being the only combination used to significantly differentiate between these groups, outperforming all the other ratios and individual biomarkers, including Aβ42 alone. The fact that CSF β-syn was already increased in preclinical AD confirms that synaptic degeneration is an early hallmark of AD pathology [5] and indicates that β-syn is more effective for detecting AD-related changes than for detecting increased synaptic dysfunction during disease progression, although we did note some value in staging disease severity with the mid-region β-syn-specific assay. We observed a strong correlation between β-syn and tau biomarkers, which suggests that they play a shared role in key pathophysiological processes in AD [14, 16–18, 20]. Our results might indicate that synaptic degeneration across the AD syndromal stages plateaus earlier than tau aggregation does, as β-syn levels are more similar across the syndromal stages, whereas tau and pTau levels continue to increase. This pattern aligns with the concept that tau aggregation continues to intensify, even as synaptic changes reach a stable phase [38]. In contrast, the weaker correlation we observed between β-syn and Aβ42 may reflect the earlier plateauing of amyloid deposition in the AD continuum than in the context of synaptic degeneration [38]. A previous study reported moderate correlations between β-syn and Aβ42 [18], but discrepancies between our findings may be due to differences in sample size or cohort characteristics.

Our results on the diagnostic accuracy of β-syn are in line with the diagnostic potential of other synaptic biomarkers, such as synaptosomal-associated protein-25 kDa (SNAP25), vesicle-associated membrane protein-2 (VAMP2), neuronal pentraxin-2 (NPTX2), glutamate ionotropic receptor-4 (GluR4), and neurogranin (Ng), in both CSF and plasma [15, 16, 39]. The advantage of β-syn (presynaptic biomarker) over Ng (postsynaptic biomarker) could be its AD specificity [15, 39] and the fact that presynaptic biomarkers are likely more affected in AD brains than are postsynaptic biomarkers [40].

While CSF measurements of β-syn have shown consistent promise as a biomarker for AD, further research is needed, e.g., to evaluate its potential as a blood-based biomarker within the ATX(N) framework [6, 41]. Although we did not find major differences in diagnostic accuracy in our study for the different CSF β-syn assays that target specific epitopes, β-syn specificity may be more important when it is measured in blood, particularly since α-syn is highly abundant in blood, it could show cross-reactivity with α-syn [14, 42]. Notably, although the N-terminus antibody *EP1646Y* did cross react with α-syn, in our specificity experiments, cross-reactivity was confuted earlier when the same N-terminus antibody was used in an immunoassay format [16].

Transforming each of our ELISA setups into ultrasensitive assays, e.g., on the Simoa HD-X, is an important next research step to determine whether epitope specificity impacts its clinical utility when β-syn is measured in blood. Additionally, exploring the timing of β-syn

changes in AD and its relationship with other emerging biomarkers in longitudinal studies could further clarify the additional context of the use of β-syn as a biomarker in (early) AD. Last, we did not investigate the biomarker potential of β-syn measured at specific regions in neurological diseases other than AD. Some studies have reported elevations of N-terminus β-syn in other neurodegenerative diseases, such as Parkinson's disease. It is worth investigating whether the detection of β-syn at specific regions results in pattern similar to that of β-syn detection at the N-terminus in non-AD [16, 19, 43].

One of the strengths of our study is that we were able to establish two CSF β-syn-specific ELISAs, which we extensively validated to have robust analytical performance. Additionally, a strength of this study is that we were able to characterize the utilized antibodies and map their epitopes. Our assays were clinically validated in a proof-of-concept cohort and subsequently in a well-characterized set of samples from the Amsterdam Dementia Cohort across the AD continuum. Although their sample sizes were small, two separate sample sets increase the confidence in the findings. A limitation is that we did not address the question of whether a mid-region or C-terminus measurement of β-syn would increase the diagnostic accuracy of AD in comparison to other dementias, such as DLB or PD-related dementia, which have α-syn pathology.

In conclusion, our study presents novel ELISAs for detecting mid-region and C-terminus β-syn in CSF, which demonstrated robust analytical performance. All β-syn measurements were effective in distinguishing AD patients from controls in our study in the earliest syndromal stages, while none of the β-syn assays had clearly superior diagnostic performance. The fact that β-syn measurements at different regions of the protein results in the same conclusion on its usefulness as a biomarker, supports of the biomarker robustness. Further validation in independent cohorts will confirm the robustness of β-syn-specific measurements as biomarkers of AD.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13195-025-01716-8.

Supplementary Material 1

Supplementary Material 2

Acknowledgements
We would like to extend our sincere gratitude to Dirk Jacobs from ADx NeuroSciences for his invaluable contributions to the development and validation of the β-syn antibodies used in this study. We also thank Kees van Uffelen, Lisanne in 't Veld, Imane Al Idrissi, and Azzam Aladdin from Amsterdam UMC for their technical support during the research process. Additionally, we appreciate the support from Abcam and Ravindran Kumaran (Abcam, Cambridge, UK) for providing the antibodies crucial to this research. Their collective efforts have been instrumental in the successful completion of this work.

Authors' contributions
Conception and design of the work: Charlotte E. Teunissen, Eugeen Vanmechelen, Julie Goossens, Inge M.W Verberk, Sherif Bayoumy. Acquisition, analysis, or interpretation of data: Sherif Bayoumy, Julie Goossens, Charlotte De Rocker, Senna Sie, Nolan J. Barrett, Wiesje M. van der Flier, Charlotte E. Teunissen, Eugeen Vanmechelen, and Inge M.W Verberk. All the authors contributed to drafting the work or revising it critically for important intellectual content and finally approved the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Funding
This work was performed under the agreement of the Marie Curie International Training Network, grant agreement No 860197 (MIRIADE). The SCIENCe project receives funding from Stichting Dioraphte and the Noaber Foundation.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All procedures involving human subjects were performed following the Helsinki Declaration of 1975. All participants provided written informed consent for the use of medical data and biomaterials for research purposes. The study was approved by the local Ethics Committee (2023.0759; Amsterdam UMC VUmc medical ethical committee).

Consent for publication
Not applicable.

Competing interests
Julie Goossens and Charlotte De Rocker are employees of ADx NeuroSciences NV. Eugeen Vanmechelen is a cofounder of ADx NeuroSciences. Wiesje van der Flier has conducted research programs funded by ZonMW, Nederlandse Organisatie voor Wetenschappelijk Onderzoek, European Union–Seventh Framework Programme, European Joint Programme on Neurodegenerative Diseases, Alzheimer Nederland, Hersenstichting CardioVascular Onderzoek Nederland, Health~Holland, Topsector Life Sciences & Health, Stichting Dioraphte, Gieskes-Strijbis Fonds, Stichting Equilibrio, Edwin Bouw Fonds, Pasman Stichting, Alzheimer & Neuropsychiatrie Foundation, Philips, Biogen MA, Amprion, Novartis-NL, Life Molecular Imaging, Avid, Roche, Fujifilm, Eisai, and Combinostics. WMvdF holds the Pasman chair; and is a recipient of ABOARD, which is a public–private partnership receiving funding from ZonMW (#73305095007) and Health~Holland, Topsector Life Sciences & Health (PPP-allowance; #LSHM20106). All funding is paid to her institution; WMvdF is a recipient of TAP-dementia, receiving funding from ZonMw (#10510032120003) in the context of the Onderzoeksprogramma Dementie, part of the Dutch National Dementia Strategy; Gieskes Strijbis Fonds also contributes to TAP-dementia. WMvdF is a consultant for the Oxford Health Policy Forum Community Interest Company, Roche, Eisai, and Biogen MA; has been an invited speaker at Boehringer Ingelheim, Biogen MA, Danone, Eisai, WebMD Neurology (Medscape), NovoNordisk, Springer Healthcare, and the European Brain Council; and has participated in advisory boards for Biogen MA, Roche, and Eli Lilly. FV has served as a consultant for Biogen (payment to Maastricht University). Charlotte E. Teunissen is a recipient of ABOARD, and Health~Holland, Topsector Life Sciences & Health (PPP-allowance; #LSHM20106). CT is also a contract researcher for ADx Neurosciences, AC-Immune, Aribio, Axon Neurosciences, Beckman-Coulter, BioConnect, Bioorchestra, Brainstorm Therapeutics, Celgene, Cognition Therapeutics, EIP Pharma, Eisai, Eli Lilly, Fujirebio, Grifols, Instant Nano Biosensors, Merck, Novo Nordisk, Olink, PeopleBio, Quanterix, Roche, Siemens, Toyama, Vivoryon, and the European Commission. CT has received payments or honoraria from Eli Lilly, Grifols, Novo Nordisk, Olink, and Roche, where all payments were made to her institution. CT also serves on the editorial boards of Medidact Neurologie/Springer and on Neurology: Neuroimmunology & Neuroinflammation. CT is the editor of Alzheimer Research and Therapy. Inge M.W. Verberk is a recipient of grants from Amsterdam UMC and Health ~ Holland. All the other authors report no conflicts of interest.

Author details
[1]Neurochemistry Laboratory, Department of Laboratory Medicine, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam Neuroscience, De Boelelaan 1117, 1018 HVm, P.O. Box 7057, 1007 MB Amsterdam, Netherlands. [2]ADx NeuroSciences NV, Technologiepark 6, B-9052 Ghent, Belgium. [3]Alzheimer Center, Department of Neurology, UMC, Vrije Universiteit Amsterdam, De Boelelaan 1117, Amsterdam 1018 HV, The Netherlands. [4]Department of Epidemiology and Data Science, UMC, Vrije Universiteit Amsterdam, De Boelelaan 1105, Amsterdam 1081 HV, The Netherlands.

Received: 14 November 2024 Accepted: 13 March 2025
Published online: 14 April 2025

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