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Short-term variability of Alzheimer's disease plasma biomarkers in a mixed memory clinic cohort

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Abstract

Background For clinical implementation of Alzheimer's disease (AD) blood-based biomarkers (BBMs), knowledge of short-term variability, is crucial to ensure safe and correct biomarker interpretation, i.e., to capture changes or treatment effects that lie beyond that of expected short-term variability and considered clinically relevant. In this study we investigated short-term intra- and inter-individual variability of AD biomarkers in the intended use population, memory clinic patients.

Methods In a consecutive sample of memory clinic patients (AD $n = 27$, non-AD $n = 20$), blood samples were collected on three separate days within a period of 36 days and analysed for plasma A β 40, A β 42, p-tau181, p-tau217, p-tau231, T-tau, neurofilament light (NfL), and glial fibrillary acidic protein (GFAP). We measured intra- and inter-individual variability and explored if the variability could be affected by confounding factors. Secondly, we established the minimum change required to detect a difference between two given blood samples that exceeds intra-individual variability and analytical variation (reference change value, RCV). Finally, we tested if classification accuracy varied across the three visits.

Results Intra-individual variability ranged from ~3% (A β 42/40) to ~12% (T-tau). Inter-individual variability ranged from ~7% (A β 40) to ~39% (NfL). Adjusting the models for time, eGFR, Hba1c, and BMI did not affect the variation. RCV was lowest for A β 42/A β 40 (~15%/+ ~17%) and highest in p-tau181 (~30/+ ~42%). No variation in classification accuracies was found across visits.

Conclusion We found low intra-individual variability, robust to various factors, appropriate to capture individual changes in AD BBMs, while moderate inter-individual variability may give rise to caution in diagnostic contexts. High RCVs may pose challenges for AD BBMs with low fold changes and consequently, short-term variability is important to take into consideration when, e.g., estimating intervention effect and longitudinal changes of AD BBM levels.

Trial registration Clinicaltrials.gov (NCT05175664), date of registration 2021–12-01.

Keywords Alzheimer's disease biomarkers, Blood-based biomarkers, Memory clinic cohort, Short-term variability, Intra- and Inter-individual variability, Reference change value (RCV), Plasma A β 42/A β 40 ratio, Phosphorylated tau (p-tau), Neurofilament light (NfL), Glial fibrillary acidic protein (GFAP)

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Background

Blood-based biomarkers (BBMs) for Alzheimer's disease (AD) are emerging and have potential to revolutionize diagnostics and prognostication of the disease. As new disease-modifying drugs are on the horizon, there is an urgent need for easily available biomarkers to detect disease pathology, select patients for advanced diagnostic work-up or treatment, and to track treatment effects in AD patients. Advances in measuring AD BBMs [1, 2] may supplement and in some cases replace lumbar puncture procedures and PET scans when a two-step diagnostic workflow is used [3], and have opened the door for repeated measurements monitoring AD pathophysiological processes. This study investigates short-term variability of AD BBMs to ensure correct interpretation of changes in biomarker concentrations as clinically relevant.

At present, AD BBMs include amyloid beta ($A\beta_{40}$, $A\beta_{42}$ and the $A\beta_{42}/A\beta_{40}$ ratio) [4, 5], phosphorylated tau variants (p-tau181, p-tau217, p-tau231) [6], total-tau (T-tau), neurofilament light (NfL) [7] and glial fibrillary acidic protein (GFAP) [8]. These constitute a panel of BBMs reflective of amyloid pathology, neurodegeneration, and inflammation, valuable as diagnostic and prognostic biomarkers and as markers of target engagement in clinical trials [9]. However, several factors such as pre-analytical and confounding factors, assay variability and short-term variability must be considered before implementation of AD BBMs. Preanalytical factors have been investigated to ensure stable biomarker concentrations [10–15] and confounding factors such as age, kidney dysfunction and body mass index are known to affect levels of BBMs [16–18]. Recently also food intake have been suggested to alter concentrations of AD BBMs [15]. Furthermore, efforts to optimize assays and minimize assay variability are ongoing [5, 19]. Indeed, low fold change and large overlap in i.e. plasma $A\beta_{42}/A\beta_{40}$ ratio values result in a too low robustness to withstand these factors in clinical routine, making this biomarker unsuitable for diagnostic use [20, 21].

However, only few studies have investigated short-term variability in AD biomarkers. One study has examined test–retest stability between two time points of plasma $A\beta_{42}/A\beta_{40}$, p-tau217, NfL and GFAP in 38 participants from a memory clinic with a 6–10 weeks interval [22]. In healthy individuals biological variation of $A\beta_{42}/A\beta_{40}$, p-tau181, p-tau217, p-tau231, NfL and GFAP have been estimated in previous studies [23–26]. Thus, there is a need, recently highlighted by an expert group, to examine biological variation (BV), of AD BBMs in patients with and without AD [27]. The term "biological variation", determined by a patient's physiology, is a fundamental concept in clinical chemistry and is usually estimated in

a reference population of healthy individuals. However, BV may be impacted by underlying pathological disease process [28], necessitating estimation of BV in the target population. Within-subject and between-subject BV quantify different aspects of BV and may be influenced by age, sex, genetic factors, chronic diseases, lifestyle factors etc. Within-subject BV is a measure of the variation of a biomarker's concentration around an individual's homeostatic set-point. Between-subject BV is a measure of the variation across individuals' homeostatic set point in a group, i.e. how much the concentration differs from one person to another within the group [26, 29]. However, when studying a disease population, biological variation, especially between-subjects, may be influenced by disease heterogeneity and varying disease severities within the group and may therefore not directly represent the inherent biological variation of the marker as in a healthy population. Therefore, throughout this article we refer to intra-individual and inter-individual variability, since the contribution of disease-related factors cannot be dismissed. Thus, intra-individual variability can be used to assess whether changes in biomarker levels in individual patients are beyond physiological variation whereas inter-individual variability can be applied to investigate whether within group physiological or disease-related variation is favorable towards e.g., application of the biomarker in a diagnostic role.

Thus, it is of great importance to examine short-term variability of AD BBMs in patients with AD to examine the impact of intra- and inter-individual variability on the interpretation of the biomarker results. Knowledge on the expected variability of BBMs is crucial to establish potential diagnostic cut-offs and to capture longitudinal changes or treatment effects that lies beyond that of intra- and inter-individual variability and thereby should be considered as clinically relevant changes.

Here we examined short-term variability of several AD BBMs at three different time points within 36 days in a real-life memory clinic setting. We measured intra- and inter-individual variability of AD BBMs in patients with and without AD. Secondly, we explored if short-term variability was affected by confounding factors. Also, we established the minimum change required to detect a difference between two given blood samples. Finally, for more clinical reliable use we tested if classification accuracy of the biomarkers differed between the three time points.

Methods

Study design and participants

This is a test–retest variability study with repeated blood sampling performed three times on the same individual within 36 days, see Fig. 1. Participants were patients with cognitive complaints, referred to the Memory Clinic

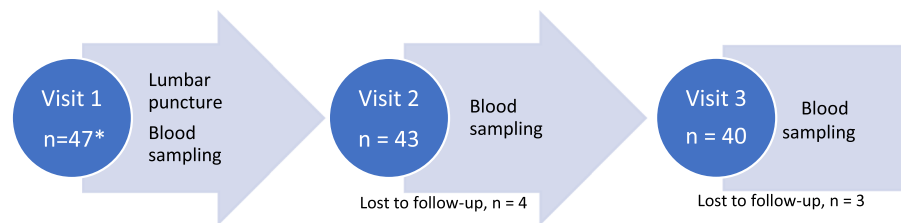


Fig. 1 Study design

Visit 1 (baseline), Visit 2 (4–16 days post LP), Visit 3 (19–36 days post LP). *Two blood samples from visit 1 were lost due to technical issues. Lost to follow-up was due to personal reasons for the participants

at Rigshospitalet, Copenhagen where physicians had requested CSF analysis of AD biomarkers as part of the routine diagnostic evaluation for suspected AD. Participants were consecutively and prospectively recruited, and the inclusion criteria were 1) a lumbar puncture as part of the diagnostic assessment and 2) Mini-Mental State Examination (MMSE) ≥ 20 . Exclusion criteria were 1) stroke within the past 3 months, 2) previous or existing major psychiatric conditions such as schizophrenia, bipolar affective disorder, or psychosis, 3) alcohol or substance abuse within the last two years, or 4) participation in intervention studies. Study inclusion was conducted between January–March 2022 and between August 2022–May 2023. The study was approved by the Danish Research Ethics Committee (H-21044863) and followed the tenets of the 1975 Helsinki Declaration. The study was registered at clinicaltrials.gov (NCT05175664). Written informed consent was obtained from each participant prior to enrolment.

Study procedure

A blood sample (baseline – denoted V1) was drawn via venipuncture immediately following the lumbar puncture procedure. The blood sampling was repeated on two separate occasions. The second blood sampling (visit 2 (V2)) occurred 4–16 days after baseline and the third (visit 3 (V3)) 19–36 days after V1. Blood samples were carried out between 9:00 AM and 15:00 PM. No information about last food intake was collected.

Sample processing

Blood was collected in ethylenediaminetetraacetic acid (EDTA)–plasma tubes according to hospital guidelines. Plasma and CSF, collected according to standard operating procedures for lumbar puncture, were centrifuged at 2,000 g, 4 °C for 10 min within 30 to 120 min after collection. Plasma and CSF were then redistributed into 250 μ l aliquots and stored at -80 °C until further analysis.

Biomarker assays

All samples were analysed at the Department of Psychiatry and Neurochemistry, the Sahlgrenska Academy, University of Gothenburg, in November 2023 to February 2024. Biomarker concentrations in both plasma and CSF were measured by Single molecule array (Simoa) technology by Quanterix HD-X [30]. The multiplex, Neurology 4-plex E assay from Quanterix was used to analyse plasma and CSF A β 42, A β 40, NfL, and GFAP. Plasma and CSF p-tau217 were measured using Simoa singleplex assay from Quanterix, ALZpath p-tau217. To analyse plasma and CSF p-tau181 and p-tau231 Simoa assays developed at University of Gothenburg were used [31, 32], while the Quanterix Simoa singleplex TAU kit was used for T-tau.

For all BBMs, calibrators were run in duplicates and obvious outlier calibrator replicates were masked before curve fitting. Plasma samples for A β 40, A β 42, NfL, GFAP and total-tau analysis were diluted fourfold, p-tau217 samples were diluted threefold, and p-tau231 and p-tau181 were diluted twofold. Results have been compensated for the dilution. A β 42/ A β 40 ratio was calculated. All samples were run in singlicates, and analyses were performed according to Simoa instructions [30]. Two QC levels were run in duplicates in the beginning and the end of each plate and determined the intermediate precision between plates, labelled inter-assay variability, CV_A . All samples were analysed in one round of experiments and all three plasma samples from each participant were run on the same plate. Concentrations lower than the calibration curve were labelled under limits of quantification (<LOQ).

Cognitive tests and diagnoses

Cognitive test results were extracted from medical files and stored in an electronic database (REDCap, Vancouver). Cognitive test results included the Mini-Mental State Examination (MMSE) [33] and Addenbrookes Cognitive Examination (ACE) [34] administered by a trained nurse and medical doctor, respectively. Patients were diagnosed based on criteria for MCI or dementia

due to AD [35, 36], dementia with Lewy bodies [37] frontotemporal dementia [38], AD with cerebrovascular pathology [36], vascular dementia [39], and cerebral amyloid angiopathy [40].

Demographics & biochemical data

Demographic data included age, sex, weight, height, body mass index (BMI), blood pressure, heart rate, medication status, and comorbidities, all extracted from medical files from the patients' visits to the Memory clinic and stored in REDCap. Biochemical data included results from the blood sample taken as part of the patient's diagnostic work-up program in the Memory clinic. Blood results included C-reactive protein, hemoglobin, hemoglobin A1c (HbA1c), alanine aminotransferase, high-density lipoprotein, low-density lipoprotein, cholesterol, triglycerides, and estimated glomerular filtration rate (eGFR). For a few patients with eGFR > 90, an exact eGFR was calculated based on age, gender, and level of creatinine, using the Modification of Diet in Renal Disease (MDRD) Study Equation [41]. CSF biomarkers used for diagnosis were analysed as part of routine analysis at Rigshospitalet for β -amyloid 1–42, tau and phosphorylated tau 181 (p-tau181) proteins. The assay used at Rigshospitalet changed during study inclusion: before June 30th 2022 the Innatest enzyme-linked immunosorbent assays (ELISA) by Fujirebio, Ghent were used, and after July 1st 2022 the Elecsys sandwich electrochemiluminescence-immunoassay by Roche, Cobas 8000, was used. $N=8$ patients CSF samples were analysed by Innatest.

Statistical analysis

Demographics and baseline characteristics are presented as means with standard deviations. Differences in baseline characteristic variables between AD and non-AD were examined with two-way sample t-tests for continuous variables and with Pearson's Chi-squared tests for categorical variables. Differences in total BBM concentration between AD and non-AD were analysed using linear mixed models with diagnosis as fixed effect and an unstructured covariance pattern to account for repeated measurements, using the *lmm* function from the *LMMstar* R package. Correlations between BBM concentrations between visits, CSF and possible confounders were examined with Spearman's correlation coefficients. We further investigated correlations between BBM and age, MMSE, ACE and all above-mentioned biochemical data.

Biological variation was examined by fitting linear mixed models (LMM) with patients as random effects, using the *lmer* function in the *lme4* package in R. The biomarkers' steady state (meaning no systematic trend (increase or decrease) in the biomarker concentration

during the study period) was evaluated with linear regression models on each biomarker with the mean concentration of the biomarker and the day of sample collection, and tests for variance of homogeneity were performed on log-10 scale-transformed data with Brown-Forsythe test. Outlier observations were detected using the interquartile range criterion. All LMM were based on log-10-transformed biomarker values. Coefficient of variation for intra-individual variability was based on the residual's standard deviation. Coefficient of variation for inter-individual variability was calculated from the square root of the total variances ($CV = \sqrt{(\text{variance}(\text{patients}) + \text{variance}(\text{residual}))}$), thereby incorporating intra-individual variability. The 95% confidence intervals for intra- and inter-individual variability were calculated from the model using the function *confint* with the *bootstrap* method. Biomarker values measured as under limit of quantification (<LOQ) (p-tau231 $n=2$ and T-tau $n=3$), were labelled as missing data (NA). The LMM models removed any observations labelled NA. All analyses were run on the total number of samples from the three visits. To investigate the effect of time and of biological confounders with a significant correlation with BBM concentrations (see Additional file 7, Figure 5), LMMs were carried out with time, eGFR, BMI, and HbA1c as a fixed effect. We considered the variables as stable over the short study period and used baseline data of the covariates. Assay-dependent analytical variation, CV_A , was based on the intermediate QC levels.

Number of samples needed to estimate an individual's homeostatic set point (n) with 15% proximity to the true value, and 95% CI was calculated by $n = (z * \sqrt{(CV_{\text{intra-individual}}^2 + CV_A^2)}) / D^2$, with $z = 1.64$ and D sat to 15%. To estimate the minimum change needed between an individual's two measurements to exceed intra-individual variability and analytical variation, CV_A , the reference change value (RCV), was calculated for each biomarker across diagnostic groups, by $RCV = 100 * (+\exp(z * \sqrt{2} * \sqrt{\log(CV_{\text{intra-individual}}^2 + CV_A^2) - 1}))$, with $z = 1.64$ (one-sided approach reflecting a unidirectional change) and CV_A based on the mean intermediate precision [42].

Classification accuracy (i.e., for the performance of each biomarker to separate AD from non-AD), was based on a predictive classification model build by splitting the dataset 50/50, using the *randomForest* package in R. Classification accuracy and corresponding 95% CI was then calculated by creating a confusion matrix, using the *caret* package, predicting the probability of an AD diagnosis at each time point.

All statistical analyses were performed using the R Statistical Software, version 4.4.1 and statistical significance

was set to $\alpha=0.05$. The R codes can be shared upon request.

Results

Participants

A total of 47 patients with cognitive dysfunction, due to AD ($n=27$) or other disorders (non-AD, $n=20$), were included in the study. Baseline characteristics for all 47 patients are presented in Table 1. In the AD group CSF A β 1–42, tau, and p-tau181 were significantly different from the non-AD group. No other significant differences in baseline demographic or clinical variables were found between the AD and non-AD group.

Concentrations of plasma & CSF biomarkers

Number of available plasma samples were V1 $n=45$, V2 $n=43$, and V3 $n=40$. The total biomarker concentrations in plasma per visit are presented in Table 2 and Fig. 2 (see Additional file 8, Figure 6 for BBMs per visit per patient). Overall, strong positive correlations of plasma concentrations between visits were found, see Additional file 9, Figure 7. A significant difference between the AD and non-AD groups was found for total plasma p-tau217 (p -value < 0.004 , 95% CI $(-0.35; -0.07)$). BBMs and CSF concentrations from each visit

are presented in Additional file 2, Table 4. CSF biomarkers were significantly different between AD and non-AD, except A β 40, NfL, and GFAP, see Additional file 2, Table 4. Correlations between CSF and plasma were only significant for NfL and p-tau217. See Additional files 5 & 10, Table 7 & Figure 8.

Intra- and inter-individual variability of plasma biomarkers

Results are presented from all participants, see Additional file 4, Table 6 for results with outliers excluded. All patients across BBMs were in steady state and no heterogeneity of variance was detected, except plasma GFAP not meeting these criteria (neither log transformed). We found a significant change in time for GFAP (p -value $= 0.02$), and the variances were not equal across samples.

Intra-individual variability

All CVs for intra-individual variability were low, see Table 2. A β markers had the lowest CV, ranging from ~ 3 to $\sim 4.5\%$ in the AD group, increasing in the p-tau variants and NfL, and was highest for T-tau and GFAP ($\sim 11\%$ in both groups). CV did not differ between the AD and the non-AD group except for

Table 1 Baseline characteristics

	AD (N=27)	non-AD (N=20)	p-value
Age, years	71.4 (5.4)	72.1 (6.2)	0.65 ⁽¹⁾
Gender, Male	15 (55.6%)	16 (80.0%)	0.08 ⁽²⁾
MMSE, Mean (SD)	26.8 (2.5)	27.0 (2.2)	0.79 ⁽¹⁾
ACE, Mean (SD)	78.8 (8.4)	78.0 (11.0)	0.77 ⁽¹⁾
Disease severity			0.42 ⁽²⁾
- MCI	13 (48.1%)	12 (60.0%)	
- Mild dementia	14 (51.9%)	8 (40.0%)	
CSF A β 1–42, pg/mL, Mean (SD) (AD $n=21$, non-AD $n=18$)*	664.8 (245.3%)	1124.2 (465.4%)	0.0004 ⁽¹⁾
CSF T-tau, pg/mL, Mean (SD) (AD $n=21$, non-AD $n=18$)*	317.2 (128.8%)	235.4 (122.7%)	0.05 ⁽¹⁾
CSF p-tau181, pg/mL, Mean (SD) (AD $n=21$, non-AD $n=18$)*	32.8 (15.2%)	20.7 (12.3%)	0.01 ⁽¹⁾
BMI, Mean (SD)	24.8 (3.4)	26.8 (5.3)	0.14 ⁽¹⁾
HbA1c, mmol/mol, Mean (SD)	40.6 (6.4)	40.8 (7.4)	0.95 ⁽¹⁾
eGFR, mL/min/1.73m ² , Mean (SD)	82.9 (13.7)	80.1 (26.1)	0.64 ⁽¹⁾
Creatinine, pmol/L, Mean (SD)	73.7 (17.7)	90.3 (42.4)	0.08 ⁽¹⁾
Diabetes II, Yes	3 (11.1%)	6 (30.0%)	0.10 ⁽²⁾

MCI mild cognitive impairment, non-AD: DLB $n=6$, Dementia with Lewy bodies, FTD $n=2$, Frontotemporal dementia, head trauma $n=1$; VD $n=1$, Vascular dementia, CAA $n=2$, cerebral amyloid angiopathy, ALS + VD $n=1$, amyotrophic lateral sclerosis, NPH $n=1$, normal pressure hydrocephalus, other tauopathy $n=1$, affective disorder $n=1$; kidney failure $n=1$; unclassified $n=3$. P-values from CSF are based on log-10-scale-transformed data. BMI 25–29.9: $n=11$, BMI > 30 : $n=8$. HbA1c > 48 mmol/mol: $n=8$. eGFR < 60 mL/min/1.73m²: $n=5$. Creatinine: Female < 46 : $n=1$, > 90 : $n=9$ & Male < 60 : $n=10$, > 105 : $n=4$

⁽¹⁾ two-way sample t-tests

⁽²⁾ Pearson's Chi-squared test

* $n=8$ patients' CSF were analyzed by Innostest (AD $n=6$ (A β 1–42 867.0 (73.1) pg/mL, T-tau 506.7 (160.2) pg/mL, p-tau 72.0 (22.1) pg/mL) and non-AD $n=2$ (A β 1–42 1176.0 (168.3) pg/mL, T-tau 255.0 (72.1) pg/mL, p-tau 39.0 (4.2) pg/mL))

Table 2 BBMs intra- & inter-variability in AD vs. non-AD

Biomarker	Population	No. of Samples	Mean plasma concentration (pg/mL, 95% CI)	Intra-individual variability CV (% 95% CI)	Inter-individual variability CV (% 95% CI)	CV _A (%)	
Aβ40	Total	128	103.2 (98.7, 107.6)	5.2 (4.4; 6.0)	10.0 (8.4; 11.9)	Low QC	4.7
	AD	71	96.9 (93.1, 100.6)	4.1 (3.3; 5.0)	7.1 (5.5; 8.7) ^c	High QC	7.5
	non-AD	57	111.0 (102.3, 119.7)	6.2 (4.9; 7.5)	12.5 (9.3; 16.2) ^c	Mean	6.1
Aβ42	Total	128	5.4 (5.1, 5.7)	5.9 (5.0; 6.7)	14.7 (12.2; 17.3)	Low QC	7.8
	AD	71	5.0 (4.7, 5.2)	4.5 (3.6; 5.5) ^c	12.2 (9.2; 15.3)	High QC	4.3
	non-AD	57	6.0 (5.3, 6.6)	7.2 (5.4; 8.8) ^c	16.9 (12.2; 21.8)	Mean	6.05
Aβ42/Aβ40	Total	128	0.052 (0.050, 0.054)	3.3 (2.8; 3.8)	10.1 (8.2; 12.0)	Mean	6.075 ^d
	AD	71	0.051 (0.049, 0.054)	3.4 (2.7; 4.1)	11.2 (8.3; 14.1)		
	non-AD	57	0.053 (0.050, 0.055)	3.2 (2.5; 3.9)	8.4 (5.9; 10.8)		
Ptau181	Total	128	8.8 (8.0, 9.6)	7.0 (6.0; 8.1)	20.5 (16.8; 24.3)	Low QC	21
	AD	71	8.6 (7.9, 9.3)	6.9 (5.6; 8.5)	15.1 (11.5; 18.8) ^c	High QC	5.8
	non-AD	57	9.1 (7.5, 10.7)	7.2 (5.5; 8.8)	26.3 (18.4; 34.0) ^c	Mean	13.4
Ptau217	Total	128	0.7 (0.6, 0.7)	9.3 (7.9; 10.8)	27.3 (21.9; 32.4)	Low QC	15.6
	AD	71	0.8 (0.7, 0.9) ^b	10.5 (8.3; 12.8)	19.7 (15.5; 24.5)	High QC	5.3
	non-AD	57	0.5 (0.4, 0.6) ^b	7.8 (6.0; 9.5)	30.9 (21.3; 40.7)	Mean	10.5
Ptau231	Total	126	7.3 (6.8, 7.8)	7.8 (6.6; 9.1)	17.4 (14.2; 20.8)	Low QC	15.6
	AD	71	7.4 (6.9, 7.9)	7.0 (5.6; 8.5)	13.4 (10.2; 16.3)	High QC	6.9
	non-AD	55 ^a	7.2 (6.3, 8.2)	8.8 (6.8; 10.9)	21.7 (15.6; 28.0)	Mean	11.3
T-tau	Total	125	1.53 (1.43, 1.66)	11.1 (9.4; 12.8)	17.7 (14.7; 20.5)	Low QC	9.2
	AD	68 ^a	1.55 (1.43, 1.66)	9.6 (7.4; 11.6)	17.4 (13.3; 21.7)	High QC	5.1
	Non-AD	57	1.50 (1.32, 1.68)	12.5 (9.7; 15.5)	18.7 (14.3; 23.2)	Mean	7.2
NfL	Total	128	31.4 (25.61, 37.23)	7.3 (6.2; 8.3)	34.1 (27.4; 41.1)	Low QC	3.2
	AD	71	24.6 (20.1, 29.1)	7.1 (5.5; 8.6)	29.6 (21.6; 37.2)	High QC	9
	non-AD	57	39.9 (28.3, 51.6)	7.5 (5.8; 9.2)	39.4 (27.1; 51.1)	Mean	6.1
GFAP	Total	128	128.9 (118.8, 139.0)	11.2 (9.3; 12.9)	20.5 (17.0; 23.8)	Low QC	5.5
	AD	71	137.3 (123.7, 150.9)	11.4 (9.2; 13.9)	19.6 (15.3; 23.9)	High QC	13.1
	non-AD	57	118.5 (103.4, 133.6)	10.9 (8.2; 13.5)	21.5 (16.4; 26.6)	Mean	9.3

Table showing the mean concentrations and 95% CI of each biomarker from all three study visits for the total number of participants as well as stratified by a diagnosis of Alzheimer's disease or other cognitive dementia diseases. Number of samples available for analyses are also presented. Intra- and inter-individual variability for each biomarker is presented together with the corresponding assay variability

Blood samples from two patients obtained at V1 were lost due to technical issues before freezing. CSF was available for those two patients. CSF from 42 patients were available for analyses collected at V1. For 5 patients there was not sufficient CSF for study analyses. In total, 38 patients had a complete set of three blood samples, and CSF was available for 33 of these patients

CI confidence interval, CV_A Assay variability (mean intermediate precision), Aβ amyloid-β, p-tau phosphorylated tau, NfL neurofilament light, GFAP glial fibrillary acidic protein

N-missing: AD: V1 *n* = 2, V2 *n* = 3, V3 *n* = 5. Non-AD: V1 *n* = 0, V2 *n* = 1, V3 *n* = 2

^a < LOQ (under limit of quantification): Plasma p-tau231 < LOQ *n* = 2 in the non-AD group, plasma T-tau < LOQ *n* = 3 in the AD group

^b Linear mixed models showed significant difference between groups

^c Non-overlapping 95% CI

^d Mean intermediate CV_A for the Aβ42/Aβ40 ratio was calculated as the mean across low and high QCs from both Aβ40 and Aβ42

a significant difference in Aβ42 CV between the two groups. Adding time as fixed effect made no notable changes to the CV, see Additional file 3, Table 5. Excluding outlier observations, Aβ42 (*n* = 6), Aβ40 (*n* = 7), T-tau (*n* = 2) and NFL (*n* = 13), in the analyses, made no change to the intra-individual variability, see Additional file 4, Table 6, for comparison.

Inter-individual variability

The lowest CVs for inter-individual variability were found in the amyloid markers ranging from ~7 to 12% in the AD group and from ~8 to ~17% in the non-AD group. CV was moderate in the p-tau variants, T-tau and GFAP and reached the highest variability in NfL. ~40% for NfL, see Table 2. There was a significant difference in CV between the AD and non-AD group for Aβ40 and

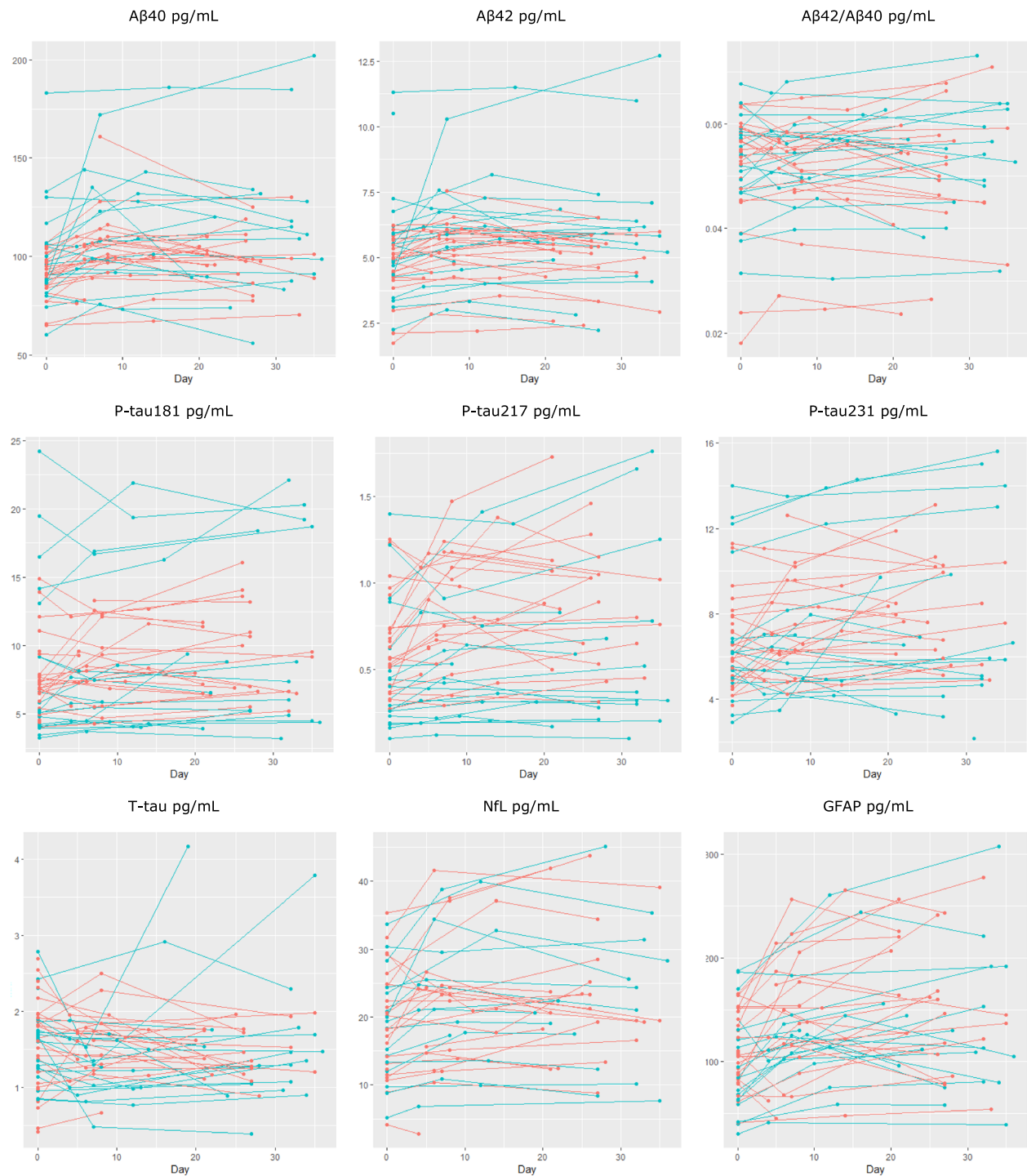



Fig. 2 BBM concentrations over time for each study participant

Spaghettiplots of all BBMs stratified by diagnosis. Outliers $n = 6$, NfL > 150 pg/mL have been removed. 

p-tau181. Adding time as a fixed effect made no notable changes to the inter-individual variability, see Additional file 3, Table 5. Excluding NfL outliers ($n = 13$), we found

a moderate effect on CV. Excluding outliers for A β 40 ($n = 7$) had a minor effect on CV, and for A β 42 ($n = 6$) a minor effect was observed in the non-AD group, and no

effect was found on CV when excluding T-tau ($n=2$) outliers, see Additional file 4, Table 6.

Inter-assay variability, CV_A

The inter-assay variability, CV_A , is based on the intermediate precision data for each biomarker and is presented in Table 2 as the low and high QC levels as well as the mean value. CV_A ranged between ~6% ($A\beta$ markers) and ~13% (p-tau181). All CV_{A_s} were lower than the corresponding intra-individual variability.

Biological confounders and its effect on intra- and inter-individual variability

No notable changes were found in intra-individual variability when adjusting for BMI, eGFR, and HbA1c. When adding BMI to the models the inter-individual variability decreased a few percent in p-tau181, p-tau217, p-tau231, NfL, and GFAP. Adjusting for eGFR, inter-individual variability in NfL and T-tau decreased a few percent. No notable differences in the variabilities were found on inter-individual variability when adjusting for HbA1c, see Additional file 3, Table 5.

Reference change value

Figure 3 shows the estimated reference change values (RCV), i.e., the minimum change (exceeding intra-individual variability and CV_A) required to detect a difference between a given patient's two blood samples. Results are based on the mean intermediate CV_A , see Additional file 6, Figure 4 for RCVs based on the lowest CV_A . The lowest RCV needed for a significant change was found in plasma $A\beta$ markers (decrease ~14% and increase ~17%). Plasma p-tau181 showed the highest RCV (increase ~42% and decrease ~-30%). Across diagnostic groups, no striking differences in RCV were observed. The number of samples needed to estimate individual homeostatic setpoints were between 1 to 3 samples per individual.

Classification accuracy across visits

Classification accuracies across visits are presented in Table 3. In general, classification accuracies were stable across visits, with T-tau and NfL showing the highest variation.

Discussion

This study examines the short-term intra- and inter-individual variability together with RCV in a large panel of AD plasma biomarkers in a memory clinic population,

including AD patients, and examined if short-term variability could be affected by known suspected confounders. We found overall low intra-individual variability and moderate to high inter-individual variability, which varied across AD biomarkers. Short-term variability was overall lowest in the $A\beta$ -markers, increasing for p-tau variants and highest in NfL and GFAP. In general, intra-individual CV was lower than inter-individual CV with comparable values across diagnostic groups. All CV estimates were robust to various confounders. Intra-individual variability was consistent regardless of outliers, however removing NfL outliers had moderate impact the inter-individual variability. RCVs were consistent across the diagnostic groups, overall lowest in the β -amyloid markers and highest in plasma GFAP.

The low intra-individual variabilities in our study are promising when tracking longitudinal changes and treatment effect for the individual patient, see Use Case 1 in Additional file 1. Inter-individual variability is important to both establish diagnostic cut-off values and to investigate if an intervention effect is consistent in the study population, and/or have different effect on different groups, see Use Case 2 in Additional file 1. Knowledge of RCV is crucial when tracking longitudinal changes and intervention effects, to estimate differences that lie beyond that of intra-individual variability and assay performance, see Use Case 1 in Additional file 1. Calculation of the patient's homeostatic set-point and use of RCVs of the given BBMs, can ensure that a given change in the plasma biomarkers is likely caused by i.e., an intervention and in fact a clinically relevant change. RCVs based on the lowest CV_A , did as expected cause narrower ranges. Though we assume the CVs for both intra- and inter-individual variability from this study to be generalizable to similar disease groups, CV_A will vary between laboratories and analytical platforms.

Furthermore, no significant variation in classification accuracies were found across visits. Some variation was observed for T-tau and NfL, which also showed high inter-individual CV, suggesting it may influence classification accuracy. In this vein, it is important to highlight, that the motivation for investigating the diagnostic ability of the biomarkers, was to examine whether the variation across a short time span, would impact these metrics which are more relatable to clinical practice.

No previous studies have investigated the short-term variability of these AD biomarkers, however few studies have examined the biological variation of some of the markers in younger healthy individuals. BV is commonly used to establish population-based reference intervals, i.e., in a non-affected population. However, previous research have addressed challenges using population-based BV and reference intervals for disease monitoring

[43] and have suggested that BV is not comparable between patients and healthy individuals, e.g. that an underlying disease may affect the variation observed in the biomarker. Therefore RCVs derived from healthy individuals may be narrower than RCVs based on the target population [28]. Meaning that if variation in a biomarker is smaller in healthy vs. the target disease, a change in a biomarker concentration could be misinterpreted as clinically relevant – whereas it is rather an effect of various physiological factors, that could include disease severity, comorbidities, medication, and clearance mechanisms in the blood. Therefore, our results of intra- and inter-individual short-term variability, which were marginally affected by confounding factors, must be seen in context of previous estimations of intra- and inter-individual variability in younger healthy individual, in which it is possible to isolate biological variation.

The low fold difference in plasma A β 42/A β 40 between individuals with or without AD pathology compared to CSF [44], makes it sensitive to even small variations [20, 21]. Plasma A β 40, A β 42, and A β 42/A β 40 showed overall low intra-individual variability, lowest for A β 42/A β 40, ~3%, consistent with results in healthy individuals [26]. Across diagnostic groups inter-individual variability and RCV were consistent and within the range of results reported in healthy individuals [26]. Therefore, despite the issues related to the low fold differences, the low variation indicates that A β measures in plasma may be relevant and affect classification into amyloid positive or negative. Furthermore, while we found significant difference in CSF A β 42/A β 40, we found no significant difference of plasma A β 42/A β 40 between groups. Whether this is an effect of underpowering, as the study was not designed to examine diagnostic performance of BBVs or poor performance of the biomarker, remains undetermined.

P-tau variants have so far shown great promise in detecting amyloid pathology in AD patients [6]. While p-tau231 may be increased in initial stages of AD [32, 45], p-tau217 seems to reflect amyloid pathology, and predict cognitive decline [46, 47], whereas p-tau181 could be indicative of more manifest tangle pathology at later disease stages [31]. Across diagnostic groups, we found the lowest intra-individual variability in p-tau181

and the highest inter-individual variability in p-tau217. In healthy individuals a previous study showed the lowest intra-individual variability for p-tau231 and the highest inter-individual variability in p-tau181 [26]. If p-tau variant concentrations are increasing at different disease stages throughout AD, an increase/decrease of inter-individual variability should be expected according to the spread of disease severity of the group. RCV for p-tau217 was consistent with results from healthy individuals [26]. The RCV for p-tau181 (–29/+42%) in the AD group was markedly lower compared to healthy individuals (–38/+62%), while RCV for p-tau231 was higher in the AD group (–26/+36%) compared to healthy individuals (–21/+26%) [26]. This difference between patients and healthy individuals could be considered to reflect that p-tau181 is more likely increased in later disease stages, narrowing the RCV index.

NfL, released following axonal damage or neuronal degeneration, has also been suggested as an early diagnostic triage biomarker [48] and can predict disease progression in AD [49–55]. Low intra-individual variability, moderate to severe inter-individual variability, and a rather wide RCV index for plasma NfL were similar across the diagnostic groups, and consistent with estimates in healthy individuals based on day-to-day and week-to-week blood samplings [24–26].

GFAP, released from activated astrocytes [8], may be elevated in pre-symptomatic AD stages [56]. Further, changes in plasma levels of GFAP following 12 weeks treatment with Donanemab were found to be decreased [9]. Our study found a reasonable intra-individual variability, consistent with results on healthy individuals [23, 26]. However, the moderate inter-individual variability was notably smaller compared to younger healthy individuals [23, 26], suggesting GFAP is a more stable marker in groups with brain pathology compared to healthy individuals. The RCV was considerably wide, ranging from –29 to +40%, consistent with RCVs based on healthy individuals [23, 26] and our results suggest at least three blood samples are needed to estimate a person's homeostatic set-point. Further, the increase over time in GFAP was unexpected and is likely to represent a spurious finding as opposed to reflecting disease progression.

(See figure on next page.)

Fig. 3 Model of reference change values, RCV %

Figures showing the amount of change for each biomarker to overcome intra-individual variability and assay variation, named Reference Change Value (RCV). The tables show the RCVs for each biomarker in patients with A) Alzheimer's disease and B) non-AD consisting of a mixed memory clinic cohort. Calculations are based on the mean intermediate CV_A, NNHS; number of samples needed to obtain homeostatic setpoint with 15% proximity to true value

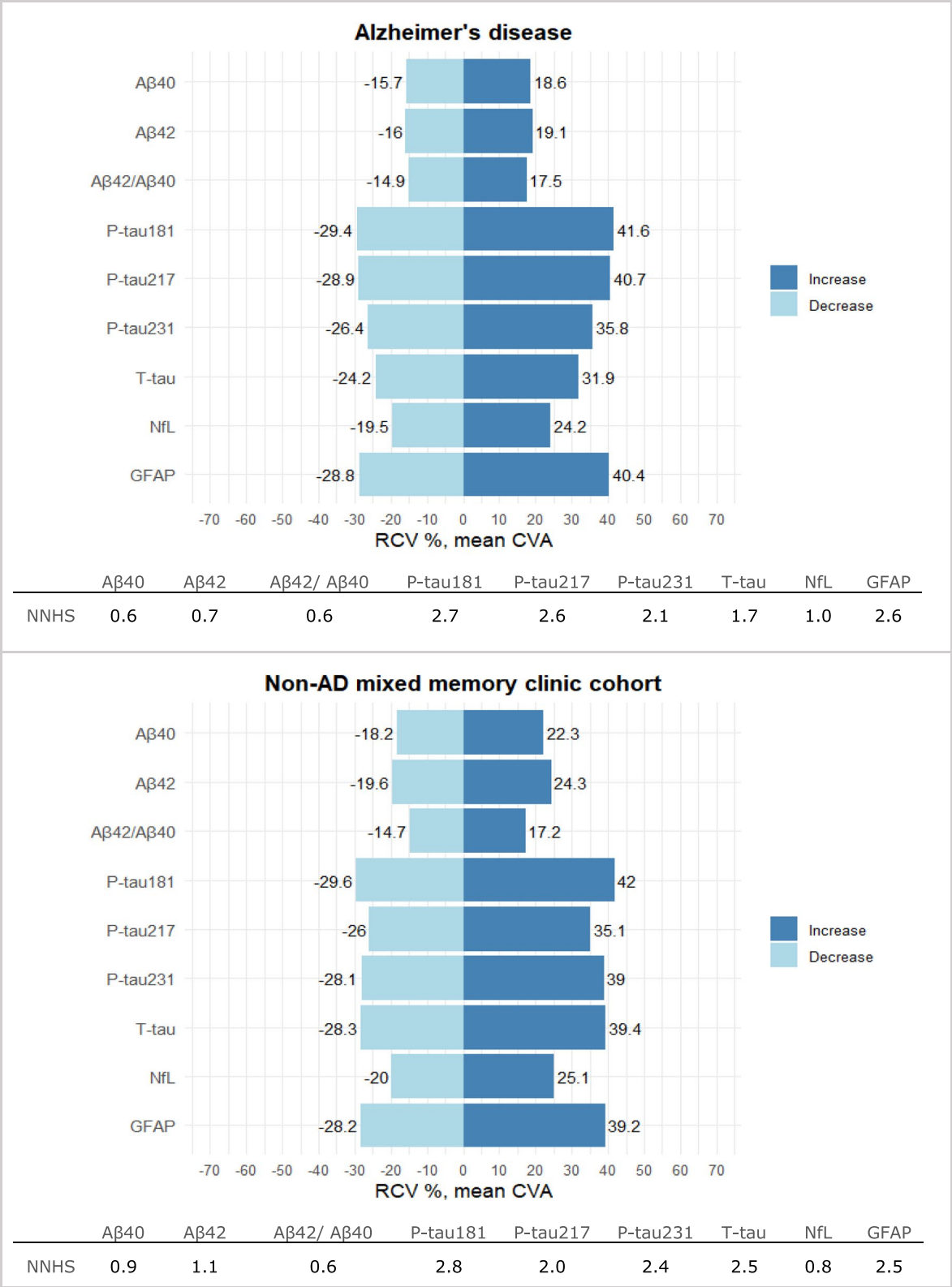


Fig. 3 (See legend on previous page.)

Table 3 Classification accuracy across visits

Biomarker	Classification Accuracy (95% CI)		
	V1	V2	V3
Aβ40	0.68 (0.45, 0.86)	0.57 (0.34, 0.78)	0.65 (0.41, 0.85)
Aβ42	0.55 (0.32, 0.76)	0.52 (0.30, 0.74)	0.5 (0.27, 0.73)
Aβ42/Aβ40	0.55 (0.32, 0.76)	0.57 (0.34, 0.78)	0.5 (0.27, 0.73)
p-tau181	0.68 (0.45, 0.86)	0.71 (0.48, 0.89)	0.60 (0.36, 0.81)
p-tau217	0.68 (0.45, 0.86)	0.67 (0.43, 0.85)	0.75 (0.51, 0.91)
p-tau231	0.57 (0.34, 0.78)	0.52 (0.30, 0.74)	0.55 (0.32, 0.77)
T-tau	0.55 (0.32, 0.76)	0.5 (0.27, 0.73)	0.26 (0.09, 0.51)
NfL	0.32 (0.14, 0.55)	0.38 (0.18, 0.62)	0.5 (0.27, 0.73)
GFAP	0.59 (0.36, 0.80)	0.57 (0.34, 0.78)	0.55 (0.32, 0.77)

Classification accuracy, based on a predictive classification model, together with their corresponding 95% CI

CI confidence interval, Aβ amyloid-β, p-tau phosphorylated tau, NfL neurofilament light, GFAP glial fibrillary acidic protein

One study has examined test–retest variability, i.e., the stability of the biomarker over time, and we notice a similar trend to our results: lowest variability in amyloid, increasing in the phosphorylated tau variants to the highest in NfL and GFAP [22].

Several biological factors such as chronic kidney disease, diabetes, history of myocardial infarction and stroke, eGFR, BMI, as well as sex and age have been reported to impact plasma level concentrations of AD BBMs [16–18]. In this study, some correlations were found between eGFR, BMI and HbA1c and baseline levels of BBMs. These potential confounders were included one by one in the LMM without considerable impact on the variation.

This study has some limitations. Though the largest study to date in a real-world clinical setting, the sample size was moderate in size and limited by patients who failed to complete all three visits. This resulted in a rather small number of plates for Simoa analysis, 3 plates in this study, which is likely to have increased the intermediate precision between plates, CV_A. The fact that all analyses were run at the same time, with all three samples from a given patient on the same plate, with the same reagent batch and in the same laboratory limits generalisability to other circumstances in which this is not the case (i.e. to most real-world clinical settings). The smaller the assay variability, the smaller the impact on RCV. However, all CV_A were less than intra-individual variability. Blood samples were collected without information on last food-intake due to the real-life clinical setting of this study. A recent study [15] investigated the impact of food intake by monitoring BMM over a duration of 3 h in groups of postprandial or fasting healthy individuals and found significant fluctuations. The clinical impact remains

unknown and further studies should collect information on food-intake prior to blood-sampling to elaborate on its potential confounding effect on BMM variability. However, in this study, postprandial physiology is considered a part of biological variation. Further, the moderate to high inter-individual variability found in this study could be due to disease heterogeneity in the non-AD group, also explaining the moderate effect when removing the few NfL outliers or may serve as an indicator of disease severity or pathological burden within the AD group. The real-life memory clinic setting with limited inclusion criteria strengthens the diversity of the study population. Though this study was not designed to test the relationship between CSF and plasma levels of AD biomarkers, we found it important to report the correlation coefficients in the AD and non-AD group, to ensure transparent research, see Additional files 5 & 10, Table 7 & Figure 8. We consider it of great strength that we had confirmatory CSF biomarkers enabling us to evaluate the effect of AD pathology of intra- and interindividual variation, which we considered to be limited. The short time span of this study is considered a strength since no pathophysiological changes are expected in AD in such a short window of time.

Conclusion

In conclusion, this is the first study to investigate short-term variability of AD BBMs in the intended use population, memory clinic patients. Across all biomarkers investigated in this study, the low intra-individual variability, robust to various factors, is promising when considering BBMs as novel prognostic markers for tracking disease progression and treatment efficacy. Moderate inter-individual variability prompts some concern for diagnostic and disease staging purposes. The relatively high RCVs may pose challenges for AD biomarkers with low fold change (plasma Aβ42/40) when i.e., estimating intervention effect and longitudinal changes. Overall, intra- and inter-individual variability of AD BBMs should be considered in both clinical and research settings. And we suggest that further research continuously assess the impact of these metrics on AD BBMs’ interpretation as this research field evolves.

Abbreviations

AD	Alzheimer’s disease
BBMs	Blood-based biomarkers
BV	Biological variation
T-tau	Total-tau
p-tau	Phosphorylated tau
NfL	Neurofilament light
GFAP	Glial fibrillary acidic protein
RCV	Reference change value
CV _A	Inter-assay variability
EDTA	Ethylenediaminetetraacetic acid

MMSE Mini-Mental State Examination
 ACE Addenbrookes Cognitive Examination
 HbA1c Hemoglobin A1c
 eGFR Estimated glomerular filtration rate

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13195-024-01658-7>.

Additional file 1: Use cases.

Additional file 2: Table 4. BBM concentrations in CSF & plasma V1-3.

Additional file 3: Table 5. BBM variability models with time, eGFR, and BMI.

Additional file 4: Table 6. Intra- and inter-individual variability excluding outliers.

Additional file 5: Table 7. Correlations between baseline plasma and CSF biomarkers.

Additional file 6: Figure 4. Model of reference change values, RCV %—Based on the lowest intermediate CV_A.

Additional file 7: Figure 5. Correlogram with BBM and confounders.

Additional file 8: Figure 6. Biomarker concentration pr. visit pr. Patient.

Additional file 9: Figure 7. Correlations in BBMs between visits.

Additional file 10: Figure 8. Scatterplots plasma and CSF correlations.

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Authors' contributions

Conceptualization: FKC, MHG, AHS, SGH, KSF. Methodology: FKC, MHG, AHS, SGH, KSF, HZ. Data Collection: FKC, MHG, HH. Formal Analysis: FKC, AHS, SGH, KSF. Writing—Original Draft: FKC. Writing—Review & Editing: FKC, MHG, AHS, NJA, HH, KB, HZ, GW, SGH, KSF. Supervision: AHS, SGH, GW, KSF. Funding Acquisition: FKC, KSF, SGH, AHS, GW, MHG. Resources: NJA, KB, HZ, GW, AHS, SGH, KSF. All authors read and approved the final manuscript.

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Data availability

Datasets from the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The study was approved by the Danish Research Ethics Committee (H-21044863) and followed the tenets of the 1975 Helsinki Declaration. The study was registered at clinicaltrials.gov (NCT05175664). Written informed consent was obtained from each participant prior to enrolment.

Consent for publication

Not applicable.

Competing interests

KB has served as a consultant and at advisory boards for Abbvie, AC Immune, ALZPath, AriBio, BioArctic, Biogen, Eisai, Lilly, Moleac Pte. Ltd, Neurimmune, Novartis, Ono Pharma, Prothena, Roche Diagnostics, and Siemens Healthineers; has served at data monitoring committees for Julius Clinical and Novartis; has given lectures, produced educational materials and participated in educational programs for AC Immune, Biogen, Celdara Medical, Eisai and Roche Diagnostics; and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, outside the work presented in this paper. HZ has served at scientific advisory boards and/or as a consultant for Abbvie, Acumen, Alector, Alzinova, ALZPath, Amylyx, Annexon, Apellis, Artery Therapeutics, AZTherapies, Cognito Therapeutics, CogRx, Denali, Eisai, LabCorp, Merry Life, Nervgen, Novo Nordisk, Optoceutics, Passage Bio, Pinteon Therapeutics, Prothena, Red Abbey Labs, reMYND, Roche, Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave, has given lectures in symposia sponsored by Alzecure, Biogen, Cellectricon, Fujirebio, Lilly, Novo Nordisk, and Roche, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work). KF serves on a scientific advisory board for Novo Nordisk and Eisai, has given lectures in symposia for Novo Nordisk, and has served or serves as principal investigator in trials for Roche, Biogen, AbbVie, Novo Nordisk and Roche Diagnostics for which fees is paid to the institution and no personal remuneration is received. KF also serves as Editor-in-Chief for Alzheimer's Research and Therapy (Springer) for which personal remuneration is paid. The other authors have no conflict of interest to report.

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