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CSF and plasma A β 42/40 across Alzheimer's disease continuum: comparison of two ultrasensitive Simoa[®] assays targeting distinct amyloid regions

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Abstract

Objectives: Decreased cerebrospinal fluid (CSF) amyloid beta 42/40 ratio (A β 42/40) is one of the core Alzheimer's disease (AD) biomarkers. Measurement of A β 42/40 in plasma has also been proposed as a surrogate marker for amyloidosis, however the validity and the diagnostic performance of this biomarker is still uncertain. Here we evaluated two immunoassays targeting distinct regions of the amyloid peptides by (a) performing a method comparison in both CSF and plasma, and (b) assessing the diagnostic performance across the AD continuum.

Methods: We used N4PE and N3PA Simoa[®] assays to measure A β 42/40 in CSF and plasma of 134 patients: preclinical AD (pre-AD, n=19), mild cognitive impairment due to AD (MCI-AD, n=41), AD at the dementia stage (AD-dem, n=35), and a control group (CTRL, n=39). The N4PE includes a detector antibody targeting the amyloid N-terminus, while the N3PA uses a detector targeting amyloid mid-region.

Results: Method comparison of N4PE and N3PA assays revealed discrepancies in assessment of plasma A β 42/A β 40. While the diagnostic performance of the two assays did not significantly differ in CSF, in plasma, N4PE assay provided better accuracy for AD discrimination than N3PA assay (AUC AD-dem vs. CTRL 0.77 N4PE, 0.68 N3PA).

Conclusions: While both A β 42/40 assays allowed for an effective discrimination between CTRL and different AD

stages, the assay targeting amyloid N-terminal region provided the best diagnostic performance in plasma. Differences observed in technical and diagnostic performance of the two assays may depend on matrix-specific amyloid processing, suggesting that further studies should be carried to standardize amyloid ratio measurement in plasma.

Keywords: Alzheimer's disease; amyloid; biomarkers; cerebrospinal fluid; plasma; single molecule array

Introduction

New disease-modifying therapies for Alzheimer's disease (AD) have the potential to revolutionize the clinical course of this neurodegenerative disease, that accounts for the majority of dementia cases [1, 2]. Development of new therapies should be accompanied by the assessment of reliable and cost-effective biomarkers to monitor treatment effect but also to support the diagnostic process in primary care settings [3–5]. Blood is the ideal biofluid for AD biomarker screening at the population level, and is rapidly becoming the sample of choice for AD molecular characterization [6], also due to the availability of ultrasensitive and automated technologies [7]. It is now clear that amyloid beta 42/40 ratio (A β 42/A β 40) and phosphorylated tau (p-tau) changes in biofluids are detectable years before the onset of AD clinical symptoms. Therefore, blood biomarker AD screening in primary care may support the early characterization of this disorder, optimizing patient outcome and reducing the burden on health care systems [5].

Among the AD biomarkers measured in blood, phosphorylated tau isoforms (p-tau181, p-tau231, p-tau217) seem to be highly reliable and closely mirror the pathogenic process in the brain [8]. On the other hand, plasma measurement of A β 42/A β 40 is still challenging and several studies reported limited diagnostic accuracy [9]. Notably, while in AD CSF A β 42/A β 40 is reduced by approximately 50 %, in plasma the difference is less prominent, ranging between 14 and 20 % [3, 10]. Accordingly, standardization of

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plasma A β 42/A β 40 measurements should be a priority for the implementation of this biomarker into clinical practice, also considering its role as therapy target [2, 11]. Here, we compared two ultrasensitive Simoa[®] A β 42/A β 40 assays, each directed toward different epitopes on the amyloid peptide (N4PE assay, targeting amyloid N-terminus and N3PA assay, targeting mid-region). Although both assays are designed to detect A β 42 and A β 40, differences in their diagnostic performance have been reported [12, 13].

Following method comparison, diagnostic performance was assessed in a well-characterized cohort of patients across the AD continuum for whom matched CSF and plasma samples were available. Our aims were: (1) to compare the technical performance of the two assays in CSF and plasma; (2) to analyze the diagnostic performance of the two assays in different biofluids and across subsequent AD stages.

Materials and methods

Study participants

A total of 134 patients from the Neurology Clinic, S. Maria della Misericordia Hospital (Perugia, Italy) were consecutively enrolled in the study following specific criteria. All patients underwent a standardized assessment including medical history, physical and neurological examination, laboratory tests, and neuropsychological evaluation, including Mini-Mental State Examination (MMSE). Brain imaging (computed tomography or MRI) or ¹⁸Fluoro-2-deoxyglucose positron emission tomography (FDG-PET), were also performed in selected cases, according to clinical suspicion. In all cases, diagnostic process was supported by the analysis of core CSF AD biomarkers (A β 42, A β 40, A β 42/A β 40, t-tau, p-tau181) measured with the Lumipulse[®] G600II (Tokyo, Fujirebio, Japan). Following principles of A/T/N system which defines three core features of the disease – brain amyloidosis, tauopathy, and neurodegeneration occurring over the disease progression [1], the patients were classified as A+/A–, T+/T–, N+/N– according to cut-off values previously established (A β 42/A β 40=0.072, 95 % CI 0.07–0.074; t-tau=50, 95 % CI 46.2–52.3; p-tau181=393, 95 % CI 359–396) [14]. The AD continuum cohort included patients with an A+/T+ CSF profile and diagnosed with AD at different phases: AD at dementia stage (AD-dem, n=35), patients with mild cognitive impairment due to AD (MCI-AD, n=41), and 19 preclinical Alzheimer's disease patients (pre-AD, n=19). The pre-AD patients were subjects with subjective cognitive complaints not confirmed by neuropsychological assessment but with A+/T+ CSF profile. The control group (CTRL, n=39) was composed of patients diagnosed with minor neurological diseases, other than inflammatory or degenerative disease of the CNS or of the peripheral nervous system. All patients categorized as CTRL were negative for classical AD CSF biomarkers (A–/T–/N– profile).

Age, gender, years of education, Clinical Dementia Rating (CDR), MMSE scores, and A/T/N profile for each clinical group are reported in Table 1. Demographic and clinical data of each patient were confidentially stored in an electronic database.

All the procedures were performed in accordance with Declaration of Helsinki and International Conference on Harmonization guidelines

for Good Clinical Practice. Informed consent was obtained from all individuals included in this study, or their legal guardians or wards and the inclusion in the study that was approved by the local Ethics Committee (Comitato Etico Aziende Sanitarie Regione Umbria 19369/AV and 20,942/21/OV).

Collection of human samples

CSF and plasma samples were collected between 2012 and 2021, following international guidelines and the same standard operating procedures throughout the study [15, 16]. Lumbar puncture was performed between 8:00 and 10:00 a.m. CSF was collected into sterile polypropylene tubes and centrifuged for 10 min at 2000 \times g at room temperature (RT). At the same time, plasma was collected into sterile polypropylene tubes containing EDTA as the anticoagulant and centrifuged for 10 min at 2000 \times g (RT). Once processed, CSF and plasma samples were stored in 0.5 mL tubes (72.730.007, Sarstedt AG & Co., Numbrecht, Germany) and immediately frozen at –80 °C, pending analysis.

Simoa[®] measurements

All CSF and plasma biomarkers were measured with use of the Simoa[®] technology [17]. All measurements were performed on HD-XTM instrument provided by Quanterix (Quanterix Corporation, Billerica, MA, USA) [18].

Single Molecule Array, acronymized as Simoa[®], is a bead-based immunoenzymatic technology enabling detection of proteins at sub-femtomolar levels [17]. Briefly, sandwich immunocomplexes are formed by incubation of a protein target with capture antibody-coated paramagnetic beads and enzyme-labelled (streptavidin beta-galactosidase, SBG) detector antibody. Immunocomplexes are resuspended in a solution of the SBG substrate – resorufin-beta-D-galactopyranoside (RGP) and then transferred to microarray Simoa[®] discs. Over 200,000 thousands of 50-fL wells distributed on each array are big enough to host only one bead per time, hence immunocomplexes are effectively separated. Once the bead loading is completed, an oily layer is spread, sealing beads in wells. As the enzyme-bound complexes and RGP substrate are mixed together, an enzymatic reaction takes place and results in generation of resorufin, a fluorescent reaction product. The increase of fluorescent signal for single beads is recorded and converted into Simoa[®] unit of measurement – Average Enzymes per Bead (AEB). In presence of the proper calibrators, AEB values are further interpolated into SI concentration units.

Here we used two different commercially available Simoa[®] kits to analyze CSF and plasma levels of A β 42 and A β 40 [19]. The first kit, Neurology 4-Plex E Advantage Kit (item 103,670) uses 21F21 (A β 42) and 2G3 (A β 40) as capture antibodies and 3D6 as a detector antibody (assay targeting N-terminus). Neurology 3-Plex A Advantage Kit, item 101,995 (N3PA) uses H31L21 (A β 42) and 2G3 (A β 40) as capture antibodies and 6E10 as a detector antibody (assay targeting mid-region).

Appropriate calibrator and controls included in each kit were run together with samples. After each run, it was checked whether the measured concentration of controls fits into lot-specific range. Samples were anonymized and the researcher completing measurements was blinded to patients' clinical profile.

Table 1: Demographic and clinical profile of the enrolled cohort.

Variable	Total	CTRL	Pre-AD	MCI-AD	AD
n	134	39	19	41	35
Age, years	71.2 ± 8.3	65.2 ± 10.4	73.6 ± 5.8	72.7 ± 5.5	74.9 ± 5.8
Mean ± SD					
Gender (female), n (%)	84 (63)	20 (51)	13 (68)	28 (68)	23 (66)
MMSE	25 (19.5, 28)	28 (27.0, 29.0)	28 (27.0, 29.0)	24 (21.5, 27.0)	17 (12.0, 20.0)
Median (IQR)					
CDR	0.5 (0.0, 1.0)	0.0 (0.0, 0.5)	0.0 (0.0, 0.5)	0.5 (0.5, 0.5)	1.0 (1.0, 1.0)
Median (IQR)					
Years of education	9.4 ± 4.6	10.5 ± 4.6	12.2 ± 3.5	9.3 ± 4.9	7.4 ± 3.9
Mean ± SD					
ATN profile, n (%)					
A–T–N–	39 (29)	39 (100)	0 (0)	0 (0)	0 (0)
A+T–N–	1 (1)	0 (0)	0 (0)	0 (0)	1 (3)
A+T+N–	10 (7)	0 (0)	7 (37)	3 (7)	0 (0)
A+T+N+	83 (62)	0 (0)	12 (63)	38 (93)	33 (94)
A–T–N+	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
A–T+N–	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
A–T+N+	1 (1)	0 (0)	0 (0)	0 (0)	1 (3)

MMSE, Mini-Mental State Examination; CDR, Clinical Dementia Rating; IQR, interquartile range; SD, standard deviation; AD, Alzheimer's disease; pre-AD, preclinical AD; MCI-AD, mild cognitive impairment due to AD; AD-dem, AD at dementia stage; CTRL, control group.

Statistical analysis

Continuous variables were represented as the median ± interquartile range (IQR). Statistical analysis was performed with the use of the GraphPad Prism software version 9.5.0 (San Diego, CA, USA) and R software version 4.2.2. (Vienna, Austria) [20]. D'Agostino-Pearson omnibus K2 test was applied to assess data normality. Non-parametric Kruskal–Wallis test was applied for multiple group comparisons, followed by Dunn's test for multiple comparisons correction. Correlation matrix was assessed by non-parametric Spearman correlation (p-value two-tailed, 95 % confidence intervals (CI)). Receiver operating characteristic curve (ROC) (95 % CI, Wilson/Brown method) analysis was applied to calculate the accuracy of each assay to discriminate between control group and different AD continuum stages (pre-AD, MCI-AD, AD-dem). A p value ≤0.05 was considered statistically significant for all the analyses. E-GFR values used for assessment of the Chronic Kidney Disease (CKD) were calculated by Chronic Kidney Disease-Epidemiology Collaboration (CKD-EPI) formula with cut-off=60 (mL/min/1.73 m²).

Results

Demographics and clinical parameters of the cohort are reported in Table 1. Statistically significant differences in age were observed among selected groups (CTRL vs. pre-AD p≤0.01, CTRL vs. MCI-AD p≤0.01, CTRL vs. AD-dem p≤0.0001; Kruskal–Wallis, followed by Dunn's *post hoc* test). No significant gender differences were present

(Fisher's exact test). As expected, baseline MMSE scores differed among the groups (CTRL vs. MCI-AD p≤0.0001, CTRL vs. AD-dem p≤0.0001, pre-AD vs. MCI-AD p≤0.01, pre-AD vs. AD-dem p≤0.0001, MCI-AD vs. AD-dem p≤0.0001; Kruskal–Wallis, followed by Dunn's *post hoc* test).

As assessed by Lumipulse® CSF measurement, all subjects within the CTRL group were characterized by A–/T–/N– CSF profile. The profile of pre-AD and MCI-AD patients was A+/T+. The majority of AD-dem patients had A+T+N+ profile with an exception of two patients having biomarker values within cut-off 95 % CI and clinical picture clearly pointing to AD at the stage of dementia [14].

CSF and plasma Aβ42/40 correlation

Spearman correlation analysis pointed to a strong positive correlation between CSF Aβ42/40 assessed by N3PA and N4PE assays, as measured in the whole cohort (CSF N4PE – CSF N3PA ρ=0.93). The correlation between the two plasma assays was less prominent (plasma N4PE – plasma N3PA, ρ=0.47). Weak positive correlation between CSF and plasma Aβ42/40 measured by both assays was observed (CSF N4PE – plasma N4PE, ρ=0.42, CSF N3PA – plasma N3PA, ρ=0.32) (Supplementary Figure S1).

Passing–Bablok regression and Bland–Altman plots analysis did not reveal major differences between the two

CSF assays (slope=1.125, CI 1.055–1.206). In plasma, higher discrepancy between the two methods was observed, Bland–Altman analysis revealed a larger bias with an increasing trend associated with higher Aβ42/40 values (Figure 1).

To evaluate the potential impact of blood-brain barrier (BBB) impairment on plasma Aβ42/40 levels, we divided the cohort into tertiles based on values of CSF/serum albumin ratio (available for 126/134 patients). The highest values of CSF/serum albumin ratio (included in the 1st tertile) were considered as indicators of increased BBB permeability. No significant inter-tertile differences were observed in correlation measures (ρ) between CSF Aβ42/40 or plasma Aβ42/40 for any of the assays (Table 2). Additionally, an increased BBB permeability was not associated with significantly different diagnostic performance for both N4PE and N3PA assays.

Information regarding renal function was available only for a subset of the cohort (69/134 patients). In that group, only 3 patients were diagnosed with a Chronic Kidney Disease (CKD) as assessed by eGFR (CDK where eGFR <60 mL/min/1.73 m²; median eGFR in CDK group 40.4 mL/min/1.73 m², non-CDK 89.15 mL/min/1.73 m²). For

Table 2: Impact of BBB permeability on agreement between CSF and plasma Aβ42/40 measurements assessed by N4PE and N3PA assays.

CSF Aβ42/40 – plasma Aβ42/40 correlation		
CSF/serum albumin ratio	N4PE	N3PA
1st tertile	ρ=0.38	ρ=0.39
2nd tertile	ρ=0.49	ρ=0.24
3rd tertile	ρ=0.38	ρ=0.37

ρ, rho values obtained for Spearman correlation analysis.

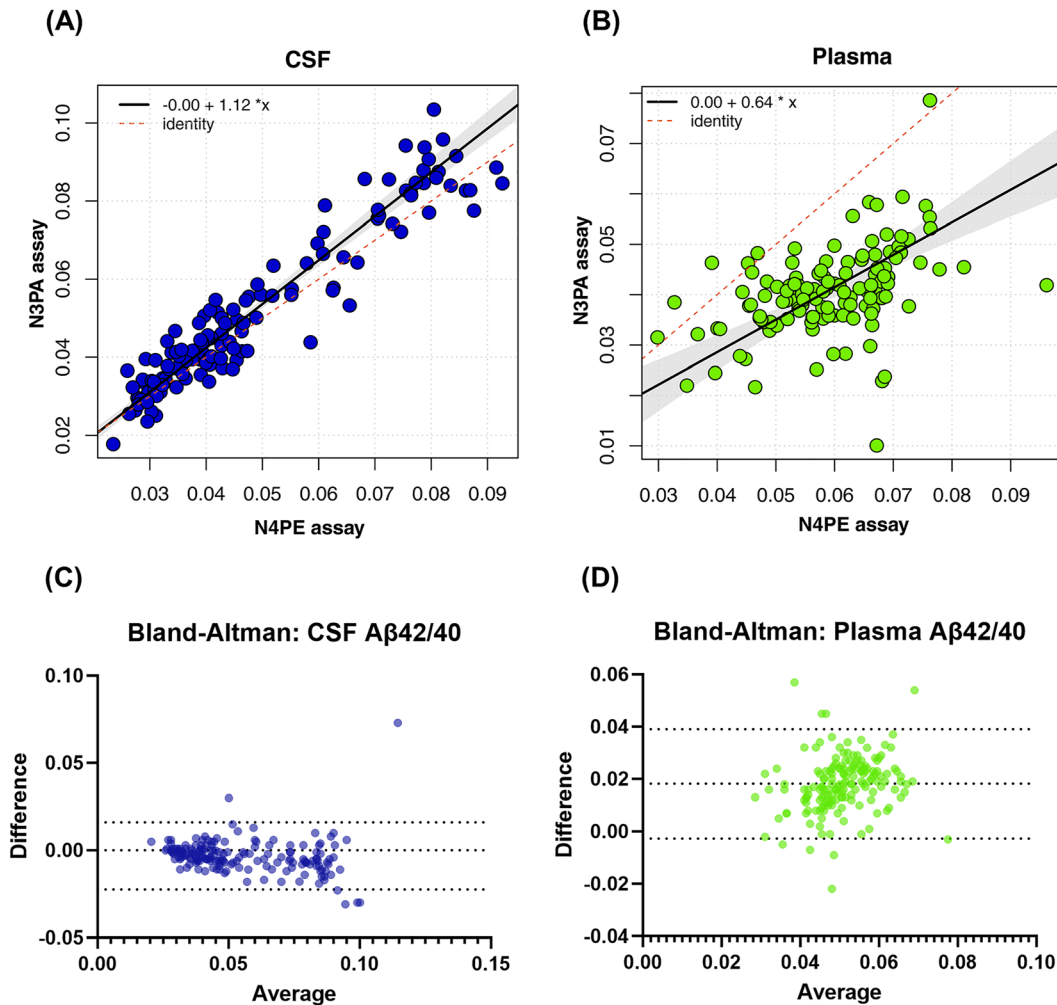


Figure 1: Comparison of N4PE and N3PA assays. (A–B) Passing–Bablok regression for comparison of CSF (A) and plasma (B) N4PE and N3PA assays in the whole cohort. (C–D) Bland–Altman plots (difference vs. average) for comparison of CSF (C) and plasma (D) N4PE and N3PA assays in the whole cohort. EST, estimate; LCI, lower confidence interval; UCI, upper confidence interval.

both N4PE and N3PA assays, the values of plasma Aβ42/40 for CKD and non-CKD patients are reported in the Supplementary Figure S2.

Diagnostic performance

CSF Aβ42/40 levels were assessed in the whole cohort by Lumipulse® and used to determine the amyloid profile within A/T/N system. However, to harmonize measurements in CSF and plasma on a single platform, all the CSF samples

underwent Simoa® Aβ42/40 assessment by N4PE and N3PA assays to be subsequently compared with related plasma Aβ42/40 Simoa® measurements.

For both N3PA and N4PE assays, CSF Aβ42/40 was significantly decreased in all AD continuum groups vs. CTRL (CSF Aβ42/40 N3PA and CSF Aβ42/40 N4PE: CTRL vs. pre-AD $p \leq 0.0001$, CTRL vs. MCI-AD $p \leq 0.0001$, CTRL vs. AD-dem $p \leq 0.0001$). For both assays, decrease in plasma Aβ42/40 was observed in all AD continuum groups vs. CTRL, however different levels of statistical significance were found (plasma Aβ42/40 N4PE: CTRL vs. pre-AD $p \leq 0.0001$, CTRL vs. MCI-AD

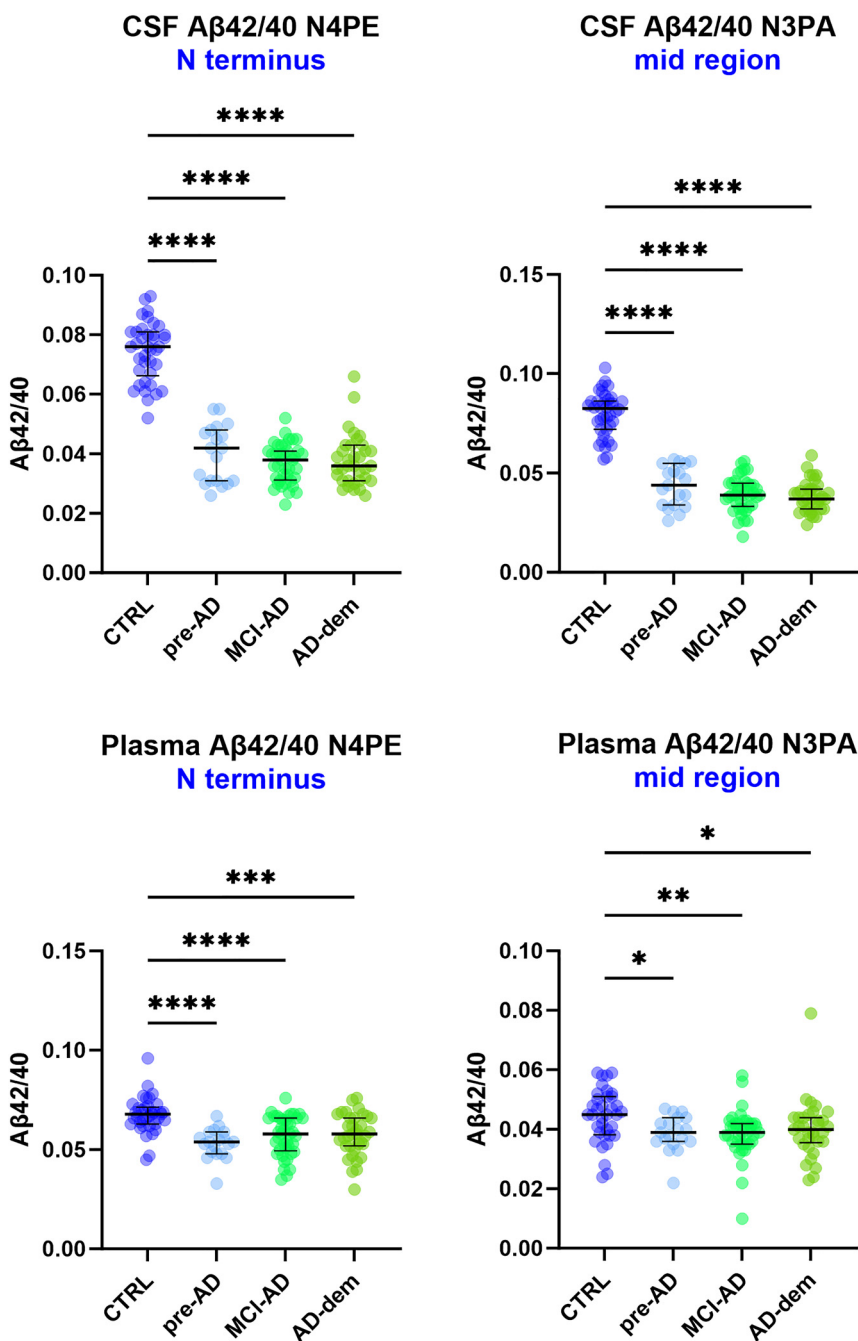


Figure 2: Diagnostic performance of N4PE (N-terminus targeted) and N3PA (mid-region targeted) assays in CSF and plasma. Kruskal–Wallis, followed by *post hoc* Dunn’s test. Horizontal line: median, error bars: interquartile range. ns= $p > 0.05$, *= $p \leq 0.05$, **= $p \leq 0.01$, ***= $p \leq 0.001$, ****= $p \leq 0.0001$, comparison vs. CTRL.

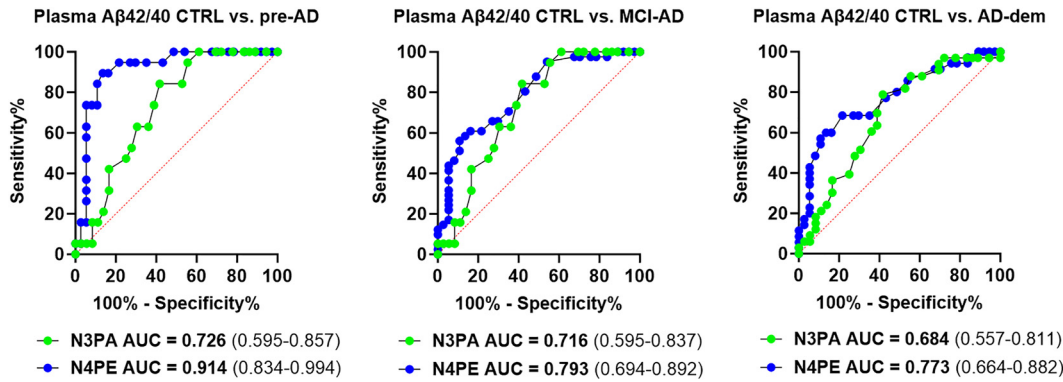


Figure 3: ROC analysis of plasma A β 42/A β 40 assessed by N3PA and N4PE assays. ROC curves were calculated for the following comparisons: CTRL vs. pre-AD, MCI-AD, and AD-dem groups. AUC, area under curve. Values in brackets represent CI 95 % (Wilson/Brown method).

$p \leq 0.0001$, CTRL vs. AD-dem $p \leq 0.001$; plasma A β 42/40 N3PA: CTRL vs. pre-AD $p \leq 0.05$, CTRL vs. MCI-AD $p \leq 0.01$, CTRL vs. AD-dem $p \leq 0.05$) (Figure 2).

As expected, ROC analysis performed for both N4PE and N3PA CSF A β 42/40 showed an excellent accuracy in discriminating between CTRL and subsequent AD continuum stages (AUC range 0.992–1; Supplementary Table S1), further underscoring the correlation between Lumipulse[®] and Simoa[®] CSF A β 42/40 measurements. Plasma A β 42/40 measured by N4PE consistently showed a better ability to differentiate between CTRL and subsequent AD continuum stages than plasma A β 42/40 assessed by N3PA assay (Figure 3). Interestingly, for both N4PE and N3PA assays, the diagnostic performance of plasma A β 42/40 was observed to decrease with the disease progression. However, such effect might be cohort-specific and due to the small sample size of pre-AD group which may drive diagnostic accuracy over-fitting.

Discussion

The concept of CSF-to-plasma transition of A β 42/40 measurements has been proposed as soon as the ultrasensitive technologies enabling its plasma detection [21–23] and standardization [24, 25] became available. However, peripheral production of A β , as well as the evidence for its physiological clearance [26–28] add another layer of difficulty in translating A β 42/40 measurement from CSF to blood plasma [9]. Studies employing immunoassay-based assessment of plasma A β 42/40 delivered conflicting results – while some authors proposed a satisfactory diagnostic performance, others reported on its moderate utility as the amyloidosis biomarker, especially at the early disease stages [12, 13, 23, 29–32]. We hypothesized that the observed differences might be due to the characteristics of the antibodies

implemented in the assays and the peptide fragments they target. Here, we performed a head-to-head method comparison of two distinct Simoa[®] assays for CSF and plasma detection of A β 42/40. Although both the assays are designed based on the same principles and are run on the same platform, different detector antibodies are used, targeting amyloid N-terminus (N4PE) or mid-region (N3PA). To evaluate the potential impact of this difference, the diagnostic performance was parallelly assessed in a cohort of AD continuum patients in matched CSF and plasma samples.

While in CSF the agreement between N4PE and N3PA was high, we observed inter-assay difference in plasma measurements, with a trend of increase accompanying higher values of A β 42/40 (Figure 1). As higher values of A β 42/40 refer to lower level of amyloid pathology, it could be interpreted that the observed method differences are more relevant mostly at the early stages of the disease.

Multiple recent studies propose that the presence of comorbidities may significantly influence the diagnostic performance of plasma A β 42/40 [33–35]. These findings are of great importance, considering the high complexity of blood plasma as well as peripheral A β expression. For instance, it has been suggested that the increased BBB permeability may improve the diagnostic performance of plasma A β 42/40 as a result of the peripheral efflux of brain-derived A β [34]. Accordingly, we analyzed the plasma A β 42/40 values in reference to CSF/serum albumin ratio. Although our results did not evidence a significant correlation, this might be due to the cohort characteristics, hence further analyses employing larger cohorts should be made. Other recent works underscore a potential link between plasma A β 42/40 and renal function [33, 35]. In the current study, we could not statistically evaluate such relation due to insufficient number of patients for whom renal function data were available. Inclusion of these parameters in future studies would be of high interest, as a strong correlation between

CKD and plasma A β 42 and A β 40 has been shown [35]. At the same time, while another study confirms this effect, the authors propose that use of A β 42/40 ratio could be an effective method to overcome it [33]. Considering the potential implementation of plasma A β 42/40 into panels of blood-based AD biomarkers for population-wide screenings, other factors potentially impacting A β 42/40 levels should be thoroughly assessed, having age-dependent differences as an example [36].

In terms of the diagnostic performance, the two CSF assays exhibited a similar accuracy. In plasma, the assay targeting A β 42 N-terminus (N4PE) performed better compared with the assay targeting A β 42 mid-region, possibly underlining a matrix effect or biofluid-specific processing. Importantly, the observed assay-specific differences in the diagnostic performance are in line with previous studies where N4PE and N3PA assays were used. In a recent study by Hirtz et al., the authors showed the mass spectrometry-based assessment of plasma A β 42/40 (AUC AD vs. OND=0.89) to be more effective than the one made by Simoa[®] N3PA assay (AUC AD vs. OND=0.82) [12]. Similarly, in the work of Janežič and colleagues, plasma A β 42/40 measured by N3PA kit exhibited a limited ability to detect abnormal brain A β status in patients with early AD (AUC=0.64) when compared with mass spectrometry based assays (AUC range=0.76–0.84) and N4PE (AUC=0.71) [13].

Considering this evidence, a thorough evaluation of the matrix effect and amyloid metabolism in plasma will be needed to fully assess the clinical utility of this blood biomarker for AD diagnosis and monitoring. Interestingly, the diagnostic performance of both A β 42/40 assays could benefit from recently described improvement of the algorithms behind Simoa[®] readout, diminishing existing quantification inaccuracies [37].

The limitations of our study are mostly related to sample size, especially of the pre-AD group. However, the inclusion of a group of functionally independent patients with no objective evidence of cognitive deficits but with positive core CSF AD biomarkers is fundamental to fully understand the early dynamics of biomarker changes in biofluids across the AD continuum. Other limitations may be linked to the limited data on CKD, as well as the nature of our control group that includes patients with neurological diseases and may influence the global diagnostic performance.

In conclusion, we propose that both A β 42/40 assays allow for an effective discrimination between the control group and different AD continuum stages, however, the assay targeting amyloid N-terminal region provides the best diagnostic performance in plasma. The observed differences in technical and diagnostic performance of the N3PA and N4PE assays in CSF and plasma indicate that the matrix-

specific amyloid processing may influence A β 42/40 determination in plasma, underscoring the need for further studies to standardize these measurements.

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Research ethics: All the procedures were performed in accordance with the Declaration of Helsinki and International Conference on Harmonization guidelines for Good Clinical Practice.

Informed consent: Informed consent was obtained from all individuals included in this study, or their legal guardians or wards and the inclusion in the study that was approved by the local Ethics Committee (Comitato Etico Aziende Sanitarie Regione Umbria 19369/AV and 20942/21/OV).

Author contributions: The authors have accepted responsibility for the entire content of this manuscript and approved its submission. Anna Lidia Wojdała: design of the work, data acquisition, analysis, and interpretation, manuscript writing; Giovanni Bellomo: design of the work, data analysis and interpretation; Andrea Toja: patients' diagnosis (neurological assessment) and cohort enrollment; Lorenzo Gaetani: patients' diagnosis (neurological assessment) and cohort enrollment; Lucilla Parnetti: design of the work, patients' diagnosis (neurological assessment) and cohort enrollment; Davide Chiasserini: design of the work, data analysis and interpretation, manuscript writing, project supervision.

Competing interests: LP served as Member of Advisory Boards for Fujirebio, IBL, Roche, and Merck. The other authors declare that they have no competing interests.

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Data availability: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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