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Original Article

Alzheimer's Disease Continuum: Evaluating the Relationship between Fluid Biomarkers and Patients' Phenotype and Profile

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ABSTRACT: The measurement of fluid biomarkers such as phosphorylated tau at threonine 181 (pTAU181), amyloid-β 40 (Aβ40), and Aβ42 is a routine medical examination that contributes to achieving an accurate diagnosis within the Alzheimer's disease (AD) clinical continuum. The aim of this study is to compare the concentration of these biomarkers in CSF and plasma and determine their relationship with the patients' clinical variant and profile. Patients were diagnosed following the NIA-AA criteria. Plasma and CSF were obtained from healthy controls (HC), mild cognitive impairment (MCI non-AD), MCI displaying positive AD markers (MCI-AD), and AD patients. Biomarker levels were assessed using the LUMIPULSE® G600II instrument (Fujirebio, Japan). Data showed a significant increase in pTAU181 concentration, specifically in the progression from MCI non-AD to AD conditions. The opposite trend was observed for Aβ42 and Aβ42/Aβ40. Furthermore, these biomarker trends appeared to change consistently with variations in the MMSE score, highlighting the relevance of plasma in detecting changes in patients' cognitive function. Considering the clinical variant, atypical patients displayed the highest pTAU181 and lowest Aβ42 levels, consistent with their lower MMSE scores. Lastly, the posterior cortical atrophy (PCA) profile showed higher pTAU181 and lower Aβ42 levels when compared to other profiles. Nevertheless, these last results are to be cautiously interpreted given the limited number of samples included in the analysis. Hence, further analyses on larger cohorts are needed to better define the role of these biomarkers in distinguishing between patients' clinical variants.

Keywords: Biomarkers, plasma, CSF, Alzheimer's disease, phenotype

INTRODUCTION

Alzheimer's Disease (AD) is the most common form of dementia and progressive cognitive decline [1]. A combination of risk factors, among which aging [2], genetics [3], and lifestyle [4], play a crucial role in determining the onset of this synaptic dysfunction disorder and contribute to its complex etiology [5]. Furthermore, given the steady increase in the elderly population, AD represents one of the major global health,

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economic, and societal burden [6], and its incidence is expected to further rise to 78 million cases by 2030 [7]. It is therefore crucial to explore novel approaches to enable early diagnosis.

AD spectrum can present in different clinical stages, which constitute a continuum of cognitive impairment, from the preclinical (asymptomatic) stage to the prodromal one (mild cognitive impairment, MCI), and eventually to full-blown dementia [8]. The terminal stage of the disease is common to all patients, nevertheless the clinical onset profile and its trajectory can be quite different, as patients with single or multidomain involvement and predominantly amnestic or non-amnestic expression can be distinguished in the early stages [9]. Moreover, in addition to the typical amnestic hippocampal presentation, other clinical variants have been described and codified, notably the frontal variant, primary progressive aphasia (PPA) (particularly the logopenic form), and posterior cortical atrophy (PCA) [10]. The availability of new fluid and neuroimaging biomarkers is increasingly broadening the spectrum of presentation of neurodegenerative diseases [11] and enhancing the understanding of their heterogeneity, particularly AD, raising questions about the mechanisms that drive the relationship between pathology and phenotype. Hence, the application of old and new biomarkers as diagnostic tools might help detect the disease at its preclinical stage, potentially allowing for a prompt therapeutic strategy that might delay cognitive decline and the onset of symptoms [12, 13].

From a biological point of view, the two hallmarks that characterize the molecular pathology of the AD continuum are the accumulation of phosphorylated tau proteins and β-amyloid (Aβ) peptides [14-16]. These are responsible for the formation of intracellular neurofibrillary tangles (NFTs) [14] and extracellular senile plaques [15, 16] in the patients' cerebral cortex, respectively. Under physiological conditions, tau proteins are usually associated with microtubules, playing an active role in stabilizing the neuronal cytoskeletal network and allowing the correct function of neurons [17]. Tau proteins interact with microtubules depending on the phosphorylation level of specific serine/threonine/tyrosine sites, with hyperphosphorylation generally associated with a decrease in the binding affinity between tau and microtubules [18]. Under pathological conditions, it has been observed that threonine-181 phosphorylated tau protein (pTAU181) is highly specific for AD, enabling its differentiation from other neurodegenerative diseases, and that high levels of pTAU181 in plasma and cerebrospinal fluid (CSF) are correlated with amyloid pathology and NFTs accumulation [19]. Other phosphorylated tau proteins, such as threonine-217 (pTAU217) or threonine-231 (pTAU231), have been found to correlate more strongly with Aβ plaque accumulation detected through amyloid PET and are hypothesized to link Aβ proteinopathy with early tau deposition [20]. As for Aβ peptides, these are generated by the cleavage of amyloid precursor protein (APP) mediated by β- and γ-secretases through the amyloidogenic pathway [15]. Although various Aβ peptide variants have been described, those that best relate to AD brain pathology are Aβ40 and Aβ42; the lower the Aβ42/Aβ40 ratio in both plasma and CSF, the greater the accumulation of cerebral Aβ plaques [21].

Given the ability to mirror the brain's pathological condition, pTAU181, Aβ40, and Aβ42 are traditionally measured in CSF during routine clinical evaluations [22]. These biomarkers are indeed essential to provide a correct diagnosis and to distinguish between different pathological stages when combined with other clinical parameters, such as psychological assessments, magnetic resonance imaging (MRI), and positron emission tomography (PET) [12, 23]. Nevertheless, CSF collection by lumbar puncture is known to be an invasive procedure [24]. For this reason, in recent years, research has increasingly focused on the potential of detecting biomarkers in plasma, using them as diagnostic tools alongside other well-established clinical parameters [20]. Starting from this, the first part of this study focused on assessing pTAU181, Aβ40, and Aβ42 levels in both CSF and plasma in a cohort comprising a total of 167 individuals. The relationship between MMSE scores and biomarker concentrations in both biofluids was also investigated. The second part of this work focused on assessing a possible association between the clinical variant and the clinical profile with the concentration of the analyzed biomarkers. The accuracy of these plasma biomarkers as disease predictors was also investigated.

MATERIALS AND METHODS

Study subjects' enrollment

All individuals enrolled in this study signed an informed consent form (Protocol n° C.E. 4407/22) in accordance with the Declaration of Helsinki. A clinical cohort of 75 patients was recruited and diagnosed at the IRCCS Mondino Foundation, Pavia (Italy), after a complete medical and instrumental examination, in accordance with the National Institute on Aging-Alzheimer's Association (NIA-AA) criteria [25]. Patients with medical history of psychiatric disease or epilepsy or any uncontrolled medical condition that could contribute to cognitive impairment (e.g., nephropathy, liver disease, brain tumor, alcohol or drug abuse, normal pressure hydrocephalus) were excluded. Based on formal neuropsychological examination, patients were first

diagnosed with dementia due to AD or MCI [26], and then categorized as amnestic, dysexecutive/attentive, or multidomain [27]. The amnestic profile was defined by the presence of memory impairment (assessed with Verbal Span, Digit Span, 15 Item Memory Test, Corsi Test and Story Recall Test), while dysexecutive/attentive profile was defined by the impairment of the logical and executive functioning or attention (examined by Raven's Colored Matrices, Frontal Assessment Battery, Trail Making Test A/B, Attentive Matrices, Stroop Test). The clinical neuropsychological profile also allowed the distinction between typical and atypical presentations of dementia due to AD, specifically primary progressive aphasia-logopenic variant (PPA-lv) [28] and posterior cortical atrophy (PCA) [29]. According to the respective diagnostic criteria, the atypical presentations were not subcategorized as amnestic, dysexecutive, or multidomain. The etiological diagnosis was made based on CSF analysis or amyloid-PET. Specifically, a blood sample was obtained from 49 AD, 14 MCI-AD (MCI presenting AD markers), and 12 MCI non-AD patients, while CSF was obtained from 42 AD, 12 MCI-AD, and 10 MCI non-AD patients. For AD patients, disease duration was calculated for those individuals with sufficient clinical information available (28 AD patients). The mean disease duration in this subset was 3.71 years ± 2.71 (standard deviation).

A second convenience sample was selected, enrolling subjects who belonged to the InveCe.Ab longitudinal population study (Brain Ageing in Abbiategrasso, ClinicalTrials.gov, NCT01345110). Briefly, all participants underwent a multidimensional assessment at baseline and after 2, 4, 8 and 12 years. The assessment includes blood sampling, social and lifestyle questionnaires administered by trained personnel, geriatric visits performed by expert geriatricians and neurologists and a comprehensive neuropsychological assessment [30]. Neuropsychologists and geriatricians independently formulate a working diagnosis within following categories after their assessment: dementia, based on DSM IV-TR criteria [31]; MCI based on Petersen criteria [27]; Cognitive Impairment Not Dementia (CIND) [32]; clinically relevant depression [33] and psychosis (ICD10: F20–F29). Those participants free from cognitive impairment, dementia, other major mental or neurological disorders were classified as cognitively normal. In the case of participants with unstable clinical condition or where there was insufficient information, no diagnosis was defined. For this study, we use samples and data of subjects assessed during the second follow-up wave receiving the following diagnoses: 2 AD, 5 MCI non-AD and 85 healthy control (HC) individuals. The diagnosis of this subject was imputed considering the aging trajectory in the 12 years of follow up (therefore MCI non-AD were subjects defined as MCI in the second follow up that have never developed dementia in the successive years). For all patients, a blood sample was collected, and plasma was retrieved and stored at -80°C until analysis.

The Mini-Mental State Examination (MMSE) was administered to all participants to determine their global cognitive status. Patients diagnosed with dementia due to AD presented a mean raw MMSE score of 20.33 ± 4.52 (standard deviation), indicating a moderate stage of dementia. Details on the recruited subjects are reported in Table 1.

Table 1. Recruited subjects' characteristics by clinical diagnosis.

	Recruited subjects			
Diagnostic group	HC	MCI non-AD	MCI-AD	AD
Recruited subjects	85	17	14	51
Age (mean ± SD, years)	75,9 ± 1,4	73,5 ± 6,8	74,8 ± 6,5	72,4 ± 7,5
Sex				
Females (%)	48,2	47,1	64,3	72,5
Males (%)	51,8	52,9	35,7	27,5

Abbreviations: HC = healthy control; MCI non-AD = mild cognitive impairment patients that do not present Alzheimer's markers; MCI-AD = mild cognitive impairment patients that present Alzheimer's markers; AD = Alzheimer's disease. SD = standard deviation.

APOE genotype determination

Starting from whole blood collected in EDTA tubes, 200 µL of blood was used to isolate genomic DNA using the Maxwell® CSC Blood DNA Kit (Promega, USA) and the Maxwell® CSC 48 instrument (Promega, USA). The NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA) was used to quantify DNA. Subsequently, the *APOE* genotype was determined using the TaqMan SNP Genotyping Master Mix (Applied Biosystems, USA) in combination with the CFX384 Touch Real-Time PCR Detection System (Bio-Rad, USA). Two probes, C___3084793_20 and C___904973_10 (TaqMan™ SNP Genotyping Assay, human, Applied Biosystems, USA), were used to analyze the *APOE* rs429358 and rs7412 polymorphisms, respectively. The *APOE* genotype was then inferred by

reading fluorescence at cycle 32 for rs429358 and at cycle 42 for rs7412.

Plasma collection and pTAU181, Aβ40, and Aβ42 analysis

Plasma was obtained from blood samples collected in sodium citrate tubes, then aliquoted into sterile polypropylene tubes (SARSTEDT AG & Co., Germany) and stored at -80 °C until analysis. The LUMIPULSE® G600II instrument (Fujirebio, Japan) was used in combination with the Lumipulse® G pTau 181 Plasma, G β-Amyloid 1-40 Plasma, and G β-Amyloid 1-42 Plasma kits (Fujirebio, Japan) to measure plasma levels of pTAU181, Aβ40, and Aβ42, respectively. Aβ42/Aβ40, pTAU181/Aβ40 and pTAU181/Aβ42 ratios were also calculated.

Cerebrospinal fluid (CSF) collection and pTAU181, Aβ40, and Aβ42 analysis

Only a subgroup of the IRCCS Mondino Foundation cohort underwent lumbar puncture for CSF collection. CSF samples were aliquoted into sterile polypropylene tubes (SARSTEDT AG & Co., Germany) and stored at -80 °C until analysis. The LUMIPULSE® G600II instrument (Fujirebio, Japan) was used to quantify pTAU181, Aβ40, and Aβ42 levels using the Lumipulse® G pTau 181, G β-Amyloid 1-40, and G β-Amyloid 1-42 assays (Fujirebio, Japan). Aβ42/Aβ40, pTAU181/Aβ40 and pTAU181/Aβ42 ratios were also calculated.

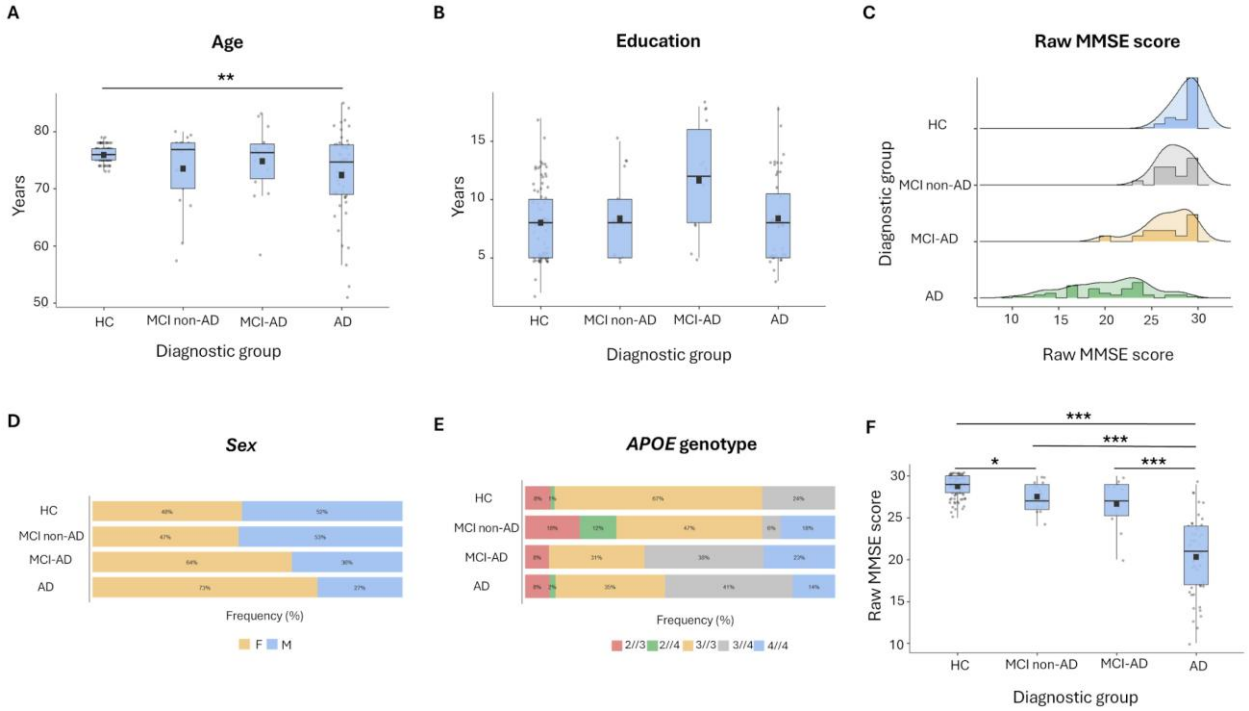

Figure 1. Cohort characterization. (**A–F)** Distribution of **A.** age, **B.** education, **C.** and **F.** raw MMSE score, **D.** sex, and **E.** *APOE* genotype across diagnostic groups: AD, MCI-AD, MCI non-AD, and HC. Group sizes (unless otherwise specified): AD (n=51), MCI-AD (n=14), MCI non-AD (n=17), HC (n=85). Exceptions: **B.** (education): AD (n=47); **C.** and **F** (raw MMSE): AD (n=49); **E.** (*APOE* genotype): MCI-AD (n=13). Games-Howell post-hoc test: *p<0.05; **p<0.01; ***p<0.001.

Statistical analysis

All statistical analyses were conducted using jamovi (Version 2.5; Sydney, Australia). Descriptive statistics, including means, standard deviations, and frequency distributions, were computed to characterize the study population and to detect potential outliers. The Shapiro-Wilk test was used to assess the normality of biomarker distributions, while Levene's test was applied to evaluate the homogeneity of variances. Since the variance assumption was not consistently met, Welch's ANOVA was employed to compare biomarker levels across diagnostic groups, clinical variants, and clinical profiles. Post-hoc pairwise comparisons were subsequently carried out using Games-Howell's test to account for unequal variances. Correlations between plasma and CSF concentrations of the same biomarkers, as well as between biomarkers and cognitive performance, were assessed

using Spearman's rank correlation coefficient. Finally, multinomial linear models were constructed to estimate the probability of belonging to each diagnostic group according to biomarker concentrations, controlling for age, sex, and education. Model diagnostics were performed to ensure model validity. All tests were two-tailed, with statistical significance set at $p < 0.05$.

RESULTS

In this study, plasma and CSF levels of pTAU181, Aβ40, and Aβ42, as well as their corresponding ratios, were measured and analyzed in relation to key clinical parameters, including diagnosis, clinical variant, clinical profile, and MMSE score. Biomarker levels were assessed in a total of 167 plasma samples, comprising 51 AD, 14 MCI-AD, 17 MCI non-AD, and 85 HC individuals. For CSF, analyses were performed on samples from 42 AD, 12 MCI-AD, and 10 MCI non-AD patients.

Cohort description and clinical parameters distribution

A cohort characterization is shown in Fig. 1. Age distribution was found to be similar among the pathological groups, with a difference observed between the HC and the AD group (Table 1) (Fig. 1A) (Supplementary Table 1). Education levels were heterogeneous across groups, but did not present a significant difference (Fig. 1B) (Supplementary Table 2). As expected, raw MMSE scores were highest in the HC group and showed a significant decline from the MCI non-AD group to the AD group (Fig. 1C, F) (Supplementary Table 3). Concerning sex distribution, the AD and MCI-AD groups displayed a predominance of female subjects, whereas both the HC and MCI non-AD groups were balanced in terms of female and male representation (Table 1) (Supplementary Table 4) (Fig. 1D). Lastly, the analysis of *APOE* genotype revealed that the pathological groups, and specifically the MCI-AD and AD subgroups, included most individuals carrying either one or two *APOE* ε4 alleles, while HC subjects predominantly carried *APOE* ε2 and ε3 alleles (Supplementary Table 5) (Fig. 1E).

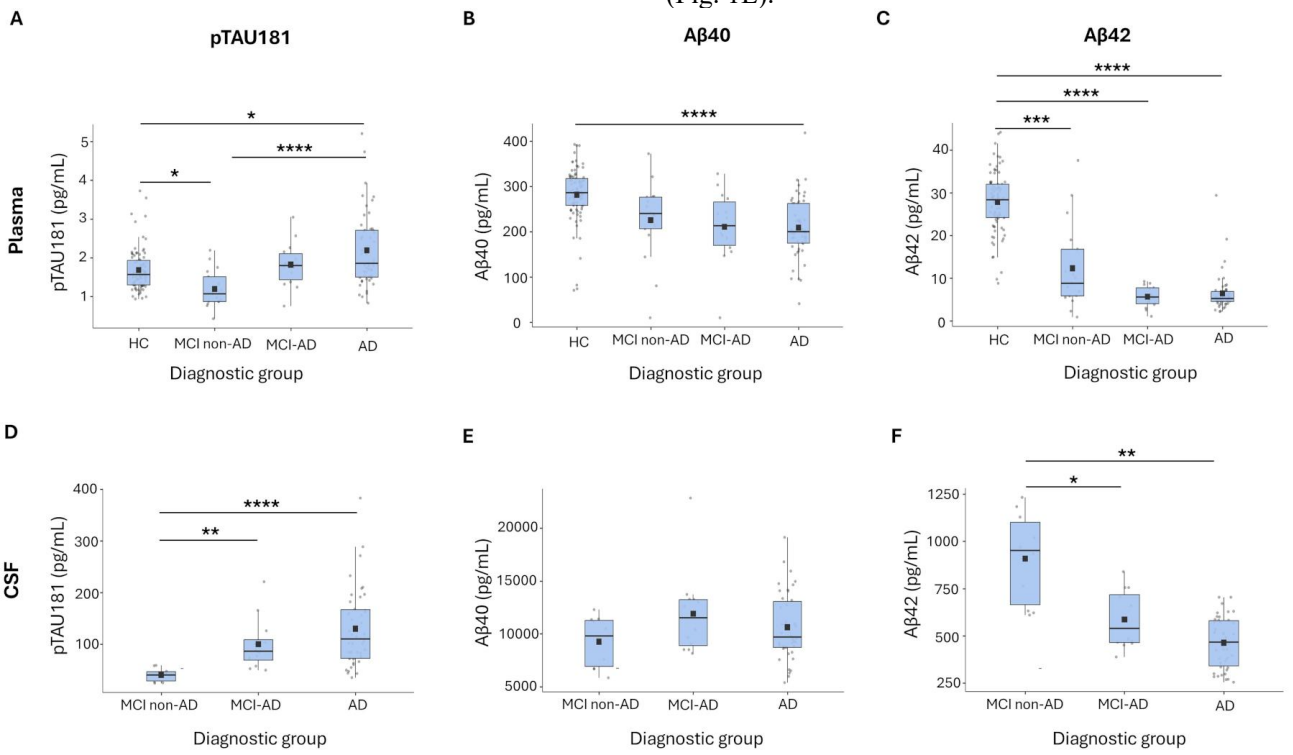

Figure 2. Comparison between plasma and CSF concentration of pTAU181, Aβ40 and Aβ42. (A–C) Plasma concentrations of **A.** pTAU181, **B.** Aβ40 and **C.** Aβ42 in AD, MCI-AD, MCI non-AD, and HC. **(D–F)** CSF concentrations of **D.** pTAU181, **E.** Aβ40 and **F.** Aβ42 in AD, MCI-AD, and MCI non-AD. Plasma cohort: AD (n=51), MCI-AD (n=14), MCI non-AD (n=17), HC (n=85). CSF cohort: AD (n=42), MCI-AD (n=12), MCI non-AD (n=10). X axis: diagnostic condition; Y axis: biomarker concentration (pg/mL). Games-Howell post-hoc test: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001

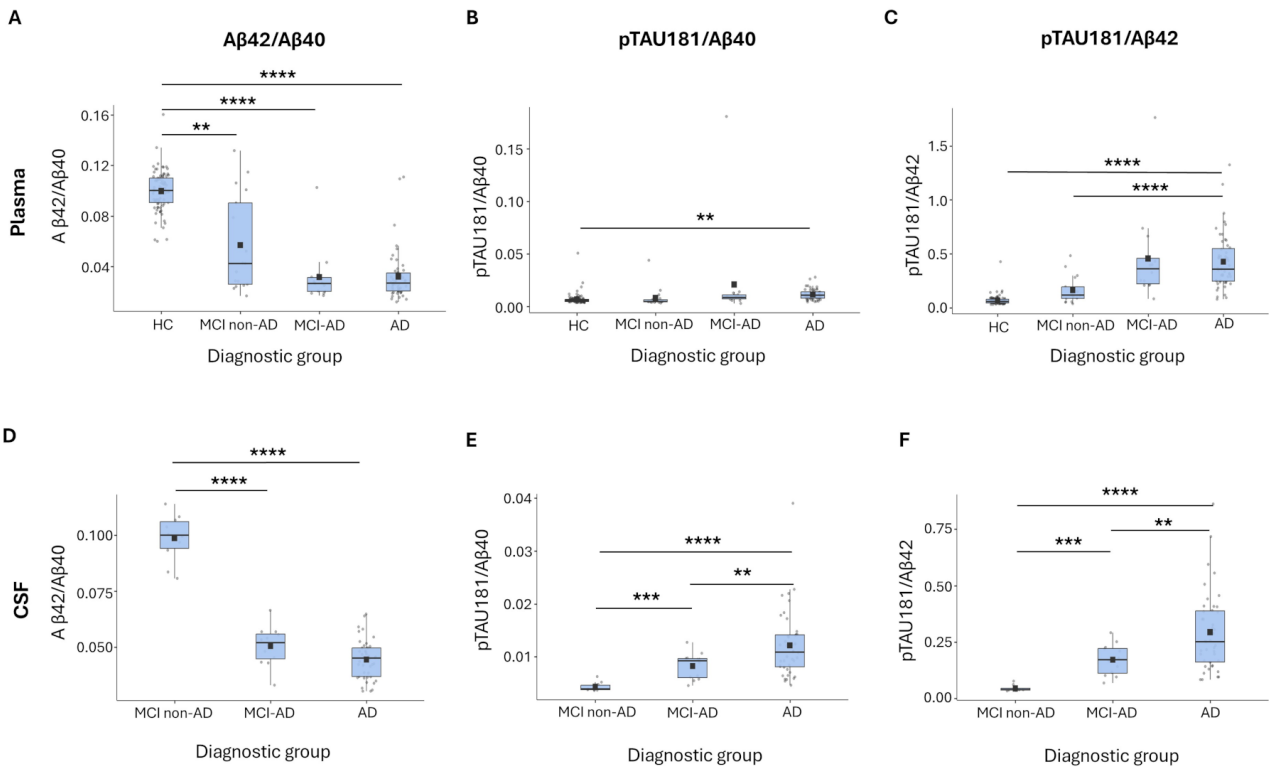

Figure 3. Comparison between Aβ42/Aβ40, pTAU181/Aβ40 and pTAU181/Aβ42 ratios in plasma and CSF. (A–C) Plasma biomarker ratios: **A.** Aβ42/Aβ40, **B.** pTAU181/Aβ40 and **C.** pTAU181/Aβ42 in AD, MCI-AD, MCI non-AD, and HC. **(D–F)** CSF biomarker ratios: **D.** Aβ42/Aβ40, **E.** pTAU181/Aβ40, and **F.** pTAU181/Aβ42 in AD, MCI-AD, and MCI non-AD. Plasma cohort: AD (n=51), MCI-AD (n=14), MCI non-AD (n=17), HC (n=85). CSF cohort: AD (n=42), MCI-AD (n=12), MCI non-AD (n=10). X axis: diagnostic condition; Y axis: biomarker ratios. Games-Howell post-hoc test: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001

Comparison between pTAU181, Aβ40, and Aβ42 levels and their ratios in plasma and CSF

The concentration of the three biomarkers in plasma and CSF is shown in Fig. 2 (Supplementary Table 6). In both biofluids, pTAU181 levels significantly increased going from MCI non-AD to AD groups (Fig. 2A, D). Specifically, in plasma, a significant difference in concentration was also observed between HC and MCI non-AD, where pTAU181 appeared reduced, and between the HC and the AD group (Fig. 2A). While little difference in Aβ40 concentration was noticed in CSF (Fig. 2E), a progressive decrease in its levels was found in plasma, with a marked contrast between HC and AD subjects (Fig. 2B). As for Aβ42, plasma measurements highlighted a great reduction going from the HC condition to the pathological states (Fig. 2C), while CSF data additionally revealed differences among the disease groups, particularly between MCI non-AD and MCI-AD, and between MCI non-AD and AD (Fig. 2F).

When considering the Aβ42/Aβ40 ratio, plasma levels in HC subjects were significantly different from those of all pathological groups (Fig. 3A). CSF data further highlighted inter-group differences within the pathological cohort (Fig. 3D). Finally, plasma showed a slight increase in both the pTAU181/Aβ40 (Fig. 3B) and pTAU181/Aβ42 ratios (Fig. 3C) from HC to AD group. These trends were further supported by CSF measurements (Fig. 3E, F). Details on the biomarker levels' mean, SD, and p-values of the multiple comparison analysis are reported in Supplementary Table 6.

Relationship between plasma and CSF biomarkers concentration

The association between plasma and CSF biomarker levels was evaluated in a total of 64 subjects (42 AD, 12 MCI-AD, and 10 MCI non-AD) (Fig. 4). Plasma pTAU181 showed a significant positive association with CSF pTAU181 levels (r: 0.440, $p < 0.001$) (Fig. 4A). Aβ42 and the Aβ42/Aβ40 ratio also displayed positive plasma-CSF associations, although these did not reach statistical significance (Aβ42 r: 0.100, p: 0.433; Aβ42/Aβ40 r: 0.042, p: 0.744) (Fig. 4B, C). Furthermore, while Aβ40 was the only biomarker whose concentration was found to be inversely related between plasma and CSF (r: -0.014, p: 0.915) (Supplementary Fig. 1A), both

pTAU181/Aβ40 and pTAU181/Aβ42 ratios appeared consistent in both analyzed biofluids (pTAU181/Aβ40 r: 0.495, p < 0.001; pTAU181/Aβ42 r: 0.594, p < 0.001) (Supplementary Fig. 1B, C).

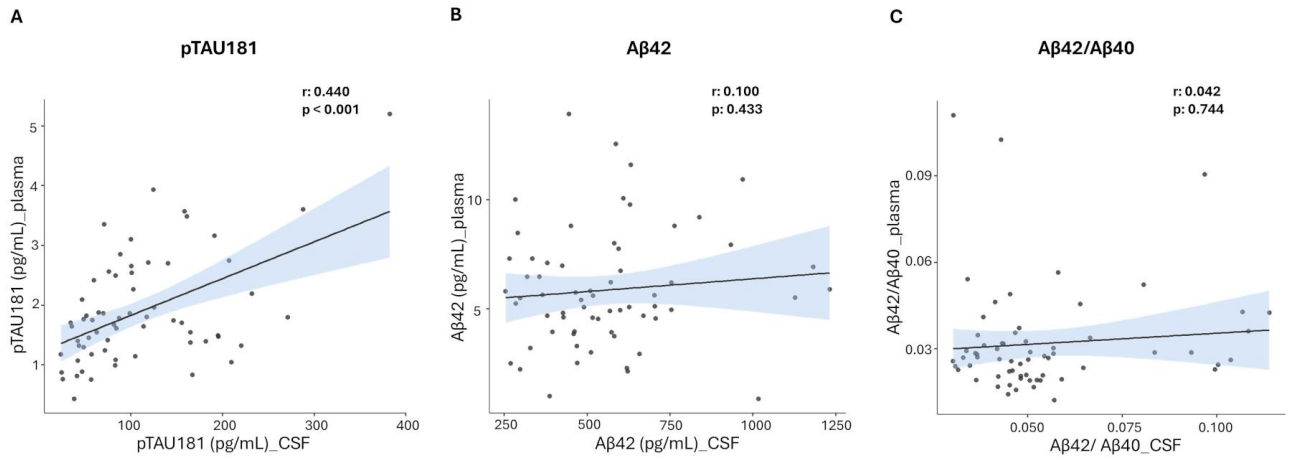

Figure 4. Association between plasma and CSF biomarker levels. A. pTAU181 plasma vs CSF association. **B.** Aβ42 plasma vs CSF association. **C.** Aβ42/Aβ40 plasma vs CSF association. Cohort n = 64 (42 AD, 12 MCI-AD and 10 MCI non-AD). X axis: biomarker CSF concentration (pg/mL) or ratio; Y axis: biomarker plasma concentration (pg/mL) or ratio. Spearman's rho test.

Relationship between the MMSE score corrected for age and education and biomarker concentration in both plasma and CSF

The association between the MMSE score corrected for age and education (MMSEc) and biomarker concentrations was assessed in both plasma and CSF (Fig. 5). In both biofluids, pTAU181 levels were inversely associated with MMSEc score, and thus with subjects' cognitive performance (plasma pTAU181 r: -0.26, p < 0.001; CSF pTAU181 r: -0.28, p: 0.028) (Fig. 5A, D). Aβ42 concentration displayed a positive association with MMSEc scores in both plasma (r: 0.54, p < 0.001) (Fig. 5B) and CSF (r: 0.56, p < 0.001) (Fig. 5E). A similar trend was observed for the Aβ42/Aβ40 ratio, both in plasma (r: 0.58, p < 0.001) (Fig. 5C) and CSF (r: 0.49, p < 0.001) (Fig. 5F), indicating that lower levels of these biomarkers correspond to reduced cognitive function. Consistent with the Aβ42 findings, Aβ40 concentration was also positively associated with MMSEc score in both biofluids (plasma Aβ40 r: 0.25, p: 0.002; CSF Aβ40 r: 0.17, p: 0.190) (Supplementary Fig. 2A, D). Finally, both the pTAU181/Aβ40 and pTAU181/Aβ42 ratios were inversely related to MMSEc scores in plasma (pTAU181/Aβ40 r: -0.39, p < 0.001; pTAU181/Aβ42 r: -0.60, p < 0.001) (Supplementary Fig. 2B, C) and in CSF (pTAU181/Aβ40 r: -0.43, p < 0.001; pTAU181/Aβ42 r: -0.48, p < 0.001) (Supplementary Fig. 2E, F). The association between the raw MMSE score and biomarkers concentration is shown in Supplementary Figure 5.

Clinical variant association with biomarker concentration in both plasma and CSF

The effect of AD patients' clinical variants on biomarker levels was investigated in both biofluids (Fig. 6) (Supplementary Table 7). Given the small subgroup sizes these analyses are exploratory and results are preliminary. While the Aβ42/Aβ40 ratio showed no differences after stratifying patients by clinical variant in either plasma (Fig. 6C) or CSF (Fig. 6F), slight differences in pTAU181 and Aβ42 concentrations were observed between the two groups. Particularly, pTAU181 levels appeared slightly higher in atypical patients compared to typical ones in both biofluids (Fig. 6A, D). Conversely, Aβ42 showed the opposite pattern, with the atypical group displaying lower concentrations than the typical group, particularly in plasma (Fig. 6B, E). Despite the lack of statistical significance, this may reflect a possible difference in pathological severity between the two groups. The same analysis was also performed for Aβ40, pTAU181/Aβ40, and pTAU181/Aβ42. While Aβ40 displayed no difference between atypical and typical groups in both analyzed biofluids (Supplementary Fig. 3A, D), both pTAU181/Aβ40 (Supplementary Fig. 3B, E) and pTAU181/Aβ42 (Supplementary Fig. 3C, F) ratios appeared to corroborate what was previously described for pTAU181 and Aβ42. Details on the biomarker levels mean, SD, and p-values of the comparison analysis are reported in Supplementary Table 7.

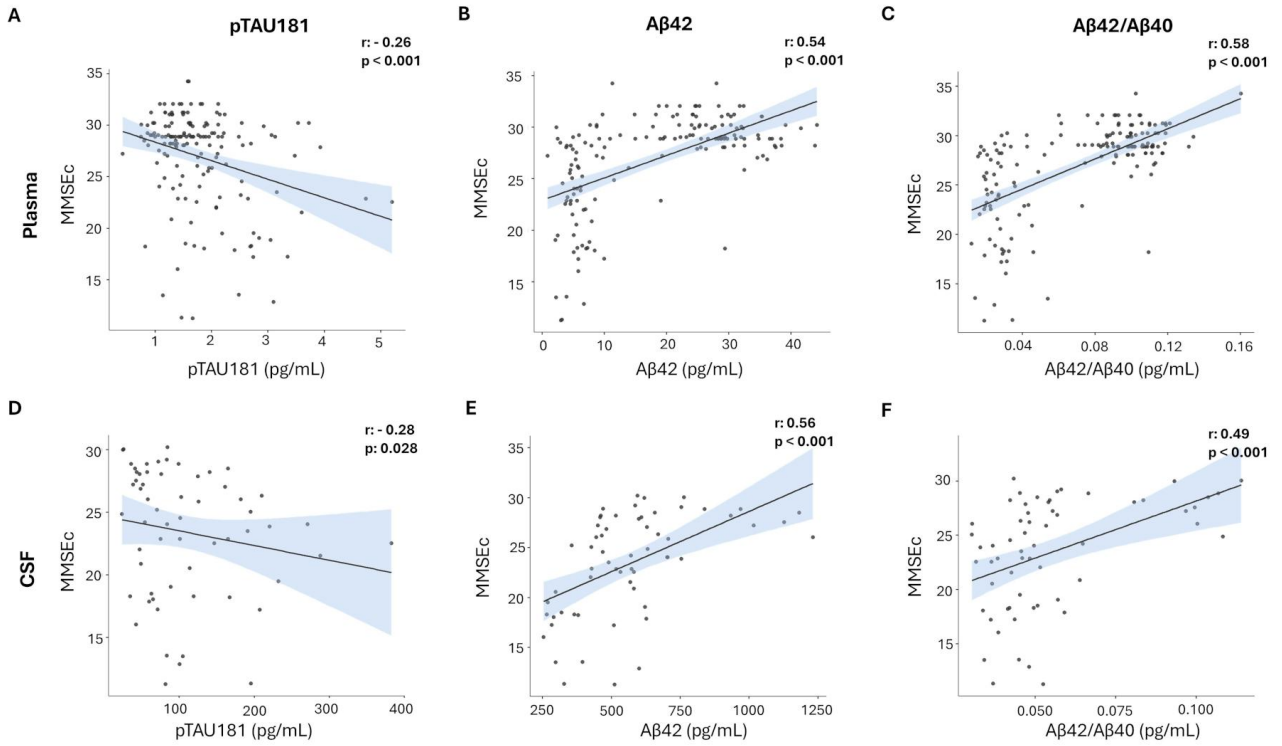

Figure 5. Association between MMSE score corrected for age and education (MMSEc) and biomarkers concentration in both plasma and CSF. A. pTAU181 plasma concentration vs raw MMSE score association. **B.** Aβ42 plasma concentration vs raw MMSE score association. **C.** Aβ42/Aβ40 plasma ratio vs raw MMSE score association. **D.** pTAU181 CSF concentration vs raw MMSE score association. **E.** Aβ42 CSF concentration vs raw MMSE score association. **F.** Aβ42/Aβ40 CSF ratio vs raw MMSE score association. Plasma cohort n = 159 (45 AD, 13 MCI-AD, 16 MCI non-AD and 85 HC). CSF cohort n = 60 (38 AD, 12 MCI-AD and 10 MCI non-AD). X axis: biomarker plasma or CSF concentration (pg/mL); Y axis: MMSEc. Spearman's rho test.

Clinical profile association with biomarker concentration in both plasma and CSF

The effect of the clinical profile on biomarker levels was investigated in both biofluids within the pathological cohort (Fig. 7). Also in this case, the analyzed subgroups had limited sample sizes. Therefore, these analyses are exploratory and should be interpreted with caution. This analysis revealed that AD patients with a PCA profile exhibited the highest pTAU181 levels and the lowest Aβ42 and Aβ42/Aβ40 concentrations, at least in plasma (Fig. 7A-C) (Supplementary Table 8). In CSF, this pattern was confirmed only for pTAU181 (Fig. 7D). Within CSF measurements, amnestic MCI-AD patients showed significantly lower Aβ42 levels compared to MCI-AD patients with a dysexecutive profile (Fig. 7E). Furthermore, AD patients with a multidomain profile exhibited a significant reduction in Aβ42 concentration compared to single domain profiles, specifically the amnestic one (Fig. 7E) (Supplementary Table 9). Both trends were not confirmed in blood. Nevertheless, the small sample size of analyzed PCA patients prevents drawing any definitive conclusions regarding the observed trend. The same analysis was also conducted on Aβ40, pTAU181/Aβ40, and pTAU181/Aβ42 (Supplementary Fig. 4). Details on the biomarker levels mean, SD, and p-values of the multiple comparison analysis are reported in Supplementary Tables 8 and 9.

Plasma biomarkers as indicators of disease status

The ability of the investigated plasma biomarkers to be associated with different disease stages was analyzed (Fig. 8). pTAU181 was confirmed as strongly associated with AD diagnosis, with higher concentrations corresponding to a higher probability of being diagnosed with AD (β estimate: -2.782, SE: 0.842, p: 0.001) (Fig. 8A). Conversely, it appeared unrelated to MCI-AD (β estimate: 0.307, SE: 0.472, p: 0.516) and MCI non-AD (β estimate: 1.058, SE: 0.316, p: 0.001) diagnosis associations. The model explained 21% of the variance in diagnostic group (R^2 = 0.21) and achieved an overall classification accuracy of 67%.

A similar pattern was observed for both Aβ42 (AD vs HC β estimate: -0.200, SE: 0.043, p < 0.001; MCI-AD vs HC β estimate: -0.443, SE: 0.121, p < 0.001; MCI non-AD vs HC β estimate: -0.345, SE: 0.059, p < 0.001) and Aβ42/Aβ40 (AD vs HC β estimate: -56.699, SE: 12.528,

$p < 0.001$; MCI-AD vs HC β estimate: -91.195, SE: 18.884, $p < 0.001$; MCI non-AD vs HC β estimate: -88.671, SE: 13.845, $p < 0.001$), with decreases in their levels being related to an increased probability of diagnosis of AD (Fig. 8B, C). While the model for Aβ42 explained 48% of the variance ($R^2 = 0.48$) with a classification accuracy of 77%, the Aβ42/Aβ40 model explained 44% of the variance ($R^2 = 0.44$) and showed an overall accuracy of 78%.

Model assumptions, including residual analysis, were checked for all models and no major violations were observed. All reported associations are cross-sectional and should not be interpreted as evidence of longitudinal prediction or causality.

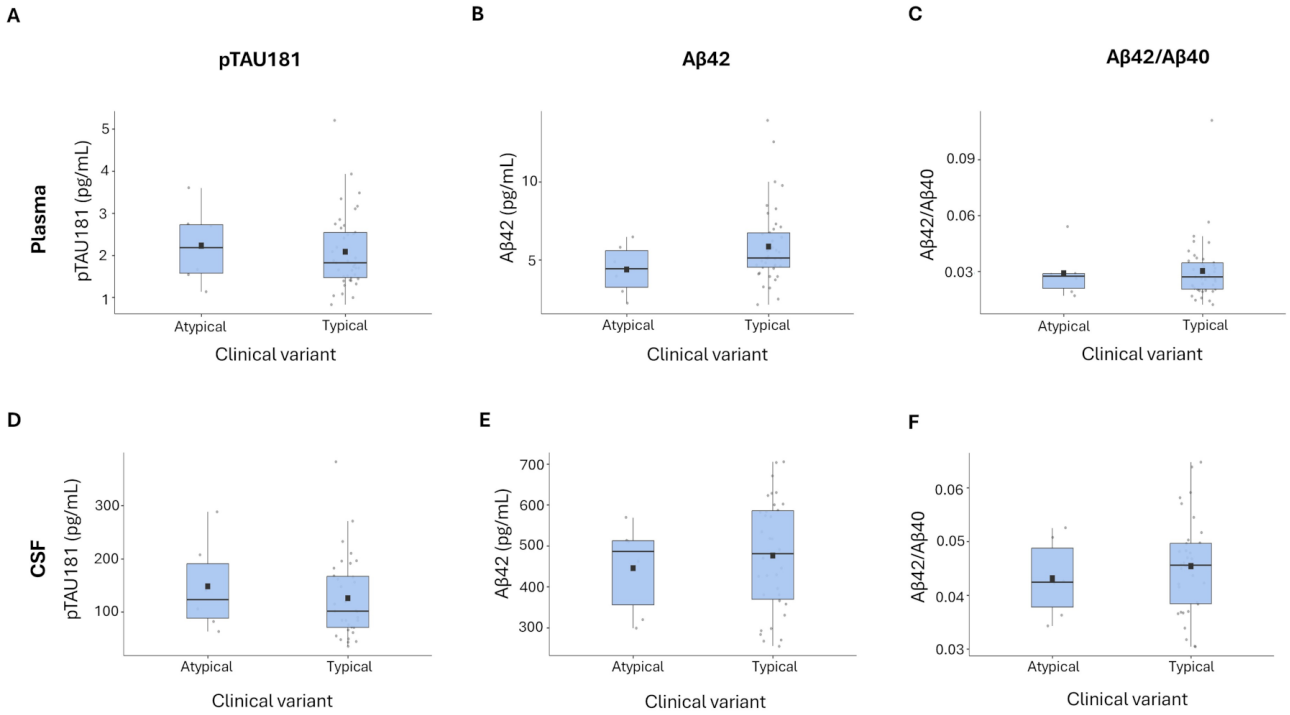

Figure 6. The effect of the clinical variant on biomarkers level in both biofluids of AD patients. (A–C) Plasma biomarkers: **A.** pTAU181, **B.** Aβ42 and **C.** Aβ42/Aβ40 ratio in typical and atypical AD. **(D–F)** CSF biomarkers: **D.** pTAU181, **E.** Aβ42 and **F.** Aβ42/Aβ40 ratio in typical and atypical AD. Plasma cohort: typical AD (n=41), atypical AD (n=6). CSF cohort: typical AD (n=34), atypical AD (n=6). X axis: AD clinical variant; Y axis: biomarker concentration (pg/mL) or ratio. Student's t-test: results not significant.

DISCUSSION

Given the significant impact of AD on the healthcare system and its expected rise in incidence in the coming years, it is crucial to focus efforts toward the identification of biomarkers that, when integrated with other diagnostic tools, may contribute to a biological definition of the disease [34, 35]. Among established AD biomarkers, plasma levels of pTAU181, Aβ40, and Aβ42 have been described as specific indicators of AD-related amyloidopathy and tauopathy [36]. Therefore, the primary aim of the present study was to measure the concentration of these biomarkers and compare their patterns in both CSF and plasma samples from an Italian cohort.

An initial characterization of the cohort evaluated the distribution of age, sex, and education across the diagnostic groups. In line with previous literature, MMSE scores showed a stage-dependent decrease from HC to AD, reflecting increasing cognitive impairment and brain atrophy in the AD group [37]. Similarly, the *APOE* ε4 allele was more prevalent in pathological groups, further confirming its role as a major genetic risk factor for AD [38].

Subsequently, the concentrations of the three biomarkers were assessed in both CSF and plasma. A significant increase in pTAU181 levels was observed in plasma when moving from the MCI non-AD to the AD condition. This pattern was further confirmed in CSF and is consistent with previous studies [39, 40]. Interestingly, when comparing plasma pTAU181 levels between HC individuals and MCI non-AD patients, a significant decrease emerged in the latter group. This trend may be explained by the patients' selection criteria adopted in the current study. Indeed, only MCI non-AD patients with relatively few comorbidities and a low or minimal presence of vascular lesions in the brain were included. In contrast, HC individuals were recruited on a voluntary

basis and were therefore less rigorously screened for mild systemic comorbidities, which could influence plasma pTAU181 levels. In this regard, a recent study reported the influence of physiological variables and comorbidities (e.g. glomerular filtration rate, hypertension, diabetes and dyslipidemia) on plasma biomarkers in cognitive unimpaired subjects; a similar trend may also be expected in patients with cognitive disorders [41]. Furthermore, HC subjects were slightly older on average, a factor that may have further contributed to the observed differences. Consistent with the literature, Aβ42 plasma concentration was significantly reduced in the AD group, while its CSF levels showed a significant and progressive decrease from MCI non-AD to AD, effectively distinguishing between the different disease stages [42, 43]. A similar trend was also observed when considering the Aβ42/Aβ40 ratio [44].

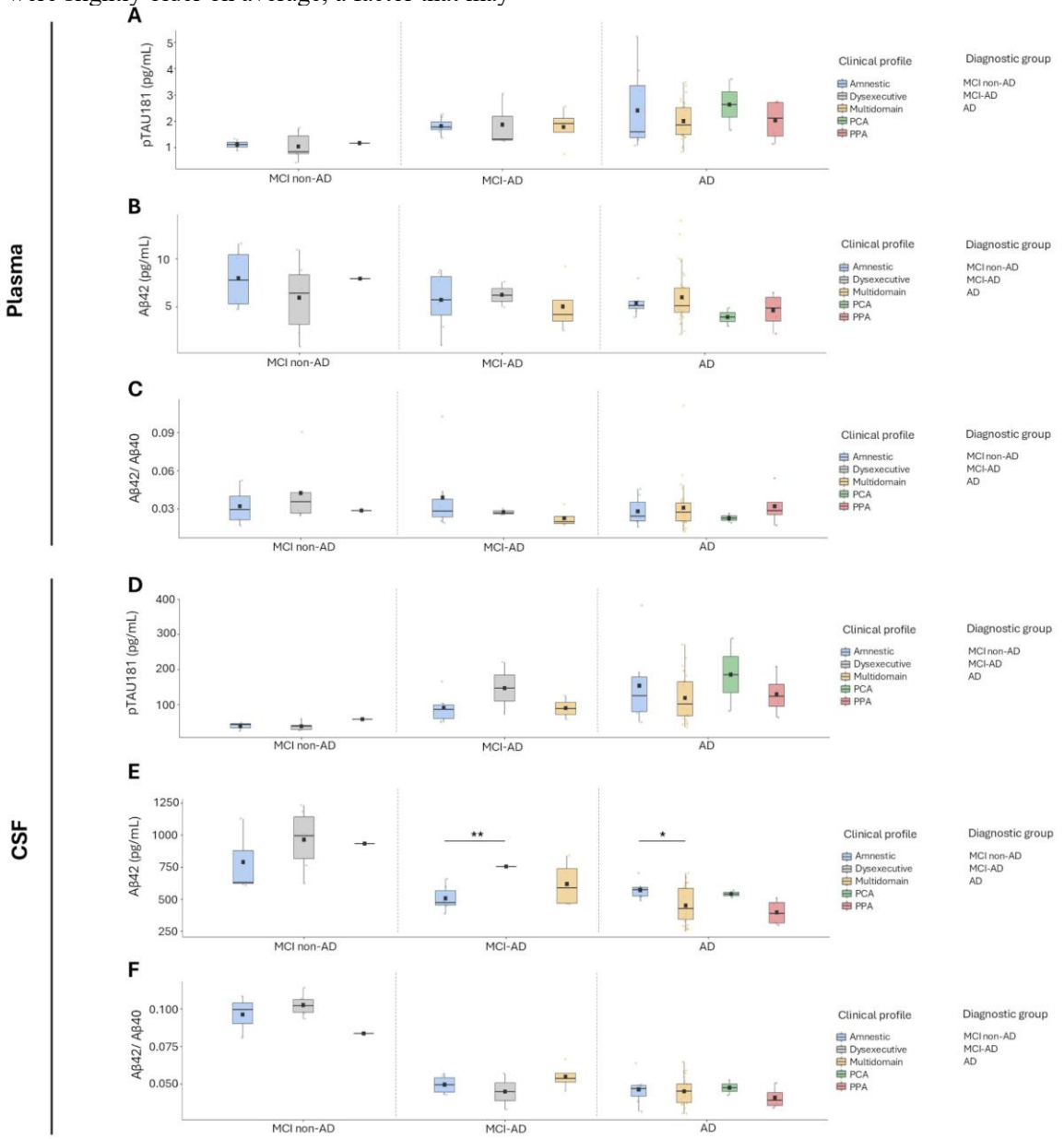

Figure 7. The effect of the clinical profile on biomarkers level in both patients' biofluids. (A–C) Plasma biomarkers: **A.** pTAU181, **B.** Aβ42 and **C.** Aβ42/Aβ40 ratio. **(D–F)** CSF biomarkers: **D.** pTAU181, **E.** Aβ42 and **F.** Aβ42/Aβ40 ratio. Plasma cohort: AD: multidomain (n=33), amnestic (n=8), PPA (n=4), PCA (n=2); MCI-AD: multidomain (n=4), amnestic (n=7), dysexecutive (n=3); MCI non-AD: multidomain (n=1), amnestic (n=4), dysexecutive (n=6). CSF cohort: AD: multidomain (n=27), amnestic (n=7), PPA (n=4), PCA (n=2); MCI-AD: multidomain (n=4), amnestic (n=6), dysexecutive (n=2); MCI non-AD: multidomain (n=1), amnestic (n=3), dysexecutive (n=6). X axis: pathological group and clinical profile; Y axis: biomarker concentration (pg/mL) or ratio. Games-Howell post-hoc test: *p<0.05; **p<0.01.

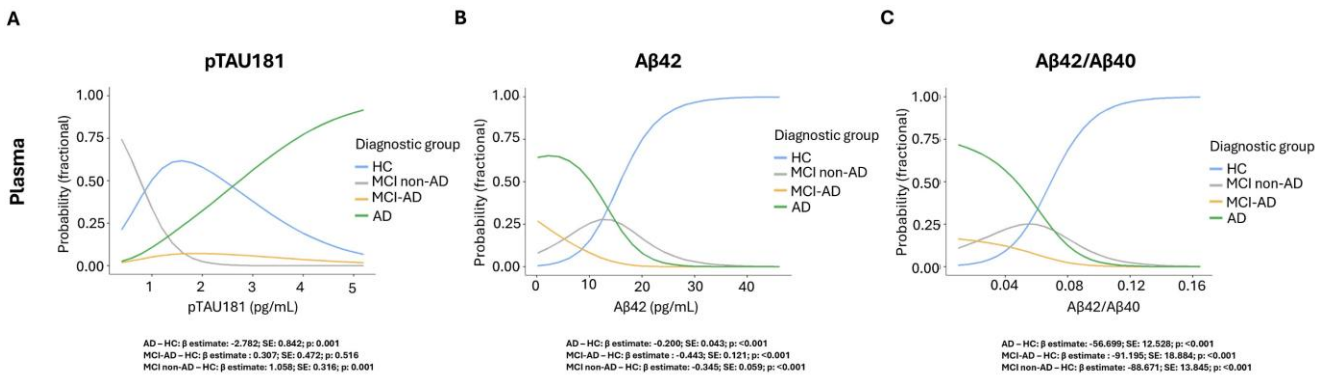

Figure 8. Plasma biomarker's ability to predict the different stages of the disease. A. pTAU181 plasma concentration vs the probability to predict the diagnosis. **B.** Aβ42 plasma concentration vs the probability to predict the diagnosis. **C.** Aβ42/Aβ40 plasma ratio vs the probability to predict the diagnosis. X axis: biomarker plasma concentration (pg/mL) or ratio; Y axis: Probability. Data were corrected for age, sex and education. Multinomial logistic regression.

Regarding the association between plasma and CSF biomarker levels, pTAU181 showed the strongest and most consistent results, with a significant correlation between the two biofluids. These findings align with previous reports in the literature [40, 45, 46]. A similar pattern was observed for both Aβ42 levels and the Aβ42/Aβ40 ratio, although the associations did not reach statistical significance [45].

This may be explained by considering that Aβ42 and Aβ40 markers in plasma generally show weaker performance compared with both plasma pTAU181 and CSF Aβ measures, largely due to their limited diagnostic range. This is consistent with previous evidence indicating that plasma Aβ42 and Aβ40 concentrations are substantially lower than in CSF, reducing their ability to capture disease-related changes with sufficient sensitivity [20]. In addition, the pathophysiological complexity of AD further contributes to the limited diagnostic power of plasma Aβ42 and Aβ42/Aβ40 [20]. In contrast, pTAU181 demonstrated stronger plasma-CSF correlation and higher diagnostic accuracy, in line with clinical guidelines. These highlight pTAU181 as a reliable biomarker of tau pathology and recommend its use in combination with other markers to improve diagnostic confidence and staging accuracy [20].

The relationship between the MMSEc score and biomarker concentrations in the two biofluids was also investigated. In both CSF and plasma, pTAU181, Aβ42, and Aβ42/Aβ40 ratio showed patterns that were consistent with variations in MMSEc. Specifically, lower MMSEc scores were associated with higher pTAU181 levels and with lower Aβ42 and Aβ42/Aβ40 ratio levels. This pattern is consistent with the association between a change in biomarker levels and cognitive impairment in AD [47–49]. Lastly, the pTAU181/Aβ42 ratio exhibited an inverse relationship with MMSEc scores, indicating that higher ratio levels correspond to more pronounced cognitive impairment. Similarly, although previous studies suggest the potential utility of the pTAU181/Aβ42 ratio in predicting early cognitive changes [50] the associations observed here should be interpreted as cross-sectional and non-predictive.

The second aim of this investigation focused on assessing whether AD clinical variants and clinical profiles could influence biomarker concentrations. Notably, both CSF and plasma pTAU181 levels appeared slightly higher in atypical AD patients compared to typical AD cases, while the opposite trend was observed for Aβ42 levels. Given the small sample sizes, these subgroups analyses are underpowered and should be interpreted as exploratory. Nonetheless, this pattern may reflect a steeper cognitive decline in atypical presentations and could potentially suggest a more pronounced tauopathy and amyloidopathy [51, 52]. A similar trend was also observed for the pTAU181/Aβ40 and pTAU181/Aβ42 ratios. However, to more accurately define the potential role of these biomarkers in distinguishing between the two clinical variants, analyses in larger and demographically heterogeneous cohorts are required.

Considering clinical profiles, the PCA group was the one that diverged most clearly from the others. Specifically, this subgroup showed the highest pTAU181 concentrations and the lowest Aβ42 and Aβ42/Aβ40 levels, particularly in plasma. However, given the very limited sample size and the evidence reported in the literature [53, 54], any direct relationship between this profile and substantial biomarker alterations remains only speculative. Interestingly, CSF analyses revealed that at earlier pathological stages, such as in MCI non-AD and MCI-AD cases, amnestic patients tended to exhibit greater amyloid accumulation, whereas dysexecutive patients showed slightly higher tau accumulation. The higher pTAU181 levels observed in dysexecutive MCI-AD cases may partially reflect a more widespread tauopathy, affecting not only posterior but also frontal

regions, as suggested by perfusion studies [55]. In this scenario, multidomain MCI patients, who present both amnestic and dysexecutive features, displayed intermediate characteristics. With disease progression, this pattern seemed to reverse, with the multidomain AD group displaying increased amyloidopathy and potentially heightened tauopathy. These findings may suggest that, in early disease stages, individual susceptibility to amyloid and tau accumulation, and the relative balance between the two, could influence the onset profile of MCI. Conversely, in more advanced stages, pathology progression may overshadow individual susceptibilities, leading to a more uniform clinical manifestation across patients, regardless of initial biomarker profiles or characteristics. It should be stressed that this interpretation remains hypothetical given the limited number of patients included in this exploratory analysis and requires further confirmation. Studies involving larger and more heterogeneous cohorts will be essential to robustly investigate the potential correlation between clinical profiles and biomarker concentrations.

Lastly, plasma pTAU181, Aβ42, and the Aβ42/Aβ40 ratio were confirmed as associated to AD diagnosis [36], while their ability to diagnose MCI non-AD or MCI-AD conditions was limited.

In conclusion, the primary aim of this study was to highlight the utility of pTAU181, Aβ42, and the Aβ42/Aβ40 ratio as consistent biomarkers of AD pathology. The observed trends in both plasma and CSF help clarify the relationship between cognitive decline and the progression of amyloidopathy and tauopathy, supporting the notion that these fluid biomarkers are associated with cognitive impairment in AD. Simultaneously, the secondary aim allowed us to explore the potential influence of clinical variants and profiles on biomarker concentrations, although further research is required to validate these findings as the interpretation remains speculative. Finally, while plasma biomarkers showed strong association with AD, their limited power in detecting preclinical stages suggests that a combined approach, integrating multiple biomarkers and diagnostic tools, is essential for accurate prognosis in early disease phases.

While further analyses on larger cohorts are needed to better define the role of these biomarkers in distinguishing clinical variants, the data suggest a potentially detectable difference between typical and atypical forms of AD.

Limitations

The recruitment of patients took place in two different centers. The vast majority of patients were enrolled at the IRCCS Mondino Foundation, with the exception of seven individuals recruited at the Golgi-Cenci Foundation. The HC cohort, instead, was entirely recruited at the Golgi-Cenci Foundation. As detailed in the Methods section, both centers adopted strict diagnostic and sampling protocols, and samples were stored and analyzed in the same way and at the same time. Nevertheless, this dual recruitment strategy may still introduce potential sources of bias. In particular, differences in clinical evaluation procedures, and especially in the administration and interpretation of neuropsychological tests, might vary across centers. This limitation is particularly relevant for the seven patients diagnosed with either AD or MCI non-AD who were enrolled at the Golgi Cenci Foundation.

Moreover, the analyses involving cognitive measures such as the MMSEc are inherently cross-sectional. Therefore, the associations observed between biomarker levels and cognitive scores should be interpreted as correlational rather than causal or predictive.

Furthermore, the limited sample size, particularly within the atypical patient group and certain clinical profiles, greatly reduced the statistical power of these analyses, that remain exploratory and preventing any robust interpretation of the observed trends. Additionally, all patients included in this study had relatively few comorbidities and were demographically homogeneous. Therefore, the findings may not be generalizable to larger, more diverse populations.

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Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data and materials availability

The datasets for this manuscript can be found at Zenodo repository DOI 10.5281/zenodo.17376829.

Author contributions

Conceptualization: RDG, MCR, AD and SG. Formal analysis: RDG, MCR, FD, EM and SG. Funding acquisition: AC, AD and SG. Investigation: RDG, EM, AD and SG. Methodology: RDG, FD, EM, BR, EZ, FRR and SG. Resources: AC, and SG. Supervision: MCR, AD, and SG. Visualization: RDG, MCR, MR and SG. Writing—original draft: RDG, MCR, EM, MR, AD and SG. Writing—review and editing: RDG, MCR, FD, EM, BR, EZ, FRR, MR, GP, AC, AD and SG. All authors contributed to the article and approved the submitted version.

Supplementary Materials

The Supplementary data can be found online at: www.aginganddisease.org/EN/10.14336/AD.2025.0971.

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