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Cerebrospinal fluid and serum D-serine concentrations are unaltered across the whole clinical spectrum of Alzheimer's disease

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ABSTRACT

The diagnosis of Alzheimer's disease (AD) relies on the presence of amyloidosis and tauopathy, as reflected in cerebrospinal fluid (CSF), independently from the clinical stage. Recently, CSF D-serine has been proposed as a possible new AD biomarker, reflecting dysfunctional activation of neuronal glutamatergic *N*-methyl-D-aspartate receptor (NMDAR). In this study, we measured blood serum and CSF concentration of two NMDAR modulators, such as D-serine and D-aspartate, in a cohort of drug-free subjects encompassing the whole AD clinical spectrum. In addition, we also analyzed D-serine levels in a cohort of *post-mortem* AD and control cortex samples. We reported unaltered serum and CSF concentrations of D-serine and D-aspartate in AD patients both during the AD progression and compared to non-demented controls. Accordingly, no correlation was detected between serum or CSF D-serine content and mini-mental state examination or Clinical Dementia Rating. Similarly, cortical D-serine levels were also unaltered in *post-mortem* samples of AD patients. Overall, our results failed to confirm previous findings indicating the CSF D-serine as a novel biomarker for AD.

1. Introduction

Worldwide, Alzheimer's disease (AD) is the most prevalent neurodegenerative disease and the main cause of dementia in the elderly population [1,2]. The main neuropathological hallmarks include synaptic loss, amyloid plaques (Aβ) deposition and neurofibrillary tangles (NFT) [3]. Presence of amyloidosis (A+) and tauopathy (T+) is reflected in the cerebrospinal fluid (CSF) by Aβ42/Aβ40 ratio and phosphorylated tau (phospho-Tau), respectively. The A + T+ molecular signature corresponds to a biochemical diagnosis of AD, independent of the clinical stage, thus encompassing the whole spectrum of the disease, from preclinical stage to overt dementia [4]. Along with the cortical Aβ deposition and NFT formation, other pathogenic mechanisms take place in AD. Aberrant glutamatergic neurotransmission at NMDA receptors (NMDARs) plays a detrimental influence on AD progression [5,6]. Specifically, extra-synaptic glutamatergic NMDAR over-stimulation has been reported to profoundly affect stress-related signaling pathways, oxidative stress, excitotoxicity and cell death [7]. Coherently with this knowledge, the uncompetitive NMDAR antagonist memantine is routinely used for treatment as potential disease-modifying therapies [1,2,8]. Recently D-serine (D-Ser), an atypical endogenous D-amino acid that binds with high affinity the co-agonist site

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of NMDAR, has been postulated as a novel molecular biomarker in AD [9]. In particular, a five-fold increase in CSF D-Ser levels has been shown in patients with probable AD, as compared with controls [9]. Nevertheless, other studies performed in the CSF and serum of AD patients, as well as in *post-mortem* AD brains, failed to confirm these striking alterations [10–12]. Besides D-Ser, also the D-amino acid D-aspartate (D-Asp) influences NMDAR-mediated transmission in the mammalian brain [13,14]. To this regard, preclinical studies in mice have shown that long-lasting exposure to non-physiological high D-Asp levels dramatically accelerates brain aging processes, including cognitive decay, precocious neuroinflammation and cell death in the hippocampus and cortex [15–17]. In line with these findings, it has been very recently shown that abnormally high concentrations of D-Asp in cortical primary neurons profoundly affect JNK signaling and tau phosphorylation, also eliciting caspase 3-dependent cell death through NMDAR-dependent mechanisms [18]. Here, in order to clarify the role of D-Ser as a potential new biomarker in AD, we measured its concentrations and D-Ser/total Ser ratio in CSF and blood serum in a cohort of anti-AD drug-free subjects encompassing the whole clinical spectrum of the AD [preclinical stage (pre-AD), mild cognitive impairment (MCI-AD) and overt dementia (AD-Dem)] and in a group of sex-matched cognitively healthy neurological controls (other neurological diseases, OND). Moreover, we also analyzed D-Asp and L- Asp content in the same CSF and blood serum samples collection. Finally, we measured D-Ser and D-Ser/total Ser ratio in *post-mortem* cortex of AD patients. Taken together, our HPLC results obtained in a cohort of drug-free subjects encompassing the whole clinical spectrum of Alzheimer's disease, failed to confirm the clinical value of CSF D-serine as a novel biomarker for this devastating neurodegenerative disease.

2. Materials and methods

2.1. Serum and cerebrospinal fluid collection

We enrolled a total number of 107 patients referring to the Center for Memory Disturbances of the University of Perugia for diagnostic work-up for the study between 2010 and 2019. All patients underwent a clinical neurological examination, neuropsychological assessment including Mini-Mental State Examination (MMSE) and Clinical Dementia Rating (CDR) scale, lumbar puncture, blood chemistry, brain CT and/or MRI scan for excluding other neurological conditions causing dementia. None of the patients was taking any cerebro-active drugs, such as acetylcholinesterase inhibitors or NMDAR agonists, at the time of diagnostic work-up. All the patients underwent a clinical follow-up of at least 1 year. According to clinical/neuropsychological characteristics and CSF biomarker profile (presence of amyloidosis (A+) and tauopathy (T+) was defining the AD-like profile), patients were subdivided into three groups: 18 pre-clinical AD, 39 MCI-AD, 50 AD-Dem) [4]. As neurological controls (other neurological diseases, OND), a total number of 59 subjects who underwent lumbar puncture for diagnostic reasons were enrolled. This group included patients with psychiatric disorders (n = 8), headache (n = 4), seizure (n = 7), transient metabolic encephalopathy (n = 5), transient global amnesia (n = 2), mononeuropathy (n = 1), or non-AD cognitive deficits (n = 32). Demographical information is reported in Table 1. Subjects did not showed difference in terms of gender distribution among groups (p = 0.674, chi-square test; Table 1), while age of MCI-AD and AD-Dem patients were significantly higher than those of OND subjects (Kruskal-Wallis, p = 0.0002; Dunn's test: OND: 66.6 ± 1.1 years *vs* MCI-AD: 72.7 ± 1.1 years, p = 0.0003; OND: 66.6 ± 1.1 years *vs* AD-Dem: 72.4 ± 1.1 years; p = 0.0002; Table 1).

CSF samples were collected according to international guidelines [19–21]. All patients and/or their legal representatives gave informed written consent. Lumbar punctures were performed from 8:00 to 10:00, after an overnight fasting. CSF (~ 12 ml) was immediately collected in sterile polypropylene tubes (Sarstedt® tubes, codes: 62.610.210) and gently mixed to avoid possible gradient effects. All samples were centrifuged at 2000 x*g* for 10 min., at room temperature and then aliquoted in 0.5 ml aliquots in sterile polypropylene tubes (Sarstedt® tubes, codes: 72.730.007). Aliquots were frozen at −80 °C pending analysis, avoiding freeze/thaw cycles. Blood-contaminated samples were excluded from the analysis (cutoff of 50 red blood cells per microliter). CSF Aβ42, Aβ40 were measured with commercially available enzyme-linked immunosorbent assays (ELISAs) purchased by Euroimmun (EUROIMMUN AG, Lübeck, Germany), while total Tau and phospho-Tau were detected using INNOTEST kits (Fujirebio Europe, Gent, Belgium). Internal quality controls were assayed in each run. Operators blinded to the diagnosis performed the measurements.

Whole blood was collected by peripheral venipuncture into clot activator tubes (Kima, code 11020) and gently mixed. Sample was stored upright for 30 min at room temperature to allow blood to clot, and centrifuged at 2000 ×*g* for 10 min at room temperature. Serum was aliquoted (0.5 ml) in polypropylene cryotubes and stored at −80 °C.

2.2. Human tissue samples collection

Human brain samples were obtained from The Netherlands Brain Bank (Netherlands Institute for Neuroscience, Amsterdam, open access: www.brainbank.nl). All material has been collected from donors for or from whom a written informed consent for a brain autopsy and the use of the material and clinical information for research purposes had been obtained by The Netherlands Brain Bank. We selected cases with a clinical diagnosis of AD (n = 10) and neuropathological staging of Braak ≥ 5. Controls (n = 10) were adults without cognitive decline and Braak ≤3 in accordance with the Braak and Braak criteria [22]. Groups did not differ significantly for both age (Ctrl *vs* AD patients, mean ± SEM of years: 84.5 ± 1.4 *vs* 85.0 ± 0.9, p = 0.97, Mann Whitney test) and *post-mortem* delay (Ctrl *vs* AD patients, mean ± SEM of hours: 6.0 ± 0.2 *vs* 5.7 ± 0.4, p = 0.34, Mann Whitney test). Details are reported in Table 4. The control subjects had no known clinical history of neurological or psychiatric disorders and were also fully neuropathologically evaluated to confirm that they were free of neurodegenerative pathologies. All patients had a clinical diagnosis of dementia or probable AD, according to National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria [23]. Tissue blocks were dissected from superior frontal gyrus of each individual (Table 4), frozen rapidly, stored at 80 °C, and handled on dry ice to avoid thawing and RNA degradation.

2.3. HPLC detection of D-/L-aspartate and D-/L-serine levels

CSF, serum and *post-mortem* brain samples were analyzed as previously reported [18,24–27]. *Post-mortem* brain samples were homogenized in 1:10 (*w/v*) 0.2 M TCA, sonicated (3 cycles, 10 s each) and centrifuged at 13,000 ×*g* for 20 min. All the precipitated protein pellets from brain samples were stored at −80 °C for protein quantification. 100 μl CSF or serum samples were mixed in a 1:10 dilution with HPLC-grade methanol (900 μl) and centrifuged at 13,000 ×*g* for 10 min. All TCA supernatants were dried, suspended in 0.2 M TCA and then neutralized with NaOH. Samples were then subjected to pre-column derivatization with o-phthaldialdehyde (OPA)/*N*-acetyl-L-cysteine in 50% methanol. Diastereoisomer derivatives were resolved on a Simmetry C8 5-μm reversed-phase column (Waters, 4.6 × 250 mm) in isocratic conditions (0.1 M sodium acetate buffer, pH 6.2, 1% tetrahydrofuran, 1 ml/min flow rate). A washing step in 0.1 M sodium acetate buffer, 3% tetrahydrofuran and 47% acetonitrile, was performed after every single run. Identification and quantification of D-Ser, L-Ser, D-Asp and L-Asp were based on retention times and peak areas and compared with those associated with external standards. In serum and CSF samples the identity of the D-Asp peak was also evaluated by selective degradation

Table 1
Demographic characteristics of subjects (total $n = 166$) enrolled in serum and cerebrospinal fluid collection.

	OND (n = 59)	*pre-AD (n = 18)*	*MCI-AD (n = 39)*	*AD-Dem (n = 50)*	*p value*
Gender (male/female)	27/32	7/11	13/26	20/30	0.6740[a]
Age (years)	66.6 ± 1.1	69.2 ± 1.3	72.7 ± 1.1	72.4 ± 1.1	0.0002[b]
CDR	–	0	0.5	1,3 ± 0.1	< 0.0001[b]
MMSE	27.3 ± 0.4 (*n* = 48)	27.3 ± 0.2	23.7 ± 0.5	16.0 ± 0.8 (*n* = 40)	< 0.0001[b]
CSF Aβ40 (pg/ml)	9330.9 ± 564.8	11,525.6 ± 646.7	12,955.7 ± 737.7	10,788.9 ± 530.5	0,0020[b]
CSF Aβ42 (pg/ml)	1076.9 ± 48.8	599.2 ± 35.2	593.6 ± 39.8	515.9 ± 31.3	< 0.0001[b]
CSF Aβ42/Aβ40 ratio (%)	12.4 ± 0.7 (*n* = 38)	5.3 ± 0,2	4.7 ± 0.2	4.8 ± 0.1	< 0.0001[b]
CSF phospho-Tau (pg/ml)	45.1 ± 1.6	70.7 ± 7.2	101.3 ± 6.0	97.4 ± 5.2	< 0.0001[b]
CSF total Tau (pg/ml)	280.7 ± 17.2	452.9 ± 52.4	698.0 ± 36.7	842.0 ± 51.2	< 0.0001[b]

Values are expressed as means ± SEM. For gender number of male and female subjects is indicated. Statistical analyses were performed by [a]Chi-square test or [b]Kruskal-Wallis test. Abbreviations: OND = Other Neurological Disease; Pre-AD = pre-clinical Alzheimer's disease; AD-MCI = Alzheimer's disease-Mild Cognitive Impairment; AD-Dem = Alzheimer's disease dementia; CDR = Clinical Dementia Rating; MMSE = Mini-Mental State Examination; CSF = Cerebrospinal fluid. Note that some MMSE scores were not available.

catalyzed by a recombinant human DDO (hDDO) [28,29]. hDDO enzyme (12.5 μg) was added to the samples, incubated at 30 °C for 3 h, and subsequently derivatized. Amino acid levels in serum and CSF samples were expressed as μM concentration. For tissue samples, total protein content of homogenates was determined by Bradford assay method, after resolubilization of the TCA precipitated protein pellets. The detected amino acids concentration was then normalized by the total protein content and expressed as nmol/mg protein. All D-amino acid/total amino acid ratio was expressed as percentage (%).

2.4. Statistical analysis

Because not all groups showed Gaussian distribution, statistical analysis was performed using Kruskal-Wallis test with Dunn's *post hoc* test for multiple comparisons, when required. p values less than 0.05 were considered statistically significant. Correlation analysis was calculated using Spearman's correlations. No outliers were removed from database for statistical analysis.

3. Results

3.1. Serum D-serine levels across the whole clinical spectrum of Alzheimer's disease

First, we assessed the blood serum concentrations of D-Ser and L-Ser in a cohort of patients showing an AD profile (A + T+) and encompassing the whole clinical spectrum of AD (preclinical, mild cognitive impairment and dementia; see Table 1 for clinical and demographic characteristics). Kruskal-Wallis analysis indicated that mean levels of D-Ser were comparable among the diagnostic groups (mean values ± SEM: OND: 4.80 ± 0.28 μM, pre-AD: 4.75 ± 0.30 μM, MCI-AD: 4.63 ± 0.22 μM, AD-Dem: 5.23 ± 0.32 μM; $p = 0.484$, Kruskal-Wallis test; Fig. 1a). Then, we assessed the levels of L-Ser, which were also unchanged among groups (mean values ± SEM: OND: 211.21 ± 8.73 μM, pre-AD: 224.97 ± 10.27 μM, MCI-AD: 209.61 ± 8.07 μM, AD-Dem: 226.85 ± 9.61 μM; $p = 0.291$, Kruskal-Wallis test; Fig. 1b). Also, D-Ser/total Ser (D + L) ratio was not significantly different among these groups (mean values ± SEM: OND: 2.26 ± 0.21%, pre-AD: 2.07 ± 0.09%, MCI-AD: 2.17 ± 0.07%, AD-Dem: 2.35 ± 0.17%; $p = 0.885$, Kruskal-Wallis test; Fig. 1c). Mean concentrations of the metabolites analyzed above are reported in Table 2. We then evaluated whether serum levels of D-Ser correlated with the severity of cognitive impairment, expressed as mini-mental state exam (MMSE) score. In this respect, no correlation between serum D-Ser levels and MMSE was observed in the whole cohort of patients ($r = -0.071$, $p = 0.396$, Spearman's correlation; Fig. 1d; Table 3). Similarly, we did not find any significant correlation between MMSE and L-Ser levels ($r = -0.072$, $p = 0.389$; Fig. 1e; Table 3) or D-Ser/total Ser ratio ($r = 0.089$, $p = 0.287$, Spearman's correlation; Fig. 1f; Table 3). Next, we assessed whether D-Ser levels were correlated with Clinical Dementia Rating (CDR) scale. To this aim, we separated subjects into groups stratified by CDR scores (0, 0.5, 1, 2). We found comparable D-Ser concentrations in all CDR groups analyzed (mean values ± SEM: CDR 0: 4.75 ± 0.30 μM, CDR 0.5: 4.63 ± 0.22 μM, CDR 1: 5.17 ± 0.40 μM; CDR 2: 5.41 ± 0.49 μM; $p = 0.422$; Kruskal-Wallis test; Fig. 1g) Likewise, L-Ser amount and D-Ser/total Ser ratio were unchanged among CDR groups (mean values ± SEM: L-Ser: CDR 0: 224.97 ± 10.27 μM, CDR 0.5: 209.61 ± 8.07 μM, CDR 1: 217.99 ± 9.61 μM; CDR 2: 252.07 ± 27.40 μM $p = 0.174$; D-Ser/total Ser: CDR 0: 2.07 ± 0.09%, CDR 0.5: 2.17 ± 0.07%, CDR 1: 2.41 ± 0.22%; CDR 1: 2.20 ± 0.20%; $p = 0.859$; Kruskal-Wallis test; Fig. 1h,i). In the same way, serum D-Ser and L-Ser levels and D-Ser/total ratio were not significantly correlated with CSF diagnostic AD biomarkers such as Aβ42/β40 ratio (D-Ser: $r = -0.050$, $p = 0.548$; L-Ser: $r = -0.078$, $p = 0.347$; D-Ser/total Ser: $r = 0.063$, $p = 0.448$; Spearman's correlation; Table 3) or phosphorylated and total Tau levels (phospho-Tau: D-Ser, $r = 0.007$, $p = 0.925$; L-Ser: $r = -0.022$, $p = 0.773$; D-Ser/total Ser: $r = -0.023$, $p = 0.709$; Total Tau: D-Ser, $r = 0.048$, $p = 0.543$; L-Ser: $r = -0.006$; $p = 0.935$; D-Ser/total Ser: $r = 0.001$; $p = 0.988$; Spearman's correlation; Table 3). Taken together these data failed to evidence any consistent changes in serum D-Ser, L-Ser levels and D-Ser/total Ser ratio among diagnosis groups.

3.2. Cerebrospinal fluid levels of D-serine in patients across the whole clinical spectrum of Alzheimer's disease

We measured CSF D-Ser and L-Ser levels in the same cohort of AD patients and controls. Statistical analysis revealed unaltered mean CSF concentrations of D-Ser among diagnosis groups (mean values ± SEM: OND: 4.79 ± 0.15 μM, pre-AD: 4.71 ± 0.22 μM, MCI-AD: 4.79 ± 0.17 μM, AD-Dem: 4.63 ± 0.20 μM; $p = 0.937$; Kruskal-Wallis test; Fig. 2a). Analogously, L-Ser levels and D-Ser/total Ser ratio did not differ among groups (mean values ± SEM: L-Ser, OND: 58.90 ± 2.08 μM, pre-AD: 60.52 ± 3.24 μM, MCI-AD: 57.11 ± 2.04 μM, AD-Dem: 56.98 ± 2.33 μM; $p = 0.772$; D-Ser/total Ser, OND: 7.75 ± 0.23%, pre-AD: 7.28 ± 0.17%, MCI-AD: 7.88 ± 0.24%, AD-Dem: 7.56 ± 0.17%; $p = 0.267$; Kruskal-Wallis test; Fig. 2b,c). Mean concentrations of the metabolites analyzed above are also reported in Table 2. We then evaluated possible correlations between CSF D-Ser or L-Ser concentrations and MMSE. Differently from what previously claimed [9], no association was observed between D-Ser and MMSE scores (D-Ser: $r = -0.112$, $p = 0.181$; Spearman's correlation; Fig. 2d; Table 3). Likewise, L-Ser and D-Ser/total Ser ratio showed no correlation with MMSE values (L-Ser: $r = -0.008$, $p = 0.918$; D-Ser/total Ser: $r = -0.129$, $p = 0.124$; Spearman's correlation; Fig. 2e,f; Table 3). Furthermore, unlike what is reported by Madeira and coworkers [9], we failed to find any significant change in CSF D-Ser concentrations among the different CDR groups analyzed

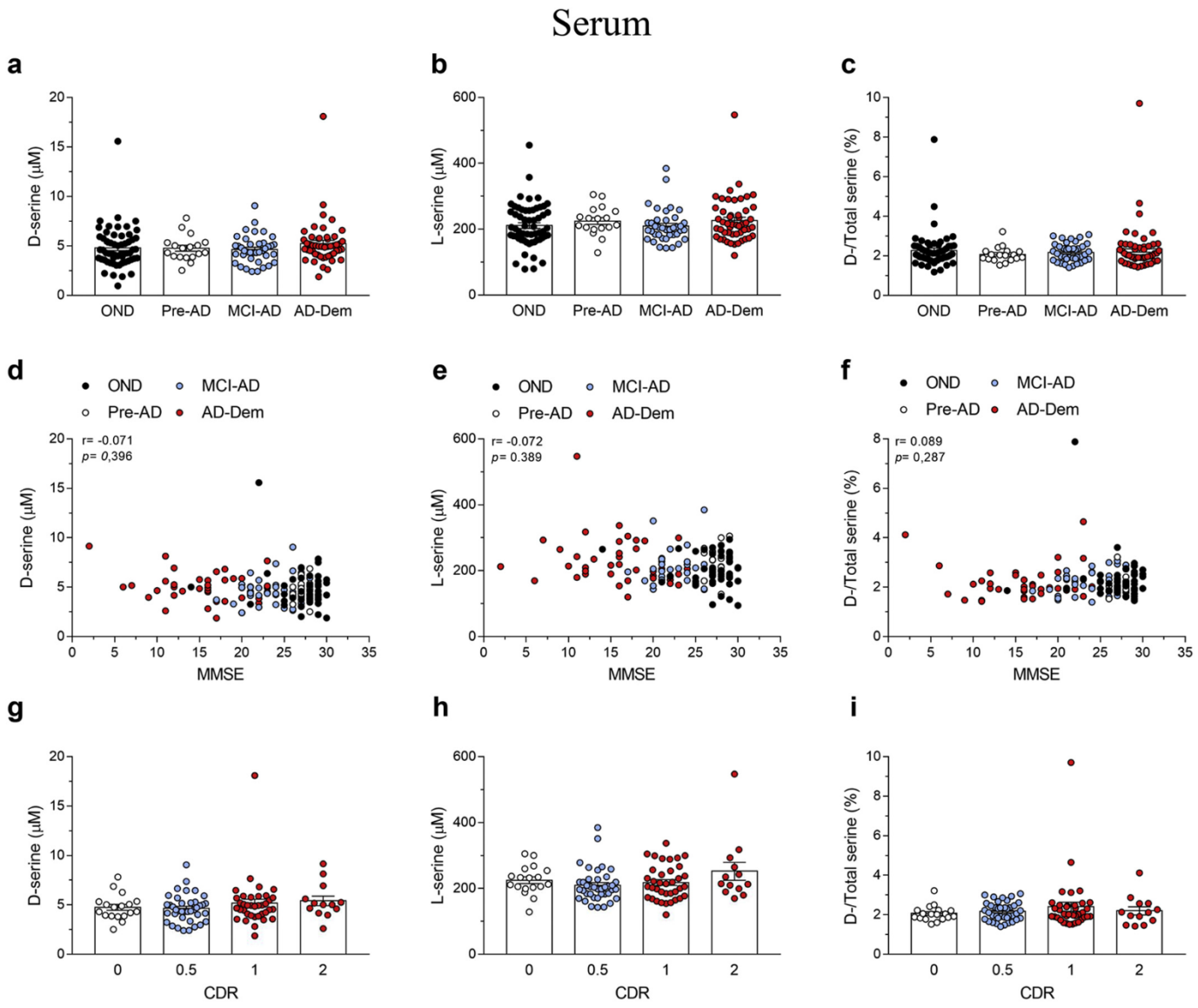

Fig. 1. Analysis of free D-serine, L-serine levels and D-serine/total serine ratio in the serum of patients encompassing the whole clinical spectrum of Alzheimer's disease. (a - c) Detection of (a) D-Ser, (b) L-Ser concentrations, and (c) D-Ser/total Ser ratio in the serum of pre-clinical AD (pre-AD, $n = 18$), Mild Cognitive Impairment-Alzheimer's disease (MCI-AD, $n = 39$) and Alzheimer's disease-Dementia (AD-Dem, $n = 50$) subjects compared to non-demented controls (other neurological disorders, OND, $n = 58$) by HPLC analysis. In each sample, all free amino acids were detected in a single run. Means were compared using Kruskal-Wallis because parameters did not show Gaussian distribution. (d-f) Correlation analysis between serum (d) D-Ser, (e) L-Ser concentrations or (f) D-Ser/total Ser ratio, and MMSE score of the entire cohort of patients. Note that some MMSE scores were not available (see Table 1). Correlation analysis was calculated using Spearman's correlations. (g-i) Analysis of (g) D-Ser, (h) L-Ser levels, and (i) D-Ser/total Ser ratio in the serum of subjects stratified within 0, 0.5, 1 and 2 CDR groups. Means were compared using Kruskal-Wallis because parameters did not show Gaussian distribution. All values are expressed as the mean ± SEM of μM concentration, while the ratio is expressed as percentage (%).

(CDR 0: 4.71 ± 0.22 μM, CDR 0.5: 4.79 ± 0.17 μM, CDR 1: 4.46 ± 0.21 μM; CDR 2: 5.13 ± 0.47 μM; $p = 0.361$; Kruskal-Wallis test; Fig. 2g). Similarly, L-Ser content and D-Ser/total Ser ratio were unchanged in all CDR groups (L-Ser, CDR 0: 60.52 ± 3.24 μM, CDR 0.5: 57.11 ± 2.04 μM, CDR 1: 54.40 ± 2.24 μM; CDR 2: 64.95 ± 6.19 μM; $p = 0.068$; D-Ser/total Ser, CDR 0: 7.28 ± 0.17%, CDR 0.5: 7.88 ± 0.24%, CDR 1: 7.57 ± 0.18%; CDR 2: 7.54 ± 0.39%; $p = 0.195$; Kruskal-Wallis test; Fig. 2h,i). In addition, we found a significant positive correlation between Aβ42/β40 ratio and CSF D-Ser or L-Ser concentrations (D-Ser: $r = 0.178$, $p = 0.033$; L-Ser: $r = 0.171$, $p = 0.040$; Spearman's correlation; Table 3), while no correlation was observed between CSF Aβ42/β40 and D-Ser/total Ser ratio ($r = 0.073$, $p = 0.386$; Spearman's correlation; Table 3). Finally, D-Ser, L-Ser concentrations and D-Ser/total Ser ratio were not significantly correlated with the CSF diagnostic AD biomarkers phospho-Tau and total Tau in the entire cohort of patients (phospho-Tau: D-Ser, $r = -0.051$, $p = 0.515$; L-Ser: $r = -0.107$, $p = 0.173$; D-Ser/total Ser: $r = 0.075$, $p = 0.340$; Total Tau: D-Ser, $r = -0.041$, $p = 0.603$; L-Ser: $r = -0.130$; $p = 0.096$; D-Ser/total Ser: $r = 0.101$; $p = 0.197$; Spearman's correlation; Table 3). Overall, the present observations failed to show any significant alteration in CSF D-Ser and L-Ser level among groups.

3.3. D-serine levels in the post-mortem brain of Alzheimer's disease patients

Different reports described controversial changes in D-Ser levels and D-Ser/total Ser ratio in *post-mortem* AD brains [9,11,30–32]. Here, we measured D-Ser in the cortical brain region of the superior frontal gyrus

Table 2
The mean values of D-serine, D-aspartate, L-serine, L-aspartate (expressed as μM), D-serine/total serine and D-aspartate/total aspartate ratio (expressed as %) in the serum or cerebrospinal fluid are compared among the different clinical conditions.

	Serum				
	OND (n = 58)	*pre-AD (n = 18)*	*MCI-AD (n = 39)*	*AD-Dem (n = 50)*	*p* value
D-Ser (μM)	4.80 ± 0.28	4.75 ± 0.30	4.63 ± 0.22	5.23 ± 0.32	0.484
L-Ser (μM)	211.21 ± 8.73	224.97 ± 10.27	209.61 ± 8.07	226.85 ± 9.61	0.291
D-Ser/total Ser (%)	2.26 ± 0.21	2.07 ± 0.09	2.17 ± 0.07	2.35 ± 0.17	0.885
D-Asp (μM)	0.48 ± 0.04	0.41 ± 0.06	0.53 ± 0.07	0.57 ± 0.05	0.105
L-Asp (μM)	23.44 ± 1.78	26.05 ± 2.85	27.76 ± 1.73	26.37 ± 2.84	0.162
D-Asp/total Asp (%)	2.77 ± 0.33	1.78 ± 0.29	2.09 ± 0.26	2.79 ± 0.27	0.091
	Cerebrospinal fluid				
	OND (n = 59)	*pre-AD (n = 18)*	*MCI-AD (n = 39)*	*AD-Dem (n = 49)*	*p* value
D-Ser (μM)	4.79 ± 0.15	4.71 ± 0.22	4.79 ± 0.17	4.63 ± 0.20	0.937
L-Ser (μM)	58.90 ± 2.08	60.52 ± 3.24	57.11 ± 2.04	56.98 ± 2.33	0.772
D-Ser/total Ser (%)	7.75 ± 0.23	7.28 ± 0.17	7.88 ± 0.24	7.56 ± 0.17	0.267
D-Asp (μM)	–	–	–	–	–
L-Asp (μM)	1.47 ± 0.17	1.41 ± 0.14	1.22 ± 0.09	1.28 ± 0.09	0.449
D-Asp/total Asp (%)	–	–	–	–	–

All values are expressed as mean ± SEM. Statistical analyses were performed by Kruscal-Wallis test. Abbreviations: D-Ser = D-serine; L-Ser = L-serine; D-Asp = D-aspartate; L-Asp L-aspartate; OND = Other Neurological Disease; Pre-AD = pre-clinical Alzheimer's disease; AD-MCI = Alzheimer's disease-Mild Cognitive Impairment; AD-Dem = Alzheimer's disease dementia.

(SFG) of AD and non-demented brains (n = 10/clinical condition) obtained from The Netherlands Brain Bank (Netherlands Institute for Neuroscience, Amsterdam, www.brainbank.nl). Demographic and clinical characteristics are reported in Table 4. Analogously to what found in the serum and CSF, we did not observe significant differences in D-Ser content between AD patients and non-demented controls (mean values ± SEM: Ctrl *vs* AD, 5.02 ± 0.47 *vs* 5.14 ± 0.42 nmol/mg protein; p = 0.811, Mann-Whitney test, Fig. 3a). Also L-Ser levels, as well as D-Ser/total Ser ratio were found unaltered between groups (mean values ± SEM: L-Ser, Ctrl *vs* AD, 18.84 ± 1.48 *vs* 20.55 ± 1.49 nmol/mg protein; p = 0.563; D-Ser/total Ser, Ctrl *vs* AD, 20.89 ± 0.89 *vs* 19.55 ± 0.68%; p = 0.218; Fig. 3b,c). Thus, in agreement with previous observations [11,31,32], our measurements carried out in *post-mortem* brains failed to demonstrate any significant alteration in D-Ser content or D-Ser/total Ser ratio in AD brains. Interestingly, in a very recent work, by using the same cohort of *post-mortem* SFG samples, we also reported unaltered D-Asp, L-Asp concentrations between AD patients and non-demented individuals [18].

3.4. Serum and cerebrospinal fluid levels of D-aspartate in patients across the whole clinical spectrum of Alzheimer's disease

As for D-Ser and L-Ser, here we measured the content of D-Asp and its L-enantiomer, L-Asp, in the serum and CSF of patients encompassing

Table 3
Correlation analysis between D-/L-serine, D-/L-aspartate concentrations and MMSE scores, Aβ42/Aβ40 ratio, phospho-Tau and total Tau levels in the serum and cerebrospinal fluid samples of entire cohort of subjects (n = 166).

Spearman's correlations		*Serum*					
		D-Ser (μM)	*L-Ser (μM)*	*D-Ser/total Ser (%)*	*D-Asp (μM)*	*L-Asp (μM)*	*D-Asp/total Asp (%)*
MMSE	*r*	−0.071	−0.072	0.089	−0.095	0.045	−0.071
	p value	0.396	0.389	0.287	0.253	0.587	0.391
Aβ42/Aβ40 ratio	*r*	−0.050	−0.078	0.063	0.041	0.032	0.084
	p value	0.548	0.347	0.448	0.616	0.704	0.318
CSF phospho-Tau (pg/ml)	*r*	0.007	−0.022	−0.023	0.134	−0.010	0.086
	p value	0.925	0.773	0.709	0.086	0.897	0.273
CSF total Tau (pg/ml)	*r*	0.048	−0.006	0.001	0.140	0.029	0.071
	p value	0.543	0.935	0.988	0.072	0.707	0.365
Spearman's correlations		*Cerebrospinal fluid*					
		D-Ser (μM)	*L-Ser (μM)*	*D-Ser/total Ser (%)*	*D-Asp (μM)*	*L-Asp (μM)*	*D-Asp/total Asp (%)*
MMSE	*r*	−0.112	−0.008	−0.129	–	0.102	–
	p value	0.181	0.918	0.124	–	0.224	–
Aβ42/Aβ40 ratio	*r*	0.178	0.171	0.073	–	0.088	–
	p value	0.033	0.040	0.386	–	0.297	–
CSF phospho-Tau (pg/ml)	*r*	−0.051	−0.107	0.075	–	0.056	–
	p value	0.515	0.173	0.340	–	0.477	–
CSF total Tau (pg/ml)	*r*	−0.041	−0.130	0.101	–	0.041	–
	p value	0.603	0.096	0.197	–	0.600	–

Statistical analyses were performed by Spearman's correlations. Abbreviations: D-Ser = D-serine; L-Ser = L-serine; D-Asp = D-aspartate; L-Asp = L-aspartate; MMSE = Mini-Mental State Evaluation.

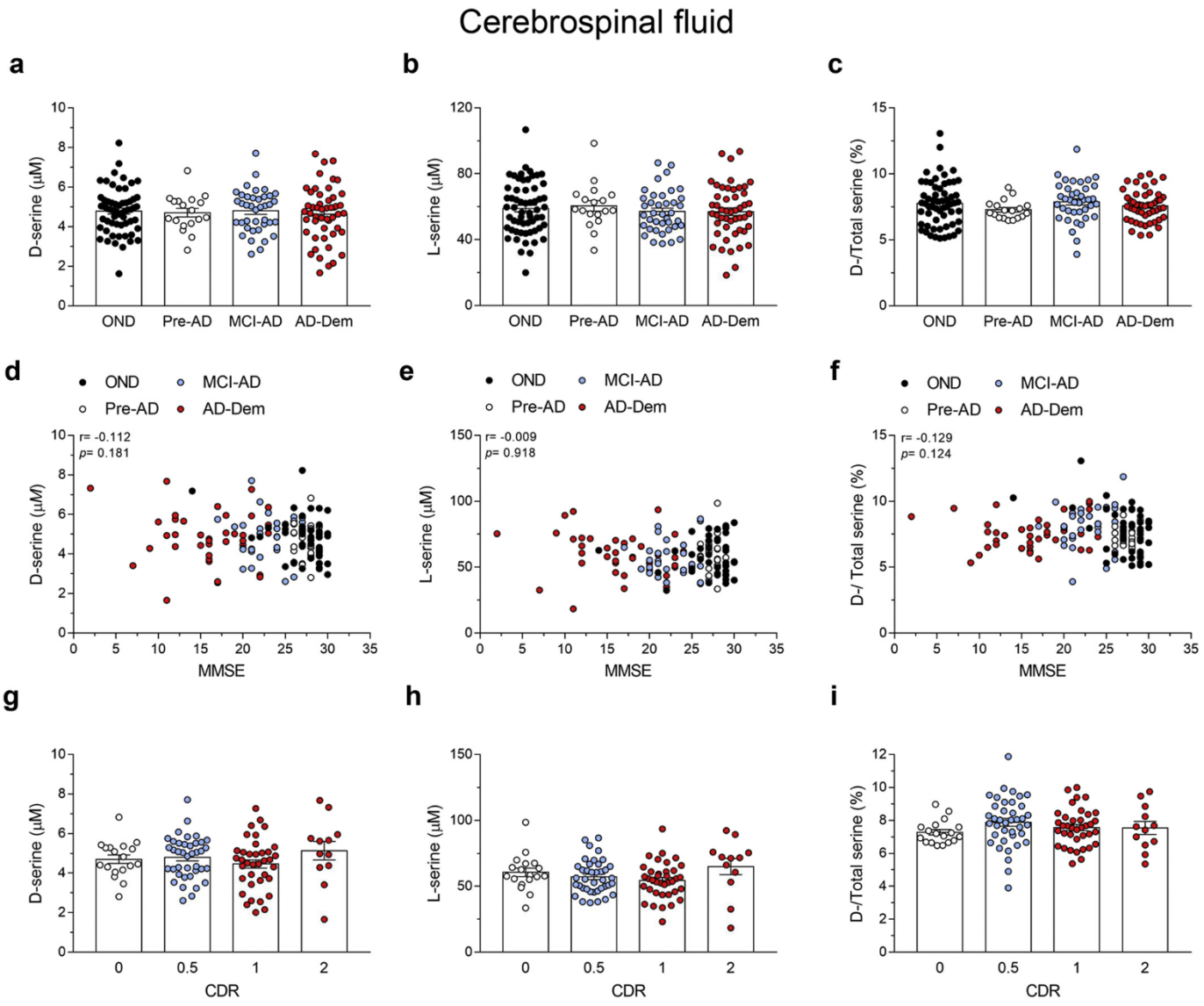

Fig. 2. Analysis of free D-serine, L-serine levels and D-serine/total serine ratio in the cerebrospinal fluid of patients encompassing the whole clinical spectrum of Alzheimer's disease. (a - c) Detection of (a) D-Ser, (b) L-Ser levels, and (c) D-Ser/total Ser ratio in the cerebrospinal fluid of pre-clinical AD (pre-AD), Mild Cognitive Impairment-Alzheimer's disease (MCI-AD) and Alzheimer's disease-Dementia (AD-Dem) subjects compared to non-demented controls by HPLC analysis. In each sample, all free amino acids were detected in a single run. D-Ser, L-Ser concentrations and D-Ser/total Ser ratio means were compared using Kruskal-Wallis because parameters did not show Gaussian distribution. (d-f) Correlation analysis between serum (d) D-Ser, (e) L-Ser concentrations or (f) D-Ser/total Ser ratio, and MMSE score of the entire cohort of patients. Note that some MMSE scores were not available (see Table 1). Correlation's analysis was calculated using Spearman's correlations. (g-i) Analysis of (g) D-Ser, (h) L-Ser levels, and (i) D-Ser/total Ser ratio in the cerebrospinal fluid of subjects stratified within 0, 0.5, 1 and 2 CDR groups. All values are expressed as the mean ± SEM of μM concentration, while the ratio is expressed as percentage (%). Means were compared using Kruskal-Wallis because parameters did not show Gaussian distribution.

Table 4
Demographic characteristics of subjects (total $n = 20$) in *post-mortem* superior frontal gyrus samples of Alzheimer's disease and non-demented controls.

	Ctrl (n = 10)	*AD (n = 10)*	*p value*
Gender (male/female)	10 / 0	10 / 0	
Age (years)	84.5 ± 1.4	85.0 ± 0.9	0.969
PMD (hours)	6.0 ± 0.2	5.7 ± 0.4	0.343
Braak stage 0-I/II-IV/V-VII	3/7/0	0/0/10	

Abbreviations: Ctrl = Non-demented control; AD = Alzheimer's disease; PMD = Post-Mortem Delay. Values are expressed as means ± SEM for Age and PMD. Statistical analyses were performed by Mann-Whitney test.

the whole clinical spectrum of AD and controls. Notably, serum D-Asp and L-Asp concentrations, and D-Asp/tot Asp ratio were not significantly different among diagnostic groups (mean values ± SEM: D-Asp, OND: 0.48 ± 0.04 μM, pre-AD: 0.41 ± 0.06 μM, MCI-AD: 0.53 ± 0.07 μM, AD-Dem: 0.57 ± 0.05 μM; $p = 0.105$; L-Asp, OND: 23.44 ± 1.78 μM, pre-AD: 26.05 ± 2.85 μM, MCI-AD: 27.76 ± 1.73 μM, AD-Dem: 26.37 ± 2.84 μM; $p = 0.162$; D-Asp/total Asp, OND: 2.77 ± 0.33%, pre-AD: 1.78 ± 0.29%, MCI-AD: 2.09 ± 0.26%, AD-Dem: 2.79 ± 0.27%; $p = 0.091$; Kruskal-Wallis test; Fig. 4a-c; Table 2). Moreover, we evaluated the possible correlation between D-Asp, L-Asp, or D-Asp/total Asp ratio with MMSE. None of these metabolites showed significant relationship with MMSE scores (D-Asp: $r = -0.095$, $p = 0.253$; L-Asp: $r = 0.045$, $p = 0.587$; D-Asp/

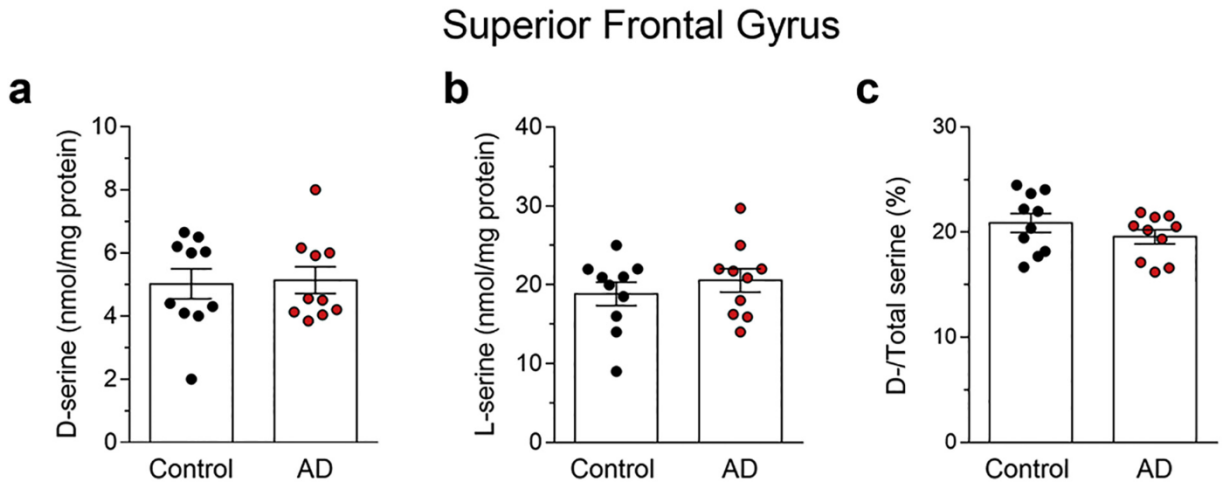

Fig. 3. Analysis of free D-serine, L-serine and D-serine/total serine ratio in the post-mortem superior frontal gyrus of Alzheimer's disease patients and non-demented controls. (a - c) HPLC measurement of (a) D-Ser, (b) L-Ser levels, and (c) D-Ser/total Ser ratio in the *post-mortem* superior frontal gyrus of Alzheimer's disease (AD, $n = 10$) subjects and non-demented controls (Ctrl, n = 10). In each tissue, all free amino acids were detected in a single run. All values are expressed as the mean ± SEM of nmol/mg protein, while the ratio is expressed as percentage (%). Data points represent values of each subject. Statistical significance is given by the Mann-Whitney *U* test.

total Asp: $r = -0.071$, $p = 0.391$; Spearman's correlation; Fig. 4d-f). We also found comparable D-Asp, L-Asp, and D-Asp/total Asp ratio among the CDR groups analyzed (mean values ± SEM: D-Asp, CDR 0: 0.41 ± 0.06 μM, CDR 0.5: 0.53 ± 0.07 μM, CDR 1: 0.57 ± 0.05 μM; CDR 2: 0.56 ± 0.10 μM; $p = 0.090$; L-Asp, CDR 0: 26.05 ± 2.85 μM, CDR 0.5: 27.76 ± 1.73 μM, CDR 1: 25.01 ± 2.56 μM; CDR 2: 30.22 ± 8.30 μM; $p = 0.342$; D-Asp/total Asp, CDR 0: 1.78 ± 0.29 μM, CDR 0.5: 2.09 ± 0.26 μM, CDR 1: 2.91 ± 0.33 μM; CDR 2: 2.41 ± 0.47 μM; $p = 0.090$; Kruskal-Wallis test; Fig. 4g-i). Finally, no significant correlation was found between D-Asp, L-Asp or D-Asp/total Asp ratio and Aβ42/β40 ratio (D-Asp: $r = 0.041$, $p = 0.616$; L-Asp: $r = 0.032$, $p = 0.704$; D-Asp/total Asp: $r = 0.084$, $p = 0.318$; Spearman's correlation, Table 3), or phospho-Tau and total Tau levels (phospho-Tau: D-Asp: $r = 0.134$, $p = 0.086$; L-Asp: $r = -0.010$, $p = 0.897$; D-Asp/total Asp: $r = 0.086$, $p = 0.273$; total Tau: D-Asp: $r = 0.140$, $p = 0.072$; L-Asp: $r = 0.029$, $p = 0.707$; D-Asp/total Asp: $r = 0.071$, $p = 0.365$; Spearman's correlation; Table 3). In line with a previous report [24], we found that D-Asp CSF content was below the detection limit of HPLC (< 0.05 μM). On the other hand, the concentration of L-Asp was unaltered among groups (mean values ± SEM: OND: 1.47 ± 0.19 μM, pre-AD: 1.41 ± 0.14 μM, MCI-AD: 1.22 ± 0.09 μM, AD-Dem: 1.28 ± 0.09 μM; $p = 0.449$; Kruskal-Wallis test; Fig. 4j; Table 2). No correlation between L-Asp CSF levels and MMSE scores ($r = 0.102$, $p = 0.224$; Spearman's correlation; Fig. 4k) or Aβ42/β40 ratio ($r = 0.088$, $p = 0.297$; Spearman's correlation; Table 3), phospho-Tau and total Tau levels (phospho-Tau: $r = 0.056$, $p = 0.477$; total Tau: D-Asp: r = 0.041, $p = 0.600$; Spearman's correlation; Table 3) was reported in the entire cohort of subjects. Finally, statistical analysis showed unaltered L-Asp levels among CDR groups (CDR 0: 1.41 ± 0.14 μM, CDR 0.5: 1.22 ± 0.09 μM, CDR 1: 1.25 ± 0.07 μM; CDR 2: 1.37 ± 0.30 μM $p = 0.449$; Kruskal-Wallis test; Fig. 4l).

4. Discussion

Knowledge on interacting pathogenic mechanisms in AD is crucial for the discovery of novel targeted therapies and prevention strategies. To this aim, the possibility to improve our diagnostic and prognostic accuracy in early pre-dementia phases through the use of novel biomarkers is crucial [33]. To this regard, consistent with dysfunctional activation of glutamatergic transmission at NMDARs in AD [6], a previous study proposed the NMDAR co-agonist, D-Ser, as a new candidate AD biomarker, even for diagnostic purposes [9]. In that work, a striking five-fold increased CSF D-Ser concentration in patients with probable AD, compared to healthy controls, was reported [9]. Due to the high translational relevance of these data, here we aimed to confirm these findings by evaluating the CSF levels of D-Ser and D-Ser/total Ser ratio in a larger and well characterized cohort of drug-free AD patients, encompassing the whole clinical spectrum of the disease (preclinical AD, MCI due to AD, and AD dementia). Differently to previous work [9], here we failed to confirm significant changes of CSF D-Ser concentrations in the three AD clinical stages. Indeed, our HPLC analysis indicated similar CSF D-Ser levels and D-Ser/total Ser ratio among the diagnostic groups. In line with this, D-Ser/total Ser ratio were correlated neither with parameters grading cognitive impairment (*i.e.*, MMSE) and staging the disease (*i.e.*, CDR), nor with β-amyloid, phospho-Tau or total Tau levels. In line with another study [10], here we were unable to replicate the Madeira's finding showing a significant negative correlation between CSF D-Ser and MMSE score [9]. Of interest, a characterizing feature of our cohort is that we have used the definition of AD according to the A+/T+ CSF profile, independently from the clinical stage, including preclinical AD [4]. To the best of our knowledge, this is the first study in which D-Ser, L-Ser, D-Asp and L-Asp have been concomitantly measured in the CSF and blood serum of fully characterized drug-free AD patients, according to CSF biomarkers.

Notably, in the current study, D-Ser concentrations detected in the CSF of non-demented control group fall within the range of control values obtained by Madeira and coworkers [mean values: 4.79 μM *vs* 2.45 μM [9]]. This similarity rules out major differences in the sensitivity of the analytical methodologies used in the two studies, both based on HPLC with fluorometric detection. Therefore, other factors might contribute to the large difference in CSF D-Ser concentrations between our and Madeira's cohorts of overt-AD patients tested [mean values: 4.63 μM *vs* 12.32 μM [9]]. A distinction between the two investigations could be related to anti-AD treatments used. Indeed, the vast majority of AD patients employed in Madeira's report were under treatment with psychoactive drugs while the whole AD cohort of our study was drug-free. However, Madeira and coworkers did not find a significant impact of these medications on CSF D-Ser levels. Therefore, other factors that could contribute to the discrepancies between the two studies might be also related to the different genetic background and/or a different number of AD patients analyzed [$n = 50$ *vs* $n = 21$ [9]]. Remarkably, our results are overall in agreement with those reported in another cohort of AD patients by Biemans et al., which showed only a

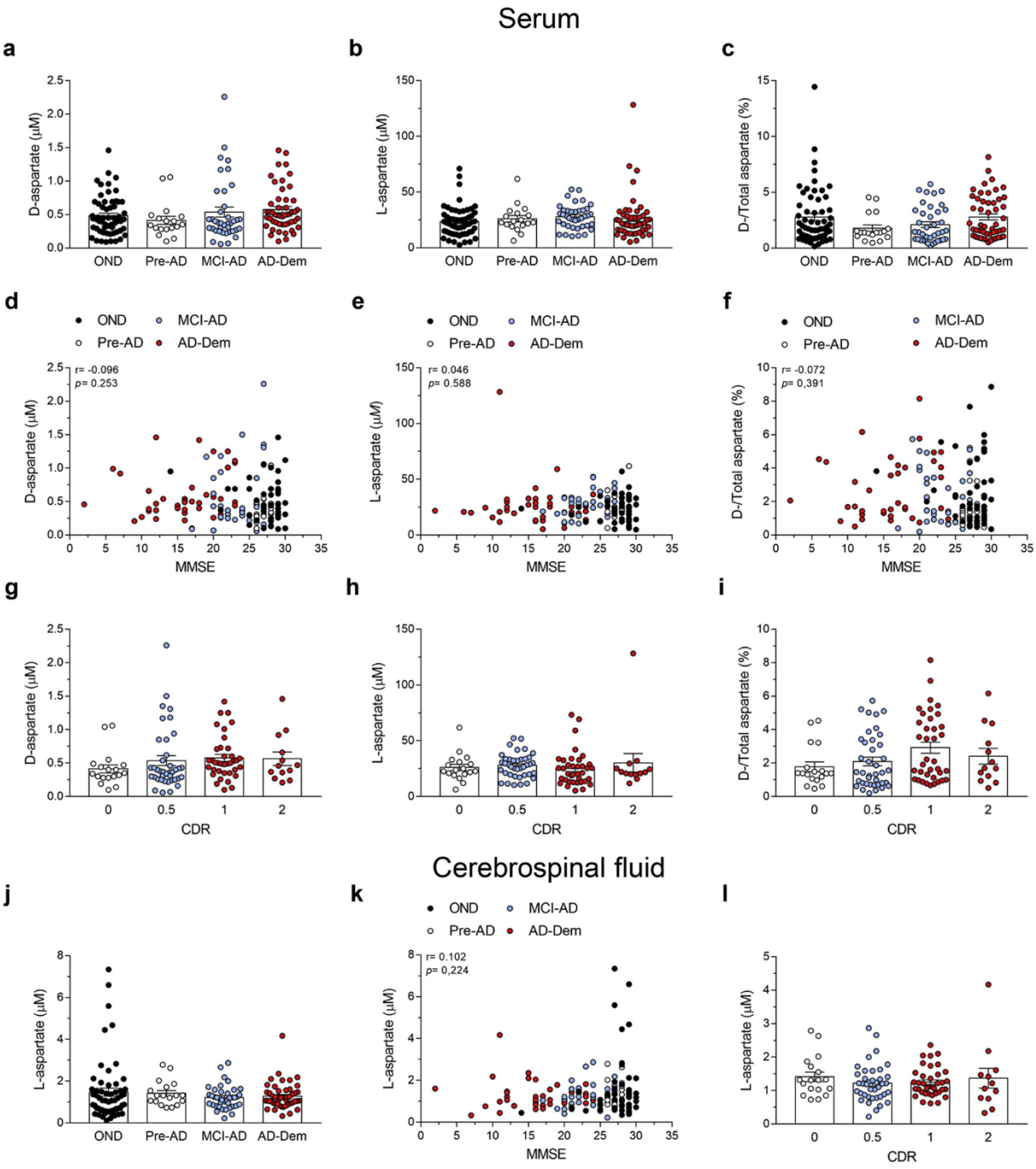

Fig. 4. Analysis of free D-aspartate, L-aspartate levels and D-aspartate/total aspartate ratio in the serum and cerebrospinal fluid of patients encompassing the whole clinical spectrum of Alzheimer's disease. (a - c) HPLC detection of (a) D-Asp, (b) L-Asp levels and (c) D-Asp/total Asp ratio in the serum of pre-clinical AD (pre-AD, $n = 18$), Mild Cognitive Impairment-Alzheimer's disease (MCI-AD, $n = 39$) and Alzheimer's disease-Dementia (AD-Dem, $n = 50$) subjects compared to non-demented controls (OND, $n = 58$). In each sample, all free amino acids were detected in a single run. (d-f) Correlation analysis between serum (d) D-Asp, (e) L-Asp concentrations and (f) D-Asp/total Asp ratio, and MMSE score of the entire cohort of patients. Note that some MMSE scores were not available (see Table 1). Correlation's analysis was calculated using Spearman's correlations. (g-i) Analysis of (g) D-Asp, (h) L-Asp levels and (i) D-Asp/total Asp ratio in the serum of subjects stratified within 0, 0.5, 1 and 2 CDR groups. (j) Content of L-Asp in the cerebrospinal fluid of pre-clinical AD (pre-AD, $n = 18$), Mild Cognitive Impairment-Alzheimer's disease (MCI-AD, $n = 39$) and Alzheimer's disease-Dementia (AD-Dem, $n = 49$) subjects compared to non-demented controls (OND, $n = 59$). In each sample, all free amino acids were detected in a single run. (k) Correlation analysis between L-Asp cerebrospinal fluid content and MMSE score of the entire cohort of patients. Note that some MMSE scores were not available (see Table 1). Correlation's analysis was calculated using Spearman's correlations. (l) Analysis of L-Asp levels in the cerebrospinal fluid of subjects stratified within 0, 0.5, 1 and 2 CDR groups. All values are expressed as the mean ± SEM of μM concentration, while the ratio is expressed as percentage (%). Means were compared using Kruskal-Wallis because parameters did not show Gaussian distribution.

very slight increase in the CSF D-Ser concentrations (13%) coupled to a non-significant change in D-Ser/total Ser ratio in AD group, compared to non-demented controls [10]. In addition, consistent with our observations, also Biemans and coworkers did not replicate the significant negative correlation between CSF D-Ser and MMSE, previously reported by Madeira and co-workers [9]. Coherently with unaltered CSF D-Ser data, we also documented overlapping blood serum D-Ser concentration and D-Ser/total Ser ratio among AD clinical stages. Likewise, serum

D-Ser levels and D-Ser/total Ser ratio were also not correlated with MMSE, CDR, β-amyloid, phospho-Tau and total Tau levels. In addition, our results are in line with those obtained by other groups showing non-significant D-Ser/L-Ser or D-Ser/total Ser ratio in patients with MCI, mild to severe AD or dementia, compared to age-matched controls [12,34]. Interestingly, in the study by Lane and coworkers, a significant increase in D-Amino Acid Oxidase (DAAO) levels, the enzyme responsible for D-Ser degradation [35], was found in MCI and AD patients, compared with elderly controls [34]. In addition, pLG72, named as DAAO activator, which has been regarded as an important modulator of DAAO activity, may also play a role in the progression of AD [36]. Consistent with these evidences and in agreement with an influence of NMDAR signaling in aging processes, the same group had previously reported in a randomized, double-blind, placebo-controlled trial, a beneficial clinical effect of the DAAO inhibitor, sodium benzoate, on the cognitive decline in patients with MCI or mild AD enrolled [37]. Very recent studies also suggest that sodium benzoate may have potential to affect cognitive function in a fraction of behavioral and psychological symptoms in dementia (BPSD) patients [38,39]. Taken together, these results suggest that the study on D-Ser metabolism in the clinical spectrum of AD deserves further evaluation, not only for its potential pathophysiological significance but also for its putative involvement in AD treatment. Differently to D-Ser, investigations on D-Asp levels in AD blood serum are still lacking. Here, we showed that serum D-Asp and L-Asp levels were unaltered among AD clinical stages and these amino acids did not show any association with MMSE, CDR, β-amyloid, phospho-Tau or total Tau levels. Therefore, these results indicate no gross alteration in the peripheral metabolism of D-Asp and L-Asp in AD. On the other hand, consistent with the well-established age-dependent decrease of D-Asp content in the brain [17,40,41], we could not detect this D-amino acid in the CSF of the AD patients and elderly controls analyzed. This observation is also in line with another work in which CSF D-Asp concentration was undetectable in both Parkinson's disease patients and elderly control subjects [24]. In addition to D-serine and D-aspartate, other D-amino acids such as D-proline, D-alanine and D-glutamate have been recently investigated in Alzheimer's disease, further suggesting a potential involvement of these atypical molecules in neurodegenerative diseases [12,42,43].

5. Conclusions

In conclusion, our observations showed indistinguishable CSF and serum D-Ser levels and D-Ser/total Ser ratio in a cohort of drug-free patients encompassing the whole clinical spectrum of AD, compared to non-demented controls. In addition, no significant correlation was found between CSF D-Ser and MMSE, CDR, β-amyloid, phospho-Tau or total Tau levels. Taken together, the current data suggest that D-Ser does not represent a suitable biomarker for AD. However, further studies are required to clarify the relation between D-Ser levels and the cognitive manifestations of AD and to understand the potential diagnostic, therapeutic and prognostic value of this D-amino acid.

Declaration of Competing Interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbapap.2020.140537.

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