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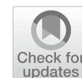

High performance liquid chromatography determination of L-glutamate, L-glutamine and glycine content in brain, cerebrospinal fluid and blood serum of patients affected by Alzheimer's disease

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Abstract

Altered glutamatergic neurotransmission is thought to play a crucial role in the progression of Alzheimer's disease (AD). Accordingly, the identification of peculiar biochemical patterns reflecting AD-related synaptopathy in blood and cerebrospinal fluid (CSF) could have relevant diagnostic and prognostic implications. In this study, we measured by High-Performance Liquid Chromatography the amount of glutamate, glutamine and glycine in post-mortem brain samples of AD patients, as well as in CSF and blood serum of drug-free subjects encompassing the whole AD clinical spectrum (pre-clinical AD, $n=18$, mild cognitive impairment-AD, $n=29$, dementia AD, $n=30$). Interestingly, we found that glutamate and glycine levels, as well as total tau protein content, were significantly reduced in the superior frontal gyrus of patients with AD, compared with non-demented controls. No significant change was also found in glutamate, glutamine and glycine CSF concentrations between AD patients and neurological controls. Remarkably, serum glutamate levels were significantly higher in patients affected by early AD phases compared to controls, and were negatively correlated with CSF total tau levels. Conversely, serum glutamine concentration was significantly increased in AD patients, with a negative correlation with MMSE performances. Finally, we reported a significant correlation between serum L-glutamate concentrations and CDR score in female but not in male cohort of AD subjects. Overall, our results suggest that serum glutamate and glutamine levels in AD patients could vary across disease stages, potentially reflecting the progressive alteration of glutamatergic signaling during neurodegenerative processes.

Keywords L-Glutamate · L-Glutamine · Alzheimer's disease · Mild cognitive impairment · Dementia

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Introduction

Alzheimer's disease (AD) represents the main cause of dementia in the elderly (Hirtz et al. 2007; Scheltens et al. 2016) and the possibility to obtain a molecular profiling of patients' biofluids has led to a biomarker-based diagnostic approach, independently from clinical stage and thus encompassing the whole spectrum of the disease, from preclinical to overt dementia (Jack et al. 2018). Specifically, neuropathological AD hallmarks like amyloid plaques (Aβ) deposition and neurofibrillary tangles (NFT) (Querfurth and LaFerla 2010) can be ex vivo assessed through cerebrospinal fluid (CSF) analysis. The presence of altered Aβ42/Aβ40 ratio and increased phosphorylated tau (phospho-Tau) in CSF are considered, respectively, as an amyloidosis (A +) and tauopathy (T +) molecular signature, corresponding to a biochemical diagnosis of AD (Jack et al. 2018). In line with this approach, it has been proposed that biofluid analysis could be useful to assess other pathological processes with potential key roles in AD pathogenesis.

Different works in experimental models of AD have suggested the presence of an imbalance between excitatory and inhibitory transmission during disease development (Jo et al. 2014; Lei et al. 2016; Sanchez et al. 2012; Verret et al. 2012). L-Glutamate (L-Glu) is the major excitatory neurotransmitter in the brain and it has been reported that during AD development it could be aberrantly released after neuronal exposure to Aβ (Harkany et al. 2000; Russell et al. 2012) and/or not properly removed from the synaptic cleft by neuronal and glial cells (Chen et al. 2011; Scott et al. 2002; Masliah et al. 1996). An excessive stimulation of glutamatergic synaptic and extra-synaptic NMDA receptors (NMDARs) could trigger calcium-dependent detrimental intracellular signaling pathways and oxidative stress, ultimately leading to excitotoxicity and cell death (Hardingham and Bading 2010; Lewerenz and Maher 2015). Accordingly, anti-glutamatergic drugs have been tested as potential disease-modifying therapies for AD patients (Zádori et al. 2014).

Although glutamate excitotoxicity is considered to play a significant role in the pathogenesis of AD (Wang and Reddy 2017) the investigation of brain L-Glu levels in patients with AD has revealed partially discordant results. Some authors reported unchanged levels of L-Glu in hippocampus and cerebral cortices obtained from controls and AD patients (Tarbit et al. 1980), while others showed increased (Procter et al. 1988a) or reduced (Sasaki et al. 1986; Ellison et al. 1986; Gueli and Taibi 2013) concentrations of this amino acid in AD brains. Interestingly, the extent of neurodegeneration and the progressive loss of glutamatergic cortical neurons during disease progression could influence the dosage of total L-Glu, as supported by the evidence that the reduction of L-Glu content in antemortem (≈ 2.7 years after disease onset) biopsy specimens of temporal cortex in AD patients was smaller than that found in AD post-mortem samples (≈ 10.2 years after disease onset) (Procter et al. 1988b). Moreover, the glial L-Glu reuptake and conversion in L-glutamine (L-Gln) by glutamine synthetase (GS), a process known as L-Glu/L-Gln cycle (Bak et al. 2006), could represent a potential rescue strategy counteracting pathological excitotoxicity during AD development (Huang et al. 2016; Zhou and Danbolt 2014). Interestingly, an alteration of such glial physiological functions has been hypothesized to contribute to AD progression, since astrocytic glutamate transporters were found to be reduced in AD brains (Lauderback et al. 2001; Masliah et al. 1996) in association with reduced GS expression in astrocytes (Robinson 2000). The combined analysis of L-Glu and L-Gln content in brain and biofluids of patients with AD could help in defining the possible changes of L-Glu/L-Gln cycle during AD.

In addition, it should be noted that other molecule including D-serine exerting a co-agonist modulatory effect on NMDAR could influence AD-related neurodegeneration and have been recently investigated as novel molecular biomarker in AD (Biemans et al. 2016; Madeira et al. 2015, Nuzzo et al. 2020a). In this scenario, glycine (Gly) represents a NMDAR co-agonist molecule with a modulatory role (Johnson and Ascher 1987) which could deeply influence the pathological activation of this glutamatergic receptor in neurological disorders. However, the changes in brain Gly levels characterizing AD patients among the different stages of the disease have been less investigated, thus representing an additional biochemical target to be analyzed.

Moving from the studies performed in brain samples, to identify possible biomarkers reflecting the imbalanced excitatory synaptic transmission, CSF and blood serum levels of L-Glu, L-Gln and Gly have been investigated in AD patients with still inconclusive results. Indeed, it has been reported that CSF L-Glu levels in patients with AD can be found as increased (Jiménez-Jiménez et al. 1998; Kaiser et al. 2010; Madeira et al. 2018; Pomara et al. 1992), unchanged (Degrell et al. 1989; Smith et al. 1985) or even decreased (Martinez et al. 1993; Tohgi et al. 1992) compared to controls. Similar conflicting results have been reported also for L-Gln (D'Aniello et al. 2005; Jiménez-Jiménez et al. 1998; Madeira et al. 2018; Pomara et al. 1992; Smith et al. 1985; Tohgi et al. 1992; Procter et al. 1988b) and Gly (D'Aniello et al. 2005; Degrell et al. 1989; Jiménez-Jiménez et al. 1998; Pomara et al. 1992; Smith et al. 1985; Tohgi et al. 1992; Madeira et al. 2015). The existing differences in patients' cohorts, both clinical and demographical, as well as in the applied inclusion criteria could explain the conflicting results obtained by the

various studies. Interestingly, the levels of these metabolites could also vary across AD stages and be useful for a prognostic evaluation. Specifically, L-Glu serum levels were found to be lower in patients with moderate/severe AD with respect to patients with amnestic MCI (Lin et al. 2017) and a prospective study involving 2067 dementia-free Framingham Offspring Cohort participants found that baseline L-Glu levels in serum were associated, at a suggestive level of significance, with risk for AD development after a mean follow-up period of 15.6 (±5.2) years (Chouraki et al. 2017).

Here, to clarify the role of L-Glu, L-Gln, Gly as potential biomarkers in AD, we measured by High-Performance Liquid Chromatography (HPLC) their concentrations in post-mortem brain samples obtained from AD patients and in CSF and blood serum of a cohort of drug-free subjects encompassing the whole clinical spectrum of AD [pre-clinical stage (pre-AD), mild cognitive impairment (MCI-AD) and overt dementia (AD-dem)] and in a group of neurological controls (other neurological diseases, OND).

Materials and methods

Post-mortem brain tissue 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 (Braak and Braak 1991). 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, $p=0.969$, Mann Whitney test) and post-mortem delay (Ctrl vs AD patients, mean ± SEM of hours: 6.0 ± 0.2 vs. 5.7 ± 0.4, $p=0.343$, Mann Whitney test). Details are reported in Table 1. 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 (McKhann et al. 1984).

Table 1 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-VI	3/7/0	0/0/10	

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

Serum and cerebrospinal fluid collection

We enrolled a total number of 77 patients referring to the Center for Memory Disturbances of the University of Perugia for diagnostic work-up for the study. 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 antagonists, 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 (CDR = 0), 29 MCI-AD (CDR = 0.5), 30 AD-Dem (CDR = 1/2) (Jack et al. 2018). As neurological controls (other neurological diseases, OND), a total number of 30 subjects who underwent lumbar puncture for diagnostic reasons were enrolled. This group included patients with epilepsy ($n=1$), psychiatric disorders ($n=2$) or non-AD cognitive deficits ($n=27$). Demographical information is reported in Table 2. Subjects did not show difference in terms of gender distribution among groups ($p=0.512$, $\chi^2=2.303$; Chi-square test; Table 2), while ages of MCI-AD and AD-Dem patients were significantly higher than those of OND subjects (Kruskal–Wallis test, $p<0.0001$; Dunn's test: OND: 66.60 ± 1.33 years vs. MCI-AD: 73.72 ± 1.12 years, $p=0.002$; OND: 66.60 ± 1.33 years vs. AD-Dem: 73.13 ± 1.35 years; $p=0.004$; Table 2).

CSF samples were collected according to international guidelines (del Campo et al. 2012; Teunissen et al. 2009; Vanderstichele et al. 2012). 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

Table 2 Demographic characteristics of subjects enrolled in serum and cerebrospinal fluid collection

	OND ($n=30$)	Pre-AD ($n=18$)	MCI-AD ($n=29$)	AD-Dem ($n=30$)	p value
Gender (Male/Female)	14/16	7/11	9/20	9/21	0.512 ($\chi^2=2.303$)[a]
Age (years)	66.60 ± 1.33	69.17 ± 1.36	73.72 ± 1.12	73.13 ± 1.35	< 0.0001[b]
MMSE score	28.21 ± 0.25 ($n=29$)	27.33 ± 0.21	24.45 ± 0.50	17.00 ± 1.00 ($n=28$)	< 0.0001[b]
CDR score		0.00 ± 0.00	0.50 ± 0.00	1.23 ± 0.08	< 0.0001[b]
CSF Aβ40 (pg/mL)	9515,50 ± 647,99 ($n=20$)	11,525.61 ± 646.76	13,196,72 ± 947.47	10,805.50 ± 713.49	0.0460[b]
CSF Aβ42 (pg/mL)	1200.47 ± 73.79	599.15 ± 35.17	637.83 ± 50.52	572.37 ± 44.23	< 0.0001[b]
CSF Aβ42/Aβ40 ratio (%)	13.42 ± 1.09 ($n=20$)	5.33 ± 0.27	5.03 ± 0.25	5.36 ± 0.19	< 0.0001[b]
CSF total Tau (pg/mL)	276.67 ± 14.61	452.91 ± 52.44	665.03 ± 33.03	860.20 ± 61.46	< 0.0001[b]
CSF phospho-Tau (pg/mL)	47.07 ± 2.25	70.70 ± 7.21	95.72 ± 4.81	95.70 ± 4.61	< 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. *OND* other neurological disease, *Pre-AD* pre-clinical Alzheimer's disease, *MCI-AD* mild cognitive impairment-Alzheimer's disease, *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

effects. All samples were centrifuged at 2000×*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 for 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 11,020) 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.

HPLC detection of L-glutamate, L-glutamine, and glycine levels

Post-mortem brain, cerebrospinal fluid and serum samples were analyzed as previously reported (Nuzzo et al. 2017, 2019ab, 2020b; Palese et al. 2020). 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. Supernatants were then dried, suspended in 0.2 M TCA and then neutralized with 0.2 M NaOH. Samples were then subjected to pre-column derivatization with o-phthaldialdehyde (OPA)/*N*-acetyl-L-cysteine in 50% methanol. Amino acids were resolved on a C8 5-µm reversed-phase column 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 L-Glu, L-Gln and Gly were based on retention times and peak areas (mean ± SEM of retention times, L-Glu = 10.50 ± 0.02 min; L-Glu = 29.88 ± 0.06 min; Gly = 44.90 ± 0.10 min) and compared with those associated with external standards. CSF and serum amino acid levels were expressed as µM concentration. For tissue samples, total protein content of homogenates was determined by Bradford assay method, after re-solubilization 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. L-Glu/Gln ratio was expressed as percentage (%).

Western blotting analysis of human post-mortem brain tissues

Preparation and immunoblotting were performed as previously described (De Rosa et al. 2020; Nuzzo et al. 2019a). Frozen, powdered samples from post-mortem *superior frontal gyrus* were sonicated in 1% SDS and boiled for 10 min. Aliquots (1 µl) of the homogenate were used for the protein determination by Bio-Rad Protein Assay kit (Bio-Rad). Equal amounts of total proteins (30 µg) for each sample were loaded on pre-cast 4–20% gradient gel (BioRad Laboratories). Proteins were separated by SDS-PAGE and transferred to PVDF membranes through Trans Blot Turbo System. Membranes were then immunoblotted overnight using anti-Tau (1:1000, MAB3420) primary antibody. Blots were then incubated in horseradish peroxidase-conjugated secondary antibodies. Immunoreactivity

was detected by enhanced chemiluminescence (ECL) (GE-Healthcare) and quantified by Quantity One software (Bio-Rad). Optical density values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1000, SC-32233) for variation in loading and transfer. Normalized values were then averaged and used as dependent variable.

Statistical analysis

Because not all groups showed Gaussian distribution, statistical analysis was performed using Mann–Whitney or 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.

Results

L-glutamate, L-glutamine and glycine in the superior frontal gyrus of Alzheimer's disease patients

Here, we measured L-Glu, L-Gln and Gly content in the post-mortem superior frontal gyrus (SFG) of AD and non-demented patients ($n = 10$/clinical condition; The Netherlands Brain Bank (Netherlands Institute for Neuroscience, Amsterdam, www.brainbank.nl). Table 1 showed demographic and clinical characteristics of patients.

HPLC analysis revealed reduced L-Glu levels in the SFG of AD patients, compared to non-demented subjects (mean values ± SEM: Ctrl vs. AD, 337.00 ± 48.92 vs. 189.30 ± 22.68 nmol/mg protein; $p = 0.0115$, Mann–Whitney test, Fig. 1a). Conversely, cortical L-Gln amount was comparable between AD and healthy subjects (mean values ± SEM: Ctrl vs. AD, 175.50 ± 19.23 vs. 139.3 ± 23.76 nmol/mg protein; $p = 0.2176$, Mann–Whitney test, Fig. 1b). In line with glutamate reduction, we reported also a ~ twofold decrease of Gly content in the AD cortex compared to non-demented controls (mean values ± SEM: Ctrl *vs* AD, 22.93 ± 2.90 vs. 8.96 ± 2.02 nmol/mg protein; $p = 0.0011$, Mann–Whitney test, Fig. 1c).

Next, to evaluate the degree of cortical cell-death, we analyzed Tau protein levels as a marker of neuronal degeneration in the same SFG samples. Western blotting analysis showed reduced Tau protein expression in AD cortex when compared to non-demented controls ($p = 0.0185$, Mann–Whitney test, Fig. 1d).

Overall, these data revealed a robust reduction of glutamate and glycine concentrations in AD cortex, probably due to cortical neuronal atrophy observed in this brain region.

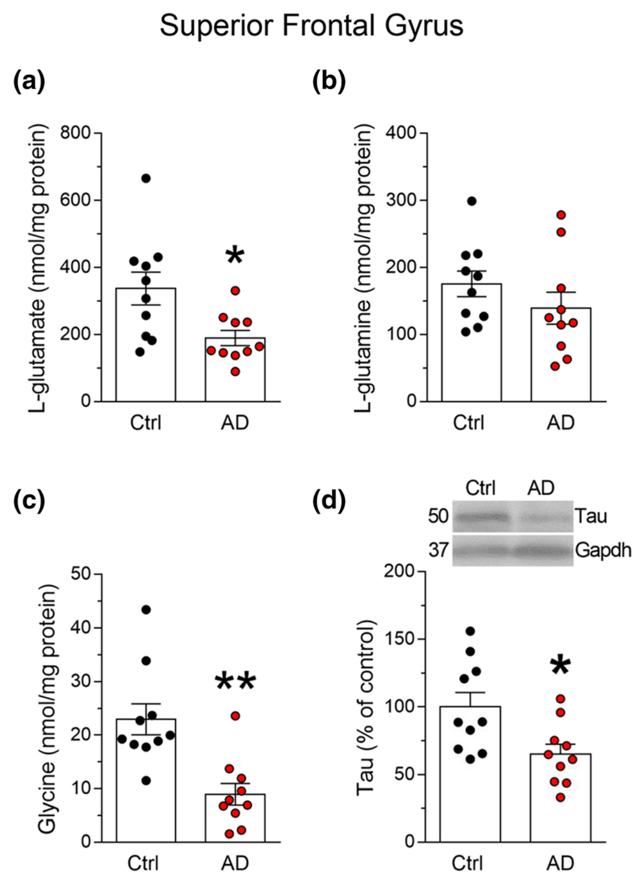

Fig. 1 Free L-glutamate, L-glutamine and glycine levels and Tau protein expression in the post-mortem superior frontal gyrus of patients affected by Alzheimer's disease. **a–c** Content of (**a**) L-glutamate (L-Glu), (**b**) L-glutamine (L-Gln) and (**c**) glycine analyzed in the superior frontal gyrus of Alzheimer's disease patients (AD, $n = 10$) and non-demented controls (Ctrl, $n = 10$) detected by HPLC analysis. In each sample, all amino acids were detected in a single run by HPLC and expressed as nmol/mg protein. **d** Tau protein levels in the post-mortem superior frontal gyrus of patients affected by Alzheimer's disease (AD) and control individuals measured by Western Blotting analysis. Representative blots are shown above the graph. Gapdh was used to normalize for variations in loading and transfer. Dots represent the single subjects' values while bars illustrate the means ± SEM. Data were analyzed by Mann–Whitney test. * $p < 0.05$, ** $p < 0.01$ compared to Ctrl

HPLC analysis of cerebrospinal fluid levels of L-glutamate, L-glutamine and glycine content across the whole clinical spectrum of Alzheimer's disease

We measured cerebrospinal fluid concentrations of L-Glu, L-Gln and Gly 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 2 for clinical and demographic characteristics). Statistical analysis revealed unaltered mean CSF concentrations of L-Glu and L-Gln among diagnosis groups (L-Glu: $p = 0.2811$; L-Gln: $p = 0.9898$; Kruskal–Wallis test; Fig. 2a, b). Consequently, L-Glu/L-Gln ratio was not significantly affected ($p = 0.1423$; Kruskal–Wallis test; Table 3). Similarly, Gly levels did not differ among groups (Gly: $p = 0.5746$; Kruskal–Wallis test; Fig. 2c). Mean concentrations of the metabolites analyzed above are reported in Table 3. In the same way, we failed to find any significant change in CSF L-Glu, L-Gln and Gly concentrations among the different CDR groups analyzed (L-Glu: $p = 0.2203$, L-Gln: $p = 0.1419$, Gly: $p = 0.1215$; Kruskal–Wallis test; Fig. 2d–f).

We then evaluated the existence of possible correlations between CSF L-Glu, L-Gln or Gly concentrations and MMSE scores or CSF diagnostic AD biomarkers (Aβ42/β40 ratio, phosphorylated and total Tau levels) in the entire cohort of patients. We reported no significant correlations between L-Glu, L-Gln or Gly content with MMSE scores or Aβ42/β40 ratio (MMSE: L-Glu, $r = 0.133$, $p = 0.178$; L-Gln, $r = 0.014$, $p = 0.886$; Gly, $r = -0.070$, $p = 0.477$; Aβ42/β40 ratio: L-Glu, $r = 0.126$, $p = 0.218$; L-Gln, $r = 0.006$, $p = 0.952$; Gly, $r = 0.133$, $p = 0.192$; Spearman's correlation; Fig. 2g–i; Table 4). Likewise, L-Gln or Gly content were not correlated

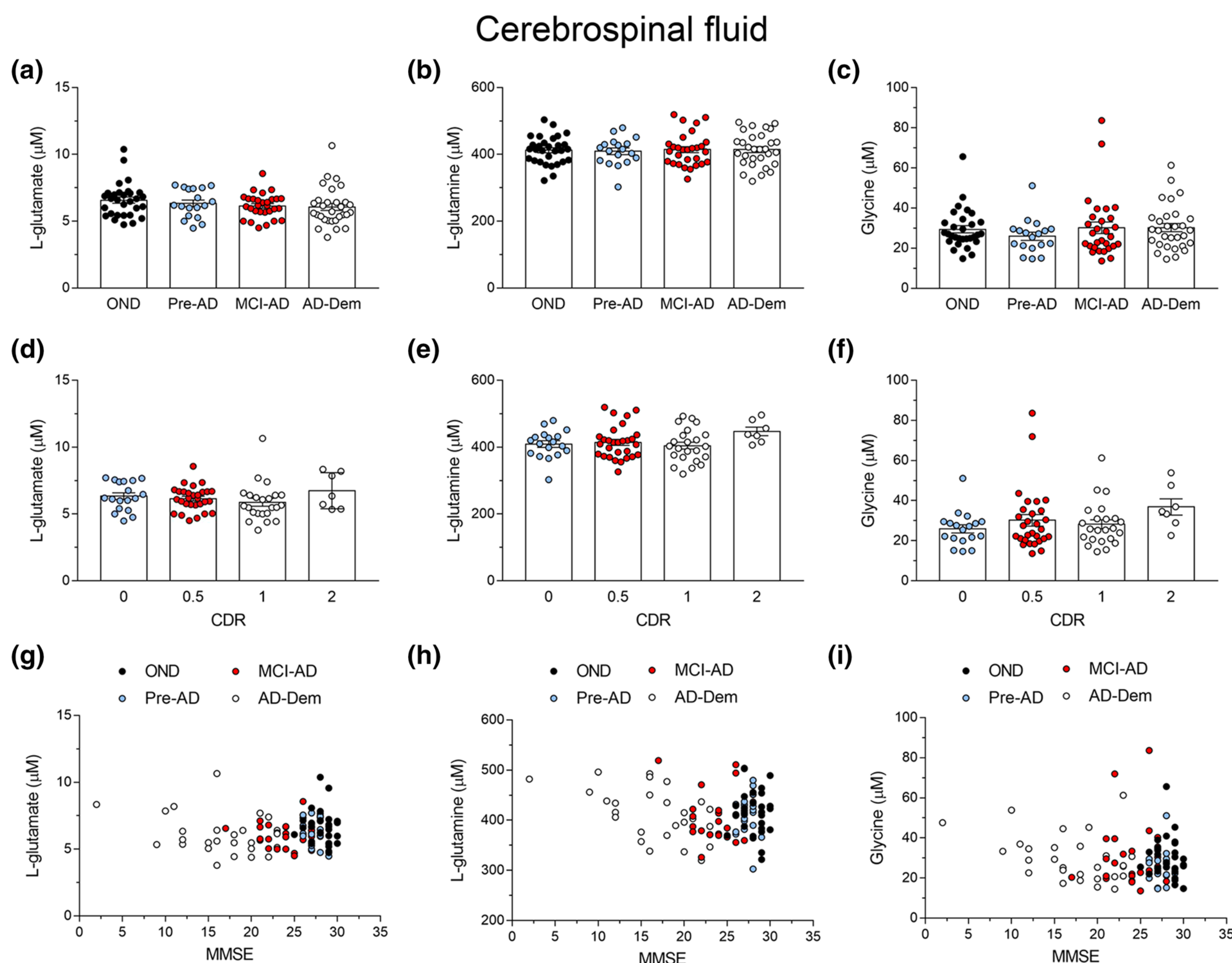

Fig. 2 Analysis of free L-glutamate, L-glutamine and glycine levels in the cerebrospinal fluid of patients encompassing the whole clinical spectrum of Alzheimer's disease. **a–c** Detection of (**a**) L-Glu, (**b**) L-Gln and (**c**) Gly concentrations in the cerebrospinal fluid of pre-clinical AD (pre-AD, $n = 18$), Mild Cognitive Impairment-Alzheimer's disease (MCI-AD, $n = 29$) and Alzheimer's disease-Dementia (AD-Dem, $n = 30$) subjects compared to non-demented controls (other neurological disorders, OND, $n = 30$) by HPLC analysis. **d–f** Analysis of (**d**) L-Glu, (**e**) L-Gln and (**f**) Gly levels in the cerebrospinal fluid of subjects stratified within 0, 0.5, 1 and 2 CDR groups. Dots represent the single subjects' values while bars illustrate the means ± SEM. All mean values are expressed as the mean ± SEM of µM concentration. Because data did not show Gaussian distribution, means were compared using Kruskal–Wallis followed by post-hoc Dunn's test, when required. **g–i** Correlation analysis between cerebrospinal fluid (**g**) L-Glu, (**h**) L-Gln or (**i**) Gly concentrations and MMSE scores of the entire cohort of patients. Note that some MMSE scores were not available (see Table 2). Correlation analysis was calculated using Spearman's correlations. In each sample, all free amino acids were detected in a single run

Table 3 The mean values of L-glutamate, L-glutamine, glycine (expressed as µM) and L-glutamate/L-glutamine ratio (expressed as %) in the serum or cerebrospinal fluid are compared among the different clinical conditions

	OND ($n=30$)	Pre-AD ($n=18$)	MCI-AD ($n=29$)	AD-Dem ($n=30$)	*p value*
Serum					
L-Glu (µM)	82.84 ± 5.07	103.67 ± 7.09[a]	86.62 ± 6.43	76.51 ± 4.90	0.0164
L-Gln (µM)	375.59 ± 16.73	425.71 ± 18.98	412.89 ± 9.44	432.6 ± 7.86[b]	0.0351
L-Glu/L-Gln ratio (%)	22.71 ± 1.44	24.87 ± 1.78	20.89 ± 1.38	17.92 ± 1.24[b]	0.0055
Gly (µM)	399.10 ± 14.64	487.83 ± 35.69	414.75 ± 20.07	451.28 ± 24.72	0.2056
CSF					
L-Glu (µM)	6.57 ± 0.23	6.33 ± 0.25	6.14 ± 0.17	6.07 ± 0.26	0.2811
L-Gln (µM)	412.58 ± 7.62	409.19 ± 9.81	414.34 ± 9.06	414.41 ± 9.03	0.9898
L-Glu/L-Gln ratio (%)	1.60 ± 0.06	1.55 ± 0.05	1.49 ± 0.03	1.46 ± 0.05	0.1423
Gly (µM)	29.41 ± 1.81	25.92 ± 2.01	30.18 ± 2.88	30.24 ± 2.06	0.5746

All values are expressed as mean ± SEM. Statistical analyses were performed by Kruskal–Wallis test followed by post-hoc Dunn's test when required

CSF cerebrospinal fluid, *L-Glu* L-glutamate, *L-Gln* L-glutamine, *Gly* glycine, *OND* other neurological disease, *Pre-AD* pre-clinical Alzheimer's disease, *MCI-AD* mild cognitive impairment-Alzheimer's disease, *AD-Dem* Alzheimer's disease dementia

[a] $p < 0.05$, compared to OND (Dunn's test)

[b] $p < 0.01$, compared to OND (Dunn's test)

Table 4 Correlation analysis between L-Glu, L-Gln and Gly 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

	Spearman's correlation	Serum (µM)			Cerebrospinal fluid (µM)		
		L-Glu	L-Gln	Gly	L-Glu	L-Gln	Gly
Age	*r*	0.027	0.13	0.053	0.031	0.116	0.222
	p value	0.781	0.184	0.59	0.751	0.234	0.021
MMSE	*r*	0.184	− 0.208	− 0.108	0.133	0.014	− 0.070
	p value	0.061	0.034	0.276	0.178	0.886	0.477
CSF Aβ42/Aβ40 ratio	*r*	− 0.108	− 0.173	0.039	0.126	0.006	0.133
	p value	0.291	0.090	0.702	0.218	0.952	0.192
CSF total Tau (pg/mL)	*r*	− 0.248	0.190	0.062	− 0.221	− 0.017	− 0.083
	p value	0.010	0.050	0.525	0.020	0.865	0.393
CSF phospho-Tau (pg/mL)	*r*	− 0.172	0.135	0.049	− 0.217	− 0.039	− 0.136
	p value	0.076	0.167	0.619	0.025	0.692	0.161

Statistical analyses were performed by Spearman's correlations

L-Glu L-glutamate, *L-Gln* L-glutamine, *Gly* glycine, *MMSE* mini-mental state evaluation

with CSF phosphorylated and total Tau levels (L-Gln: pospho-Tau, $r = -0.039$, $p = 0.692$; total Tau: $r = -0.017$, $p = 0.865$; Gly: pospho-Tau, $r = -0.136$, $p = 0.161$; total Tau: $r = -0.083$, $p = 0.393$; Spearman's correlation; Table 4). Conversely, L-Glu amount showed a negative correlation with CSF phosphorylated and total Tau levels in the entire cohort of patients (pospho-Tau: $r = -0.217$, $p = 0.025$; total Tau: $r = -0.221$ $p = 0.020$; Spearman's correlation; Table 4).

HPLC analysis of serum levels of L-glutamate, L-glutamine and glycine in patients across the whole clinical spectrum of Alzheimer's disease

We measured serum L-Glu, L-Gln and Gly levels in the same cohort of AD patients and controls. Kruskal–Wallis analysis indicated a significant diagnosis effect on serum L-Glu levels ($p = 0.0164$; Kruskal–Wallis test; Fig. 3a). In particular, we reported increased L-Glu amount in the serum of pre-AD subjects compared to controls (means ± SEM of µM: OND, 82.84 ± 5.07 vs. pre-AD, 103.67 ± 7.09; $p = 0.0408$; Dunn's test; Fig. 3a). Then, we assessed the levels of L-Gln among groups. Statistical analysis indicated a significant diagnosis effect on serum L-Gln content ($p = 0.0351$; Kruskal–Wallis

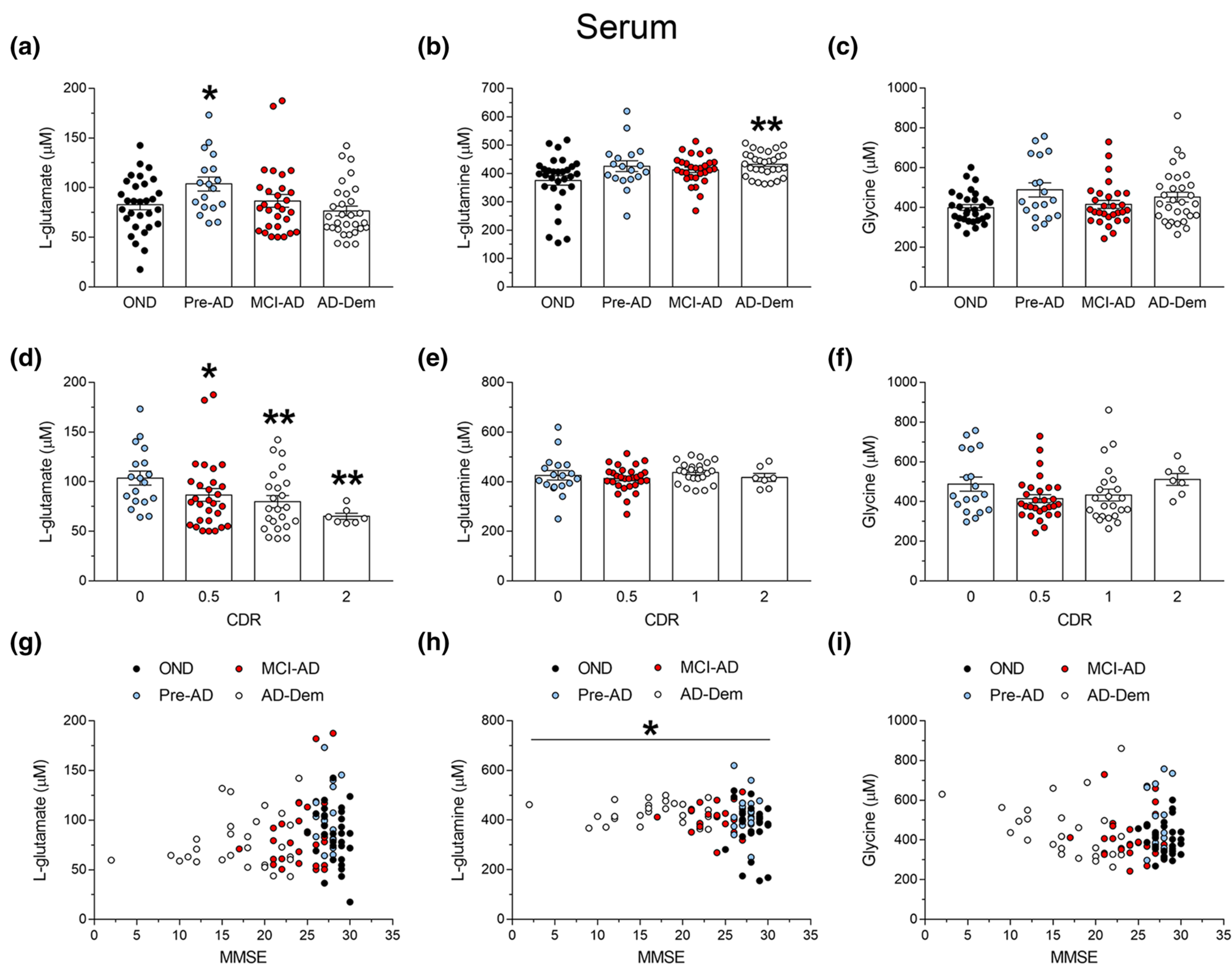

Fig. 3 Analysis of free L-glutamate, L-glutamine and glycine levels in the serum of patients encompassing the whole clinical spectrum of Alzheimer's disease. **a–c** Detection of (**a**) L-Glu, (**b**) L-Gln and (**c**) Gly concentrations in the serum of pre-clinical AD (pre-AD, $n=18$), Mild Cognitive Impairment-Alzheimer's disease (MCI-AD, $n=29$) and Alzheimer's disease-Dementia (AD-Dem, $n=30$) subjects compared to non-demented controls (other neurological disorders, OND, $n=30$) by HPLC analysis. Because data did not show Gaussian distribution, means were compared using Kruskal–Wallis followed by post-hoc Dunn's test, when required. **d–f** Analysis of (**d**) L-Glu, (**e**) L-Gln and (**f**) Gly levels in the serum of subjects stratified within 0, 0.5, 1 and 2 CDR groups. Dots represent the single subjects' values while bars illustrate the means ± SEM. All mean values are expressed as the mean ± SEM of µM concentration. Because data did not show Gaussian distribution, means were compared using Kruskal–Wallis followed by post-hoc Dunn's test, when required. $*p<0.05$, $**p<0.01$ compared to OND or CDR 0. **g–i** Correlation analysis between serum (**g**) L-Glu, (**h**) L-Gln or (**i**) Gly concentrations and MMSE scores of the entire cohort of patients. Note that some MMSE scores were not available (see Table 2). Correlation analysis was calculated using Spearman's correlations ($*p<0.05$). In each sample, all free amino acids were detected in a single run

test; Fig. 3b). Following Dunn's test showed increased L-Gln content in the serum of AD-Dem patients compared to controls (means ± SEM of µM, OND: 375.59 ± 16.73 vs AD-Dem: 432.60 ± 7.86; $p=0.0037$; Dunn's test; Fig. 3b). Interestingly, we reported a significant main effect of diagnosis on the calculated L-Glu/L-Gln ratio ($p=0.0055$; Kruskal–Wallis test; Table 3). In particular, L-Glu/L-Gln ratio was reduced in AD-Dem patients compared to OND subjects (means ± SEM of %, OND: 22.71 ± 1.44 vs. AD-Dem: 17.92 ± 1.24; $p=0.0089$; Dunn's test; Table 3). In contrast, we found unaltered Gly levels among groups ($p=0.2056$; Kruskal–Wallis test; Fig. 1c). Mean concentrations of the metabolites analyzed above are reported in Table 2. Next, we assessed whether in patients L-Glu, L-Gln or Gly 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). Interestingly, we reported a significant main effect of CDR scores on L-Glu amount ($p=0.2056$; Kruskal–Wallis test; Fig. 3d). Indeed, CDR 0.5, 1 and 2 groups showed significantly lower levels of L-Glu compared to CDR 0 (cognitively normal) group (means ± SEM of µM, CDR 0: 103.67 ± 7.10 vs CDR 0.5:

86.62 ± 6.43, $p = 0.0263$; CDR 0: 103.67 ± 7.10 vs. CDR 1: 79.97 ± 6.18, $p = 0.0081$; CDR 0: 103.67 ± 7.10 vs. CDR 2: 65.17 ± 3.12, $p = 0.0023$; Dunn's test; Fig. 3d). Conversely, we found comparable L-Gln and Gly concentrations in all CDR groups analyzed (L-Gln, $p = 0.3559$; Gly, $p = 0.0657$; Kruskal–Wallis test; Fig. 3e, f).

To evaluate the gender effect, we also analyzed the amino acids levels among the clinical conditions within males and female. Interestingly, we found that serum L-Glu is significantly increased in pre-AD female subjects compared to female controls ($p = 0.004$; Kruskal–Wallis test; means ± SEM of µM: OND, 80.78 ± 7.70 vs. pre-AD, 112.31 ± 9.35; $p = 0.022$; Dunn's test; Table 5). Furthermore, in female AD patients, L-Glu serum levels were also significantly correlated with CDR values ($p = 0.004$; Kruskal–Wallis test; means ± SEM of µM, CDR 0: 112.31 ± 9.35 vs. CDR 0.5: 77.03 ± 4.94, $p = 0.004$; CDR 0: 112.31 ± 9.35 vs CDR 1: 74.17 ± 8.14, $p = 0.001$; CDR 0: 112.31 ± 9.35 vs CDR 2: 66.22 ± 3.48, $p = 0.005$; Dunn's test; Table 5). In addition, also L-Glu/L-Gln ratio showed a significant reduction with increasing CDR values ($p = 0.021$; Kruskal–Wallis test; means ± SEM of %, CDR 0: 25.79 ± 2.57 vs. CDR 0.5: 18.92 ± 1.21, $p = 0.050$; CDR 0: 25.79 ± 2.57 vs. CDR 1: 17.27 ± 2.14, $p = 0.005$; CDR 0: 25.79 ± 2.57 vs. CDR 2: 15.94 ± 0.81, $p = 0.019$; Dunn's test; Table 5). Yet, we showed that serum Gly levels were significantly higher in pre-AD female subjects compared to controls ($p = 0.024$; Kruskal–Wallis test; means ± SEM of µM: OND, 428.36 ± 22.32 vs pre-AD, 567.66 ± 42.10; $p = 0.022$; Dunn's test; Table 5). In particular, we reported a significant reduction of Gly content only in female patients with CDR 0.5 compared to their sex-matched CDR 0 group ($p = 0.024$; Kruskal–Wallis test; means ± SEM of µM: CDR 0: 567.66 ± 42.10 vs. CDR 0.5: 435.31 ± 26.09; $p = 0.035$; Dunn's test; Table 5). On the other hand, in the cohort of male subjects we failed to find any significant alteration of L-Glu, L-Gln or Gly levels between clinical conditions or CDR scores (Table 5).

One possible explanation could be related to the lower sample size of male subjects compared to females. However, further studies are mandatory to confirm such gender-specific serum L-Glu and Gly alterations in AD patients. Next, we evaluated whether serum levels of L-Glu, L-Gln or Gly correlated with the severity of cognitive impairment, expressed as mini-mental state examination (MMSE) score or with CSF diagnostic AD biomarkers such as Aβ42/Aβ40 ratio, phosphorylated and total Tau levels (Table 4). Despite statistical analysis failed to reach significance, we showed that serum L-Glu content was positively correlated to MMSE score ($r = 0.184$; $p = 0.061$, Spearman's correlation; Fig. 3g). Conversely, we reported negative correlations between serum L-Glu amount and CSF total Tau levels (total Tau: $r = -0.248$; $p = 0.010$; Spearman's correlation; Table 4). Otherwise, no correlation between L-Glu levels and CSF Aβ42/β40 ratio or phosphorylated Tau levels was observed (Aβ42/β40 ratio: $r = -0.108$; $p = 0.291$; phospho-Tau: $r = -0.172$; $p = 0.076$; Spearman's correlation; Table 4). In contrast to L-Glu, we described negative correlation between L-Gln concentrations and MMSE score ($r = -0.208$; $p = 0.034$; Spearman's correlation; Fig. 3h) while positive correlation was observed between L-Gln content and CSF total Tau ($r = 0.190$; $p = 0.050$; Spearman's correlation; Table 4), but not with CSF phospho-Tau ($r = 0.135$; $p = 0.167$; Spearman's correlation; Table 4) or Aβ42/β40 ratio ($r = -0.173$; $p = 0.090$; Spearman's correlation; Table 4). Serum Gly content was not significantly correlated with MMSE scores ($r = -0.108$; $p = 0.276$; Spearman's correlation; Fig. 3i) or CSF diagnostic AD biomarkers such as Aβ42/β40 ratio ($r = 0.039$; $p = 0.702$; Spearman's correlation; Table 4) or phosphorylated and total Tau levels (phospho-Tau: $r = 0.049$; $p = 0.619$; total Tau: $r = 0.062$; $p = 0.525$; Spearman's correlation; Table 4).

Discussion

The application of a biomarker-based diagnostic and prognostic work-up, assessing amyloidosis (A +) and tauopathy (T +) CSF molecular signature, has significantly improved the possibility to identify AD patients since the early pre-dementia phase (Jack et al. 2018). Peripheral biofluids can mirror the pathological processes taking place in CNS during the development AD. To this regard, it has been proposed that the systematic analysis of small molecule metabolites in CSF and blood could lead to an accurate metabolomic profiling of AD, potentially unveiling novel diagnostic and prognostic biomarkers, as well as promising new therapeutic targets (Ellis et al. 2015; Trushina and Mielke 2014. Accordingly, we aimed to investigate the L-Glu/L-Gln/Gly levels in brain tissue of AD patients and their metabolic fingerprint in peripheral biofluids of a cohort of drug-free AD patients, enrolled according to the A + /T + CSF profile and encompassing the whole clinical spectrum of the disease (pre-clinical AD, MCI due to AD, and AD dementia), and neurological controls.

Notably, we showed that total concentrations of L-Glu in post-mortem brain samples, specifically in superior frontal gyrus, were significantly reduced in AD patients with respect to non-demented controls. This result is in line with what has been reported by other research groups (Ellison et al. 1986; Gueli and Taibi 2013; Sasaki et al. 1986; Procter et al. 1988b) and potentially reflects the progressive loss of cortical glutamatergic neurons during disease progression, as suggested by other authors (Procter et al. 1988b) and supported by the reduction in total Tau levels that we have found in AD superior frontal gyrus. Surprisingly, the analysis of

Table 5 L-glutamate, L-glutamine, glycine levels (expressed as µM) and L-glutamate/L-glutamine ratio (expressed as %) in the serum and CSF of male or female patients

	Gender	OND ($n=16$F; $n=14$ M)	Pre-AD ($n=11$F; $n=7$ M)	MCI-AD ($n=20$F; $n=9$ M)	AD-Dem ($n=21$F; $n=9$ M)	*p value*[a]	CDR 0 ($n=11$F; $n=7$ M)	CDR 0.5 ($n=20$F; $n=9$ M)	CDR 1 ($n=15$F; $n=8$ M)	CDR 2 ($n=6$F; $n=1$ M)	*p value*[a]
Serum											
L-Glu (µM)	F	80.78 ± 7.7	112.31 ± 9.35[#]	77.03 ± 4.94	71.9 ± 5.88	0.004	112.31 ± 9.35	77.03 ± 4.94**	74.17 ± 8.14**	66.22 ± 3.48**	0.004
	M	85.2 ± 6.62	90.08 ± 9.33	107.94 ± 15.99	87.29 ± 8.24	0.766	90.08 ± 9.33	107.94 ± 15.99	90.85 ± 8.43	58.84	0.531
L-Gln (µM)	F	388.31 ± 20.28	448.22 ± 23.79	408.32 ± 9.28	433.47 ± 9.98	0.132	44.22 ± 23.79	408.32 ± 9.28	439.73 ± 11.71	417.83 ± 19.14	0.23
	M	361.05 ± 27.66	390.34 ± 28.31	423.03 ± 23.07	430.57 ± 12.83	0.124	390.34 ± 28.31	423.03 ± 23.07	432.54 ± 14.37	414.81	0.693
L-Glu/L-Gln ratio (%)	F	21.03 ± 2.12	25.79 ± 2.57	18.92 ± 1.21	16.89 ± 1.53	0.021	25.79 ± 2.57	18.92 ± 1.21*	17.27 ± 2.14**	15.94 ± 0.81*	0.025
	M	24.63 ± 1.88	23.43 ± 2.26	25.29 ± 3.19	20.34 ± 1.92	0.477	23.43 ± 2.26	25.29 ± 3.19	21.11 ± 1.99	14.18	0.379
Gly (µM)	F	428.36 ± 22.32	567.66 ± 42.1[##]	435.31 ± 26.09	494.29 ± 29.71	0.024	567.66 ± 42.1	435.31 ± 26.09**	482.4 ± 39.81	524.01 ± 31.84	0.035
	M	365.65 ± 14.39	362.38 ± 18.55	369.07 ± 23.88	350.92 ± 20.9	0.887	362.38 ± 18.55	369.07 ± 23.88	340.13 ± 20.3	437.22	0.381
CSF											
L-Glu (µM)	F	6.73 ± 0.39	6.16 ± 0.32	5.97 ± 0.18	5.78 ± 0.25	0.173	6.16 ± 0.32	5.97 ± 0.18	5.48 ± 0.25	6.55 ± 0.56	0.192
	M	6.39 ± 0.24	6.59 ± 0.41	6.54 ± 0.34	6.74 ± 0.6	0.962	6.59 ± 0.41	6.54 ± 0.34	6.6 ± 0.66	7.86	0.481
L-Gln (µM)	F	400.15 ± 8.77	412.32 ± 15.63	405.8 ± 11.69	404.16 ± 9.49	0.807	412.32 ± 15.63	405.8 ± 11.69	390.3 ± 10.68	438.81 ± 11.23	0.07
	M	426.79 ± 12.13	404.28 ± 7.41	433.32 ± 11.76	438.34 ± 18.93	0.222	404.28 ± 7.41	433.32 ± 11.76	431.1 ± 19.83	496.26	0.111
L-Glu/L-Gln ratio (%)	F	1.68 ± 0.09	1.5 ± 0.06	1.48 ± 0.04	1.43 ± 0.05	0.08	1.5 ± 0.06	1.48 ± 0.04	1.41 ± 0.06	1.49 ± 0.11	0.586
	M	1.5 ± 0.05	1.63 ± 0.1	1.51 ± 0.05	1.53 ± 0.1	0.604	1.63 ± 0.1	1.51 ± 0.05	1.52 ± 0.11	1.58	0.64
Gly (µM)	F	30.9 ± 3.03	28.35 ± 2.84	29.81 ± 2.93	30.82 ± 2.3	0.922	28.35 ± 2.84	29.81 ± 2.93	29.53 ± 2.92	34.02 ± 3.42	0.502
	M	27.71 ± 1.76	22.09 ± 2.1	31 ± 6.93	28.9 ± 4.48	0.488	22.09 ± 2.1	31 ± 6.93	25.77 ± 3.64	53.89	0.417

In the left side of the table, mean values (± SEM) of Pre-AD, MCI-AD or AD-Dem patients were compared with those of control subjects (OND). In the right side of the table, mean values (± SEM) of patients with CDR score of 0.5, 1 or 2 were compared with those of patients with CDR 0. All statistical analyses were performed within gender (male or female) by Kruskal–Wallis test (a), followed by post-hoc Dunn's test, when required.

CSF cerebrospinal fluid, *F* female, *M* male, *L-Glu* L-glutamate, *L-Gln* L-glutamine, *Gly* glycine, *OND* other neurological disease, *Pre-AD* pre-clinical Alzheimer's disease, *MCI-AD* mild cognitive impairment-Alzheimer's disease, *AD-Dem* Alzheimer's disease dementia

[#] $p < 0.05$, [##] $p < 0.01$, compared with OND (Dunn's test)

* $p < 0.05$, ** $p < 0.01$, compared with CDR 0 (Dunn's test)

CSF levels of l-Glu in our cohort of patients, encompassing the whole AD clinical spectrum, did not reveal significant differences with respect to controls. However, HPLC analysis of serum samples suggested that l-Glu content could vary across AD stages. We showed that serum l-Glu amount was significantly higher in pre-AD patients compared to controls, but not significantly altered when compared to MCI-AD and AD-dem patients. Intriguingly, l-Glu serum levels were found to negatively correlate with CSF total Tau, which is considered a biomarker reflecting the ongoing neuronal/axonal loss, and CDR 0.5, 1 and 2 groups showed significantly lower levels of l-Glu compared to CDR 0 group. Overall, our data suggest that serum l-Glu levels were higher during early pre-clinical phases and tend to decrease in concomitance with the progression of cognitive impairment.

In line with this assumption, it has been shown that patients with amnestic MCI are characterized by higher serum l-Glu levels relative to more advanced AD stages (Lin et al. 2017). Moreover, baseline serum l-Glu levels in a large cohort of non-demented patients were associated with a significantly increased risk of AD-dementia development after a mean follow-up period of 15.6 (± 5.2) years (Chouraki et al. 2017).

Indeed, an abnormal neuronal and astrocytic release of l-Glu is considered an early pathogenic event in AD pathogenesis, potentially driving progressive neuronal loss through the aberrant stimulation of synaptic and extra-synaptic glutamatergic receptors (Hardingham and Bading 2010; Lewerenz and Maher 2015; Parsons and Raymond 2014). In line with changes in glutamatergic neurotransmission associated to cognitive decline, alterations of l-Glu levels were recently reported in the CSF and serum of patients affected by frontotemporal dementia (Palese et al. 2020). Of note, even if some previous reports are in line with our findings, showing that serum l-Glu levels are unaltered in MCI-AD and AD-dem patients compared to controls (Martinez et al. 1993), other authors reported significantly reduced serum l-Glu levels in both MCI-AD and AD-dem patients compared to non-demented controls (Olazarán et al. 2015). This discrepancy could be explained by the influence of clinical and demographic characteristics of the different cohorts of patients (i.e., age, drug assumption) and by different criteria used for AD diagnosis (clinical vs, biomarker-based criteria). Moreover, "paradoxically" normal serum l-Glu levels in patients with diagnosis of advanced AD stages do not rule out an excessive release of this neurotransmitter by cortical neurons, since AD-related progressive glutamatergic neuronal death and glutamate metabolism through other metabolic pathways could mask the absolute increase of this neurotransmitter, as suggested also by other authors (Manyevitch et al. 2018).

Indeed, pathological studies have suggested that AD progression could be characterized by an in vivo adaptation of physiological l-Glu-clearing molecular pathways. Although it has been proposed that the astrocytic expression of GS, catalyzing the ATP-dependent condensation of ammonia and l-Glu to form l-Gln (Ramonet et al. 2004; Rose et al. 2013; Chen and Herrup 2012), could be reduced during AD development (Robinson 2000), other studies have reported partially conflicting results. Some authors showed that GS activity in AD brains, with respect to age-matched controls, can be found either decreased or unchanged in relation to the brain area analyzed (Smith et al. 1991). Moreover, GS levels were found to be increased in prefrontal cortex (Burbaeva et al. 2005) or in CSF of AD patients (Tumani et al. 1999; Gunnersen and Haley 1992), but other authors did not confirm this last observation (Timmer et al. 2015). In addition, pathological studies showed that the reduced astrocytic GS expression could be associated with an increased neuronal expression of this enzyme (Robinson 2001; Chen and Herrup 2012), with still unknown functional consequences.

In this context, it has been reported that l-Gln content is higher in post-mortem brain samples of AD patients with respect to controls, in parallel with a reduction of l-Glu levels thus suggesting an increased activity of l-Glu/l-Gln cycle (Gueli and Taibi 2013). However, in line with a previous report (Procter et al. 1988b), we did not detect significant differences in l-Gln content in post-mortem brain samples obtained from AD and non-demented patients. Moreover, l-Gln levels were found to be unchanged in cerebrospinal fluid of AD patients and neurological controls. Conversely, we showed that serum l-Gln levels were increased in AD-dem patients compared to controls and negatively correlated with MMSE performances. This evidence is in line with previous findings showing increased serum l-Gln content in AD patients compared to non-demented patients (Cui et al. 2020). Therefore, the higher circulating l-Gln levels in AD patients were associated with worse cognition and increased risk of AD in large cohorts of population-based studies with different ethnicity (Cui et al. 2020; van der Lee et al. 2018).

We speculate that the adaptation of l-Glu/l-Gln cycle during AD progression could explain why serum l-Glu was found not increased in advanced AD stages. In line with this observation, previous findings showed decreased plasmatic l-Glu/l-Gln ratio in patients with amnestic MCI and AD compared to controls (Wang et al. 2014). Remarkably, here, we reported reduced l-Glu/l-Gln ratio in AD-Dem but not in MCI-AD patients compared to OND subjects, thus confirming its usefulness in distinguishing AD patients from non-demented controls (Cui et al. 2020).

Lastly, we focused on the analysis of Gly levels since this amino acid acts as a NMDAR co-agonist and could be involved in AD-related excitotoxicity (Yu and Lau 2018). Gly levels in post-mortem brain samples obtained from AD patients have been less investigated, with a report showing unchanged Gly levels in AD brains with respect to controls

(Lowe et al. 1990). Of interest, we have shown that Gly concentration is significantly reduced in the post-mortem superior frontal gyrus of patients affected by AD with respect to non-demented controls. However, CSF and serum samples HPLC analysis did not reveal significant differences in Gly levels among diagnosis groups. This result is in contrast with previous reports showing slightly increased serum Gly content in AD patients (Lin et al. 2017; Olazarán et al. 2015). However, more recent studies did not detect differences in Gly levels between AD patients and non-demented subjects, also showing that lower serum Gly levels were associated with increased risk of developing AD (Cui et al. 2020). Considering the differences among studies in terms of demographic and clinical characteristics of enrolled patients, further investigations are needed to clarify the possible usefulness of serum Gly as possible biomarker in AD.

Overall, the HPLC analysis of CSF analysis revealed no significant differences in L-Glu, L-Gln and Gly levels among all the experimental groups. Various studies have investigated CSF levels of these metabolites obtaining conflicting and inconclusive results (Degrell et al. 1989; Jiménez-Jiménez et al. 1998; Kaiser et al. 2010; Madeira et al. 2015, 2018; Martinez et al. 1993; Pomara et al. 1992; Smith et al. 1985; Tohgi et al. 1992; Adage et al. 2008; Procter et al. 1988b). Interestingly, a recent meta-analysis performed a statistical comparison of all available pooled data on CSF L-Glu/L-Gln/Gly levels in studies enrolling AD patients (Manyevitch et al. 2018). In line with our observations, the analysis performed by the authors did not reveal significant differences in CSF levels of these metabolites among AD and non-AD patients. The comparison of the analyzed studies could have been limited by many confounding factors, related to methodological differences and patients' clinical characteristics, also taking into account that analyzed studies are spread over a wide span of time. In apparent contrast with our findings, two of the most recently published studies on the theme showed an increased concentration of Gly (Madeira et al. 2015), L-Glu and L-Gln (Madeira et al. 2018) in a group of patients with clinically probable AD with respect to controls. However, it should be noted that these observations have been made in small sample size and some confounding factors could limit the generalization and comparison of the results. Indeed, the probable AD patients enrolled by the authors were mainly under treatment with psychoactive drugs, some acting on the glutamatergic neurotransmission, while the whole AD cohort of our study was drug-free and fully characterized according to CSF biomarker profile. A proper selection of AD patients and controls on the basis of CSF biomarker profile, together with the application of internationally shared guidelines for CSF withdrawal, storage and analysis (del Campo et al. 2012; Teunissen et al. 2009; Vanderstichele et al. 2012), will limit the potential confounding factors in future studies aimed at testing the clinical value of the proposed biomarkers.

The lack of consistency between brain, CSF and serum analysis for L-Glu, L-Gln and Gly levels could be due to the presence of potential pre-analytical confounding factors. It is possible to hypothesize that the metabolic fingerprint observed in the superior frontal gyrus of AD patients could be region-specific and not properly reflected in CSF or blood serum, due to a "dilution" effect or because of compensatory metabolic pathways in other brain regions. Moreover, the potential different clearance of the metabolite from the medium (CSF, blood serum) and the possible presence of a rostro-caudal gradient for brain-derived molecules could influence the analysis performed from lumbar CSF (Reiber 2001). The issue of consistency of metabolomic studies among different fluids is still under investigation (Trushina and Mielke 2014). Interestingly, an extensive metabolomic study performed in AD patients showed that only 30% of the metabolic pathways altered in the CSF of AD-MCI patients were also affected in plasma, and this percentage was of about 60% in AD-dem patients, suggesting that the number of pathways found as being affected in both CSF and plasma tends to increase with disease severity for still unknown reasons (Trushina et al. 2013). Finally, it should be noted that the possible influence exerted by peripheral metabolic pathways could confound analyses in blood serum for some compounds.

Overall, our results suggest that serum L-Glu levels could reflect the pathologic imbalance in excitatory neurotransmission occurring in patients with pre-clinical AD. Interestingly, the normalization of serum L-Glu levels characterizing late disease stages is paralleled by an increment in serum L-Gln concentrations. It could be hypothesized that this variation in serum L-Glu/L-Gln ratio across AD stages could mirror the extent of glutamatergic cortical neuronal loss and the in vivo adaptation of the mechanisms counteracting aberrant L-Glu release, but further investigations are required to test this hypothesis. The potential diagnostic and prognostic value of these biomarkers should be tested by cross-sectional and, hopefully, longitudinal investigations of L-Glu/L-Gln ratio and GS levels in biofluids of drug-free pre-clinical and MCI-AD patients, fully characterized on the basis of CSF biomarker profile. The analysis of the longitudinal variations of L-Glu/L-Gln ratio in serum and CSF of each AD patient could help in clarify the relationship existing between this biomarker and disease progression, and if such alterations can be detected in both biofluids. We assume that a combined analysis of these metabolites could improve the diagnostic and prognostic work-up of patients affected by AD, since the early phases of the disease.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent Informed consent was obtained from all individual participants included in the study.

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