Received: 21 February 2023 | Revised: 17 April 2023 | Accepted: 28 May 2023

DOI: 10.1111/jnc.15884

Journal of Neurochemistry JNC The Official Journal of the International Society for Neurochemistry WILEY

ORIGINAL ARTICLE

Cerebrospinal fluid, brain, and spinal cord levels of L-aspartate signal excitatory neurotransmission abnormalities in multiple sclerosis patients and experimental autoimmune encephalomyelitis mouse model

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Abstract

The neuroinflammatory process characterizing multiple sclerosis (MS) is associated with changes in excitatory synaptic transmission and altered central concentrations of the primary excitatory amino acid, L-glutamate (L-Glu). Recent findings report that cerebrospinal fluid (CSF) levels of L-Glu positively correlate with pro-inflammatory cytokines in MS patients. However, to date, there is no evidence about the relationship between the other primary excitatory amino acid, L-aspartate (L-Asp), its derivative D-enantiomer, D-aspartate, and the levels of pro-inflammatory and anti-inflammatory cytokines in the CSF of MS. In the present study, we measured by HPLC the levels of these amino acids in

Abbreviations: AST, aspartate aminotransferase; B-H, Benjamini-Hockberg; BMI, body mass index; CNS, central nervous system; CSF, cerebrospinal fluid; D-Asp, D-aspartate; EAE, experimental autoimmune encephalomyelitis; EAAT1, excitatory amino acid transporter 1; EDSS, Expanded Disability Status Scale; FGF, Fibroblast growth factor; G-CSF, Granulocyte colony-stimulating factor; Gd, gadolinium; GLAST-1, gluatamate aspartate transporter-1; hDDO, human D-aspartate oxidase; IFN-, interferon-; IL, interleukin; IL-1ra, IL-1 receptor antagonist; IP-10, Interferon-γ-induced protein 10; IQR, interquartile range; L-Asp, L-aspartate; L-Gln, L-glutamine; L-Glu, L-glutamate; LP, lumbar puncture; MCP1, Monocyte chemoattractant protein 1; MIP, macrophage inflammatory protein; MRI, magnetic resonance imaging; MS, multiple sclerosis; NAC, N-acetyl-L-cysteine; NMDAR, N-methyl-D-aspartate receptor; OCB, oligoclonal bands; OND, other neurological disorder; OPA, o-phthaldialdehyde; PDGF, Platelet-derived growth factor; RANTES, regulated upon activation, normal T cell expressed and presumably secreted; RR, relapsingremitting; SD, standard deviation; SP/PP, secondary progressive/primary progressive; TCA, trichloroacetic acid; TNF, tumor necrosis factor; VEGF, Vascular endothelial growth factor.

Francesco Errico, Luana Gilio and Andrea Mancini have contributed equally to this work.

Funding information
Fondazione Italiana Sclerosi Multipla, Grant/Award Number: 2019/S/1; Ministero della Salute, Grant/Award Number: RF-2018-12366144; Ministero dell'Istruzione, dell'Università e della Ricerca, Grant/Award Number: 2017K55HLC, 2017M42834 and 2020K53E57

the cortex, hippocampus, cerebellum, and spinal cord of mice affected by experimental autoimmune encephalomyelitis (EAE). Interestingly, in support of glutamatergic neurotransmission abnormalities in neuroinflammatory conditions, we showed reduced L-Asp levels in the cortex and spinal cord of EAE mice and increased D-aspartate/total aspartate ratio within the cerebellum and spinal cord of these animals. Additionally, we found significantly decreased CSF levels of L-Asp in both relapsing–remitting ($n=157$) MS (RR-MS) and secondary progressive/primary progressive ($n=22$) (SP/PP-MS) patients, compared to control subjects with other neurological diseases ($n=40$). Importantly, in RR-MS patients, L-Asp levels were correlated with the CSF concentrations of the inflammatory biomarkers G-CSF, IL-1ra, MIP-1β, and Eotaxin, indicating that the central content of this excitatory amino acid, as previously reported for L-Glu, reflects a neuroinflammatory environment in MS. In keeping with this, we revealed that CSF L-Asp levels were positively correlated with those of L-Glu, highlighting the convergent variation of these two excitatory amino acids under inflammatory synaptopathy occurring in MS.

KEYWORDS
aspartate, cerebrospinal fluid, inflammation, multiple sclerosis, NMDA receptors

1 | INTRODUCTION

Multiple sclerosis (MS) is an inflammatory and degenerative disease of the central nervous system (CNS) (Filippi et al., 2018). Although its etiology is still unclear, a large bulk of evidence supports a pathogenic role for diffuse immune-mediated synaptic damage contributing to disease progression and disability accumulation (Bellingacci et al., 2021; Di Filippo et al., 2018; Mandolesi, Gentile, Musella, Fresegna, et al., 2015). Consistent with this, in experimental autoimmune encephalomyelitis (EAE) animals, the most used preclinical model of MS, increased intrathecal production of pro-inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-17 (IL-17), and tumor necrosis factor (TNF), was associated with remarkable abnormalities in excitatory synaptic transmission and synaptic plasticity in different brain areas (Centonze et al., 2009; Di Filippo et al., 2013, 2021; Mandolesi, Gentile, Musella, & Centonze, 2015). Furthermore, clinical studies also showed that abnormally greater cerebrospinal fluid (CSF) levels of pro-inflammatory cytokines are associated with severe alterations in excitatory synaptic transmission and plasticity and with a higher risk of disease progression in MS patients (Rossi et al., 2012, 2014; Stampanoni Bassi et al., 2019, 2020). In line with this, independent findings unveiled the existence of a direct correlation between pro-inflammatory cytokines and L-glutamate (L-Glu) concentrations in the CSF of MS patients (Kostic et al., 2014; Stampanoni Bassi et al., 2021), further suggesting that excitatory synaptic transmission and neuroinflammatory processes are intimately linked in a complex dynamic interaction that contributes to MS clinical phenotypes and disease progression (Levite, 2017).

L-Glu and L-aspartate (L-Asp) are the most abundant excitatory amino acids in the mammalian CNS, responsible for a wide range of metabolic and physiological functions, including brain development, synaptic transmission/plasticity, and cognition (Egbenya et al., 2021; Curtis & Watkins, 1960; Zhou & Danbolt, 2014; Andersen et al., 2021).

In addition to these L-amino acids, also the right-handed derivative of L-Asp, D-Asp, is known to affect glutamatergic neurotransmission by activating N-Methyl-D-Aspartate receptors (NMDARs) at their glutamate binding site (Grasselli et al., 2013; Ota et al., 2012; Usiello et al., 2020). However, unlike L-Glu, little evidence is available on the relationship linking both Asp enantiomers with MS disability progression and central inflammation.

Here, we investigated the potential intrathecal changes of L-Asp and D-Asp during neuroinflammatory processes by assessing their levels through HPLC in the CSF of a large cohort of MS patients and different CNS regions of mice affected by EAE. Specifically, we determined the concentrations of both Asp enantiomers in the CSF of MS patients with different clinical phenotypes, compared to control subjects with non-inflammatory/non-degenerative other neurological disorders (OND). Moreover, we explored the possible correlations between the CSF Asp enantiomer levels and clinical and radiological characteristics (neurological disability, inflammatory disease activity, disease duration, and disease progression) or other biochemical CSF features (L-Glu and various pro- and anti-inflammatory cytokines levels) of MS patients. Our results indicate that intrathecal L-Asp levels positively correlate with inflammatory cytokines, suggesting the involvement of this amino acid in the modulation of inflammatory synaptopathy occurring in MS.

2 | METHODS

2.1 | Patients' enrolment and cerebrospinal fluid collection

In the present retrospective study, clinical data and CSF samples collected in a group of 179 newly diagnosed MS patients enrolled between 2017 and 2019 (Stampanoni Bassi et al., 2021) were analyzed.

This study was not pre-registered with any official preregistration sites comparable to clinicaltrials.gov for clinical studies or OSF registries. Patients were admitted to the Neurology Unit of IRCCS Neuromed in Pozzilli (IS) and later diagnosed as suffering from relapsing–remitting MS (RR-MS) and secondary progressive/primary progressive MS (SP/PP-MS). All patients underwent for diagnostic purposes blood tests, complete neurological evaluation, brain, and spinal magnetic resonance imaging (MRI) scan and CSF withdrawal within 24 h. Patients were drug-free before CSF withdrawal. Corticosteroids or other MS-specific immunoactive therapies were initiated later when appropriate. The control group comprised 40 patients with non-inflammatory/non-degenerative CNS disorders or peripheral nervous system disorders, such as vascular leukoencephalopathy, metabolic and hereditary polyneuropathies, normal pressure hydrocephalus, functional neurological disorder, and spondylotic myelopathy.

All patients and/or their legal representatives gave informed written consent to the study. The protocol was authorized by the ethics committee of IRCCS Neuromed (cod. 10–17). All procedures were carried out in accordance with the Declaration of Helsinki.

The diagnosis of RR-MS or SP/PP-MS was established by clinical, laboratory, and MRI parameters, and matched published criteria (Thompson et al., 2018). Demographic and clinical information was derived from medical records. MS disease onset was defined as the first episode of focal neurological dysfunction indicative of MS. Disease duration was estimated as the number of months from onset to the time of diagnosis. Disability was determined by a specially trained (Neurostatus training and documentation DVD for a standardized neurological examination and assessment of Kurtzke's functional systems and Expanded Disability Status Scale for MS patients. Basel, Switzerland: Neurostatus, 2006; available at http://www.neurostatus.net) and certified examining neurologist using Expanded Disability Status Scale (EDSS) (Kurtzke, 1983).

MRI examination consisted of 3 Tesla dual-echo proton density, fluid-attenuated inversion recovery (FLAIR), T2-weighted spin-echo images, and pre-contrast and post-contrast (gadolinium, Gd, 0.2 mL/Kg e.v.) T1-weighted spin-echo images. All images were acquired in the axial orientation with 3 mm-thick contiguous slices. Radiological activity was defined as the presence of Gd-enhancing lesions evaluated by a neuroradiologist who was unaware of the patients' clinical details. CSF samples were collected according to international guidelines (del Campo et al., 2012; Teunissen et al., 2009; Vanderstichele et al., 2011). Lumbar puncture (LP) was performed from 8:00 to 10:00, after an overnight fasting. CSF was immediately collected in sterile polypropylene tubes (Sarstedt® tubes, codes: 62.610.210) and gently mixed to avoid possible gradient effects. Two mL of CSF were used for total cell count. All samples were centrifuged at 2000xg 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 (cut-off of 50 red blood cells/μL). Internal quality controls were assayed in each run. Operators blinded to the diagnosis performed the measurements.

2.2 | EAE protocol

EAE was induced in six to eight-week-old wild-type C57Bl/6 male mice (Cat# 027C57BL/6, Charles River, Italy) through the subcutaneous inoculation of 200 μg MOG_{35-55} emulsified in Freund's incomplete adjuvant, supplemented with 8 mg/mL *M. tuberculosis* H37Ra at day 0 (Di Filippo et al., 2021). Mice were also intra-peritoneally injected with 500 ng of Pertussis toxin at day 0 and day 2. In the EAE group, animals were monitored and weighed daily, from day 10 post-inoculation (p.i.) onwards, to assess the development of relapsing–remitting paralysis. Clinical signs of the experimental disease were scored as follows: 0 = normal; 1 = fully flaccid tail; 2 = impaired righting reflex; 3 = hind limb paresis; 4 = complete hindlimb paresis; 5 = moribund/death (Di Filippo et al., 2021). Mice affected by the first episode of neurological deficit, suggestive of CNS inflammation (total mice subjected to EAE induction: $n = 10$; $n = 2$ animals did not develop neurological deficits and were thus excluded from the study; $n = 8$ mice developed EAE and were included in the study, mean clinical neurological score 2, day 20 p.i.) have been sacrificed by cervical dislocation for HPLC analysis, with the surgical isolation of the cortex, hippocampus, cerebellum and spinal cord, that have been snap frozen in dry-ice and stored at −80°C pending analysis. Six to eight-week-old male wild-type C57Bl/6 mice (Cat# 027C57BL/6, Charles River, Italy), subjected to the same feeding and housing conditions for the same period as EAE mice, were utilized as controls (total control mice: $n = 6$; no animal was excluded). The study involved a total number of 16 animals. Sample size calculation was not performed; the number of mice was based on a previous study of a similar nature (Musgrave et al., 2011). We performed a *post hoc* power analysis for a posteriori validation of our study, which showed an effect size of 0.8 (level of significance, $p < 0.05$). Animals were arbitrarily assigned by the researchers to the experimental groups at the beginning of the study. Animals were housed at the University of Perugia with standard housing conditions and free access to food and water (Center Authorization N: 08/2018-UT; 24/07/2018). Animals were monitored on a daily basis, assessing specific and non-specific EAE disease parameters (i.e., neurological score, body weight, fur status) with humane end-points aimed at minimizing animal suffering. All procedures involving animals were performed in conformity with the European Directive 2010/63/EU, in accordance with protocols approved by the Animal Care and Use Committee at the University of Perugia.

2.3 | Analysis of CSF pro- and anti-inflammatory cytokines

CSF samples were analyzed using a Bio-Plex multiplex cytokine assay (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. Concentrations were calculated according to a standard curve generated for the specific target and expressed as pg/mL. All samples were analyzed in triplicate. The CSF molecules examined included: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8,

IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, granulocyte colony-stimulating factor (G-CSF), eotaxin, fibroblast growth factor-basic (FGF-Basic), monocyte chemoattractant protein 1 (MCP1), interferon (IFN)-γ-induced protein 10 (IP-10), macrophage inflammatory protein-1α (MIP-1α), MIP-1β, IL-1 receptor antagonist (ra), TNF, IFN-γ, platelet-derived growth factor (PDGF)-BB, regulated upon activation, normal T cell expressed and presumably secreted (RANTES), and vascular endothelial growth factor (VEGF).

2.4 | HPLC analysis

Brain and spinal cord tissues and CSF samples deriving from EAE mice and MS patients, respectively, were analyzed as previously reported (Nuzzo et al., 2019, 2020, 2021; Palese et al., 2020; Stampanoni Bassi et al., 2021). Mice brain and spinal cord samples were homogenized in 1:10 (w/v) 0.2 M trichloroacetic acid (TCA), sonicated (3 cycles, 10 s each), and centrifuged at 13000xg for 20 min. All the precipitated protein pellets from brain and spinal cord samples were stored at −80°C for protein quantification. CSF samples (100 μL) were mixed in a 1:10 dilution with HPLC-grade methanol (900 μL) and centrifuged at 13000xg for 10 min; supernatants were dried and then suspended in 0.2 M TCA. TCA supernatants were then neutralized with NaOH and subjected to pre-column derivatization with o-phthaldialdehyde (OPA)/N-acetyl-L-cysteine (NAC). Diastereoisomer derivatives were resolved on a UHPLC Nexera system (Shimadzu, Kyoto, Japan) using a Shim-pack GIST C18 3-μm reversed-phase column (Shimadzu, 4.0×160 mm) under isocratic conditions (0.1 M sodium acetate buffer, pH 6.2, 1% tetrahydrofuran, and 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 amino acids were based on retention times and peak areas, compared with those associated with external standards. The identity of the D-Asp peak was also evaluated by selective degradation catalyzed by a recombinant human D-Aspartate oxidase (hDDO) (Katane et al., 2017, 2018). Briefly, samples were incubated with hDDO enzyme (12.5 μg) at 30°C for 3 h, and subsequently derivatized. Total protein content of brain and spinal cord tissue homogenates was determined by Bradford assay method, after solubilization of the TCA-precipitated protein pellets in 1% SDS solution. The detected amino acids concentration in tissue homogenates was normalized by the total protein content and expressed as nmol/mg protein; amino acids level in the CSF was expressed as μM. HPLC data analyses were carried out by an experimenter that was unaware of the study groups.

2.5 | Statistical analysis

Since it was a retrospective study, no sample size evaluations were performed. Clinical data and cerebrospinal fluid samples collected in a group of 179 newly diagnosed MS patients enrolled between 2017 and 2019 (previously published in doi: 10.1111/jnc.15518) were analyzed. Normality distribution was tested using the Kolmogorov–Smirnov test. Data were shown as mean (± standard deviation, SD) or, as median (± interquartile range, IQR) if not normally distributed. Categorical variables were presented as number (n). Differences in continuous variables among the two groups were evaluated by parametric *t*-test or, if necessary, non-parametric Mann–Whitney test. A *p*-value <0.05 was considered as statistically significant. Spearman's non-parametric correlation was used to test possible associations between non-parametric variables. When exploring correlations between L-Asp and various inflammatory CSF molecules, Benjamini-Hockberg (B-H) procedure was used to decrease the false discovery rate and avoid type I errors (false positives). To explore associations between L-Asp and different variables, after adjustment for possible confounding factors [i.e., age at LP, disease duration, sex, body mass index (BMI), oligoclonal band (OCB) presence, radiological disease activity], linear regression models were used. Box plot was used to depict statistically significant differences between groups. All analyses were performed using IBM SPSS Statistics for Windows (IBM Corp., Armonk, NY, USA).

3 | RESULTS

3.1 | Altered L-aspartate and D-aspartate levels in the CNS of symptomatic EAE mice

First, we measured by HPLC the levels of D-Asp and L-Asp in the cortex, hippocampus, cerebellum, and spinal cord of symptomatic EAE mice and relative controls (Figure 1a shows a representative chromatogram obtained from the cortex homogenate).

Statistical analysis revealed a reduction of both D-Asp and L-Asp levels in the cortex of EAE mice, compared to controls (median [IQR] of nmol/mg protein, D-Asp: Ctrl = 2.07 [1.58; 2.46] vs EAE = 1.17 [0.99; 1.58], $p = 0.003$; L-Asp: Ctrl = 102.20 [77.24; 114.70] vs EAE = 52.04[44.30; 62.68], $p = 0.0003$; unpaired Student's *t*-test; Figure 1b,c). In line with the reduction of both stereoisomers, we reported comparable cortical D-Asp/total Asp ratio, regarded as an index of D-Asp metabolism, in EAE mice and their controls (Ctrl = 1.99 [1.9; 2.34] vs EAE = 2.23 [2.14; 2.49], $p = 0.1533$; Figure 1d).

Conversely, D-Asp and L-Asp content was unchanged in the hippocampus (D-Asp: Ctrl = 0.92 [0.72; 1.28] vs EAE = 1.04 [0.94; 1.17], $p = 0.6856$; L-Asp: Ctrl = 46.4 [32.34; 71.37] vs EAE = 45.15 [38.33; 49.09], $p = 0.4057$; D-Asp/total Asp: Ctrl = 2.105 [1.714; 2.274] vs EAE = 2.243 [2.016; 2.522], $p = 0.1298$; Figure 1e–g) and cerebellum (D-Asp: Ctrl = 0.28 [0.23; 0.34] vs EAE = 0.31[0.19; 0.37], $p = 0.6990$; L-Asp: Ctrl = 65.11 [49.29; 80.38] vs EAE = 54.44[38.86; 69.28], $p = 0.2144$; D-Asp/total Asp: Ctrl = 0.44 [0.32; 0.54] vs EAE = 0.56 [0.48; 0.61], $p = 0.0426$; Figure 1h–j) of EAE mice, compared to controls.

Notably, we found a significant L-Asp reduction in the spinal cord of EAE mice, compared to controls, with unchanged D-Asp levels (D-Asp: Ctrl = 0.13 [0.11; 0.15] vs EAE = 0.16 [0.13; 0.20], $p = 0.1410$;

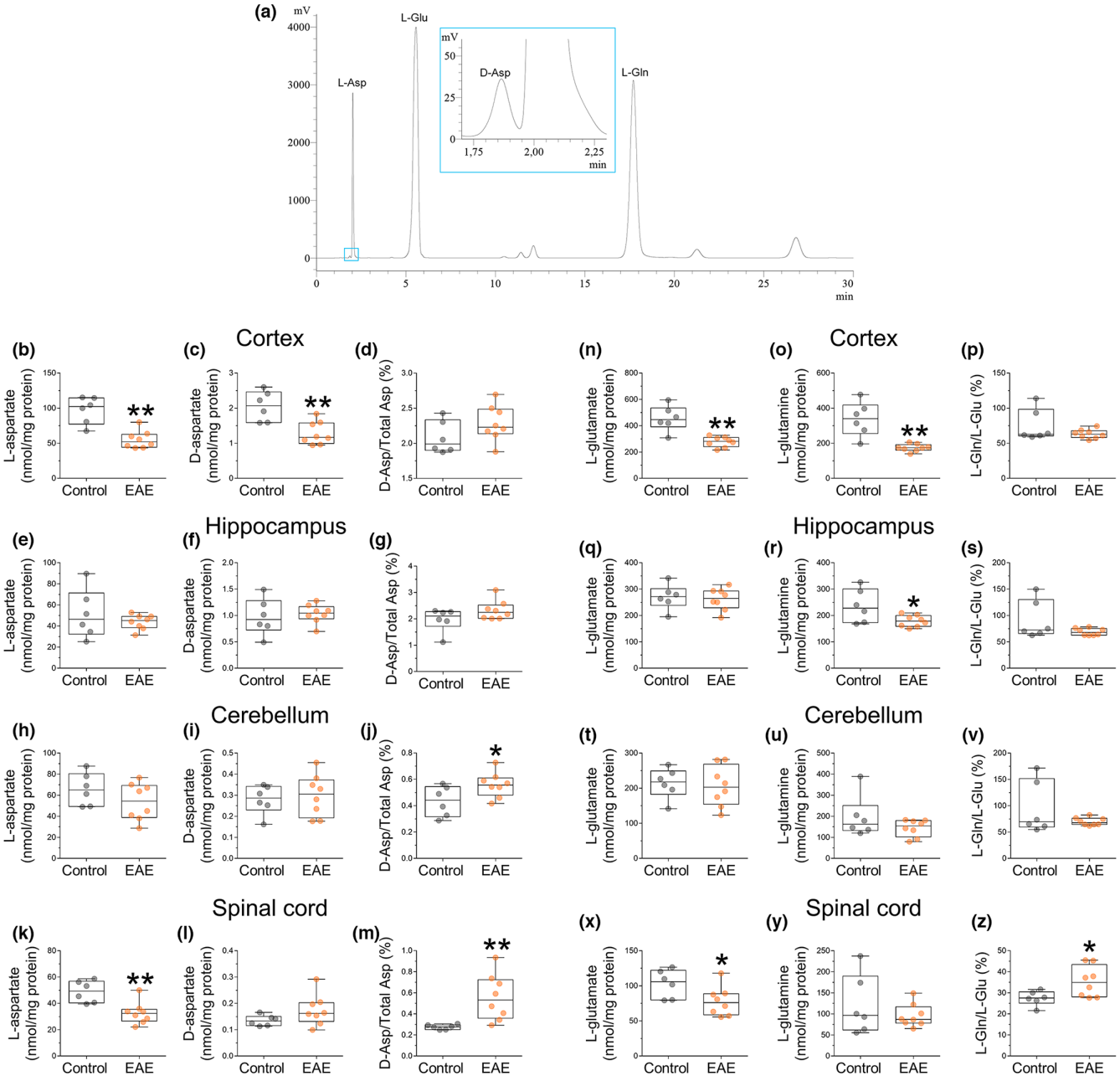

FIGURE 1 L-aspartate, D-aspartate, L-glutamate, and L-glutamine content in the CNS of symptomatic EAE mice. (a) Representative HPLC chromatogram showing L-aspartate (L-Asp), D-aspartate (D-Asp), L-glutamate (L-Glu), and L-glutamine (L-Gln) peaks obtained from the cortex homogenate of an EAE mouse. (b–m) Box and whisker plots representing (b, e, h, k) L-Asp and (c, f, i, l) D-Asp levels, and (d, g, j, m) D-Asp/total Asp ratio, (n, q, t, x) L-Glu and (o, r, u, y) L-Gln levels, and (p, s, v, z) L-Gln/L-Glu ratio in the (b–d, n–p) cortex, (e–g, q–s) hippocampus, (h–j, t–v) cerebellum, and (k–m, x–z) spinal cord of adult EAE ($n=8$) and control (Ctrl, $n=6$) mice. Box and whisker plots represent the median with an interquartile range, the dots represent individual mice values. Amino acid levels are expressed as nmol/mg protein. $^{*}p<0.05$, $^{**}p<0.01$, compared to control mice (Student's *t*-test).

L-Asp: Ctrl = 49.32 [40.36; 56.86] vs EAE = 32.32 [26.50; 35.44], $p=0.0034$; Figure 1k,l). In line with this, we observed a significantly increased D-Asp/total Asp ratio in the spinal cord of EAE mice, compared to controls (spinal cord: Ctrl = 0.28 [0.25; 0.30] vs EAE = 0.53 [0.36; 0.72], $p=0.0091$; Figure 1m). Moreover, we also reported a greater D-Asp/total Asp ratio in the cerebellum of EAE mice, compared to controls (cerebellum: Ctrl = 0.44 [0.32; 0.54] vs EAE = 0.56 [0.48; 0.61], $p=0.0464$; Figure 1j).

Next, we analyzed the levels of L-Glu and L-glutamine (L-Gln) in the CNS samples of the same EAE and control mice. HPLC data indicated a significant decrease of both L-Glu and L-Gln levels in the cortex of EAE animals, compared to controls (L-Glu: Ctrl = 445.9 [391.1; 534.7] vs EAE = 281.7 [239.8; 310], $p=0.0005$; L-Gln: Ctrl = 340.1 [255; 417.3] vs EAE = 174 [160.6; 192.2], $p=0.0006$; Figure 1n,o). Interestingly, as reported for L-Asp, we observed a reduction of L-Glu content in the spinal cord of EAE animals compared to control

mice, while L-Gln levels were unaltered (L-Glu: Ctrl = 105.8 [79.8; 122] vs EAE = 76.09 [58.7; 88.8], $p = 0.0418$; L-Gln: Ctrl = 96.6 [61.7; 189.9] vs EAE = 87.1 [78.1; 116.9], $p = 0.3849$; Figure 1x,y). A selective significant increase in the L-Gln/L-Glu ratio, regarded as an index of glutamine-glutamate cycle and glutamatergic neurotransmission (Andersen et al., 2021), was found in the spinal cord of EAE mice compared to control mice (Ctrl = 27.5 [25.08; 30.47] vs EAE = 34.89 [28.06; 43.41], $p = 0.0320$; Figure 1z). Moreover, we observed a selective reduction of L-Gln levels in the hippocampus of EAE animals (L-Gln: Ctrl = 227.9 [172.9; 300.6] vs EAE = 178.4 [158.7; 200.1], $p = 0.0332$; L-Glu: Ctrl = 271.3 [238.3; 301.9] vs EAE = 265 [229.1; 292.9], $p = 0.7410$; Figure 1q,r), and unaltered levels of both amino acids in the cerebellum of EAE mice, compared to their controls (L-Glu: Ctrl = 217.8 [182.4; 249] vs EAE = 202.8 [154.3; 268.3], $p = 0.7787$; L-Gln: Ctrl = 162.4 [132.3; 251] vs EAE = 154 [102; 179.3], $p = 0.2025$; Figure 1t,u). The L-Gln/L-Glu ratio was found as unchanged in all the investigated brain areas (cortex: Ctrl = 62.72 [60.45; 98.36] vs EAE = 62.85 [57.99; 67.78], $p = 0.1734$; hippocampus: Ctrl = 72.5 [65.6; 130.2] vs EAE = 67.99 [62.59; 75.46], $p = 0.1098$; cerebellum: Ctrl = 69.37 [59.54; 151.5] vs EAE = 67.76 [64.4; 76.24], $p = 0.1800$; Figure 1p,s,v). Overall, these data indicate the occurrence of pronounced alterations in the CNS levels of excitatory amino acids and L-Gln/L-Glu ratio in EAE condition, with a potential influence on glutamatergic neurotransmission under neuroinflammatory and neurodegenerative insults.

3.2 | Altered L-aspartate content in the CSF of MS patients

We recently showed a mild reduction of CSF L-Glu levels in MS patients, compared with OND subjects (Stampanoni Bassi et al., 2021). Here we sought to evaluate the levels of the excitatory amino acids L-Asp and D-Asp by performing HPLC analysis in the same CSF cohort of MS patients ($n = 157$ RR-MS, $n = 22$ SP/PP-MS) and OND control individuals ($n = 40$) (Table 1; Figure 2a) previously used to detect L-Glu levels (Stampanoni Bassi et al., 2021). Statistical analysis revealed a trend to decrease of L-Asp content in the CSF of the whole cohort of MS patients, compared to the OND group (median [IQR] of μM, OND = 1.62 [0.98–2.05] vs MS = 1.27 [0.83–1.79]; $p = 0.053$, Mann–Whitney test; Figure 2b). Such reduction was significant when comparing alone RR-MS (~10%) or SP/PP patients (~28%) to OND subjects (OND = 1.62 [0.98–2.05] vs RR-MS = 1.21 [0.82–1.70]; $p = 0.034$, Mann–Whitney test; OND = 1.62 [0.98–2.05] vs SP/PP-MS = 1.03 [0.80–1.59]; $p = 0.019$; Figure 2c). On the other side, CSF D-Asp levels were below the limit of HPLC detection in all MS and OND subjects analyzed. Then, we investigated the association between L-Asp levels and radiological activity in RR-MS patients, assessed by the evaluation of Gd-enhancing lesions at MRI. Despite CSF L-Asp levels showed a non-significant trend toward reduction in Gd^- patients, compared with controls, this reduction resulted as statistically significant in Gd^+ patients (OND = 1.62 [0.98–2.05] vs RR-MS Gd^+ = 1.00 [0.78–1.57], $p = 0.015$; OND = 1.62 [0.98–2.05] vs RR-MS Gd^- = 1.34 [0.84–1.84], $p = 0.123$; RR-MS Gd^+ = 1.00 [0.78–1.57] vs RR-MS Gd^- = 1.34 [0.84–1.84], $p = 0.265$; Figure 2d). Moreover, we observed that CSF L-Asp content in RR-MS patients during the active phase of the disease was slightly decreased, compared to OND subjects, while its levels were well aligned to those of RR-MS patients in stable phase (OND = 1.62 [0.98–2.05] vs stable RR-MS = 1.32 [0.83–2.01], $p = 0.146$; OND = 1.62 [0.98–2.05] vs relapse RR-MS = 1.06 [0.79–1.51], $p = 0.010$; stable RR-MS = 1.32 [0.83–2.01] vs relapse RR-MS = 1.06 [0.79–1.51], $p = 0.133$; Figure 2e).

Next, to assess whether CSF L-Asp changes were influenced by demographic and clinical features, we correlated L-Asp content with age, disease duration, EDSS at LP and 1 year after LP (Table 2). In

TABLE 1 Demographic and clinical characteristics of MS and OND patients.

Demographic/clinical characteristic	OND ($N = 40$) Median (min; max)	RR-MS ($N = 157$) Median (min; max)	SP/PP-MS ($N = 22$) Median (min; max)	Total MS ($N = 179$) Median (min; max)
Sex (Female/Male)	24/16	109/48	9/13	118/61
Age at LP (years)	41.76 (16.91; 64.86)	35.61 (16.30; 75.55)	48.62 (32.73; 72.95)	37.99 (16.3;75.55)
Radiological activity (Gd^-/Gd^+)	–	75/67	16/5	91/72
OCB presence (No/Yes)	–	37/114	2/20	39/134
MS disease duration (months)	–	6.98 (0; 371.53) $N = 150$	20.57 (6.33; 175.40)	9.83 (0.06;371.53) $N = 172$
EDSS score at LP	–	1.50 (0; 6.00)	4.25 (1.50; 6.50) $N = 20$	2 (0; 6.50) $N = 177$
EDSS score at 1 year from LP	–	1 (0; 6.50) $N = 118$	4.75 (1; 6.50) $N = 18$	1.50 (0; 6.50) $N = 136$
CSF L-glutamate (μM)[a]	7.03 (5.75; 8.83)	5.57 (4.53; 6.76)	5.71 (4.51; 6.56)	5.71 (4.76; 7.08)

Abbreviations: CSF, cerebrospinal fluid; EDSS, Expanded Disability Status Scale; Gd, gadolinium; LP, lumbar puncture; MS, multiple sclerosis; OCB, oligoclonal bands; body mass index (BMI), OND, other neurological disorder; RR, relapsing–remitting; SP/PP, secondary progressive/primary progressive.

[a]Data for L-glutamate content are referred to Stampanoni Bassi et al. (2021).

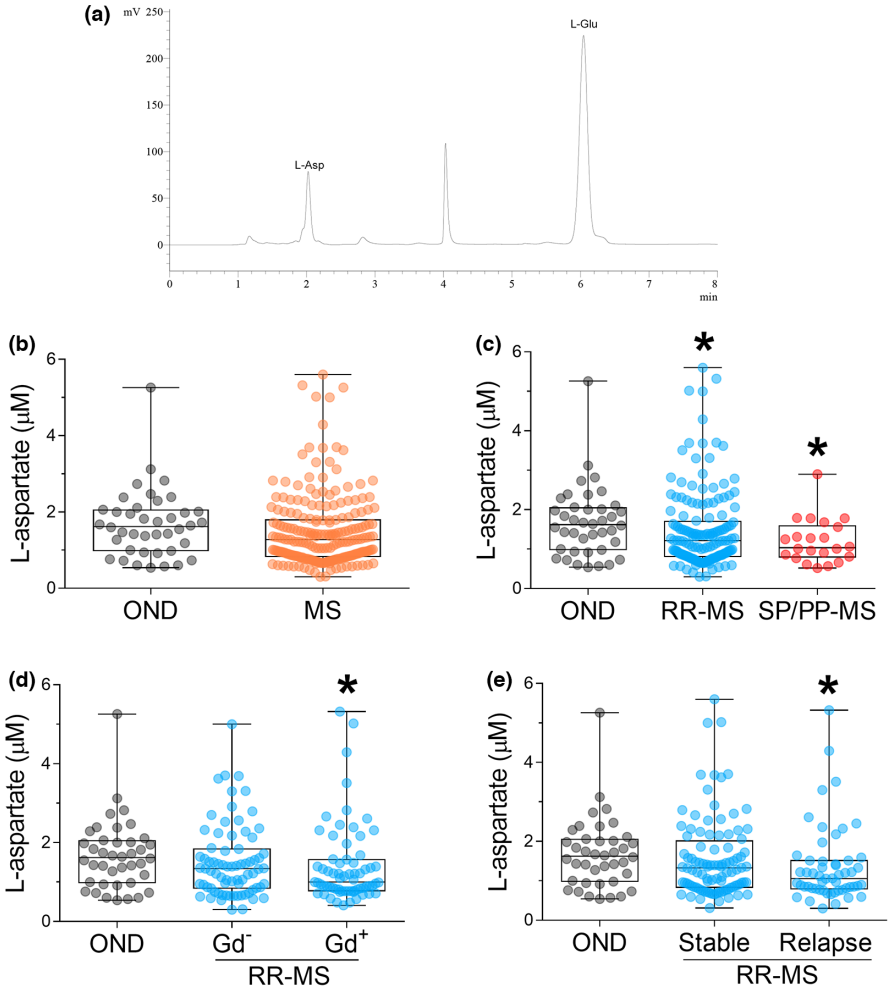

FIGURE 2 Decreased L-aspartate content in the CSF of RR-MS and SP/PP-MS patients. (a) Representative HPLC chromatogram illustrating L-aspartate (L-Asp) and L-glutamate (L-Glu) peaks obtained from the cerebrospinal fluid (CSF) sample of an OND control subject. (b–e) Box and whisker plots of L-Asp levels in (b) the whole cohort of multiple sclerosis (MS) patients ($n=179$), (c) separate cohorts of relapsing–remitting (RR-MS, $n=157$) and secondary progressive/primary progressive MS patients (SP/PP-MS, $n=22$), (d) RR-MS patients with (Gd^+, $n=67$) or without (Gd^-, $n=75$) gadolinium-enhancing lesions at magnetic resonance imaging, (e) RR-MS patients in stable phase ($n=100$) and relapse phase ($n=54$) of the disease, compared with control subjects with other neurological disorder (OND, $n=40$). Box and whisker plots represent the median with an interquartile range, and dots represent individual patients' values. L-Asp concentration is expressed as μM. *$p<0.05$, compared with OND (Mann–Whitney test).

contrast to other investigations (Sarchielli et al., 2003), we did not find any significant correlation of L-Asp amount with any of these parameters (Table 2). In particular, differently to L-Glu (Stampanoni Bassi et al., 2021), we found that L-Asp amount was not correlated with disability progression (measured as EDSS at 1-year follow-up) in both RR-MS and SP/PP-MS patients (Table 2).

3.3 | CSF L-aspartate levels correlate with inflammation biomarkers in RR-MS patients

According to the previously documented relationship between CSF levels of L-Glu and pro-inflammatory secreted molecules (Kostic et al., 2014; Stampanoni Bassi et al., 2021), we investigated the possible association between CSF L-Asp content and inflammatory cytokines concentrations using the same cohort of MS patients recently analyzed (Stampanoni Bassi et al., 2021).

Statistical analysis in OND control subjects revealed that the CSF L-Asp levels were not correlated with any of the detected cytokines (OND: G-CSF: $r=-0.1216$; $p=0.4548$; Eotaxin: $r=-0.3059$; $p=0.0549$; IL-1ra: $r=0.1024$; $p=0.5297$; MIP-1β: $r=0.05704$; $p=0.7266$; Figure 3a–d). Conversely, RR-MS patients evidenced a significant negative correlation between the CSF L-Asp levels and those of IL-1ra, an anti-inflammatory protein that antagonizes the biological effects of IL-1, or G-CSF, a pleiotropic cytokine exerting an immunoregulatory effect on adaptive immunity (Xiao et al., 2007) (IL-1ra: $r=-0.2213$; $p=0.0053$, B-H $p=0.0077$; G-CSF: $r=-0.360$; $p<0.0001$, B-H $p=0.00004$; Figure 3e,f). Moreover, we highlighted a direct correlation between L-Asp and the levels of MIP-1β, a chemotactic factor crucial for the recruitment of T cells to sites of inflammation (Maurer & von Stebut, 2004) or of eotaxin, an eosinophil-attracting protein that can also modulate lymphocyte activation (MIP-1β: $r=0.1989$; $p=0.0125$, B-H $p=0.0096$; eotaxin: $r=0.3363$; $p<0.0001$, B-H $p=0.00008$; Figure 3g,h). All these correlations remained significant also after correction for multiple comparisons and after controlling for the effect of confounding factors like age, sex, BMI, disease duration, EDSS at LP, OCB presence, and radiological disease activity (G-CSF: B = −0.014, Beta = −0.289, CI [−0.023; −0.005], $p=0.002$; IL-1ra: B = −0.008, Beta = −0.212, CI [−0.016; −0.001], $p=0.026$; MIP-1β: B = 0.057, Beta = 0.190, CI [0.0001; 0.113], $p=0.049$; Eotaxin: B = 0.515, Beta = 0.296, CI [0.160; 0.870], $p=0.005$).

In contrast to RR-MS, CSF L-Asp levels were not correlated with any of these cytokines in SP/PP-MS patients (G-CSF: $r=-0.3695$; $p=0.0906$, IL-1ra: $r=0.4661$; $p=0.0288$; MIP-1β: $r=0.4634$; $p=0.0298$; Eotaxin $r=0.3209$; $p=0.1454$; Figure 3i–l).

TABLE 2 Correlation analysis between CSF L-aspartate content and demographic and clinical parameters of MS and OND patients.

Demographic and clinical parameters	OND					RR-MS					SP/PP-MS				
		L-aspartate (μM)		L-glutamate (μM)[a]			L-aspartate (μM)		L-glutamate (μM)[a]			L-aspartate (μM)		L-glutamate (μM)[a]	
	N	r	p-value	r	p-value	N	r	p-value	r	p-value	N	r	p-value	r	p-value
Age at LP (years)	40	−0.108	0.505	−0.046	0.78	157	−0.036	0.657	0.189	0.018[b]	22	0.496	0.019[b]	0.374	0.086
MS disease duration (months)						150	−0.019	0.815	0.089	0.28	22	0.152	0.5	−0.206	0.357
EDSS score at LP						157	0.017	0.828	0.036	0.65	20	0.491	0.028[b]	0.34	0.143
EDSS score at 1 year from LP						118	0.123	0.186	0.205	**0.026**[c]	18	0.459	0.055	0.344	0.162

Note: Spearman's non-parametric correlation was used to test possible associations between variables.

Abbreviations: EDSS, Expanded Disability Status Scale; LP, lumbar puncture; MS, multiple sclerosis; OND, other neurological disorder; RR, relapsing–remitting; SP/PP, secondary progressive/primary progressive.

[a]Data for L-glutamate content are referred to Stampanoni Bassi et al. (2021).

[b]*p*-value was not significant after controlling for the effect of confounding factors.

[c]*p*-value was significant after controlling for the effect of confounding factors (in bold).

For the first time, the present data indicate that CSF levels of L-Asp are significantly associated with soluble inflammatory mediators in RR-MS, as previously described for L-Glu (Kostic et al., 2014; Stampanoni Bassi et al., 2021), supporting the hypothesis that the presence of a neuroinflammatory process might directly influence the metabolism of these excitatory amino acids.

3.4 | CSF levels of L-aspartate are directly correlated with those of L-glutamate

Next, we investigated the degree of correlation between the CSF levels of L-Asp and L-Glu in the CSF of OND, RR-MS, and SP/PP-MS patients. Interestingly, we found a significant positive correlation between L-Asp and L-Glu content in the CSF of OND ($r=0.338$, $p=0.0329$, $n=40$; Figure 4a), RR-MS and SP/PP-MS patients (RR-MS: $r=0.57$, $p<0.0001$, $n=157$; SP/PP-MS: $r=0.523$, $p=0.0154$, $n=22$; Figure 4b,c). In RR-MS and SP/PP-MS patients, these results were confirmed even after controlling for the effect of demographic and clinical confounding factors, including age, sex, BMI, disease duration, EDSS at LP and OCB presence and radiological disease activity (RR-MS: B = 0.267, Beta = 0.627, CI [0.203;0.330], $p<0.0001$; SP/PP-MS: B = 0.159, Beta = 0.874, CI [0.005;0.313], $p=0.045$). Overall, the present HPLC results are in line with the knowledge that L-Glu and L-Asp metabolism is intimately linked by transaminase activity (Andersen et al., 2021).

4 | DISCUSSION

Although there is still some controversy regarding the status of L-Asp as a neurotransmitter (Herring et al., 2015), this molecule is considered as the secondary excitatory amino acid in the CNS, with some findings suggesting that L-Asp and L-Glu may be co-released (Andersen et al., 2021; Docherty et al., 1987; Fleck et al., 1993). In particular, neuropharmacological studies indicate a role for L-Asp as a selective endogenous agonist of NMDARs, since its application in rodent brain slices elicits inward currents blocked selectively by NMDAR antagonists (Balazs et al., 2012; Patneau & Mayer, 1990). Hence, alterations of neuronal or glial L-Asp metabolism may contribute to the pathogenesis of glutamatergic synaptic dysfunction and, ultimately, excitotoxicity in MS. In agreement with this possibility, previous work has demonstrated that the enzyme aspartate aminotransferase (AST) and the malate–aspartate shuttle system, which regulate L-Asp metabolism (Satrustegui & Bak, 2015), play a critical role in the microglial pro-inflammatory activation during neuroinflammatory processes (Zhou et al., 2021). Additionally, it has been demonstrated that neuroinflammatory condition occurring in EAE mouse brain triggers reduced expression and functioning of glutamate aspartate transporter 1 (GLAST-1)/excitatory amino acid transporter 1 (EAAT1) in glial cells (Mandolesi, Gentile, Musella, Fresegna, et al., 2015), potentially altering not only L-Glu but also L-Asp and

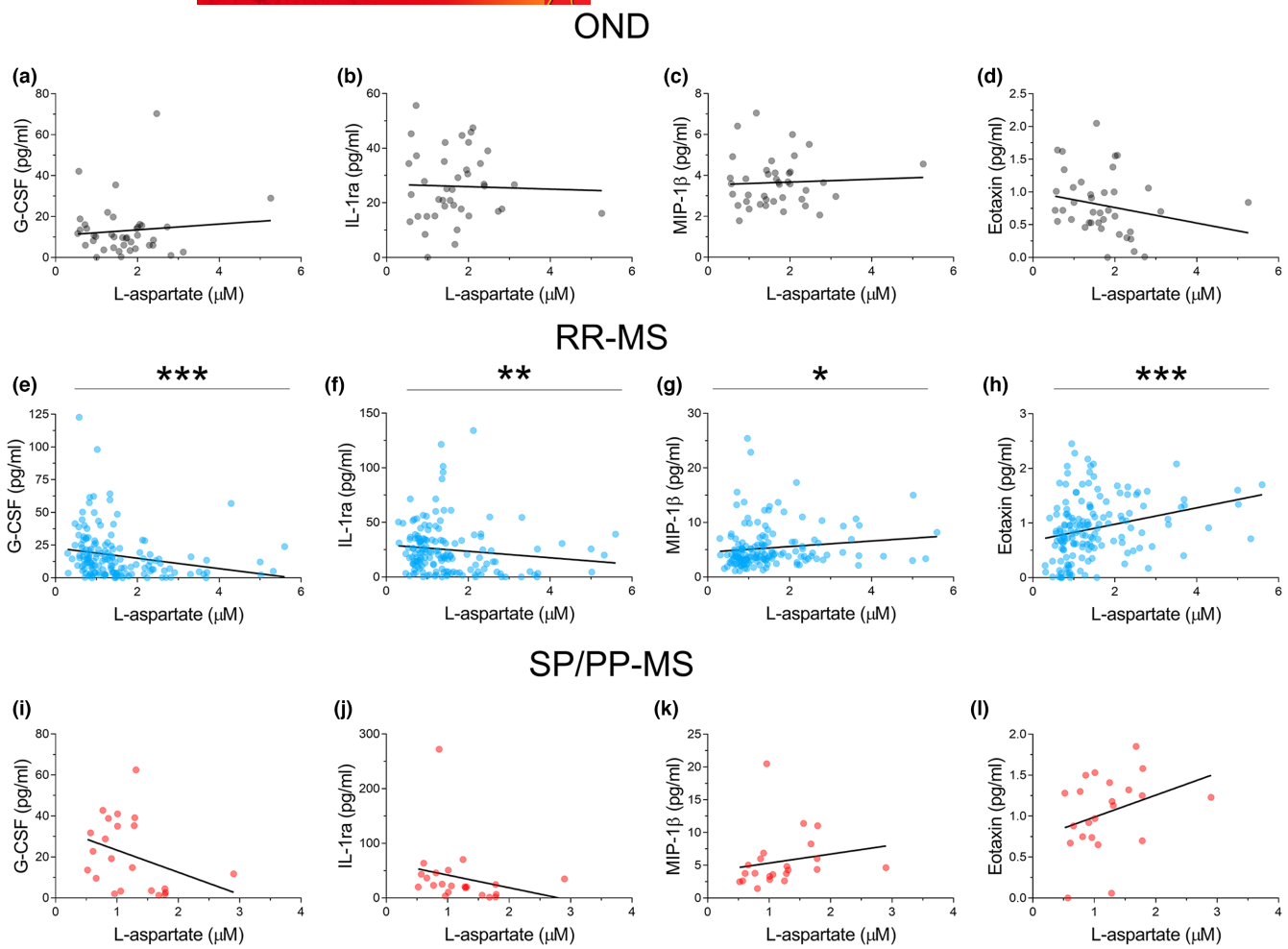

FIGURE 3 CSF L-aspartate levels signal neuroinflammation in RR-MS patients. Correlation between the CSF levels of L-aspartate and the cytokine (a, e, i) G-CSF, (b, f, j) IL-1ra, (c, g, k) MIP-1β or (d, h, l) Eotaxin in (a–d) control subjects with other neurological disorders (OND, $n=40$), patients with (e–h) relapsing–remitting (RR-MS, $n=157$) and (i–l) secondary progressive/primary progressive multiple sclerosis (SP/PP-MS, $n=22$). L-aspartate and cytokines levels are expressed as μM and pg/ml, respectively. Spearman's correlation coefficient (r) and relative p-value are shown on the top of each panel. $^{*}p<0.05$, $^{**}p<0.01$; $^{***}p<0.0001$ (linear regression after adjustment for confounding factors).

D-Asp reuptake, as EAAT system recognizes both Asp enantiomers (Palacin et al., 1998). In keeping with this, during CNS inflammatory processes, HPLC measurements showed significantly decreased L-Asp levels in the spinal cord and brainstem of EAE mice, compared to controls (Musgrave et al., 2011). Similarly, NMR spectroscopy experiments confirmed L-Asp downregulation in the spinal cord (Battini et al., 2018) and brain (Brenner et al., 1993) of animal models affected by the neuroinflammatory disorder.

In line with previous data, our HPLC results confirmed a significant reduction of L-Asp levels in the cortex and spinal cord of EAE mice, compared to controls. Noteworthy, we showed that also L-Glu levels were concomitantly reduced in both CNS regions of EAE mice. This neurochemical evidence suggests that neuroinflammatory condition in EAE CNS induces similar changes in both excitatory amino acids. The synaptic consequences induced by such alterations are surely complex and worthy of specific studies, considering both the potential effect on pre- and post-synaptic glutamatergic receptors, as well as the potential receptor desensitization and the dynamic regulation of membrane expression of glutamatergic receptors.

Nonetheless, our analysis in homogenized brain samples does not allow us to discriminate between intra- or extracellular content of L-Glu or L-Asp, and to identify a specific cell population in which such deregulations occur. Of note, the levels of L-Asp and L-Glu were not significantly different from controls in other CNS regions, such as the hippocampus and cerebellum. Such heterogeneity in amino acids concentrations in the CNS during the course of EAE has been shown also by other authors (Musgrave et al., 2011) and could rely on a variable extent of EAE-related immune infiltrates, glial activation, or neuroaxonal damage across different CNS areas. The concordance in showing a greater alteration of these excitatory amino acids in the spinal cord is indeed in line with the preferential and earlier involvement of this structure in most EAE models (Gold et al., 2006; Lassmann & Bradl, 2017). Further studies are required to characterize how the EAE pathogenic events induce L-Asp and L-Glu metabolism changes in different CNS areas.

Besides L-Glu and L-Asp, another excitatory amino acid in the atypical D-form, D-Asp, acts as an endogenous agonist at the

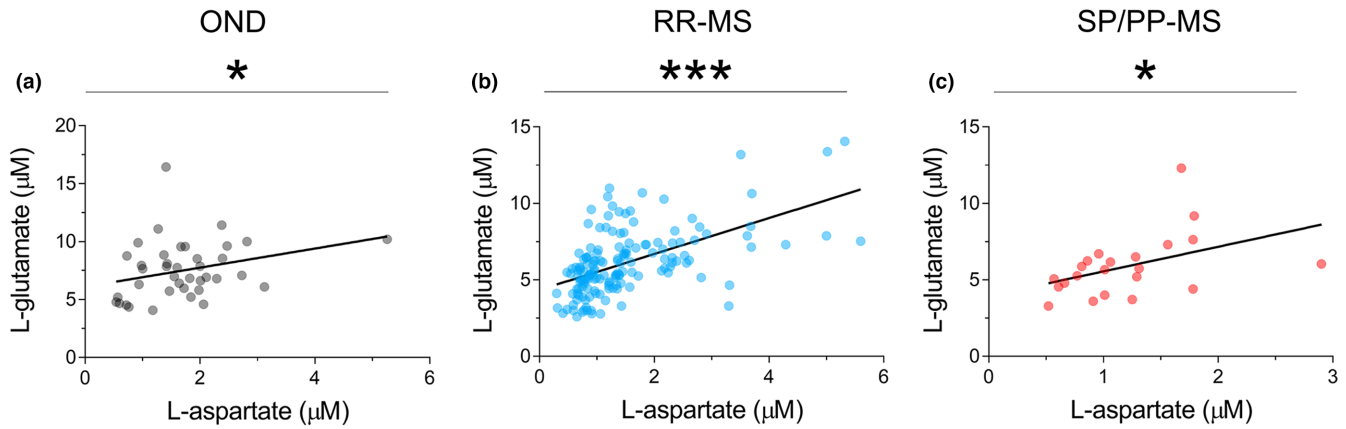

FIGURE 4 L-aspartate and L-glutamate levels are positively correlated in the CSF of MS patients. Correlation between the CSF levels of L-aspartate and L-glutamate in (a) control subjects with other neurological disorders (OND, $n=40$), patients with (b) relapsing–remitting (RR-MS, $n=157$), and (c) primary progressive/secondary progressive multiple sclerosis (SP/PP-MS, $n=22$). L-aspartate and L-glutamate levels are expressed as μM. Spearman's correlation coefficient (r) and relative *p-value* are shown on the top of each panel. OND: $^{*}p<0.05$ (Spearman's correlation); RR-MS and SP/PP: $^{*}p<0.05$, $^{***}p<0.0001$ (linear regression after adjustment for confounding factors).

glutamate binding site of NMDARs (Errico et al., 2020). Based on its neuropharmacological features, previous works have demonstrated that D-Asp treatment enhances glutamatergic cortical transmission and slightly mitigates fatigue symptoms in progressive MS patients (Nicoletti et al., 2020), while in animal models of MS, it delays the onset of motor symptoms, attenuates their severity, promotes myelin recovery and decreases IL-6 serum levels (Afraei et al., 2017; de Rosa et al., 2019). On the contrary, exaggerated high levels of D-Asp in a genetic mouse model lacking the DDO enzyme that selectively catabolizes this NMDAR agonist, exacerbate EAE symptoms (Grasselli et al., 2013). Interestingly, our HPLC analysis unveiled for the first time a significant reduction of D-Asp levels within the cortex of EAE mice, suggesting a brain-specific alteration in the metabolism of this neuroactive amino acid. This evidence may explain the beneficial effect of its supplementation previously reported in EAE condition (Afraei et al., 2017; de Rosa et al., 2019). Differently from the brain tissue, CSF D-Asp levels were below the HPLC detection limit in the whole cohort of MS patients tested. Therefore, the present data indicate that HPLC might not be sensitive enough to quantify D-Asp in the CSF of MS patients.

Notably, our preclinical observations indicating concomitant L-Asp and L-Glu reductions in the cortex and spinal cord of EAE mice are in line with present and previous HPLC analyses reporting, respectively, reduced L-Asp and L-Glu levels (Stampanoni Bassi et al., 2021) in the CSF of a common cohort of RR-MS and SP-PP/MS patients, compared with OND controls. Similarly, other independent studies reported decreased CSF levels of L-Asp and/or L-Glu in MS patients (Garseth et al., 2001; Qureshi & Baig, 1988). However, it remains still puzzling whether CSF concentrations of these excitatory amino acids represent per se a reliable biochemical signature of MS given that other previous works revealed unaltered or increased L-Asp and/or L-Glu levels in the CSF of MS patients (Klivenyi et al., 1997; Launes et al., 1998; Sarchielli et al., 2003; Stover et al., 1997). However, differences in age, gender, disease duration, sample sizes and criteria of patients' enrollment, and/or the substantial variability in L-Asp and L-Glu levels measured in our and others' neurological control groups may account for such neurochemical discrepancies. Also, CSF sampling site, diet, and circadian rhythms may contribute to the reported differences among studies (Arciero et al., 2008; Janssens et al., 2019); therefore, in the present work, to avoid possible confounding influences on metabolite levels from these factors, the LP procedures in MS patients and OND were performed from 8:00 to 10:00 am, after overnight fasting.

Our results also showed a mild selective reduction of CSF L-Asp levels in RR-MS patients with evidence of radiological activity, defined as Gd-enhancing lesions, or acute clinical relapses compared to OND individuals. Considering the key role of L-Asp in the regulation of energy homeostasis processes (i.e., gluconeogenesis and malate–aspartate shuttle), we speculate that such slight reduction may reflect enhanced central utilization of this amino acid because of the greater metabolic expenditure occurring under acute inflammation, defined either radiologically or clinically. Moreover, it should be noted that people with stable MS or without Gd-enhancing lesions showed a non-significant trend of reduced L-Asp levels in CSF with respect to OND. This could be explained by the presence of a gradient of active inflammation between ONDs, stable/Gd- MS, and active/Gd + MS that results in a more pronounced alteration of L-Asp metabolism in the case of new focal brain lesions. Indeed, MS patients with no Gd-enhancing lesions or acute clinical manifestations are nonetheless characterized by the presence of a subtle intrathecal inflammatory process. However, further clinical investigations are warranted to clarify this issue.

Immunoinflammatory processes in MS are intimately associated with the activation of the glutamatergic system in the CNS (Levite, 2017). Consistent with this knowledge, a significant positive correlation between L-Glu levels and IL-17-mediated inflammatory and excitotoxic events was reported in the CSF of RR-MS patients (Kostic et al., 2014). In line with this, we have previously reported a strong negative correlation between CSF L-Glu and

anti-inflammatory cytokine IL-1ra concentrations in the CSF of RR-MS patients (Stampanoni Bassi et al., 2021). Here, using the same cohort of subjects tested in this previous work (Stampanoni Bassi et al., 2021), we assessed whether also L-Asp levels might be influenced by central inflammatory environments by analyzing the relationship between CSF L-Asp and different pro- and anti-inflammatory cytokines concentrations. Remarkably, we found that CSF L-Asp levels of RR-MS patients are positively correlated with pro-inflammatory cytokines MIP-1β and Eotaxin, and negatively correlated with anti-inflammatory IL-1ra, as previously reported for L-Glu (Stampanoni Bassi et al., 2021). Taken together, the present and previous findings (Kostic et al., 2014; Stampanoni Bassi et al., 2021) highlight that both CSF L-Glu and L-Asp levels change during the neuroinflammatory process characterizing RR–MS, with a direct correlation with the amount of inflammatory mediators produced. Accordingly, we documented a direct correlation between L-Asp and L-Glu levels in the CSF of MS patients and OND controls, which is also in line with their metabolic interaction through the AST pathway (Andersen et al., 2021).

Intriguingly, previous data obtained in the same cohort of MS patients unveiled that L-Glu levels detected at MS diagnosis correlate with EDSS after 1 year of follow-up, thus suggesting that the levels of this excitatory neurotransmitter predict disability worsening (Stampanoni Bassi et al., 2021). Although other investigations are mandatory, differently from L-Glu, CSF levels of L-Asp were not significantly correlated with EDSS. Altogether, these findings highlight a distinct involvement of L-Asp and L-Glu in modulating disability progression.

In conclusion, our results indicate that CSF L-Asp and L-Glu levels reliably signal CNS events linked to inflammation-mediated excitatory synaptopathy in MS patients.

AUTHOR CONTRIBUTIONS

FE: Drafting/revision of the manuscript; interpretation of data; LG: acquisition of data; AM: generation of EAE mice and dissection; drafting/revision of the manuscript for content, interpretation of data; TN: acquisition of data; MSB: Drafting/revision of the manuscript, interpretation of data; LB: generation of EAE mice and dissection; FB: acquisition of data; ED: acquisition of data; AB: acquisition of data; GG: acquisition of data; RF: acquisition of data; AF: acquisition of data; ADM acquisition of data; MDF: Drafting/revision of the manuscript for content, including medical writing for content, Analysis or interpretation of data; DC: Study concept; Analysis and interpretation of data, Drafting/revision of the manuscript for content, including medical writing for content; AU: Study concept; Analysis and interpretation of data, writing the manuscript.

ACKNOWLEDGMENTS

We thank Alessia Casamassa, Mattia Miroballo, Arianna De Rosa, Giorgia Donati, Giulia Sansone, Giada Torresi, and Silvia Santangeli for their valuable technical support. We thank Hiroshi Homma and Masumi Katane for the generous gift of an aliquot of the hDDO recombinant enzyme.

All experiments were conducted in compliance with the ARRIVE guidelines.

FUNDING INFORMATION

The study was supported by Ministero della Salute (Ministry of Health, Italy): Diego Centonze; Ministero della Salute (Ministry of Health, Italy): Fabio Buttari GR-2018-12366154; Ministero della Salute (Ministry of Health, Italy): Progetto Ricerca Corrente to IRCCS Neuromed; Fondazione Italiana Sclerosi Multipla (FISM): Diego Centonze cod. 2019/S/1 and financed or co-financed with the '5 per mille' public funding; Project 'Nuovi Biomarker Diagnostici e Terapeutici delle Malattie Neurodegenerative'—ADOPT co-funded by FOE 2020—funding from CNR to Diego Centonze. A.U. was supported by #NEXTGENERATIONEU (NGEU) and funded by the Ministry of University and Research (MUR), National Recovery and Resilience Plan (NRRP), project MNESYS (PE0000006) – A Multiscale integrated approach to the study of the nervous system in health and disease (DN. 1553 11.10.2022).

CONFLICT OF INTEREST STATEMENT

The authors declare the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: F.B. acted as an Advisory Board member of Teva and Roche and received honoraria for speaking or consultation fees from Merck Serono, Teva, Biogen Idec, Sanofi, and Novartis and non-financial support from Merck Serono, Teva, Biogen Idec, and Sanofi. R.F. received honoraria for serving on scientific advisory boards or as a speaker from Biogen, Novartis, Roche, and Merck and funding for research from Merck. D.C. is an Advisory Board member of Almirall, Bayer Schering, Biogen, GW Pharmaceuticals, Merck Serono, Novartis, Roche, Sanofi-Genzyme, Teva, Protagon, Sandoz, Bristol-Myers Squibb, and Alexion and received honoraria for speaking or consultation fees from Almirall, Bayer Schering, Biogen, GW Pharmaceuticals, Merck Serono, Novartis, Roche, Sanofi-Genzyme, and Teva. He is also the principal investigator in clinical trials for Bayer Schering, Biogen, Merck Serono, Mitsubishi, Novartis, Roche, Sanofi-Genzyme, and Teva. His preclinical and clinical research was supported by grants from Bayer Schering, Biogen Idec, Celgene, Merck Serono, Novartis, Roche, Sanofi-Genzyme, and Teva. A.M. participated on advisory boards for, and received writing honoraria and travel grants from Almirall, Biogen, Merck, Mylan, Novartis, Sanofi. M.D.F. participated on advisory boards for and received speaker or writing honoraria, funding for traveling, and research support from Alexion, Bayer, Biogen Idec, Sanofi, Siemens Healthineers, Merck, Mylan, Novartis, Roche, Teva, and Viatris. The other authors declare that no conflict of interest exists.

DATA AVAILABILITY STATEMENT

The datasets used and analyzed in the current study are available from the corresponding authors upon reasonable request.

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How to cite this article: Errico, F., Gilio, L., Mancini, A., Nuzzo, T., Bassi, M. S., Bellingacci, L., Buttari, F., Dolcetti, E., Bruno, A., Galifi, G., Furlan, R., Finardi, A., Di Maio, A., Di Filippo, M., Centonze, D., & Usiello, A. (2023). Cerebrospinal fluid, brain, and spinal cord levels of L-aspartate signal excitatory neurotransmission abnormalities in multiple sclerosis patients and experimental autoimmune encephalomyelitis mouse model. *Journal of Neurochemistry*, *166*, 534–546. https://doi.org/10.1111/jnc.15884