Received: 24 June 2021 | Revised: 15 September 2021 | Accepted: 17 September 2021

DOI: 10.1111/jnc.15518

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

ORIGINAL ARTICLE

Cerebrospinal fluid levels of L-glutamate signal central inflammatory neurodegeneration in multiple sclerosis

Mario Stampanoni Bassi[1] | Tommaso Nuzzo[2,3] | Luana Gilio[1] | Mattia Miroballo[4] | Alessia Casamassa[4] | Fabio Buttari[1] | Paolo Bellantonio[1] | Roberta Fantozzi[1] | Giovanni Galifi[1] | Roberto Furlan[5] | Annamaria Finardi[5] | Arianna De Rosa[2,3] | Anna Di Maio[3,6] | Francesco Errico[3,7] | Diego Centonze[1,8] | Alessandro Usiello[2,3]

[1]Unit of Neurology, IRCCS Neuromed, Pozzilli (IS), Italy

[2]Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, University of Campania "Luigi Vanvitelli", Caserta, Italy

[3]CEINGE Biotecnologie Avanzate, Naples, Italy

[4]IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy

[5]Clinical Neuroimmunology Unit, Division of Neuroscience, Institute of Experimental Neurology, San Raffaele Scientific Institute, Milan, Italy

[6]Department of Experimental Medicine, Sapienza University of Rome, Rome, Italy

[7]Department of Agricultural Sciences, University of Naples "Federico II", Portici, Italy

[8]Department of Systems Medicine, University of Rome Tor Vergata, Rome, Italy

Correspondence
Diego Centonze, Unit of Neurology, IRCCS Neuromed, Via Atinense, 18, 86077 Pozzilli (IS), Italy.
Emails: centonze@uniroma2.it; diego.centonze@neuromed.it

Funding information
The study was supported by: FISM grants [Fondazione Italiana Sclerosi Multipla-cod. 2019/S/1 to D.C.]; National funding of the Italian Ministry of Universities and Research [MIUR-PRIN 2017-cod. 2017K55HLC to D.C.; MIUR-PRIN 2017-Project nr 2017M42834 to A.U., T.N., and A.D.R.]; and of the Italian Ministry of Health [RF-2018-12366144 to D.C.; Ricerca corrente and '5 per mille' public funding to IRCCS Neuromed]; and Fondazione Cariplo grant [Project nr 2017–0575 to A.U. and T.N.].

Abstract

Excessive extracellular concentrations of L-glutamate (L-Glu) can be neurotoxic and contribute to neurodegenerative processes in multiple sclerosis (MS). The association between cerebrospinal fluid (CSF) L-Glu levels, clinical features, and inflammatory biomarkers in patients with MS remains unclear. In 179 MS patients (relapsing remitting, RR, $N = 157$; secondary progressive/primary progressive, SP/PP, $N = 22$), CSF levels of L-Glu at diagnosis were determined and compared with those obtained in a group of 40 patients with non-inflammatory/non-degenerative disorders. Disability at the time of diagnosis, and after 1 year follow-up, was assessed using the Expanded Disability Status Scale (EDSS). CSF concentrations of lactate and of a large set of pro-inflammatory and anti-inflammatory molecules were explored. CSF levels of L-Glu were slightly reduced in MS patients compared to controls. In RR-MS patients, L-Glu levels correlated with EDSS after 1 year follow-up. Moreover, in MS patients, significant correlations were found between L-Glu and both CSF levels of lactate and the inflammatory molecules interleukin (IL)-2, IL-6, and IL-1 receptor antagonist. Altered expression of L-Glu is associated with disability progression, oxidative stress, and inflammation. These findings identify CSF L-Glu as a candidate neurochemical marker of inflammatory neurodegeneration in MS.

Abbreviations: AMPARs, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid/kainate receptors; BMI, body mass index; CNS, central nervous system; CSF, cerebrospinal fluid; EAE, experimental autoimmune encephalomyelitis; EDSS, Expanded Disability Status Scale; FLAIR, fluid-attenuated inversion recovery; GLAST, glutamate aspartate transporter; IFN-γ, interferon-γ; IL, interleukin; IL-1ra, IL-1 receptor antagonist; L-Glu, L-glutamate; LP, Lumbar puncture; MIP-1a, macrophage inflammatory protein 1-alpha; MS, multiple sclerosis; NAC, N-acetyl-L-cysteine; NMDARs, N-methyl-D-aspartate receptors; OCB, oligoclonal bands; OPA, o-phthaldialdehyde; OPCs, oligodendrocytes precursor cells; PP, primary progressive; RR, relapsing remitting; SP, secondary progressive; TNF, tumor necrosis factor.

Mario Stampanoni Bassi, Tommaso Nuzzo and Luana Gilio contributed equally to this work.

KEYWORDS
cerebrospinal fluid, glutamate, inflammation, lactate, multiple sclerosis

1 | INTRODUCTION

Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system (CNS) (Levite, 2017). Neuroinflammation is a hallmark of MS, characterized by abundant release of cytokines, such as interleukin-1β (IL-1β), interferon-γ (IFN-γ), and tumor necrosis factor (TNF) by activated T lymphocytes, astrocytes, and microglia (Kothur et al., 2016). Inflammatory cytokines influence brain function by altering neuronal synaptic transmission and plasticity (Stampanoni Bassi et al., 2017). Increased cerebrospinal fluid (CSF) levels of inflammatory cytokines induce profound alterations of glutamatergic neurotransmission both in experimental autoimmune encephalomyelitis (EAE) models and in MS patients (Centonze et al., 2009; Kostic et al., 2014; Levite, 2017; Mandolesi et al., 2013; Rossi et al., 2012, 2014).

Besides its main role in regulating neuronal development, differentiation, and function, L-Glu released by neurons activates α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid/kainate receptors (AMPARs), and N-methyl-D-aspartate receptors (NMDARs) expressed by myelin-producing oligodendrocytes precursor cells (OPCs), influencing their proliferation, differentiation, and myelination capacity (Bonetto et al., 2020; Fields et al., 2002; Gautier et al., 2015; Kolodziejczyk et al., 2010). While physiological signaling through L-Glu receptors is fundamental for the proper development and function of gray and white matter, an abnormal increase in extracellular concentrations of L-Glu trigger axonal damage as well as neuronal and oligodendrocyte death, contributing to neurological impairment in MS (Levite, 2017; Stojanovic et al., 2014). Consistent with this, administration of AMPAR and NMDAR antagonists attenuates synaptic hyperexcitability, excitotoxic neurodegeneration and motor deficits in EAE mice (Centonze et al., 2009).

These findings functionally link aberrant activation of L-Glu receptors with clinical features of MS, however, it remains unclear whether the concentration of L-Glu in the CSF represents a suitable biochemical signature of MS. In fact, while some reports have shown either comparable or reduced levels of L-Glu in the CSF of MS-affected subjects compared to control patients (Gårseth et al., 2001; Klivényi et al., 1997; Launes et al., 1998; Qureshi et al., 1988; Rossi et al., 2014), others have documented increased amounts of this amino acid (Sarchielli et al., 2003; Stover et al., 1997).

Based on the discrepancies among studies to date, here we sought to evaluate the concentrations of L-Glu in the CSF of a large cohort of MS patients with different clinical phenotypes (relapsing remitting (RR)-MS, secondary progressive/primary progressive (SP/PP)-MS) compared to patients with other non-inflammatory/non-degenerative disorders. We also explored potential correlations between the CSF levels of L-Glu and those of various pro- and anti-inflammatory molecules.

2 | MATERIALS AND METHODS

2.1 | Patients enrollment and cerebrospinal fluid collection

A group of 179 consecutive MS patients enrolled between 2017 and 2019 participated in this cohort study. No randomization was performed to allocate subjects in the study. Inclusion criteria were the established MS diagnosis and the ability to provide written informed consent to the study. The study was not pre-registered. Patients were admitted to the Neurology Unit of IRCCS Neuromed in Pozzilli (IS) and later diagnosed as suffering from RR and SP/PP-MS. All patients underwent for diagnostic purposes blood tests, complete neurological evaluation, brain and spinal MRI scan and CSF withdrawal within 24 h. No patients were treated with corticosteroids or other MS-specific immunoactive therapies before CSF withdrawal and neurophysiologic evaluation. Such medications were started later, if appropriate. The control group comprised 40 patients with non-inflammatory/non-degenerative CNS disorders or peripheral nervous system disorders, such as vascular leukoencephalopathy ($N = 21$ patients), metabolic and hereditary polyneuropathies ($N = 4$), normal pressure hydrocephalus ($N = 2$), pseudotumor cerebri ($N = 3$), functional neurological disorder ($N = 5$), migraine ($N = 1$), and spondylotic myelopathy ($N = 4$).

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 (CE number 6/17). All procedures were carried out in accordance with approved guidelines.

The diagnosis of RR-MS or 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 three Tesla dual-echo proton density, fluid-attenuated inversion recovery (FLAIR), T2-weighted spin-echo images and pre-contrast and post-contrast 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 gadolinium (Gd) (0.2 ml/Kg e.v.) 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., 2012). Lumbar puncture (LP) was performed from 8:00 to 10:00, after an overnight fasting. 2 ml of CSF sample were used for biochemistry analysis including total cell count and lactate levels. CSF was immediately collected in sterile polypropylene tubes (Sarstedt® tubes, codes: 62.610.210) and gently mixed to avoid possible gradient effects. All samples were centrifuged at 2000 *g* for 10 min at room temperature and then aliquoted in 0.5 ml aliquots in sterile polypropylene tubes (Sarstedt® tubes, codes: 72.730.007). Aliquots were frozen at −80°C pending analysis, avoiding freeze/thaw cycles. Blood-contaminated samples were excluded from the analysis (cut-off of 50 red blood cells per microliter). Internal quality controls were assayed in each run. Operators blinded to the diagnosis performed the measurements.

2.2 | HPLC detection of CSF L-glutamate levels

CSF samples were analyzed as previously reported (Nuzzo et al., 2021), with minor modifications. The HPLC system comprised a Shimadzu Nexera X2 ultra high-performance liquid chromatography system. 100 μl CSF sample were mixed with 900 μl HPLC-grade methanol and centrifuged at 13 000 *g* for 10 min. The supernatants were then dried and suspended in 0.2 M TCA. CSF supernatants were then neutralized with 0.2 M NaOH and subjected to precolumn derivatization with o-phthaldialdehyde (OPA)/N-acetyl-L-cysteine (NAC) in 50% methanol. Amino acids derivatives were then resolved on a Simmetry C8 5 μm reversed-phase column (Waters, 4.6 × 250 mm), in isocratic conditions (0.1 M sodium acetate buffer, pH 6.2, 1% tetrahydrofuran, 1 ml/min flow rate). A washing step in 0.1 M sodium acetate buffer, 3% tetrahydrofuran and 47% acetonitrile, was performed after every single run. Identification and quantification of L-Glu was based on retention time (L-Glu = 10.8 ± 0.1 min) and peak areas, compared with those associated with external standard. L-Glu levels were expressed as μM concentration.

2.3 | Analysis of CSF inflammatory molecules

CSF samples were analyzed using a Bio-Plex multiplex cytokine assay (Bio-Rad Laboratories), according to the manufacturer's instructions. Concentrations were calculated according to a standard curve generated for the specific target and expressed as picograms/milliliter. 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-1 receptor antagonist (ra), TNF, macrophage inflammatory protein 1-alpha (MIP-1a), IFN-γ.

2.4 | Statistical analysis

The 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 two groups were evaluated by parametric t-test or, if necessary, non-parametric Mann–Whitney test. A p value ≤ 0.05 was considered statistically significant. Spearman's non-parametric correlation was used to test possible associations between non-parametric variables. When exploring correlations between L-Glu 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-Glu and different variables, after adjustment for possible confounding factors (i.e. age at LP, disease duration, sex, radiological disease activity), linear regression models were used. Box plot was used to depict statistically significant differences between groups. No test for outliers was conducted.

In this exploratory study, predetermined primary and secondary outcomes were not used. Sample size calculation was not performed; the number of participants was based on previous studies of a similar nature (Sarchielli et al., 2003; Stover et al., 1997). All analyses were performed using IBM SPSS Statistics for Windows (IBM Corp.). The data that support the findings of this study are available from the corresponding author upon reasonable request.

3 | RESULTS

3.1 | HPLC determination of L-glutamate levels in the CSF of MS and control patients

Here, by HPLC analysis we determined the CSF levels of L-Glu in a group of 179 MS patients ($n = 157$ RR-MS, $n = 22$ SP/PP) and 40 control individuals with other non-inflammatory/non-degenerative disorders (clinical and demographic characteristics shown in Table 1). First, we explored the correlations between L-Glu concentration and demographic characteristics at the time of LP in both MS and control patient groups. Our results showed that L-Glu levels did not significantly change with aging in control patients ($r = -0.042$; $p = 0.793$; $N = 40$ patients; Figure 1a). Also, in the control group no significant correlations emerged between L-Glu CSF concentrations and other demographic characteristics, such as sex and body mass index (BMI). In the whole cohort of MS patients we reported a significant correlation between CSF L-Glu content and age ($r = 0.191$; $p = 0.010$; $N = 179$; Figure 1b), while no significant correlation emerged with sex, BMI, and disease duration. In addition, a significant association was found between CSF L-Glu amounts and oligoclonal bands (OCB) presence in MS patients (OCB+median [IQR] of μM = 5.46 [4.54–7.14] vs. OCB- median [IQR] of μM = 6.24 [5.39–7.41]; $p = 0.014$).

When comparing CSF L-Glu concentrations in MS patients and controls, statistical analysis showed a mild L-Glu reduction in the CSF of whole cohort of MS patients compared with controls (median [IQR]

TABLE 1 Demographic and clinical characteristics of multiple sclerosis and control subjects

		Multiple sclerosis		
Demographic and clinical characteristics	**Controls ($N = 40$) median (IQR)**	**RR-MS ($n = 157$) median (IQR)**	**SP/PP-MS ($n = 22$) median (IQR)**	**Total MS ($n = 179$) median (IQR)**
Gender (Female/Male)	24/16	109/48	9/13	118/61
Age at LP (years)	41.76 (33.59–49.04)	35.61 (27.34–45.99)	48.62 (38.41–56.79)	37.99 (28.62–47.32)
BMI	23.89 (21.44–26.73) $n = 37$	24.44 (21.80–28.67) $n = 136$	26.21 (22.9–29.51) $n = 20$	24.67 (21.98; 28.84) $n = 156$
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 (1.70–36.64) $n = 150$	20.57 (11.08–50.8)	9.83 (2.20–36.74) $n = 172$
EDSS score at LP	–	1.50 (1–2.5)	4.25 (2.12–5.37) $n = 20$	2 (1–3) $n = 177$
EDSS score at 1 year from LP	–	1 (1–2) $n = 118$	4.75 (2.75–6) $n = 18$	1.50 (1–2) $n = 136$
CSF lactate (mmol/l)	1.40 (1.3–1.6) $n = 39$	1.50 (1.3–1.7) $n = 153$	1.55 (1.4–1.8)	1.50 (1.4–1.7) $n = 175$

Abbreviations: BMI, body max index; CSF, cerebrospinal fluid; EDSS, expanded disability status scale; Gd, gadolinium; LP, lumbar puncture; MS, multiple sclerosis; *n*, number of patients; OCB, oligoclonal bands; PP, primary progressive; RR, relapsing-remitting; SP, secondary progressive.

of μM, Ctrl = 7.03 [5.75–8.83] vs. MS = 5.71 [4.76–7.08]; $p < 0.01$; Figure 1c), while comparable levels were found between RR-MS and SP/PP-MS patients (RR-MS median [IQR] of μM = 5.73 [4.76–7.13] vs. SP/PP-MS median [IQR] of μM = 5.71 [4.51–6.55]; Figure 1d).

Of interest, in RR-MS patients, similar L-Glu CSF levels were detected regardless their radiological activity (Gd+ median [IQR] of μM = 5.66 [4.8–6.74] vs. Gd- median [IQR] of μM = 5.72 [4.66–7.31]; Figure 1e).

3.2 | L-glutamate concentration correlates with EDSS after 1 year follow-up in RR-MS patients

While in RR-MS patients no significant association emerged between CSF L-Glu concentration and EDSS at the time of LP ($r = 0.037$, $p = 0.648$; $N = 157$; Figure 2a), we found a significant correlation between this neurotransmitter and EDSS after 1 year follow-up ($r = 0.205$; $p = 0.026$; $N = 118$; Figure 2b). This association was significant after correcting for all other clinical and demographic variables (age, gender, disease duration, radiological activity, EDSS at LP, and OCB presence) (Beta = 0.244; CI 0.054–0.201; $p = 0.001$). In contrast, no significant correlation between L-Glu CSF levels and EDSS at the time of LP ($r = 0.339$, $p = 0.143$; $N = 20$) or EDSS after 1 year ($r = 0.344$, $p = 0.162$; $N = 18$; Figure 2c,d) was observed in SP/PP-MS group.

3.3 | L-glutamate directly correlates with lactate levels in the CSF of MS patients

Statistical analysis indicated no significant differences in CSF lactate concentrations between MS patients and control subjects (Ctrl = 1.4 [1.3–1.6] vs. MS = 1.5 [1.4–1.7]; $p = 0.116$). Also comparing RR-MS, SP/PP-MS, and control patients no changes were found in CSF lactate concentrations. In the controls, a significant correlation emerged between CSF lactate and age at LP ($r = 0.440$, $p = 0.005$; $N = 40$, whereas no significant correlation was found between L-Glu and lactate ($r = 0.105$; $p = 0.523$; Figure 3a). As found in controls, also in the whole MS group a significant correlation emerged between CSF lactate and age at LP ($r = 0.298$, $p < 0.001$). Interestingly, a significant positive correlation was found between L-Glu CSF levels and lactate in the whole cohort of MS patients ($r = 0.175$; $p = 0.02$; $N = 175$; Figure 3b). This association was significant also controlling for the effect of age, gender, disease duration, EDSS at LP, and OCB presence (Beta =0.205, CI 0.006–0.040, $p = 0.008$). On the other hand, no significant correlations were observed between CSF L-Glu and lactate concentration, when analyzing separately RR-MS patients and SP/PP-MS patients.

3.4 | L-glutamate concentration is associated with IL-2, IL-6, and IL-1ra in MS patients

Finally, in MS patients we explored the existence of possible correlations between the CSF L-Glu concentration and the levels of a set of proinflammatory and anti-inflammatory cytokines.

We found that CSF L-Glu levels positively correlated with inflammatory cytokines IL-2 ($r = 0.239$; $p = 0.003$, B-H $p = 0.01$; Figure 4a) and IL-6 ($r = 0.253$; $p = 0.001$, B-H $p = 0.006$; Figure 4b) after correction for multiple comparisons. In addition, in RR-MS patients we observed a negative correlation between L-Glu and IL-5 ($r = -0.246$; $p = 0.002$, B-H $p = 0.009$; Figure 4c) or IL-1ra ($r = -0.375$; $p < 0.0001$, B-H $p = 0.0009$; Figure 4d), after correction for multiple comparisons. No other significant correlations were found between L-Glu and CSF inflammatory molecules in the RR-MS group

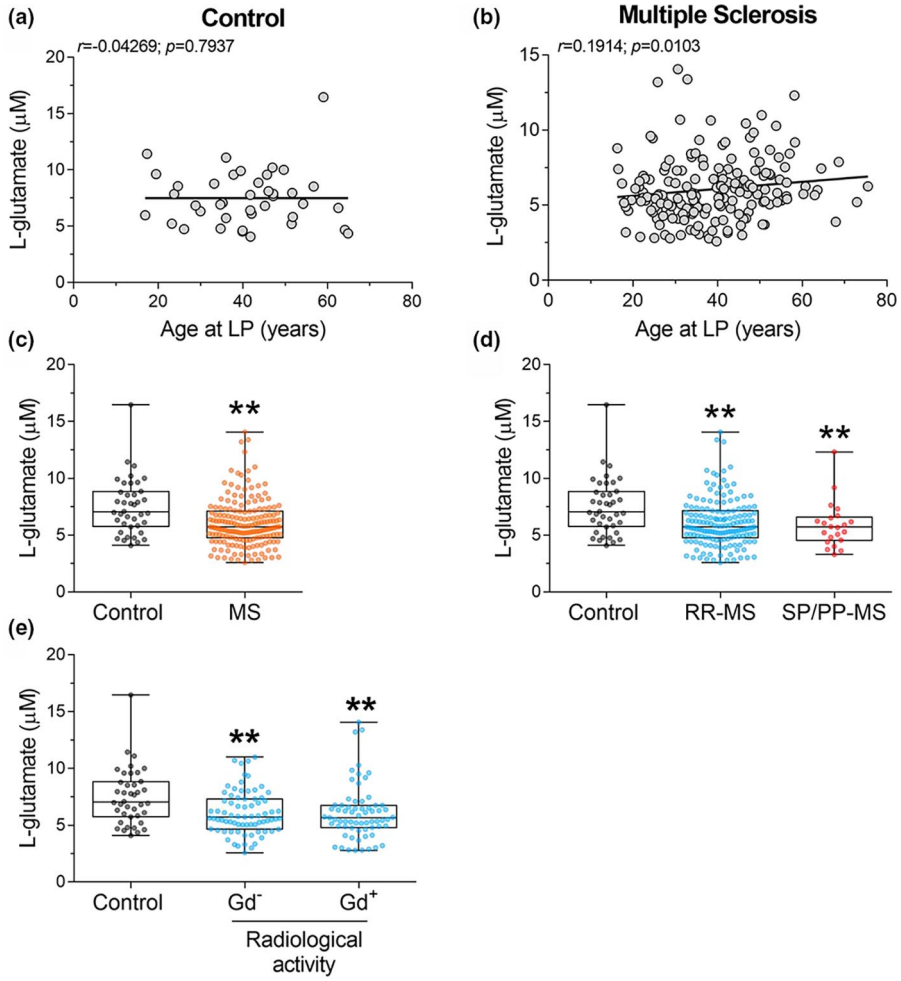

FIGURE 1 Analysis of L-glutamate levels in the CSF of MS and control patients ($N = 40$ Ctrl; $N = 179$ MS). Panel (a, b): Correlation analysis between CSF concentrations of L-glutamate and age at the time of LP in controls (a) and in the whole cohort of MS patients (b). Panel (c): L-glutamate CSF levels in control patients and in the whole cohort of MS patients. Panel (d): L-glutamate CSF levels in controls ($N = 40$), RR-MS ($N = 157$), and SP/PP-MS ($N = 22$) patients. Panel (e): L-glutamate CSF levels in controls ($N = 40$) and in RR-MS patients with (Gd^+, $N = 67$) or without (Gd^-, $N = 75$) gadolinium enhancing lesions at MRI. **<0.01 (Mann–Whitney test), compared to control group. Abbreviations: CSF, cerebrospinal fluid; Gd, gadolinium; LP, lumbar puncture; MRI, magnetic resonance imaging; MS, multiple sclerosis; RR, relapsing remitting; SP/PP, secondary progressive/primary progressive

(Supplementary Figure, panel a). Linear regressions confirmed significant correlations between L-Glu and IL-2 (Beta = 0.528, CI 0.176–0.318, $p < 0.001$), IL-6 (Beta = 0.132, CI 0.089–0.613, $p = 0.009$), IL-5 (Beta = −0.248, CI −0.479 to −0.092, $p = 0.004$), and IL-1ra (Beta = −0.277, CI −4.917 to −1.148, $p = 0.002$) also controlling for the effect of age, gender, disease duration, EDSS at LP, OCB presence, and radiological disease activity.

Similar to what was observed for RR-MS, also in SP/PP-MS patients, we found significant positive correlations between L-Glu and IL-6 ($r = 0.640$, $p = 0.001$, B-H $p = 0.009$; Figure 4g), along a negative correlation between the concentrations of this excitatory amino acid and those of IL-1ra ($r = -0.583$, $p = 0.004$, B-H $p = 0.024$; Figure 4i) and IL-8 ($r = -0.675$, $p = 0.001$, B-H $p = 0.009$; Figure 4j) after correction for multiple comparisons. Conversely, correlation between L-Glu and IL-2 in SP/PP-MS patients was not significant after controlling for multiple comparisons ($r = 0.478$; $p = 0.024$, B-H $p = 0.086$; Figure 4f). No other significant correlations were found between L-Glu and CSF inflammatory molecules in the SP/PP-MS group (Supplementary Figure, panel b).

4 | DISCUSSION

It is well-established that CSF inflammation negatively influences the disease course in MS patients, being associated with increased prospective neurodegeneration and disability (Rossi et al., 2014; Stampanoni Bassi et al., 2019). In animal models and in MS patients it has been consistently demonstrated that inflammation alters glutamatergic transmission in several brain areas, promoting neuronal hyperexcitability and excitotoxic damage (Stampanoni Bassi et al., 2017). In vivo brain evidence of elevated glutamate in MS using MRI (Srinivasan et al., 2005; Tisell et al., 2013), along immunohistochemical investigation in *post-mortem* specimens confirmed

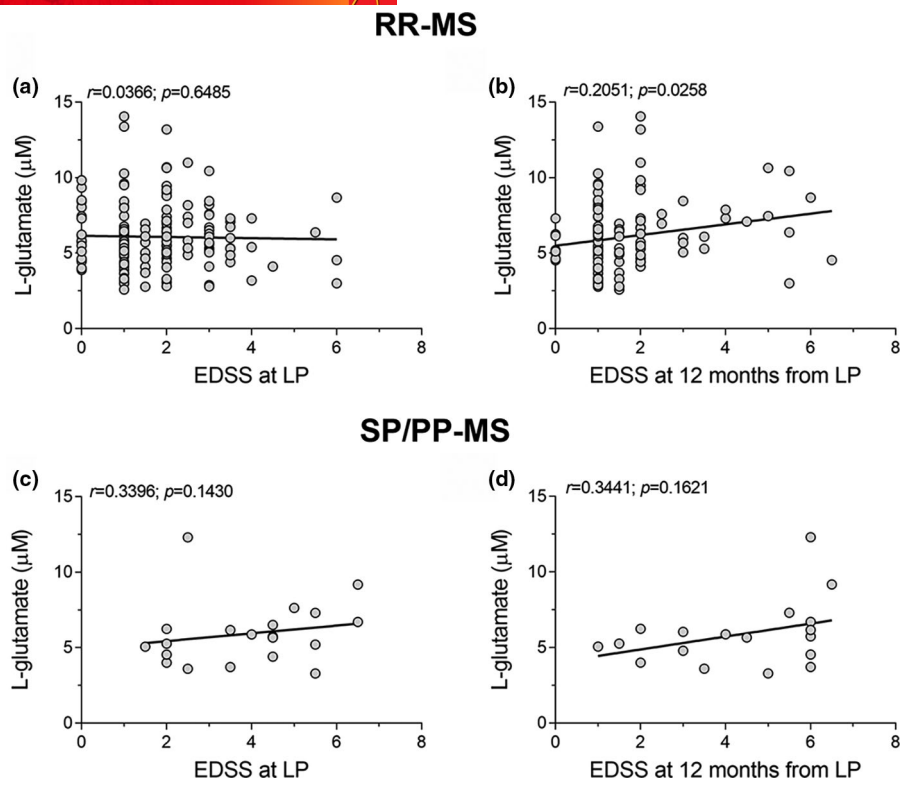

FIGURE 2 Association between L-Glutamate CSF concentration and EDSS in RR-MS and SP/PP-MS patients. Panels (a, b): Correlation between L-Glutamate CSF levels and EDSS at the time of LP (N= 157) and EDSS after 12 months from LP (N = 118) in the RR-MS group. Panels (c, d): Correlation between L-Glutamate CSF levels and EDSS at the time of LP (N = 20) and EDSS after 12 months from LP (N = 18) in the SP/PP-MS group. Abbreviations: CSF, cerebrospinal fluid; EDSS, expanded disability status scale; LP, lumbar puncture; MS, multiple sclerosis; RR, relapsing remitting; SP/PP, secondary progressive/primary progressive

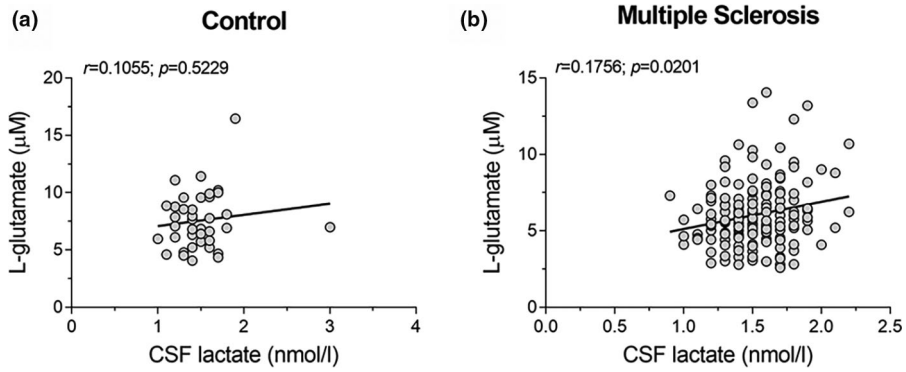

FIGURE 3 Analysis of CSF lactate levels in MS and control patients. Correlation analysis between CSF concentrations of lactate and L-glutamate in controls (N = 40; panel a) and in the whole MS cohort (N = 175; panel b). Abbreviations: CSF, cerebrospinal fluid; MS, multiple sclerosis

homeostasis dysfunction of L-Glu transmission within the CNS (Werner et al., 2001). In agreement with this, decrease in L-Glu transporters together with defects in enzymes regulating L-Glu metabolism in activated microglia and astrocytes localized within demyelinating lesions and in the surrounding areas, have been reported in EAE model and in MS patients (Levite, 2017; Stojanovic et al., 2014). Although these findings strongly indicate a substantial increase in glutamatergic signaling within demyelinating lesions of MS patients it remains unclear whether glutamate homeostasis dysregulation could directly impact on its CSF levels. In fact, data from the literature remain highly controversial.

Consistent with other reports (Gårseth et al., 2001; Klivényi et al., 1997; Kostic et al., 2014; Launes et al., 1998; Qureshi et al., 1988), we failed to find higher levels of L-Glu in the CSF of RR-MS and SP/PP-MS patients, when compared to controls. In the attempt to reconcile the different observations reported by others

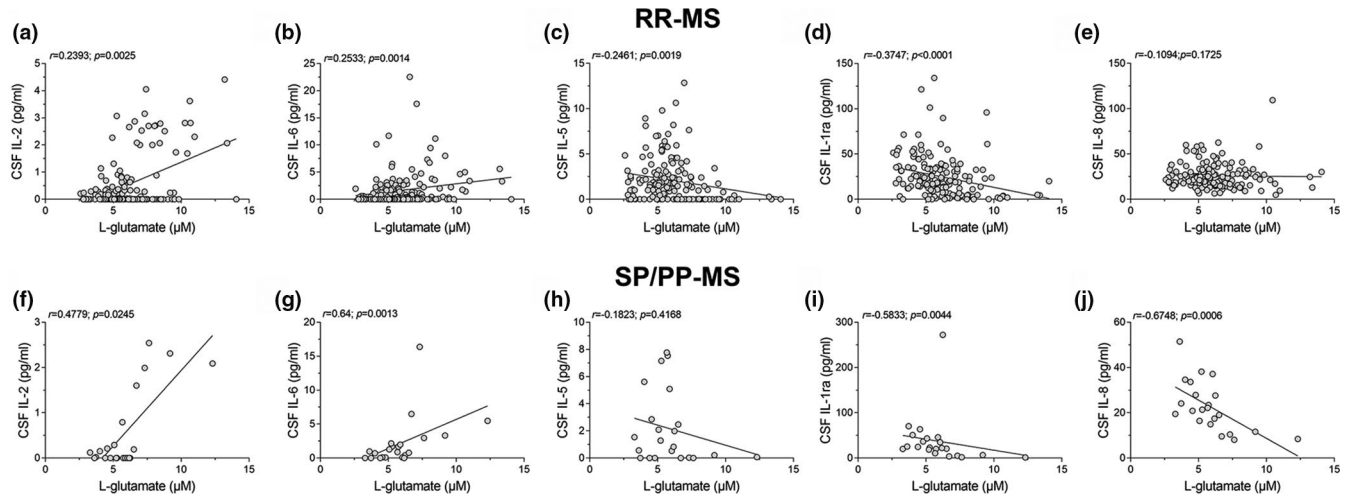

FIGURE 4 Association of L-glutamate content with inflammatory cytokines in the CSF of MS patients. Panels (a–e): Correlation between CSF L-glutamate levels and cytokines IL-2, IL-6, IL-1ra, IL-8, and IL-5 in RR-MS patients ($N = 157$). Panels (f–j): Correlation between CSF L-glutamate levels and cytokines IL-2, IL-6, IL-1ra, IL-8, and IL-5 in SP/PP-MS patients ($N = 22$). Abbreviations: CSF, cerebrospinal fluid; IL, interleukin; MS, multiple sclerosis; RR, relapsing remitting; SP/PP, secondary progressive/primary progressive

(Sarchielli et al., 2003; Stover et al., 1997), it is important to consider that any L-Glu measurement in the CSF mirrors a complex equilibrium of its cellular synthesis, catabolism, and transport between the blood, the CSF and the CNS. Furthermore, L-Glu levels are also affected by metabolic processes in the body and cerebral tissues as well as by Blood Brain Barrier leakage, reported in MS (Minagar et al., 2003). Therefore, CSF glutamate levels might be significantly affected by several factors. Also, we argue that a not negligible source of variability among studies is linked to the clinical features of control patients because subjects affected by non-inflammatory/non-degenerative neurological disorders suffer from heterogeneous clinical diseases that may involve glutamatergic dysfunction, this potential bias may directly influence the comparisons performed with MS patient group. Notably, taking into account this caveat, we documented that L-Glu concentrations in our control patients were considerably greater compared to those indicated by others (Sarchielli et al., 2003; Stover et al., 1997). Yet, other factors that may also contribute to explain the divergent published data are the difference in samples size and age of MS patients enrolled among distinct studies. Although our group of 179 MS patients is larger than all of those used in previous HPLC determination of L-Glu levels in the CSF these factors gave cause for concern, and suggest that highly controlled studies on large cohort of patients are warranted to investigate this issue.

Analysis of the association between the levels of L-Glu and clinical/demographic characteristics of RR-MS patients revealed that L-Glu at diagnosis was positively correlated with EDSS after 1 year of follow-up. This result suggests that L-Glu concentrations might predict disability worsening, however, the short duration of follow-up and lack of prospective radiological measures represent two major limitations of the present investigation, and further studies are needed to better elucidate the impact of L-Glu CSF levels at diagnosis on the course of MS disease.

To better characterize the pathophysiological role of dysfunctional L-Glu transmission in MS, here we explored the correlation between L-Glu and CSF biomarkers of oxidative stress and inflammation. Mitochondrial dysfunction and oxidative stress may contribute to MS progression by promoting axonal and neuronal damage (Campbell et al., 2011; Su et al., 2013; Trapp et al., 2009). It has been proposed that CSF lactate may represent a suitable biomarker of disease severity in MS (Albanese et al., 2016). Notably, a study involving 118 MS patients showed that higher CSF lactate levels were associated with enhanced long-term disability and increased levels of neurofilament light protein in the CSF (Albanese et al., 2016). Remarkably, here we found a significant direct correlation between CSF L-Glu and lactate concentrations in the whole cohort of MS patients. These findings mirror those obtained in EAE mice, showing that excessive activation of L-Glu receptors was associated with enhanced mitochondrial oxidative stress and neuronal swelling (Levite, 2017; Stover et al., 1997).

The relationship between altered CSF levels of L-Glu, clinical features, and levels of central inflammation represents an open question. One study previously reported a correlation between CSF L-Glu and the CSF levels of the proinflammatory molecule IL-17 in MS patients (Kostic et al., 2014). Here, we documented that in the CSF of MS patients L-Glu concentrations positively correlated with the levels of specific proinflammatory cytokines IL-2 and IL-6, while negatively correlated with the anti-inflammatory cytokine IL-5 and the competitive inhibitor IL-1ra. No other significant correlations were found with the other CSF molecules analyzed. Remarkably, IL-2 and IL-6 represent major proinflammatory molecules previously associated with MS pathogenesis (Kimura et al., 2017; Kothur et al., 2016; Maimone et al., 1997). Particularly, it has been shown that IL-6 impaired synaptic plasticity and exacerbated disease course in MS

(Stampanoni Bassi et al., 2019). IL-1β is another important inflammatory molecule involved in MS pathogenesis (Lin et al., 2017). IL-1β activities are mediated by the IL-1 receptor type 1, which also represents the site of binding of the competitive inhibitor IL-1ra (Dinarello, 2002). Increased expression of IL-1β has been previously associated with increased neurodegeneration in MS (Rossi et al., 2014). We previously documented that IL-1β promotes neuronal swelling along with synaptic hyperexcitability, enhancing glutamatergic transmission, and reducing GABAergic transmission in EAE and MS (Centonze et al., 2009; Rossi et al., 2011, 2012). In the cerebellum of EAE mice, IL-1β alters the expression of the glutamate aspartate transporter (GLAST), reducing glutamate reuptake, and leading to excitotoxic neuronal damage (Mandolesi e al., 2013). The present findings, in line with preclinical evidence in EAE, support the role of inflammation in excitotoxic neurodegeneration in MS.

Our results suggest that L-Glu may negatively influence disability progression in MS by interacting with fundamental mechanisms, such as oxidative stress and inflammation.

ACKNOWLEDGMENTS

We thank Giorgia Donati, Giulia Sansone, Giada Torresi, and Silvia Santangeli for the valuable technical support.

CONFLICT OF INTEREST DISCLOSURE

The authors declare the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: FB acted as Advisory Board members 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. RFu received honoraria for serving on scientific advisory boards or as a speaker from Biogen, Novartis, Roche, and Merck and funding for research from Merck. DC is an Advisory Board member of Almirall, Bayer Schering, Biogen, GW Pharmaceuticals, Merck Serono, Novartis, Roche, Sanofi-Genzyme, and Teva 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. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. MSB, TN, LG, MM, AC, PB, RFA, GG, AF, ADR, ADM, FE, and AU: nothing to report.

AUTHOR CONTRIBUTIONS

Conceived and designed study: MSB, TN, LG, DC, AU. Performed the experiments: MM, AC, RFu, AF, ADR, ADM and FE. Acquisition of data: LG, FB, RFa, PB and GG. Analysis of data: MSB and TN. Interpreted the data: MSB, TN, LG, DC and AU. Prepared the manuscript: MSB, TN and LG. Corrected and modified the manuscript: all authors. The authors read and approved the final manuscripts.

OPEN RESEARCH BADGES

This article has received a badge for *Open Materials* because it provided all relevant information to reproduce the study in the manuscript. More information about the Open Science badges can be found at https://cos.io/our-services/open-science-badges/

DATA AVAILABILITY STATEMENT

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

ORCID

Diego Centonze https://orcid.org/0000-0002-8390-8545

REFERENCES

Albanese, M., Zagaglia, S., Landi, D., Boffa, L., Nicoletti, C. G., Marciani, M. G., Mandolesi, G., Marfia, G. A., Buttari, F., Mori, F., & Centonze, D. (2016). Cerebrospinal fluid lactate is associated with multiple sclerosis disease progression. *Journal of Neuroinflammation*, 13, 36. https://doi.org/10.1186/s12974-016-0502-1.

Bonetto, G., Kamen, Y., Evans, K. A., & Káradóttir, R. T. (2020). Unraveling myelin plasticity. *Frontiers in Cellular Neuroscience*, 14, 156. https://doi.org/10.3389/fncel.2020.00156.

Campbell, G. R., & Mahad, D. J. (2011). Mitochondria as crucial players in demyelinated axons: Lessons from neuropathology and experimental demyelination. *Autoimmune Diseases*, 2011, 262847, https://doi.org/10.4061/2011/262847.

Centonze, D., Muzio, L., Rossi, S., Cavasinni, F., De Chiara, V., Bergami, A., Musella, A., D'Amelio, M., Cavallucci, V., Martorana, A., Bergamaschi, A., Cencioni, M. T., Diamantini, A., Butti, E., Comi, G., Bernardi, G., Cecconi, F., Battistini, L., Furlan, R., & Martino, G. (2009). Inflammation triggers synaptic alteration and degeneration in experimental autoimmune encephalomyelitis. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 29(11), 3442–3452. https://doi.org/10.1523/JNEUROSCI.5804-08.2009.

del Campo, M., Mollenhauer, B., Bertolotto, A., Engelborghs, S., Hampel, H., Simonsen, A. H., Kapaki, E., Kruse, N., Le Bastard, N., Lehmann, S., Molinuevo, J. L., Parnetti, L., Perret-Liaudet, A., Sáez-Valero, J., Saka, E., Urbani, A., Vanmechelen, E., Verbeek, M., Visser, P. J., & Teunissen, C. (2012). Recommendations to standardize preanalytical confounding factors in Alzheimer's and Parkinson's disease cerebrospinal fluid biomarkers: an update. *Biomarkers in Medicine*, 6(4), 419–430. https://doi.org/10.2217/bmm.12.46.

Dinarello, C. A. (2002). The IL-1 family and inflammatory diseases. *Clinical and Experimental Rheumatology*, 20(5 Suppl 27), S1–S13.

Fields, R. D., & Stevens-Graham, B. (2002). New insights into neuron-glia communication. *Science (New York, N.Y.)*, 298(5593), 556–562. https://doi.org/10.1126/science.298.5593.556.

Gårseth, M., White, L. R., & Aasly, J. (2001). Little change in cerebrospinal fluid amino acids in subtypes of multiple sclerosis compared with acute polyradiculoneuropathy. *Neurochemistry International*, 39(2), 111–115. https://doi.org/10.1016/s0197-0186(01)00020-1.

Gautier, H. O., Evans, K. A., Volbracht, K., James, R., Sitnikov, S., Lundgaard, I., James, F., Lao-Peregrin, C., Reynolds, R., Franklin,

R. J., & Káradóttir, R. T. (2015). Neuronal activity regulates remyelination via glutamate signalling to oligodendrocyte progenitors. *Nature Communications*, 6, 8518. https://doi.org/10.1038/ncomms9518.

Kimura, A., Takemura, M., Saito, K., Serrero, G., Yoshikura, N., Hayashi, Y., & Inuzuka, T. (2017). Increased cerebrospinal fluid progranulin correlates with interleukin-6 in the acute phase of neuromyelitis optica spectrum disorder. *Journal of Neuroimmunology*, 305, 175–181. https://doi.org/10.1016/j.jneuroim.2017.01.006.

Klivényi, P., Kékesi, K., Juhász, G., & Vécsei, L. (1997). Amino acid concentrations in cerebrospinal fluid of patients with multiple sclerosis. *Acta Neurologica Scandinavica*, 95(2), 96–98. https://doi.org/10.1111/j.1600-0404.1997.tb00076.x.

Kolodziejczyk, K., Saab, A. S., Nave, K. A., & Attwell, D. (2010). Why do oligodendrocyte lineage cells express glutamate receptors? *F1000 Biology Reports*, 2, 57. https://doi.org/10.3410/B2-57.

Kostic, M., Dzopalic, T., Zivanovic, S., Zivkovic, N., Cvetanovic, A., Stojanovic, I., Vojinovic, S., Marjanovic, G., Savic, V., & Colic, M. (2014). IL-17 and glutamate excitotoxicity in the pathogenesis of multiple sclerosis. *Scandinavian Journal of Immunology*, 79(3), 181–186. https://doi.org/10.1111/sji.12147.

Kothur, K., Wienholt, L., Brilot, F., & Dale, R. C. (2016). CSF cytokines/chemokines as biomarkers in neuroinflammatory CNS disorders: A systematic review. *Cytokine*, 77, 227–237. https://doi.org/10.1016/j.cyto.2015.10.001.

Kurtzke, J. F. (1983). Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology*, 33(11), 1444–1452. https://doi.org/10.1212/wnl.33.11.1444.

Launes, J., Sirén, J., Viinikka, L., Hokkanen, L., & Lindsberg, P. J. (1998). Does glutamate mediate brain damage in acute encephalitis? *NeuroReport*, 9(4), 577–581. https://doi.org/10.1097/00001756-199803090-00003.

Levite, M. (2017). Glutamate, T cells and multiple sclerosis. *Journal of Neural Transmission*, 124(7), 775–798. https://doi.org/10.1007/s00702-016-1661-z.

Lin, C. C., & Edelson, B. T. (2017). New insights into the role of IL-1β in experimental autoimmune encephalomyelitis and multiple sclerosis. *The Journal of Immunology*, 198(12), 4553–4560. https://doi.org/10.4049/jimmunol.1700263.

Maimone, D., Guazzi, G. C., & Annunziata, P. (1997). IL-6 detection in multiple sclerosis brain. *Journal of the Neurological Sciences*, 146(1), 59–65. https://doi.org/10.1016/s0022-510x(96)00283-3.

Mandolesi, G., Musella, A., Gentile, A., Grasselli, G., Haji, N., Sepman, H., Fresegna, D., Bullitta, S., De Vito, F., Musumeci, G., Di Sanza, C., Strata, P., & Centonze, D. (2013). Interleukin-1β alters glutamate transmission at purkinje cell synapses in a mouse model of multiple sclerosis. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 33(29), 12105–12121. https://doi.org/10.1523/JNEUROSCI.5369-12.2013.

Minagar, A., & Alexander, J. S. (2003). Blood-brain barrier disruption in multiple sclerosis. *Multiple Sclerosis (Houndmills, Basingstoke, England)*, 9(6), 540–549. https://doi.org/10.1191/1352458503ms965oa.

Nuzzo, T., Mancini, A., Miroballo, M., Casamassa, A., Di Maio, A., Donati, G., Sansone, G., Gaetani, L., Paoletti, F. P., Isidori, A., Calabresi, P., Errico, F., Parnetti, L., & Usiello, A. (2021). 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. *Amino Acids*, 53(3), 435–449. https://doi.org/10.1007/s00726-021-02943-7.

Qureshi, G. A., & Baig, M. S. (1988). Quantitation of free amino acids in biological samples by high-performance liquid chromatography. Application of the method in evaluating amino acid levels in cerebrospinal fluid and plasma of patients with multiple sclerosis. *Journal of Chromatography*, 459, 237–244. https://doi.org/10.1016/s0021-9673(01)82032-x.

Rossi, S., Furlan, R., De Chiara, V., Motta, C., Studer, V., Mori, F., Musella, A., Bergami, A., Muzio, L., Bernardi, G., Battistini, L., Martino, G., & Centonze, D. (2012). Interleukin-1β causes synaptic hyperexcitability in multiple sclerosis. *Annals of Neurology*, 71(1), 76–83. https://doi.org/10.1002/ana.22512.

Rossi, S., Motta, C., Studer, V., Barbieri, F., Buttari, F., Bergami, A., Sancesario, G., Bernardini, S., De Angelis, G., Martino, G., Furlan, R., & Centonze, D. (2014). Tumor necrosis factor is elevated in progressive multiple sclerosis and causes excitotoxic neurodegeneration. *Multiple Sclerosis (Houndmills, Basingstoke, England)*, 20(3), 304–312. https://doi.org/10.1177/1352458513498128.

Rossi, S., Muzio, L., De Chiara, V., Grasselli, G., Musella, A., Musumeci, G., Mandolesi, G., De Ceglia, R., Maida, S., Biffi, E., Pedrocchi, A., Menegon, A., Bernardi, G., Furlan, R., Martino, G., & Centonze, D. (2011). Impaired striatal GABA transmission in experimental autoimmune encephalomyelitis. *Brain, Behavior, and Immunity*, 25(5), 947–956. https://doi.org/10.1016/j.bbi.2010.10.004.

Sarchielli, P., Greco, L., Floridi, A., Floridi, A., & Gallai, V. (2003). Excitatory amino acids and multiple sclerosis: Evidence from cerebrospinal fluid. *Archives of Neurology*, 60(8), 1082–1088. https://doi.org/10.1001/archneur.60.8.1082.

Srinivasan, R., Sailasuta, N., Hurd, R., Nelson, S., & Pelletier, D. (2005). Evidence of elevated glutamate in multiple sclerosis using magnetic resonance spectroscopy at 3 T. *Brain: A Journal of Neurology*, 128(Pt 5), 1016–1025. https://doi.org/10.1093/brain/awh467.

Stampanoni Bassi, M., Iezzi, E., Mori, F., Simonelli, I., Gilio, L., Buttari, F., Sica, F., De Paolis, N., Mandolesi, G., Musella, A., De Vito, F., Dolcetti, E., Bruno, A., Furlan, R., Finardi, A., Marfia, G. A., Centonze, D., & Rizzo, F. R. (2019). Interleukin-6 disrupts synaptic plasticity and impairs tissue damage compensation in multiple sclerosis. *Neurorehabilitation and Neural Repair*, 33(10), 825–835. https://doi.org/10.1177/1545968319868713.

Stampanoni Bassi, M., Mori, F., Buttari, F., Marfia, G. A., Sancesario, A., Centonze, D., & Iezzi, E. (2017). Neurophysiology of synaptic functioning in multiple sclerosis. *Clinical Neurophysiology: Official Journal of the International Federation of Clinical Neurophysiology*, 128(7), 1148–1157. https://doi.org/10.1016/j.clinph.2017.04.006.

Stojanovic, I. R., Kostic, M., & Ljubisavljevic, S. (2014). The role of glutamate and its receptors in multiple sclerosis. *Journal of Neural Transmission*, 121(8), 945–955. https://doi.org/10.1007/s00702-014-1188-0.

Stover, J. F., Pleines, U. E., Morganti-Kossmann, M. C., Kossmann, T., Lowitzsch, K., & Kempski, O. S. (1997). Neurotransmitters in cerebrospinal fluid reflect pathological activity. *European Journal of Clinical Investigation*, 27(12), 1038–1043. https://doi.org/10.1046/j.1365-2362.1997.2250774.x.

Su, K., Bourdette, D., & Forte, M. (2013). Mitochondrial dysfunction and neurodegeneration in multiple sclerosis. *Frontiers in Physiology*, 4, 169. https://doi.org/10.3389/fphys.2013.00169.

Teunissen, C. E., Petzold, A., Bennett, J. L., Berven, F. S., Brundin, L., Comabella, M., Franciotta, D., Frederiksen, J. L., Fleming, J. O., Furlan, R., Hintzen, MD, R. Q., Hughes, S. G., Johnson, M. H., Krasulova, E., Kuhle, J., Magnone, M. C., Rajda, C., Rejdak, K., Schmidt, H. K., … Deisenhammer, F. (2009). A consensus protocol for the standardization of cerebrospinal fluid collection and biobanking. *Neurology*, 73(22), 1914–1922. https://doi.org/10.1212/WNL.0b013e3181c47cc2.

Thompson, A. J., Banwell, B. L., Barkhof, F., Carroll, W. M., Coetzee, T., Comi, G., Correale, J., Fazekas, F., Filippi, M., Freedman, M. S., Fujihara, K., Galetta, S. L., Hartung, H. P., Kappos, L., Lublin, F. D., Marrie, R. A., Miller, A. E., Miller, D. H., Montalban, X., … Cohen, J. A. (2018). Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *The Lancet Neurology*, 17(2), 162–173. https://doi.org/10.1016/S1474-4422(17)30470-2.

Tisell, A., Leinhard, O. D., Warntjes, J. B., Aalto, A., Smedby, Ö., Landtblom, A. M., & Lundberg, P. (2013). Increased concentrations of glutamate

and glutamine in normal-appearing white matter of patients with multiple sclerosis and normal MR imaging brain scans. *PLoS One*, 8(4), e61817. https://doi.org/10.1371/journal.pone.0061817.

Trapp, B. D., & Stys, P. K. (2009). Virtual hypoxia and chronic necrosis of demyelinated axons in multiple sclerosis. *The Lancet. Neurology*, 8(3), 280–291. https://doi.org/10.1016/S1474-4422(09)70043-2.

Vanderstichele, H., Bibl, M., Engelborghs, S., Le Bastard, N., Lewczuk, P., Molinuevo, J. L., Parnetti, L., Perret-Liaudet, A., Shaw, L. M., Teunissen, C., Wouters, D., & Blennow, K. (2012). Standardization of preanalytical aspects of cerebrospinal fluid biomarker testing for Alzheimer's disease diagnosis: a consensus paper from the Alzheimer's Biomarkers Standardization Initiative. *Alzheimer's & Dementia: The Journal of the Alzheimer's Association*, 8(1), 65–73. https://doi.org/10.1016/j.jalz.2011.07.004.

Werner, P., Pitt, D., & Raine, C. S. (2001). Multiple sclerosis: altered glutamate homeostasis in lesions correlates with oligodendrocyte and axonal damage. *Annals of Neurology*, 50(2), 169–180. https://doi.org/10.1002/ana.1077.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Stampanoni Bassi, M., Nuzzo, T., Gilio, L., Miroballo, M., Casamassa, A., Buttari, F., Bellantonio, P., Fantozzi, R., Galifi, G., Furlan, R., Finardi, A., De Rosa, A., Di Maio, A., Errico, F., Centonze, D., & Usiello, A. (2021). Cerebrospinal fluid levels of L-glutamate signal central inflammatory neurodegeneration in multiple sclerosis. *Journal of Neurochemistry*, 159, 857–866. https://doi.org/10.1111/jnc.15518