Journal of Neuroinflammation

RESEARCH

Open Access

The effect of cladribine tablets on intrathecal inflammation in relapsing-remitting multiple sclerosis

Damiano Marastoni[1*†], Chiara Eccher[1†], Alessio Signori[2†], Daniela Anni[1†], Matteo Foschi[3,4], Francesco Crescenzo[1], Valentina Mazziotti[1], Federica Virla[1], Monica Sicchieri[1], Ermanna Turano[1], Agnese Tamanti[1], Valentina Camera[1], Stefano Ziccardi[1], Giulia Zanetti[1], Francesca Benedetta Pizzini[5], Silvia Martini[6], Anna Fratucello[6], Bruno Bonetti[7], Maria Pia Sormani[2] and Massimiliano Calabrese[1*]

Abstract

Background To investigate the intrathecal effect of cladribine tablets on cerebrospinal fluid (CSF) inflammatory proteomic profile, cortical lesions (CLs), paramagnetic rim lesions (PRLs) number and cognition after 2 years of treatment.

Results Forty-two patients with RRMS treated with cladribine tablets were enrolled in a prospective longitudinal 2-year study. All patients were scheduled to undergo a lumbar puncture before treatment initiation and after 2 years of treatment, a clinical evaluation, including Expanded Disability Status Scale (EDSS) assessment, every 6 months, and a 3T MRI every year. White matter lesion number and volume, CLs, PRLs number and neuropsychological status were evaluated. CSF levels of 17 inflammatory markers were assessed by multiplex immune assay. No evidence of disease activity (NEDA) was defined as the absence of relapses, MRI activity and 6-months confirmed disability progression, defined as an increase of ≥ 1 point in the EDSS. Thirty-nine patients completed the study, and 33 agreed to repeat the lumbar puncture. After two years, 23 (59%) patients retained the NEDA status. Cladribine tablets reduced most of the inflammatory markers in the CSF of patients, with a significant reduction, after correction for multiple comparisons, in levels of sTNFR1, Pentraxin3 and CCL22. No patients accumulated new CLs, and no significant changes in PRLs and cognition were observed over the follow-up.

Conclusions Cladribine tablets administration led to a reduction of intrathecal inflammatory markers. These findings, along with the absence of CLs and PRLs accumulation, suggest a potential effect of the drug on intrathecal compartmentalized inflammation.

[†]Damiano Marastoni, Chiara Eccher, Alessio Signori and Daniela Anni contributed equally to this work.

*Correspondence:
Damiano Marastoni
damiano.marastoni@univr.it
Massimiliano Calabrese
massimiliano.calabrese@univr.it

Full list of author information is available at the end of the article

Keywords Multiple sclerosis, Cladribine, Cerebrospinal fluid, Paramagnetic rim lesions, Cortical lesions, Intrathecal inflammation

Introduction

Relapsing multiple sclerosis (RMS) is characterized by the occurrence of new neurological symptoms with or without disability accumulation, associated in many cases with a slow accumulation of irreversible disability that defines the progression independent of relapse activity (PIRA, [1, 2]).

Among factors contributing to irreversible disability accumulation, intrathecal compartmentalised inflammation has been suggested to play a crucial role, leading to extensive demyelination in white and grey matter (GM) and axonal and neuronal loss [3]. Intrathecal chronic inflammatory processes occur both in the meningeal compartment, with inflammatory aggregates associated with demyelination in the adjacent GM [4–6], as well as on the chronically activated microglial-rich edge of the chronic active lesions in the inflamed Central Nervous System (CNS) parenchyma [7, 8].

Cerebrospinal fluid (CSF) markers are an easily accessible and reasonable surrogate of intrathecal inflammation [9]. In vivo and ex vivo evaluation of CSF inflammatory markers provided evidence of an association between meningeal inflammation, GM damage, and disease activity in early MS [5, 6, 10, 11]. Similarly, efforts have been done to characterize molecules implicated in chronic microglial activation, such as CHIT-1L3 and sCD163, that could act as good surrogate markers of chronic microglial activity at the edge of demyelinating lesions [12–14]. Notably, a longitudinal assessment of CSF inflammatory markers could provide insight into intrathecal drug's efficacy [15, 16] as well as suggest biomarkers associated with the non-relapsing biology of the disease [17].

Cladribine is an oral drug licensed for the treatment of adults with RMS, including relapsing-remitting (RRMS) and active secondary progressive MS (SPMS) in the US and for adults with highly active RMS in the European Union. The molecule is an adenosine prodrug thought to function by causing cytotoxic effects on B and T cells by impairing DNA synthesis, resulting in lymphocyte depletion. Due to its small size and lipophilic features, it could cross the blood-brain barrier [18, 19], thus potentially directly acting on intrathecal compartmentalized inflammatory processes.

According to this evidence, we designed a longitudinal prospective trial aimed to evaluate the drug efficacy on (i) CSF inflammatory markers, assessed after a two-year follow-up; (ii) MRI biomarkers of chronic intrathecal inflammation such as cortical lesions (CLs) and paramagnetic rim lesions (PRLs); (iii) longitudinal changes in neuropsychological status.

Materials and methods

Study population

Forty-two patients with a diagnosis of RRMS according to revised McDonald's Criteria [20], who started cladribine tablets at the MS Centre of Verona University Hospital, were recruited to participate in a phase IV, single-arm, longitudinal prospective study (CLAD19, n°2018-004947-21, https://www.clinicaltrialsregister.eu/ctr-search/). Along with a diagnosis of RRMS, inclusion criteria were (i) the absence of any other inflammatory disease; (ii) the availability of at least 1 ml of CSF collected by lumbar puncture performed before treatment initiation. All the patients had to be therapy-free at the time of the lumbar puncture and, after the study's inclusion, started the treatment with cladribine. Medical history review and laboratory analysis excluded concurrent infections and systemic disorders.

Study design

Each patient underwent a clinical and radiological 2-years follow-up, including neurological evaluations every six months with additional examinations in case of relapses. A relapse was defined as a worsening of neurological impairment or appearance of a new symptom or abnormality attributable to MS, lasting at least 24 h, in the absence of fever, and preceded by the stability of at least 1 month. The neurological evaluation included the Expanded Disability Status Scale (EDSS) assessment, by two certified raters (MC, DM).

The combined three-domain status of 'no evidence of disease activity' (NEDA-3) was defined by no evidence of clinical relapses, magnetic resonance imaging (MRI) activity (new or enlarged white matter T2 hyperintense lesions, gadolinium-enhancing lesions), and 6-months confirmed disability progression (CDP), defined as a six-months confirmed increase of ≥ 1 point in Expanded Disability Status Scale (EDSS). Occurrence of PIRA was defined if: (i) a baseline/reference score, applied to set a new reference score every time the EDSS or individual measure of the composite was higher than the previous measure and confirmed at the following visit or if a relapse caused residual disability; (ii) a clinically significant increase of EDSS of 1.5 points or more from an EDSS of 0, 1.0 points or more from an EDSS of 1.0 to 5.0, or 0.5 points or more from an EDSS score of 5.5 or more; (iii) an increase of EDSS not determined within 30 days before and 90 days after the onset of an investigator-reported

relapse; (iv) a confirmation EDSS assigned at least 6 months after the initial disability increase [21].

All patients were scheduled to undergo a brain 3T-MRI at 6 (re-baseline), 12 and 24 months after cladribine administration. All the patients were proposed to undergo a second lumbar puncture with a CSF examination after 24 months after treatment initiation.

Adverse events, including the occurrence of lymphopenia, were recorded. Lymphopenia was defined in accordance with the Common Terminology Criteria for Adverse Events (CTCAE version 5.0) as follows: grade 0 ($\geq 910 \times 10^9$/L), grade 1 ($\geq 800 \times 10^9$/L), grade 2 (< 800–500×10^9/L), grade 3 (< 500–200×10^9/L), and grade 4 ($< 200 \times 10^9$/L).

CSF protein analysis

CSF samples were obtained before and after 2 years of Cladribine tablets treatment according to Consensus Guidelines for CSF and Blood Biobanking [22]. After centrifugation, the CSF supernatant was collected and stored at -80 °C until use. The CSF analysis was optimized and performed by two independent investigators (DA, FV), blinded with respect to the patients' clinical and MRI features. The levels of 17 inflammatory mediators, that potentially reflect chronic inflammatory processes and a severe disease course [5, 6, 10–14] were assessed using a Human 40- (#171AK99MR2) and 37-Plex (#171AL001M) magnetic beads panels in a Bio-Plex X200 Luminex instrument equipped with a magnetic workstation (Biorad, Hercules, CA, USA), as previously described [5, 6]. The CSF level of each protein was normalized to the total protein concentration of each CSF sample measured by the Bradford protocol [6], and expressed as pg/ml/mg. Finally, CSF/serum albumin quotient, Immunoglobulin G (IgG) index and the presence or absence of oligoclonal bands (OCB) were evaluated in each patient.

MRI analysis

MRI acquisition protocol

All 3T MRI scans were acquired using a Philips Achieva 3T MRI Scanner at the Neuroradiology Unit of the University Hospital of Verona. A manual quality check was carried out to exclude significant artifacts.

A standardized protocol was employed to acquire the following sequences: (i) 3D-T1 weighted Turbo Field Echo (TFE) (Repetition Time (TR)/Echo Time (TE) = 8.4/3.7 ms, voxel size of $1 \times 1 \times 1$ mm, acquisition time of 5:51 min); (ii) 3D-Double Inversion Recovery (DIR, TR/TE = 5500/292 ms, Inversion Times (TI) TI1/TI2 = 525/2530 ms voxel size of $1 \times 1 \times 1$ mm, acquisition time of 10:49 min); (iii) 3D-Fluid Attenuated Inversion Recovery (FLAIR) (TR/TE = 5500 /292 ms, TI = 1650 ms voxel size of $1 \times 1 \times 1$ mm, acquisition time of 4:48 min); (iv) 3D-T1 weighted TFE post-contrast with the same parameters of the pre-contrast sequence (TR/TE = 8.4/3.7 ms, voxel size of $1 \times 1 \times 1$ mm, acquisition time of 5:51 min); (v) 3D echo-planar imaging (EPI) phase ($0.55 \times 0.55 \times 0.55$ mm) sequences.

MRI analysis

All MRI scans were anonymized prior to evaluation, and all readers were blinded to clinical data and MRI acquisition timepoint (baseline vs. follow-up).

Lesion detection

The number of white matter (WM) lesions (WMLn) at re-baseline and the new and enlarging WM lesions during and at the end of the study were assessed on FLAIR images by a neuroradiologist with extensive experience with MS (FBP). The number of total cortical lesions (CLn) and the new CLs were assessed by two independent raters (CE and GZ), who were blinded to clinical data and MRI timepoint, on DIR images based on recent recommendations [23]. Inter-rater agreement was 75% at baseline and 78% at follow-up. Owing to the suboptimal performance of the MRI in visualizing subpial lesions, the present analysis could have considered mainly the intracortical and leukocortical lesions. In case of disagreement, CLs classification was resolved by joint re-evaluation and consensus, involving an expert neuroradiologist (FBP).

Paramagnetic rim lesions

3D EPI was coregistered to the other MRI modalities using Advanced Normalization Tools (ANTs), and itk-snap [24] was used for lesion visualization, identification, and classification purposes. The 3D EPI phase image processing consisted of phase unwrapping using a Laplacian filter and Gaussian filtering with a filter size of 32 pixels and full width at a maximum of 8 pixels [25]. The susceptibility-weighted images (SWI) were reconstructed using the phase-filtered image, and a final SWI image was created and included in the itk-snap workspace with other modalities. Supratentorial MS lesions were evaluated by visual inspection by two independent investigators blinded to timepoint and clinical data (MC and GZ, inter-rater agreement of 79% and 77% at baseline and follow-up, respectively) and classified as PRLs when showing a hypointense rim on the lesion edge on phase-filtered images obtained from 3D EPI sequence, according to recently adopted criteria [7]. In case of discordant ratings, images were jointly re-evaluated and a final classification was reached by consensus, involving an expert neuroradiologist (FBP).

Neuropsychological assessment

A comprehensive battery of neuropsychological tests was administered to all patients at the time of cladribine tablets initiation and after 24 months. Patients underwent the Brief Repeatable Battery of Neuropsychological Tests [26] and some executive functioning tests such as the Stroop Test [27], the Phonemic Semantic Alternate Verbal Fluency [28], and the Modified Five Point Test [29]. Raw scores were adjusted for age, education, and sex. Adjusted scores below the cut-off (5th percentile) of the Italian normative data of each test/battery were considered as failed. Patients were divided into three groups based on their cognitive performance on all neuropsychological tests [30]: cognitively normal (CN, 0 failed subtests), mildly cognitively impaired (mCI, one or 2 failed subtests), and severely cognitive impaired (sCI, 3 or more failed subtests). Occurrence of cognitive PIRA was also evaluated, considering a significant cognitive decline (using a reliable change index methodology) in the Symbol Digit Modalities Test (SDMT) occurred in absence of relapse activity 9 months before and 9 months after the cognitive decline [31].

Statistical analysis

Clinical and demographical variables were reported as mean with standard deviation or median with interquartile range, accordingly to their distribution. Protein values at baseline, two years after cladribine tablets administration, and their percentage difference, were reported as median with interquartile ranges. Differences after two years of treatment were compared to baseline values for the analysed CSF markers. All CSF markers were considered exploratory. Differences between the two-years follow-up and baseline values were statistically tested using the non-parametric two-sided sign test for matched pairs. Furthermore, a composite intrathecal inflammation score was derived as the mean of baseline-standardized z-scores of the markers exhibiting the strongest trend ($p < 0.1$ after adjustment for multiple comparisons) toward reduction following treatment. Baseline and follow-up values were compared using a two-sided Wilcoxon signed-rank test. Additionally, patients who achieved NEDA were compared with those who did not on the percentage change, using the non-parametric Mann-Whitney test. P-values were adjusted using the false-discovery rate method to account for multiple comparisons, with a significance level set at 0.05. Stata (v.16; StataCorp) was used for all statistical analyses.

Results

Study cohort

Thirty-nine of the 42 patients completed the study and achieved a follow-up of 2 years (Fig. 1). Three patients dropped out for personal reasons. Of those who completed the study, 22 patients (58%) began cladribine tablets as their first disease-modifying drug. All these patients had at least two relapses with concurrent disability accumulation and radiological activity in the year before treatment started. Seventeen patients (45%) were switched to cladribine tablets after experiencing a previous treatment (12 from dimethyl fumarate, 5 from interferon beta1a) due to the occurrence of new disease activity despite the ongoing treatment. The demographic and clinical characteristics of the 39 patients who ended the study are reported in Table 1. After two years of follow-up, 23 patients retained the NEDA status. Notably, CDP occurred in 4 patients, with only one patient experiencing PIRA. No severe adverse drug reactions leading to discontinuation were reported, nor did grade 4 lymphopenia occur. Two cutaneous Varicella Zoster Virus reactivation events leading to antiviral therapy administration were reported in the first year of follow-up. All patients regularly underwent a second year treatment (third and fourth cycles of cladribine tablets) without delay.

Cladribine tablets reduce intrathecal inflammatory milieu

Thirty-three out of 39 patients agreed to undergo a second lumbar puncture after 24 months of treatment initiation. The interval since the last treatment administration was the same for all participants (11 months) since the regular two-year treatment schedule was respected. Cladribine tablets reduced most of the examined CSF inflammatory markers, except OPN, CCL21 and APRIL, which didn't decrease after treatment (Fig. 2, Table 2). In particular significant reductions in levels of TNF, TNFR1, Pentraxin3, CCL22, Chitinase 3like1 was observed before adjustment for multiple comparisons (Table 2). After correction for multiple comparisons, TNFR1, Pentraxin3, and CCL22 levels were significantly reduced after treatment, while TNF showed a trend toward reduced levels without reaching statistical significance (Fig. 3). The composite intrathecal inflammation score, which included TNFR1, Pentraxin3, CCL22 and TNF values, showed a significant reduction from baseline to follow-up (median [IQR]: −0.27 [−0.43 to 0.09] vs. −0.49 [−0.70 to −0.26], $p = 0.004$).

When stratified by disease activity, no significant different in percentage change of reduced markers were observed between groups. Reductions in selected CSF inflammatory markers were more consistently observed in patients maintaining NEDA, whereas patients with evidence of disease activity showed smaller and more heterogeneous changes (Table 3).

No changes in marker levels were noticed according to patients' exposure to cladribine tablets as a first therapeutic approach. Regarding OCBs status, OCBs disappeared in five patients while were detected in two patients who previously turned out negative. The albumin quotient

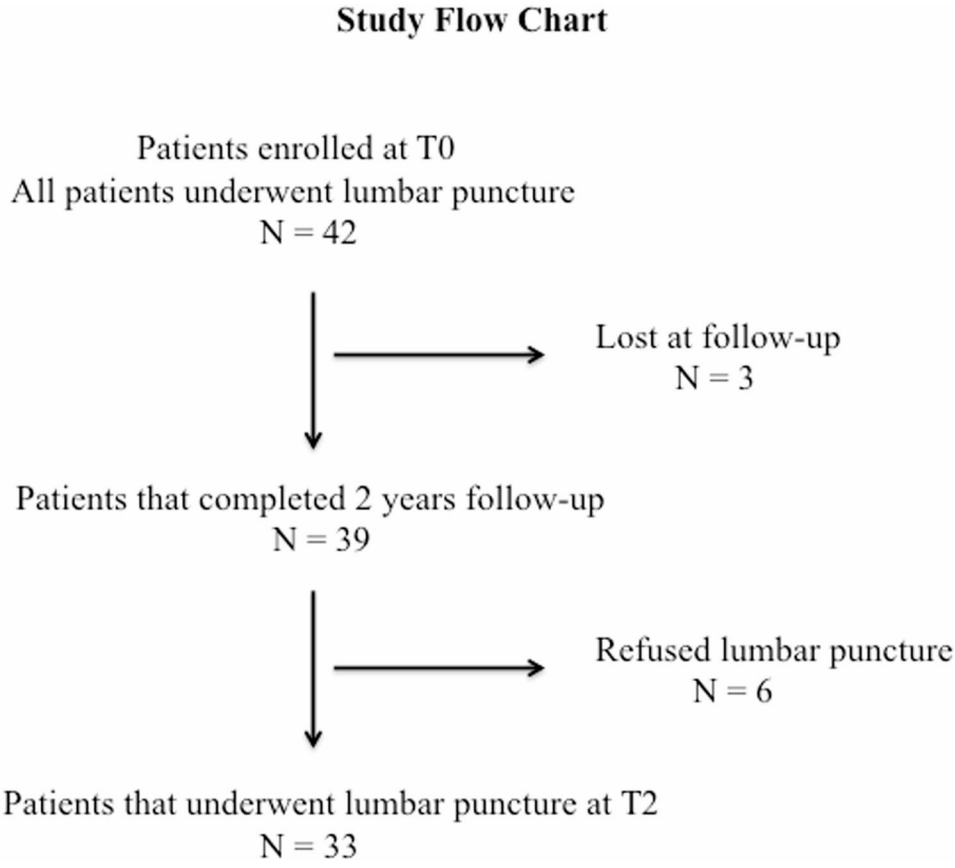

Fig. 1 Schematic illustration of the study flow-chart. Of forty-two patients enrolled (T0), 39 completed the two years follow-up and 33 underwent a second lumbar puncture after two years of treatment with cladribine tablets (T2)

Table 1 Baseline demographic, clinical and MRI characteristics of the whole population

	n **= 39**
Age - yr	34 ± 11.0
Female– no. (%)	28 (71.8)
EDSS score - median (range)	2.0 (0.0–5.0)
Disease duration - mean ± SD	2.44 ± 4.3
Previous treatment – yes (%)	17 (43.6)
Relapses in previous year - mean ± SD	1.5 ± 0.6
BMI (Kg/m^2)	23.7 ± 2.1
WMLn - mean ± SD	11.2 ± 5.6
Spinal Cord lesion number - mean ± SD	1.2 ± 1.5
Gd+ lesions - mean ± SD	0.5 ± 0.9
CLn - mean ± SD	2.9 ± 5.0
PRLs - mean ± SD	0.9 ± 2.1
CI – number (%)	15 (39.5)
CSF OCBs (yes/not)	32/7

Abbreviations: *EDSS* Expanded Disability Status Scale, *WMLn*, White Matter Lesion Number, *Gd+ lesions* Gadolinium enhancing lesions, *CLn* Cortical lesion number, *PRLs* Paramagnetic Rim Lesions, *CI* Cognitive Impaired, *CSF* Cerebrospinal fluid, *OCBs* Oligoclonal bands

was 5.10 ± 2.18 at the time of the first lumbar puncture and 5.76 ± 2.39 ($p = 0.157$) at the second lumbar puncture. IgG index changed from 0.77 ± 0.32 to 0.71 ± 0.20 ($p = 0.958$).

Cladribine efficacy on cortical and paramagnetic rim lesions

Over the follow-up, no patients developed new CLs. Regarding PRLs, no significant changes have been observed during the follow-up (0.94 ± 2.07 vs. 0.94 ± 1.95, $p = 0.707$). Patients with disease activity showed a not significant trend toward an increased baseline CLs number (4.0 ± 6.4 vs. 1.8 ± 2.5, $p = 0.280$) and number of PRLs at treatment initiation (1.3 ± 2.7 vs. 0.4 ± 1.0, $p = 0.429$).

Neuropsychological evaluation

Thirty-eight out of 39 patients completed the neuropsychological testing before and after cladribine tablets administration. Of these, at the time of treatment initiation, 23 (60.5%) were classified as CN while the remaining 15 (39.5%) were classified as cognitively impaired (7 were mCI and 8 were sCI). After the completion of the two-year cladribine cycle, the proportion between cognitive normal and impaired patients was the same: 23

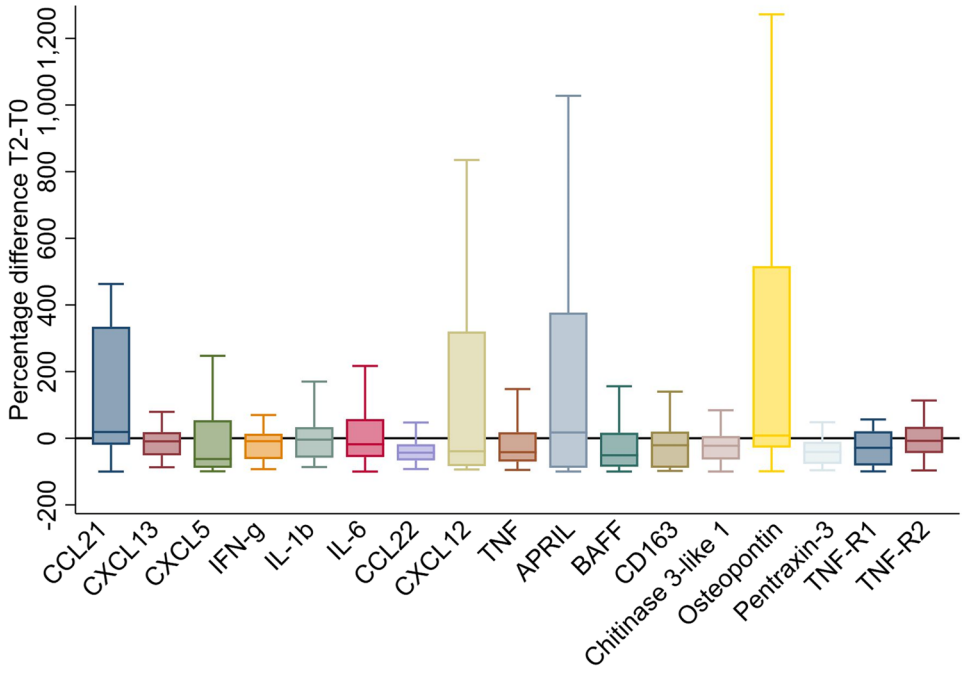

Fig. 2 Percentage decrement in cerebrospinal fluid levels of 17 inflammatory markers. After two years of treatment (T2) with cladribine tablets, most of inflammatory markers showed a reduction in their CSF levels compared with paired baseline values (T0). *Abbreviations*: CCL21, chemokine (C-C motif) ligand 21; CXCL13, C-X-C motif chemokine 13; CXCL5, C-X-C motif chemokine 5; IFN-g, interferon gamma; IL-1b, interleukin-1beta; IL-6, interleukin-6; CCL22, chemokine (C-C motif) ligand 22; CXCL12, C-X-C motif chemokine 12; TNF, tumor necrosis factor; APRIL, a proliferation-inducing ligand; BAFF, B-cell activating factor; CD163, soluble-CD163 (Cluster of Differentiation 163); TNFR1, soluble-tumor necrosis factor-receptor 1; TNFR2, soluble-tumor necrosis factor-receptor 2

Table 2 Levels of inflammatory markers before and after cladribine tablets administration

	T0	T2	Percentage difference	*p*-value unadjusted	*p*-value adjusted for m.c
CCL21	1926 (657–3296)	3265 (2278–4294)	18.8 (-19.4, 334.4)	0.061	0.17
CXCL13	1.55 (0.83–2.52)	1.24 (0.60–1.57)	-9.3 (-51.3, 18.3)	0.093	0.18
CXCL5	120 (54–209)	67 (15–88)	-62.4 (-88.8, 53.9)	0.077	0.17
IFN-g	4.07 (2.36–6.56)	3.25 (2.38–4.21)	-8.9 (-62.5, 13.4)	0.85	0.85
IL-1b	0.28 (0.16–0.63)	0.25 (0.14–0.44)	-4.2 (-58.3, 33.3)	0.52	0.63
IL-6	8.80 (6.80–19.50)	9.49 (6.65–14.59)	-18.0 (-56.4, 57.5)	0.26	0.44
CCL22	9.38 (5.40–15.00)	5.32 (3.77–8.33)	-43.3 (-66.5, -18.0)	**0.004**	**0.023**
CXCL12	372 (125–775)	257 (62–619)	-39.1 (-83.6, 320.2)	0.44	0.58
TNF	4.64 (3.14–5.58)	3.11 (1.04–4.52)	-41.8 (-69.9, 17.8)	**0.023**	0.098
APRIL	62951 (15249–136267)	72755 (15518–145244)	17.3 (-88.6, 377.1)	0.73	0.78
BAFF	4843 (1123–9132)	1434 (723–5443)	-51.1 (-85.4, 16.3)	0.080	0.17
sCD163	12811 (5412–23268)	4188 (1845–14614)	-21.0 (-88.6, 19.9)	0.30	0.46
Chitinase 3-like 1	13563 (5169–19984)	8715 (2633–15974)	-22.5 (-63.7, 6.5)	**0.035**	0.12
Osteopontin	13523 (4344–38534)	27698 (5883–57184)	8.0 (-28.0, 516.4)	0.73	0.78
Pentraxin-3	126 (84–163)	79 (23–119)	-41.2 (-77.0, -10.8)	**<0.001**	**0.002**
TNF-R1	2980 (1739–4816)	1983 (499–3115)	-28.7 (-81.6, 20.9)	**0.001**	**0.012**
TNF-R2	229 (151–374)	219 (153–302)	-7.8 (-44.3, 34.1)	0.38	0.54

Values are expressed as pg/ml/mg^{Prot}. Results are reported as median (25th -75th percentile). Significant comparisons ($p < 0.05$) between cerebrospinal fluid markers values obtained before (T0) and after two years of treatment (T2) are reported in bold

Abbreviations: *m.c.* Multiple comparisons, *CCL21* Chemokine (C-C motif) ligand 21, *CXCL13* Chemokine (C-X-C motif) ligand 13, *CXCL5* C-X-C motif chemokine 5, *IFNgamma* Interferon gamma, *IL1beta* Interleukin-1 beta, *IL6* Interleukin-6, *CCL22* C-C motif chemokine 22, *CXCL12* C-X-C motif chemokine 12, *TNF* Tumor necrosis factor, *APRIL* A proliferation-inducing ligand, *BAFF* B-cell activating factor, *sCD163* Soluble-CD163 (Cluster of Differentiation 163), *sTNFR1* Soluble- tumor necrosis factor-receptor 1, *sTNFR2* Soluble- tumor necrosis factor-receptor 2

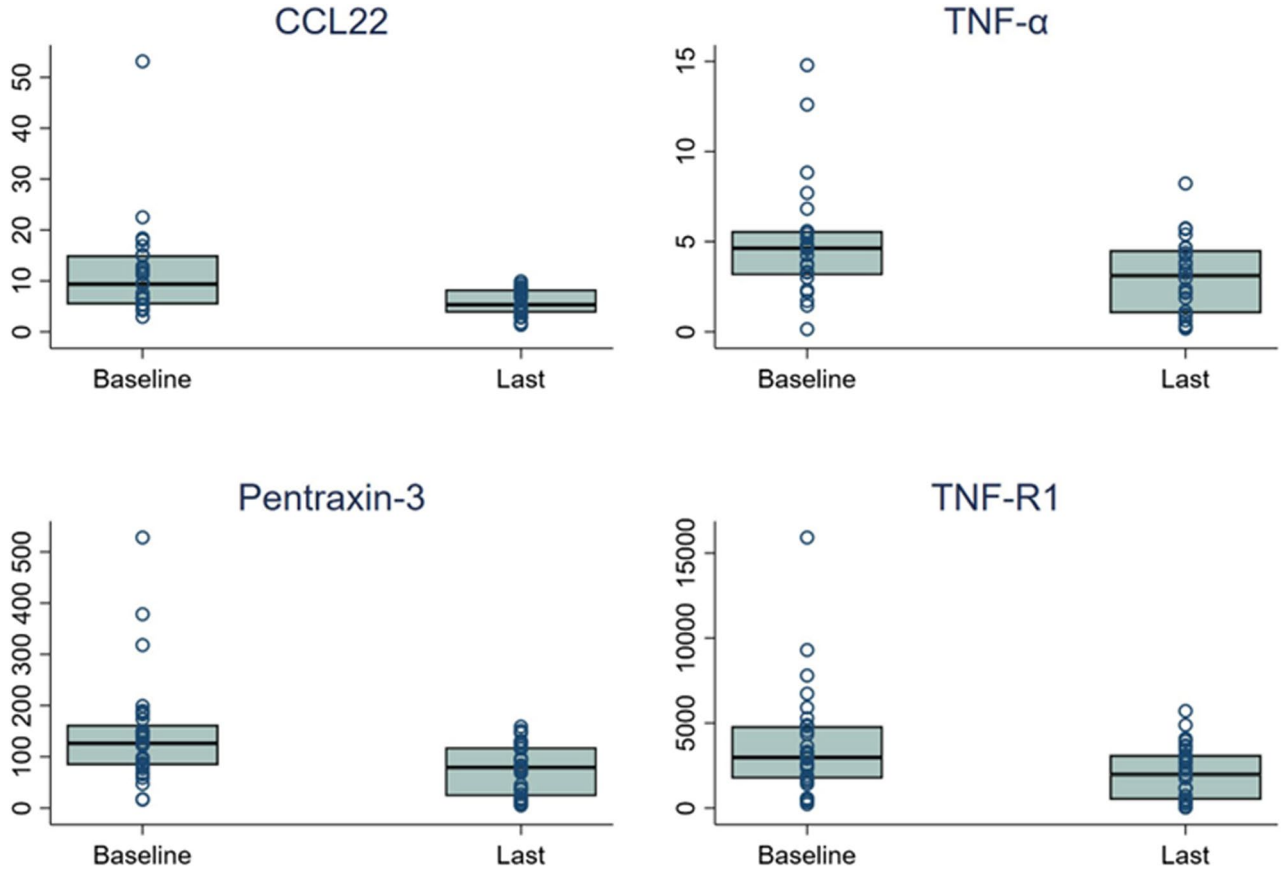

Fig. 3 Box-plot illustrating differences in cerebrospinal fluid levels of selected molecules after two years of cladribine treatment (Last) when compared to baseline values. TNFR1 ($p=0.012$), Pentraxin3 ($p=0.002$), and CCL22 ($p=0.023$) levels were significantly reduced after treatment, while TNF showed a trend toward reduced levels without reaching statistical significance ($p=0.098$). A composite intrathecal inflammation score derived as the mean of baseline-standardized z-scores of these markers showed a significant reduction from baseline to follow-up ($p=0.004$). The CSF level of each protein was normalized to the total protein concentration of each CSF sample (expressed as pg/ml/mg^{Prot}). P-values were adjusted using the false-discovery rate method to account for multiple comparisons, with a significance level set at 0.05. *Abbreviations*: CCL22, chemokine (C-C motif) ligand 22; TNF-R1, soluble-tumor necrosis factor-receptor 1; TNF-α, tumor necrosis factor

(60.5%) were classified as CN, while 15 (39.5%) were classified as cognitively impaired (9 mCI and 6 sCI). Only one patient experienced cognitive PIRA at the follow-up evaluation.

Discussion

Our findings showed that cladribine tablets reduces multiple inflammatory mediators in the CSF of patients with RRMS and helps prevent the formation of new cortical and paramagnetic rim lesions. These observations suggest that the drug potentially impacts on inflammatory processes underlying chronic disability.

This is in line with the molecule size that permits the crossing of the human BBB [18, 19], thus potentially acting directly into the CNS compartment. Cladribine, an adenosine analogue prodrug, is considered an immune reconstitution therapy [32]. This is suggested by initial cytotoxic effects on B and T cells through the impairment of DNA synthesis resulting in lymphocyte depletion and subsequent repopulation of the immune system that regains the ability to respond to infections without commensurate return of MS disease activity [33]. Such attenuation in MS activity is compatible with the reduction in inflammatory markers we observed after the two-years follow-up. In particular we observed significant changes in the sTNFR1 levels, a marker that have been previously associated with cortical damage and MS severity [6, 34]. An unbalanced TNF signaling between TNFR1 and 2, towards an increased pro-inflammatory TNFR1, associated with chronic meningeal inflammation, has been detected by gene expression of both cortical lesions and normal appearing GM [35]. In line with this evidence, subpial demyelination and neuronal loss have been associated with the persistent meningeal production of TNF and IFNγ in the animal model [36]. According to a previous observation in a 10-years longitudinal study [37] we detected an effect on the intrathecal humoral response, with the disappearance of oligoclonal bands

Table 3 CSF markers values according to NEDA status before and after cladribine tablets administration

	NEDA ($n=23$)				EDA ($n=10$)				NEDA vs. EDA (p-value)
	T0	T2	p-value unadjusted	p-value adjusted for m.c	T0	T2	p-value unadjusted	p-value adjusted for m.c	
CCL21	1926 (657–3296)	3224 (2278–4143)	0.078	0.19	2012 (669–3066)	4002 (2191–4477)	0.73	0.99	0.68
CXCL13	1.53 (0.89–2.53)	1.06 (0.59–1.45)	0.21	0.36	1.55 (0.63–2.32)	1.55 (0.60–1.83)	0.45	0.99	0.41
CXCL5	75 (28–182)	32 (14–77)	0.23	0.36	179 (70–355)	88 (76–112)	0.38	0.99	0.74
IFN-g	4.17 (2.60–6.56)	2.96 (2.15–3.79)	0.65	0.72	3.73 (2.28–16.6)	3.86 (2.83–4.52)	0.99	0.99	0.33
IL-1b	0.42 (0.17–0.70)	0.36 (0.13–0.47)	0.99	0.99	0.22 (0.16–0.42)	0.22 (0.14–0.28)	0.45	0.99	0.86
IL-6	11.90 (7.18–20.5)	9.36 (6.34–14.35)	**0.041**	0.12	8.78 (4.79–13.59)	12.32 (7.72–14.59)	0.51	0.99	0.15
CCL22	10.31 (6.46-15.00)	4.99 (3.66–8.33)	**0.001**	**0.022**	6.84 (5.40–18.00)	6.82 (4.89–8.75)	0.99	0.99	0.24
CXCL12	356 (125–550)	323 (76–556)	0.36	0.51	386 (92–811)	181 (57–928)	0.99	0.99	0.86
TNF	4.58 (3.33–5.18)	2.22 (0.95–3.86)	**0.013**	0.055	5.59 (2.33–12.6)	4.65 (2.97–5.69)	0.99	0.99	0.46
APRIL	74929 (24429–139741)	34584 (13159–138971)	0.68	0.72	25427 (14463–77778)	106241 (72755–161521)	0.11	0.94	**0.042**
BAFF	2966 (785–7574)	1281 (575–5427)	0.093	0.20	8432 (5457–11348)	3133 (1138–5750)	0.75	0.99	0.71
CD163	11787 (4136–23268)	3874 (1636–8969)	0.21	0.36	14195 (11312–25793)	15879 (1943–25126)	0.99	0.99	0.29
Chitinase 3-like 1	16333 (7625–21257)	8175 (3482–15974)	**0.035**	0.12	12687 (1723–19938)	11731 (1467–16955)	0.75	0.99	0.69
Osteopontin	8918 (4344–46392)	19328 (5699–64268)	0.68	0.72	19539 (4199–38534)	40832 (5882–49154)	0.99	0.99	0.83
Pentraxin-3	132 (77–187)	75 (19–121)	**0.004**	**0.037**	114 (87–144)	82 (37–115)	**0.022**	0.37	0.56
TNF-R1	2896 (1572–4892)	1169 (352–2815)	**0.011**	0.055	3264 (2493–3647)	2920 (2247–3616)	0.75	0.99	0.55
TNF-R2	200 (115–376)	207 (152–319)	0.52	0.68	248 (219–357)	226 (167–292)	0.75	0.99	0.46

Values are expressed as pg/ml/mg^{Prot}. Results are reported as median (25th -75th percentile). Significant comparisons ($p<0.05$) between cerebrospinal fluid markers values obtained before (T0) and after two years of treatment (T2) are reported in bold

Abbreviations: *CSF* Cerebrospinal fluid, *NEDA* No Evidence of disease activity, *m.c.* Multiple comparisons, *CCL21* Chemokine (C-C motif) ligand 21, *CXCL13* Chemokine (C-X-C motif) ligand 13, *CXCL5* C-X-C motif chemokine 5, *IFNgamma* Interferon gamma, *IL1beta* Interleukin-1 beta, *IL6* Interleukin-6, *CCL22* C-C motif chemokine 22, *CXCL12* C-X-C motif chemokine 12, *TNF* Tumor necrosis factor, *APRIL* A proliferation-inducing ligand, *BAFF* B-cell activating factor, *CD163* Soluble-CD163 (Cluster of Differentiation 163), *TNFR1* soluble- tumor necrosis factor-receptor 1, *TNFR2* soluble-tumor necrosis factor-receptor 2

in five patients who showed no disease activity over the follow-up.

Whether the effect of the drug in the CSF compartment reflects a direct action on chronic inflammatory parenchymal processes remains to be elucidated. It has been suggested that cladribine reduces the recruitment of inflammatory cells into the CNS by acting on adhesion molecule secretion by immune cells [38]. Nevertheless, exposure to cladribine in vitro reduced the granularity and phagocytic activity of microglia [39]. Furthermore, cladribine inhibits microglial proliferation and release of proinflammatory cytokines and induces apoptosis [40, 41]. In the EAE model, cladribine administration led to reduced immune cell infiltration into the CNS and partially restored cortical neuronal network function, potentially suggesting a CNS neuroprotective effect after the cross of the blood-brain barrier [42]. Accordingly, intracerebroventricular administration of the drug ameliorated glutamatergic synaptopathy associated with central inflammation and blocked EAE synaptic alterations by interfering with interleukin-1β effects [43]. Finally, oral cladribine treatment significantly attenuated clinical deficits in EAE mice. Nevertheless, given that these observations originate from preclinical studies, interpretations regarding anti-inflammatory or neuroprotective effects of cladribine independent of its peripheral immunosuppressive action should be regarded as exploratory and speculative.

In line with the need to provide evidence on the ability of available therapies to halt chronic intrathecal inflammation and neuro-axonal damage, previous human studies in patients with MS have suggested beneficial effects of high-efficacy treatments. In particular, the antiCD20 monoclonal antibody rituximab (RTX) administration in patients with RRMS resulted in the expected marked reduction of CSF B cells, along with a decrease in CSF T cells and reduced levels of CXCL13 and CCL19 [44]. These observations support the central role of B-cell-mediated immunity in MS and highlighted how targeted therapies may modulate the production of chemotactic molecules (particularly those involved in lymphoid recruitment) that are not directly produced by B cells. RTX was also associated with a reduction in CSF markers of neuroaxonal damage, such as neurofilament light chain (NfL), while neurofilament heavy chain (NfH) levels were significantly reduced only in patients achieving NEDA status [45]. Additional evidence supporting the modulation of intrathecal immunity derives from studies of patients switching from first-line therapies to fingolimod, which demonstrated reduced inflammatory activity (lower CSF levels of CXCL13, CHI3L1, and CHIT1), as well as decreased NfL concentrations [46]. Finally, similar findings have been reported in progressive MS, where reductions in CXCL13 and NfL, but not in the astroglial marker GFAP, suggested an effect of mitoxantrone and RTX on axonal damage, particularly in patients with active disease [47]. Notably, defining biomarkers that reflect the non-relapsing, progressive, MS biology, represent a major need. In the current study we focused on inflammatory markers, also previously associated with cortical damage [5] and a worse disease outcome including accumulation of brain atrophy [6], while we did not assess NfL, NfH, and GFAP levels, that would have provided additional evidence about the drug effect on the non-relapsing biology of the disease and its efficacy on the intrathecal compartment. Indeed, a recent study based on different cohorts suggested GFAP and NfH as specifically associated with non-relapsing progressive disease outcomes, potentially providing complementary information to NfL, which was more closely related to acute disease activity [17]. Notably, in a cohort of patients from the MAGNIFY-MS study, a reduction of NfL in both serum and CSF have been detected after 2 years from cladribine tablets administration, suggesting a protective effect on neuro-axonal damage that occurred along with a potential reduction of intrathecal inflammation [48].

We observed, after cladribine tablets, a potential beneficial effect on specific MRI markers associated with disability progression independent of relapse-related mechanisms, such as CLs and PRLs [8]. In our cohort, no patients developed new CLs, and no significant changes were detected in PRLs throughout the 2-year follow-up period. This findings point to a potential, albeit partial, impact on the inflammatory processes underlying chronic disability. Indeed, PRLs, a subset of chronic active lesions characterized by a rim of activated microglia with iron accumulation detectable with susceptibility-sensitive MRI, are considered surrogate marker of persistent parenchymal inflammation, neuro-axonal damage, and a severe disease course [7]. Similarly, CLs represent a significant marker associated with disability accumulation [49–51]. Of note, the accumulation of CLs in the first years of the disease represents a negative prognostic factor for disability accumulation [52]. The absence of CLs accumulation potentially aligns with the experienced reduction in cortical atrophy in patients exposed to cladribine. In a previous study, cladribine treatment was associated with reduced brain atrophy over two years compared with placebo, closely linked with a lower risk of disability progression [53]. Similarly, cladribine tablets reduce GM atrophy accumulation compared to placebo [54], further supporting its effect on cortical pathology. Of note, in the absence of a comparator group and given the absence of MRI lesions evolution, our findings can only suggest a potential efficacy of the drug on MRI markers of compartmentalized inflammation, not

supporting causal or mechanistic conclusions. Future studies confirming this hypothesis are then needed.

The present study has several limitations. The relatively small sample size may limit the generalizability of the findings. Of note, the present findings should be regarded as exploratory, hypothesis-generating, and not a definitive proof of intrathecal efficacy of the drug. Indeed, the single-arm design of the study precludes causal inference and prevents treatment-related effects on intrathecal inflammation from being fully disentangled from regression to the mean or non-specific temporal fluctuations in CSF inflammation. The pattern of CSF changes was highly selective, with reductions in TNFR1, Pentraxin-3 and CCL22, with other markers remaining stable, reducing the probability of a uniform temporal shift. Furthermore, when stratified by disease activity, reductions in selected CSF inflammatory markers were more consistently observed in patients maintaining NEDA, whereas patients with evidence of disease activity showed smaller and more heterogeneous changes. Nevertheless, these observations are descriptive and not intended to imply a differential causal effect. Notably, defining group comparisons according to NEDA status assessed at two years inherently conditions the analyses on post-baseline outcomes that are themselves linked to inflammatory activity, thereby limiting the interpretability of the associations between CSF biomarker changes and clinical response. Biological analyses were limited to patients who consented to a second lumbar puncture. Because this selection occurred after treatment initiation and during follow-up, it may have introduced selection bias i.e. favoring patients with greater clinical stability or treatment adherence. Serum samples were not included, as our primary aim was to focus on the intrathecal inflammatory compartment. While previous studies have already demonstrated a reduction in various serum inflammatory markers following cladribine treatment [55, 56], it remains unclear how faithfully the serum compartment reflects CNS-specific immune activity. In a large, treatment-naïve MS cohort, we showed only weak and inconsistent correlations between serum and CSF inflammatory patterns, with markers displaying minimal cross-compartment association [57]. Future studies directly comparing serum and CSF trajectories of markers may nonetheless provide valuable insights into the relationship between peripheral and intrathecal immune modulation that occurs under cladribine treatment.

In conclusion, our findings suggest that cladribine tablets exert a measurable effect on the intrathecal compartment, leading to a selective reduction of CSF inflammatory markers. The observed stability of MRI markers underscores the need for further controlled studies to confirm and better characterize the impact of cladribine on compartmentalized CNS inflammation and the chronic evolution of the disease.

Abbreviations

CCL21	Chemokine (C-C motif) ligand 21
CXCL13	C-X-C motif chemokine 13
CXCL5	C-X-C motif chemokine 5
IFN-g	Interferon gamma
IL-1b	Interleukin-1beta
IL-6	Interleukin-6
CCL22	Chemokine (C-C motif) ligand 22
CXCL12	C-X-C motif chemokine 12
TNF	Tumor necrosis factor
APRIL	A proliferation-inducing ligand
BAFF	B-cell activating factor
CD163	Soluble-CD163 (Cluster of Differentiation 163)
TNFR1	Soluble-tumor necrosis factor-receptor 1
TNFR2	Soluble-tumor necrosis factor-receptor 2

Author' contributions

MC, DM, AS contributed to the conception and design of the study. DM, CE, DA, AS, MF, FC, VM, FV, MS, ET, AT, VC, SZ, GZ, FBP, SM, AF, BB, MPS, MC contributed to the acquisition and analysis of data and drafting and revising the text. All authors approved the manuscript and agree both to be personally accountable for their own contribution and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

Funding

This research was supported by grant MS700568_0091 from Merck Serono S.p.A, Rome, Italy, an affiliate of Merck KGaA (CrossRef Funder ID: https://doi.org/10.13039/100009945). Damiano Marastoni was supported by the GR-2021-12373041 grant from Italian Ministry of Health #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). Massimiliano Calabrese was supported by the GR-2013-02-355322 grant from the Italian Ministry of Health.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The local ethics committee (Comitato etico per la sperimentazione clinica delle province di Verona e Rovigo) approved the study (CLAD19 Study, 2179CESC), and written informed consent was obtained from all the patients. Clinical trials registration: EudraCT Number: 2018-004947-21.

Competing interests

DM received honoraria for research or speaking and funds for travel from Biogen Idec, Roche, Sanofi-Genzyme, Novartis, and Merck-Serono. VC received research grant from European Charcot Foundation, received support for scientific meetings from Biogen, Janssen, Novartis, BMS, Roche and speaking honoraria from Novartis. MPS received consulting fees from Biogen, Merck, Novartis, Sanofi, Roche, Immunic, Alexion, Bristol-Meyer Squibb. MC received speaker honoraria from Biogen, Bristol Myers Squibb, Celgene, Genzyme, Merck Serono, Novartis, and Roche and received research support from the Progressive MS Alliance, Italian Minister of Health, the Novartis Pharma, Roche, Bristol Myers Squibb and Merck Serono.

Author details

[1]Neurology B, Department of Neurosciences, Biomedicine and Movement Sciences, University of Verona, Policlinico "G.B. Rossi" Borgo Roma, Piazzale L. A. Scuro, 10, Verona 37134, Italy
[2]Department of Health Sciences - Section of Biostatistics, University of Genoa, Genoa, Italy

[3]Department of Neuroscience, Neurology Unit, Multiple Sclerosis Center, S.Maria delle Croci Hospital, AUSL Romagna, Ravenna, Italy
[4]Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, L'Aquila, Italy
[5]Department of Engineering for Innovation Medicine, University of Verona, Verona, Italy
[6]Clinical Research Unit, Azienda Ospedaliera Universitaria Integrata (AOUI) Verona, Verona, Italy
[7]Neurology A, Azienda Ospedaliera Universitaria Integrata di Verona, Verona, Italy

Received: 2 December 2025 / Accepted: 3 February 2026
Published online: 12 February 2026

References

1. Kappos L, Wolinsky JS, Giovannoni G, et al. Contribution of Relapse-Independent progression vs Relapse-Associated worsening to overall confirmed disability accumulation in typical relapsing multiple sclerosis in a pooled analysis of 2 randomized clinical trials. JAMA Neurol. 2020;77(9):1132–40. https://doi.org/10.1001/jamaneurol.2020.1568.
2. Lublin FD, Häring DA, Ganjgahi H, et al. How patients with multiple sclerosis acquire disability. Brain. 2022;145(9):3147–61. https://doi.org/10.1093/brain/awac016.
3. Lassmann H. Pathogenic mechanisms associated with different clinical courses of multiple sclerosis. Front Immunol. 2018;9:3116. https://doi.org/10.3389/fimmu.2018.03116.
4. Howell OW, Reeves CA, Nicholas R, et al. Meningeal inflammation is widespread and linked to cortical pathology in multiple sclerosis. Brain. 2011;134(Pt 9):2755–71. https://doi.org/10.1093/brain/awr182.
5. Magliozzi R, Howell OW, Nicholas R, et al. Inflammatory intrathecal profiles and cortical damage in multiple sclerosis. Ann Neurol. 2018;83(4):739–55. https://doi.org/10.1002/ana.25197.
6. Magliozzi R, Scalfari A, Pisani AI, et al. The CSF profile linked to cortical damage predicts multiple sclerosis activity. Ann Neurol. 2020;88(3):562–73. https://doi.org/10.1002/ana.25786.
7. Absinta M, Sati P, Fechner A, Schindler MK, Nair G, Reich DS. Identification of chronic active multiple sclerosis lesions on 3T MRI. AJNR Am J Neuroradiol. 2018;39(7):1233–8. https://doi.org/10.3174/ajnr.A5660.
8. Calabrese M, Preziosa P, Scalfari A, et al. Determinants and biomarkers of progression independent of relapses in multiple sclerosis. Ann Neurol. 2024;96(1):1–20. https://doi.org/10.1002/ana.26913.
9. Heming M, Borsch AL, Wiendl H, Meyer Zu Horste G. High-dimensional investigation of the cerebrospinal fluid to explore and monitor CNS immune responses. Genome Med. 2022;14(1):94. https://doi.org/10.1186/s13073-022-01097-9.
10. Marastoni D, Magliozzi R, Bolzan A, et al. CSF levels of CXCL12 and osteopontin as early markers of primary progressive multiple sclerosis. Neurol Neuroimmunol Neuroinflamm. 2021;8(6). https://doi.org/10.1212/nxi.0000000000001083.
11. Marastoni D, Foschi M, Eccher C, et al. CSF levels of Chitinase3like1 correlate with early response to cladribine in multiple sclerosis. Front Immunol. 2024;15:1343892. https://doi.org/10.3389/fimmu.2024.1343892.
12. Pinteac R, Montalban X, Comabella M. Chitinases and chitinase-like proteins as biomarkers in neurologic disorders. Neurol Neuroimmunol Neuroinflamm. 2021;8(1). https://doi.org/10.1212/nxi.0000000000000921.
13. Stilund M, Reuschlein AK, Christensen T, Møller HJ, Rasmussen PV, Petersen T. Soluble CD163 as a marker of macrophage activity in newly diagnosed patients with multiple sclerosis. PLoS ONE. 2014;9(6):e98588. https://doi.org/10.1371/journal.pone.0098588.
14. Hofmann A, Krajnc N, Dal-Bianco A, et al. Myeloid cell iron uptake pathways and paramagnetic rim formation in multiple sclerosis. Acta Neuropathol. 2023;146(5):707–24. https://doi.org/10.1007/s00401-023-02627-4.
15. Mellergard J, Edstrom M, Vrethem M, Ernerudh J, Dahle C. Natalizumab treatment in multiple sclerosis: marked decline of chemokines and cytokines in cerebrospinal fluid. Mult Scler. 2010;16(2):208–17. https://doi.org/10.1177/1352458509355068.
16. Talbot J, Hojsgaard Chow H, Holm Hansen R, von Essen MR, Sellebjerg F. Immunological effects of dimethyl fumarate treatment in blood and CSF of patients with primary progressive MS. J Neuroimmunol. 2021;361:577756. https://doi.org/10.1016/j.jneuroim.2021.577756.
17. Cross AH, Gelfand JM, Thebault S, et al. Emerging cerebrospinal fluid biomarkers of disease activity and progression in multiple sclerosis. JAMA Neurol. 2024;81(4):373–83. https://doi.org/10.1001/jamaneurol.2024.0017.
18. Liliemark J. The clinical pharmacokinetics of cladribine. Clin Pharmacokinet. 1997;32(2):120–31. https://doi.org/10.2165/00003088-199732020-00003.
19. Kearns CM, Blakley RL, Santana VM, Crom WR. Pharmacokinetics of cladribine (2-chlorodeoxyadenosine) in children with acute leukemia. Cancer Res. 1994;54(5):1235–9.
20. Thompson AJ, Banwell BL, Barkhof F, et al. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. Lancet Neurol. 2018;17(2):162–73. https://doi.org/10.1016/S1474-4422(17)30470-2.
21. Muller J, Cagol A, Lorscheider J, et al. Harmonizing definitions for progression independent of relapse activity in multiple sclerosis: A systematic review. JAMA Neurol. 2023;80(11):1232–45. https://doi.org/10.1001/jamaneurol.2023.3331.
22. Teunissen CE, Petzold A, Bennett JL, et al. A consensus protocol for the standardization of cerebrospinal fluid collection and biobanking. Neurology. 2009;73(22):1914–22. https://doi.org/10.1212/WNL.0b013e3181c47cc2.
23. Geurts JJ, Roosendaal SD, Calabrese M, et al. Consensus recommendations for MS cortical lesion scoring using double inversion recovery MRI. Neurology. 2011;76(5):418–24. https://doi.org/10.1212/WNL.0b013e31820a0cc4.
24. Yushkevich PA, Yang G, Gerig G. ITK-SNAP: an interactive tool for semi-automatic segmentation of multi-modality biomedical images. Annu Int Conf IEEE Eng Med Biol Soc. 2016;2016:3342–5. https://doi.org/10.1109/EMBC.2016.7591443.
25. Sati P, Thomasson DM, Li N, et al. Rapid, high-resolution, whole-brain, susceptibility-based MRI of multiple sclerosis. Mult Scler. 2014;20(11):1464–70. https://doi.org/10.1177/1352458514525868.
26. Amato MP, Portaccio E, Goretti B, et al. The rao's brief repeatable battery and Stroop test: normative values with age, education and gender corrections in an Italian population. Mult Scler. 2006;12(6):787–93. https://doi.org/10.1177/1352458506070933.
27. Caffarra P, Vezzadini G, Francesca D, Zonato F, Venneri A. A short version of the Stroop test: normative data in an Italian population sample. Nuova Rivista Di Neurologia. 2002;07/01:12:111–5.
28. Costa A, Bagoj E, Monaco M, et al. Standardization and normative data obtained in the Italian population for a new verbal fluency instrument, the phonemic/semantic alternate fluency test. Neurol Sci. 2014;35(3):365–72. https://doi.org/10.1007/s10072-013-1520-8.
29. Cattelani R, Dal Sasso F, Corsini D, Posteraro L. The modified Five-Point test: normative data for a sample of Italian healthy adults aged 16–60. Neurol Sci. 2011;32(4):595–601. https://doi.org/10.1007/s10072-011-0489-4.
30. Ziccardi S, Pisani AI, Schiavi GM, et al. Cortical lesions at diagnosis predict long-term cognitive impairment in multiple sclerosis: A 20-year study. Eur J Neurol. 2023;30(5):1378–88. https://doi.org/10.1111/ene.15697.
31. Ziccardi S, Fuchs TA, Guandalini M, Marastoni D, Benedict RH, Calabrese M. Cognitive progression independent of relapse and MRI activity in multiple sclerosis. Neurol Open Access. 2025;1(e0011). https://doi.org/10.1212/WN9.0000000000000005.
32. Wiendl H. Cladribine - an old newcomer for pulsed immune reconstitution in MS. Nat Rev Neurol. 2017;13(10):573–4. https://doi.org/10.1038/nrneurol.2017.119.
33. Baker D, Pryce G, Herrod SS, Schmierer K. Potential mechanisms of action related to the efficacy and safety of cladribine. Mult Scler Relat Disord. 2019;30:176–86. https://doi.org/10.1016/j.msard.2019.02.018.
34. Fresegna D, Bullitta S, Musella A, et al. Re-Examining the role of TNF in MS pathogenesis and therapy. Cells. 2020;9(10). https://doi.org/10.3390/cells9102290.
35. Magliozzi R, Pezzini F, Pucci M, et al. Changes in cerebrospinal fluid balance of TNF and TNF receptors in Naive multiple sclerosis patients: early involvement in compartmentalised intrathecal inflammation. Cells. 2021;10(7). https://doi.org/10.3390/cells10071712.
36. James RE, Schalks R, Browne E, et al. Persistent elevation of intrathecal pro-inflammatory cytokines leads to multiple sclerosis-like cortical demyelination and neurodegeneration. Acta Neuropathol Commun. 2020;8(1):66. https://doi.org/10.1186/s40478-020-00938-1.
37. Rejdak K, Stelmasiak Z, Grieb P. Cladribine induces long lasting oligoclonal bands disappearance in relapsing multiple sclerosis patients: 10-year observational study. Mult Scler Relat Disord. 2019;27:117–20. https://doi.org/10.1016/j.msard.2018.10.006.

38. Leist TP, Weissert R. Cladribine: mode of action and implications for treatment of multiple sclerosis. Clin Neuropharmacol. 2011;34(1):28–35. https://doi.org/10.1097/WNF.0b013e318204cd90.
39. Jorgensen LO, Hyrlov KH, Elkjaer ML, et al. Cladribine modifies functional properties of microglia. Clin Exp Immunol. 2020;201(3):328–40. https://doi.org/10.1111/cei.13473.
40. Singh V, Voss EV, Benardais K, Stangel M. Effects of 2-chlorodeoxyadenosine (Cladribine) on primary rat microglia. J Neuroimmune Pharmacol. 2012;7(4):939–50. https://doi.org/10.1007/s11481-012-9387-7.
41. Aybar F, Julia Perez M, Silvina Marcora M, et al. 2-Chlorodeoxyadenosine (Cladribine) preferentially inhibits the biological activity of microglial cells. Int Immunopharmacol. 2022;105:108571. https://doi.org/10.1016/j.intimp.2022.108571.
42. Schroeter CB, Rolfes L, Gothan KSS, et al. Cladribine treatment improves cortical network functionality in a mouse model of autoimmune encephalomyelitis. J Neuroinflammation. 2022;19(1):270. https://doi.org/10.1186/s12974-022-02588-7.
43. Musella A, Mandolesi G, Gentile A, et al. Cladribine interferes with IL-1beta synaptic effects in experimental multiple sclerosis. J Neuroimmunol. 2013;264(1–2):8–13. https://doi.org/10.1016/j.jneuroim.2013.08.009.
44. Piccio L, Naismith RT, Trinkaus K, et al. Changes in B- and T-lymphocyte and chemokine levels with rituximab treatment in multiple sclerosis. Arch Neurol. 2010;67(6):707–14. https://doi.org/10.1001/archneurol.2010.99.
45. Alvarez E, Piccio L, Mikesell RJ, et al. Predicting optimal response to B-cell depletion with rituximab in multiple sclerosis using CXCL13 index, magnetic resonance imaging and clinical measures. Mult Scler J Exp Transl Clin. 2015;1:2055217315623800. https://doi.org/10.1177/2055217315623800.
46. Axelsson M, Malmeström C, Gunnarsson M, et al. Immunosuppressive therapy reduces axonal damage in progressive multiple sclerosis. Mult Scler. 2014;20(1):43–50. https://doi.org/10.1177/1352458513490544.
47. Novakova L, Axelsson M, Khademi M, et al. Cerebrospinal fluid biomarkers of inflammation and degeneration as measures of Fingolimod efficacy in multiple sclerosis. Mult Scler. 2017;23(1):62–71. https://doi.org/10.1177/1352458516639384.
48. Schmierer K, Wiendl H, Barkhof F, et al. Clinical and mechanistic effects of cladribine in relapsing multiple sclerosis: 2-year results from the MAGNIFY-MS study. Ther Adv Neurol Disord. 2025;18:17562864251351760. https://doi.org/10.1177/17562864251351760.
49. Haider L, Prados F, Chung K, et al. Cortical involvement determines impairment 30 years after a clinically isolated syndrome. Brain. 2021;144(5):1384–95. https://doi.org/10.1093/brain/awab033.
50. Calabrese M, Battaglini M, Giorgio A, et al. Imaging distribution and frequency of cortical lesions in patients with multiple sclerosis. Neurology. 2010;75(14):1234–40. https://doi.org/10.1212/WNL.0b013e3181f5d4da.
51. Calabrese M, Reynolds R, Magliozzi R, et al. Regional distribution and evolution of Gray matter damage in different populations of multiple sclerosis patients. PLoS ONE. 2015;10(8):e0135428. https://doi.org/10.1371/journal.pone.0135428.
52. Scalfari A, Romualdi C, Nicholas RS, et al. The cortical damage, early relapses, and onset of the progressive phase in multiple sclerosis. Neurology. 2018;90(24):e2107–18. https://doi.org/10.1212/WNL.0000000000005685.
53. De Stefano N, Giorgio A, Battaglini M, et al. Reduced brain atrophy rates are associated with lower risk of disability progression in patients with relapsing multiple sclerosis treated with cladribine tablets. Mult Scler. 2018;24(2):222–6. https://doi.org/10.1177/1352458517690269.
54. Cortese R, Battaglini M, Sormani MP, et al. Reduction in grey matter atrophy in patients with relapsing multiple sclerosis following treatment with cladribine tablets. Eur J Neurol. 2023;30(1):179–86. https://doi.org/10.1111/ene.15579.
55. Monif M, Sequeira RP, Muscat A, et al. CLADIN- cladribine and innate immune response in multiple sclerosis - A phase IV prospective study. Clin Immunol. 2024;265:110304. https://doi.org/10.1016/j.clim.2024.110304.
56. Wiendl H, Barkhof F, Montalban X, et al. Blood biomarker dynamics in people with relapsing multiple sclerosis treated with cladribine tablets: results of the 2-year MAGNIFY-MS study. Front Immunol. 2025;16:1571978. https://doi.org/10.3389/fimmu.2025.1571978.
57. Pezzini F, Pisani A, Mazziotti V, et al. Intrathecal versus peripheral inflammatory protein profile in MS patients at diagnosis: A comprehensive investigation on serum and CSF. Int J Mol Sci. 2023;24(4):3768. https://doi.org/10.3390/ijms24043768.

Publisher's note