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Insights in multiple sclerosis and neuroimmunology 2021

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Insights in multiple sclerosis and neuroimmunology: 2021

Topic editors

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Editorial: Insights in multiple sclerosis and neuroimmunology: 2021

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Editorial on the Research Topic
Insights in multiple sclerosis and neuroimmunology: 2021

A total of 19 articles are published in the Frontiers Research Topic "*Insights in multiple sclerosis and neuroimmunology 2021*." These cover a wide aspect of multiple sclerosis (MS)-related themes as well as themes in autoimmune encephalitis.

There is a need for consensus criteria about the identification of certain types of MS. Meca-Lallana et al. show in their article "*Consensus on early detection of disease progression in patients with multiple sclerosis*" that such consensus statements could help clinicians to find early in the disease course patients with secondary progressive MS (SPMS). Such an early identification is important to perform adequate therapeutic management. Standardized clinical assessments are meaningful in MS care. In the article "*Making every step count: minute-by-minute characterization of step counts augments remote activity monitoring in people with multiple sclerosis*," Block et al. used a model to predict disease progression over the longer term (>2 years) based on obtained measurements. These findings will be used to develop further descriptive metrics for activity. In their article "*Current status and future opportunities in modeling clinical characteristics of multiple sclerosis*," Liu et al. suggest that there is a strong need to develop validated models of MS clinical outcomes by using cellular or/and molecular biomarkers. In the article titled "*Models of care in multiple sclerosis: a survey of Canadian health providers*," Marrie, Donkers et al. claim that the ideal MS service is multidisciplinary in nature, ideally integrated, and with prompt access to care.

The myxovirus resistance protein A (MxA) has been long used as a marker for exogenous interferon-beta efficacy in MS treatment. Coerver et al. show in the article "*The association between blood MxA mRNA and long-term disease activity in early multiple sclerosis*" that MxA mRNA is expressed in inflammatory pathology in MS that is dependent on the endogenous type-1 interferon system and that this might be a prognostic biomarker for long-term inflammatory disease activity in MS. In the article "*Genetic risk variants for multiple sclerosis are linked to differences in alternative pre-mRNA splicing*" by Putscher et al., the authors show that genetic variants from MS risk loci affect pre-mRNA splicing. Amoriello et al. investigated soluble HLA-G (sHLA-G) levels in MS in the article "*Investigating serum sHLA-G cooperation with MRI activity and disease-modifying treatment outcome in relapsing-remitting multiple sclerosis*."

They found that the HLA-G genotype strongly influences sHLA-G levels. Autoantibodies are of importance in various neuroimmunological disorders, and their role and mechanism of action are partly undefined. In the article "*Peptidylarginine deiminase 2 autoantibodies are linked to less severe disease in multiple sclerosis and post-treatment Lyme disease*," Kim et al. make the case that anti-peptidylarginine deiminase 2 (PAD2) antibodies may attenuate inflammation. This effect is observable in tissues with high expression of PAD2. The role of hemolysis was analyzed in the article "*Peripheral hemolysis in relation to iron rim presence and brain volume in multiple sclerosis*" by Krajnc et al. The authors found an influence of hemolysis on the brain volume but not on the presence of iron rim lesions in progressive MS. Investigations about metabolomics in neuroimmunological disorders is of increasing interest. In their study "*Metabolomics of cerebrospinal fluid in multiple sclerosis compared with healthy controls: a pilot study*," Židó et al. investigated cerebrospinal fluid (CSF) from MS patients compared to controls regarding metabolomic profiles. They found differences in amino and fatty acids in the CSF of newly diagnosed patients with MS in comparison with controls. The most significant changes were seen in levels of arginine, histidine, and palmitic acid. They concluded that such a metabolomic profile may predict inflammatory disease activity in MS. In the article "*Effects of vascular comorbidity on cognition in multiple sclerosis are partially mediated by changes in brain structure*," Marrie, Patel et al. showed that vascular comorbidity leads to changes in brain macrostructure and microstructure. In addition, this is associated with lower cognitive function in patients with MS.

In "*Bridging therapies with injectable immunomodulatory drugs in the management of multiple sclerosis: a Delphi survey of an Italian expert panel of neurologists*," Marfia et al. suggest that the value of bridging therapy with injectable immunomodulatory drugs in MS disease conditions is underscored. The article focuses on patients with MS who plan to become pregnant and patients with MS at risk for cancer recurrence. Ozanimod is a selective sphingosine-1-phosphate (S1P)-receptor 1 (S1P1) and S1P5 modulator used for the treatment of active forms of relapsing-remitting MS (RRMS). Ziemssen et al. present their real-world and long-term study "*OzEAN study to collect real-world evidence of persistent use, effectiveness, and safety of ozanimod over 5 years in patients with relapsing-remitting multiple sclerosis in Germany*." The results of this study will add to the safety profile and efficacy profile of ozanimod in the treatment of RRMS. In the study "*Safety, adherence and persistence in a real-world cohort of German MS patients newly treated with ocrelizumab: first insights from the CONFIDENCE study*," Weber et al. describe the safety profile of ocrelizumab in the CONFIDENCE real-world MS population study. The findings were consistent with the findings in pivotal clinical trials for the anti-CD20 B cell-depleting antibody ocrelizumab used for the treatment of patients with RRMS and patients with primary progressive MS (PPMS). Importantly, high treatment persistence and adherence were seen in this real-world MS population study. Fathi et al. suggested in their article, "*Dynamic changes in kynurenine pathway metabolites in multiple sclerosis: a systematic review*," that quinolinic acid is a possible player in the pathogenesis of MS. This conclusion is mainly based on the finding that quinolinic acid levels in CSF were higher in patients with MS than in healthy controls. The value of disease models induced in mice and rats on certain novel MS therapeutic approaches is outlined by Jayaraman and Jayaraman in their article "*Impact of histone modifier-induced protection against autoimmune encephalomyelitis on multiple sclerosis treatment*" about histone deacetylase (HDAC) inhibitors. HDAC inhibitors such as valproic acid and hydroxamates as well as others are possible candidates for future treatment of MS.

It has been shown that in paraneoplastic forms of autoimmune encephalitis, the removal of the associated cancer entity is of primary importance in long-term disease outcomes. For teratoma, Zhang et al. show in their article "*Long-term prognosis of patients with anti-N-methyl-D-aspartate receptor encephalitis who underwent teratoma removal: an observational study*" that early detection and removal of teratoma resulted in a favorable long-term prognosis in patients with anti-NMDAR encephalitis. Case studies can be of importance for defining potential new disease entities and for the description of rare disease variants. In the case study "*Acute cerebellitis associated with anti-homer 3 antibodies: a rare case report and literature review*" by Miao et al., the authors underscore the need for immune-mediated causes to be considered in acute cerebellitis. Importantly, immunotherapy can contribute to the improvement of cerebellar syndrome. Neuropsychological assessment is important in phenotyping and care of patients with neuroimmunological disorders and especially autoimmune encephalitis. In the article by Chan et al., "*Cognitive and mood profiles among patients with stiff person syndrome spectrum disorders*," it is clarified that neuropsychological testing in stiff person syndrome should include testing of verbal learning and recall, phonemic verbal fluency, attention, and processing speed.

In conclusion, the Research Topic "*Insights in multiple sclerosis and neuroimmunology 2021*" gives novel insight into current research themes on MS and autoimmune encephalitis.

Author contributions

RW outlined and wrote the Editorial.

Conflict of interest

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Publisher's note

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CASE REPORT
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Acute Cerebellitis Associated With Anti-homer 3 Antibodies: A Rare Case Report and Literature Review

Ailiang Miao [1,2], Chuanyong Yu [1,2], Yulei Sun [3], Lingling Wang [1,2], Jianqing Ge [1,2] and Xiaoshan Wang [1,2]*

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Acute cerebellitis associated with Homer-3 antibodies is very rare. Here we present a 20-year-old woman who suffered from uncontrollable head shaking quickly from side to side and an unsteady gait for 2 days after the cold. Antibodies were screened by cell-based assays. The indirect immunofluorescence technique results revealed anti-Homer-3 antibody titers of 1:3.2 in the CSF and 1:100 in the serum. The woman was obviously improved after antiviral and immunosuppression (immunoglobin, methylprednisolone and mycophenolate mofetil) treatment. Our report indicated immune-mediated causes should be considered in the acute cerebellitis. Immunotherapy can contribute to the improvement of cerebellar syndrome.

Keywords: cerebellitis, anti-Homer 3 antibody, head shaking, cerebellar syndrome, immunotherapeutic treatment

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INTRODUCTION

In 2013, Hoftberger et al. reported a 38-year-old man with anti-Homer 3 antibodies who presented with symptoms of acute encephalopathy including headache, nausea, vomiting, and confusion, and cerebellar syndrome (1). Furthermore, seven patients with subacute or insidious "idiopathic cerebellar ataxia," not acute cerebellitis, were reported (2, 3). Acute cerebellitis associated with anti-Homer 3 antibodies is very rare. Here, we report a female with acute cerebellitis associated with anti-Homer 3 antibodies.

CASE REPORT

A 20-year-old woman suffered from uncontrollable head shaking twice quickly from side to side (**Supplementary Video 1** and **Figure 1A**) for 2 days after the cold (**Table 1**). In other words, the head swayed twice quickly and slightly from side to side. The unsteady gait was also observed (**Supplementary Video 2**). The patient had a history of allergic rhinitis for 2 years. Neurological examination demonstrated bilateral horizontal nystagmus, moderate limb dysmetria, Romberg sign positivity and gait ataxia. The patient was admitted to Nanjing Brain Hospital. On day 1, brain magnetic resonance imaging (MRI) showed an increased signal in the right cerebellar hemisphere without enhancement (**Figures 1B,C**). On day 3, lumbar puncture was performed, and a pressure of 180 mmH_2O, a WBC count of 139 $\times$ 10^6 /L (**Figure 2**), and a protein level of 1.67 g/L were observed. The oligoclonal band was positive. On day 5, the indirect immunofluorescence technique (IIFT) results revealed anti-Homer-3 antibody titers of 1:3.2 in the CSF and 1:100 in the

TABLE 1 | The symptoms and treatment in the patient according to timeline.

Symptoms and examination	Time	Treatment
Intermittent headache; fatigue;	2021-10-7	Without treatment
Intermittent headache, neck pain; fatigue	2021-11-1	
Gait ataxia.	2021-11-16	
Head shaking uncontrollably from side to side (**Supplementary Video 1** and **Figure 1A**); Gait ataxia (**Supplementary Video 2**).	2021-11-18	
The patient could not complete heel-knee-tibia test and finger-nose test stably, and presented moderate limb dysmetria, Romberg sign and horizontal nystagmus. The patient also could not walk in a straight line.	2021-11-20 Hospitalization	Ganciclovir for injection was administered and sustained by 0.375 g twice a day.
Increased signal in the right cerebellar hemisphere without enhancement	2021-11-20 Brain magnetic resonance imaging (MRI)	
WBC count:139 × 10^6 /L (**Figure 2**); protein level:1.67 g/L.	2021-11-22 Lumbar puncture	
	2021-11-24	Anti-Homer-3 antibody titers of 1:3.2 in the CSF and 1:100 in the serum (**Figure 1**).
	2021-11-24	Methylprednisolone for injection was administered by 1,000 mg per day, and reduced by half every three days. Immunoglobin was administered by 25 g per day for 5 days (2 g per kilogram).
Gait ataxia, head shaking and horizontal nystagmus improved.	2021-11-30	
Gait ataxia, head shaking and horizontal nystagmus still improved.	2021-12-3	Mcophenolate mofetil was given and sustained by 0.5 g twice a day. Methylprednisolone was administered by 120 mg per day for 3 days.
The heel-knee-tibia test, finger-nose test and moderate limb dysmetria improved.	2021-12-6	Prednison was given by 60mg per day, and reduced by 5mg every two week.
Anti-Homer-3 antibody titers of 1:3.2 in the CSF and 1:100 in the serum. WBC count: 55 × 10^6 /L; protein level: 0.91 g/L. Horizontal nystagmus was not observed. Gait ataxia and head shaking was still observed (**Supplementary Video 2**).	2021-12-9 Lumbar puncture	
Head shaking disappeared.	2021-12-15	
The improvement of gait ataxia was not remarkable. The patient still could not walk in a straight line.	2021-12-25	Another immunoglobin was administered by 25 g per day for 5 days (2 g per kilogram).
Normal	2022-1-5 Brain MRI	
WBC count: 29 × 10^6 /L; protein level: 0.75g/L.	2022-1-13 Lumbar puncture	Ganciclovir and mcophenolate mofetil was administered and sustained.
Although the patient improved remarkably, mild gait ataxia and unbalance during walking in a straight line were still observed (**Supplementary Video 2**). The patient could complete both hands alternating movement test, heel-knee-tibia test and finger-nose stably, and did not present Romberg sign. Another lumbar puncture was not received by the patient.	2022-1-25 Hospital discharge	Acyclovir tablets were given by 0.4 g three times a day for two weeks. Mcophenolate mofetil was given and sustained by 0.5 g twice a day. Prednison was given by 50 mg daily, and reduced by 5 mg every two weeks.

serum (**Figures 1D,E**). The gynecological sonography was normal. On day 4, mild diffuse waves were observed on electroencephalography. After 66 days of antiviral and immunosuppression (immunoglobin, methylprednisolone and mycophenolate mofetil) treatment, the woman was obviously improved (**Supplementary Video 2**, **Table 1**).

DISCUSSION

We described a rare case of cerebellitis associated with Homer-3 antibodies. This patient was positive for the anti-Homer 3 antibody in the CSF and serum, but negative for anti-ATP1A3, ARHGAP26, ITPR1, Hu, Yo, Ri, CV2, Ma2, amphiphysin, Tr(DNER), Zic4, Ma1, GAD65, PKCγ, SOX1, NMDAR, AMPA1, AMPA2, GABAB, LG1, CASPR2, DPPX, lolON5, mGluR5, GlyRα1, GABAARα1, and GABAARβ3.

The Homer family includes Homer-1, Homer-2, and Homer-3, all of which have several isoforms as a result of alternative splicing (4). Homer proteins can be divided into the two structurally distinct groups of short and long Homer proteins. Short Homers include Homer-1A, Homer-2C, Homer-2D, Homer-3C and Homer-3D. Long Homer proteins include Homer-1B, Homer-1C, Homer-2A, Homer-2B, Homer-$3A_{xx}$ and Homer-$3B_{xx}$. The short∼35 amino

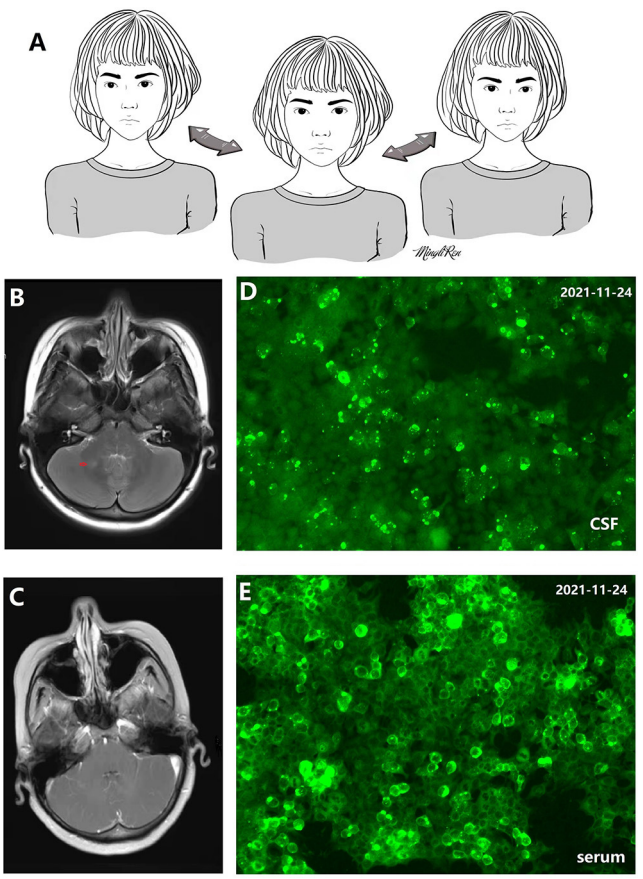

FIGURE 1 | (A) 2D picture associated with **Supplementary Video 1** showing uncontrollable and quick head shaking. **(B,C)** Increased signal in the right cerebellar hemisphere without enhancement. **(D)** Antibody in cerebrospinal fluid (CSF) recognizes Homer-3 antigens in fifixed HEK293 cells. Anti-Homer-3 antibody titers in CSF: 1:3.2. **(E)** Antibody in serum recognizes Homer-3 antigens in fifixed HEK293 cells. Anti-Homer-3 antibody titers in serum: 1:100.

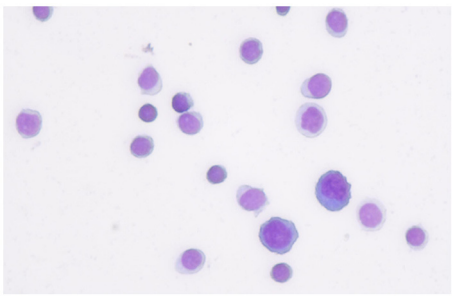

FIGURE 2 | The first cerebrospinal fluid cytology from the patient showed that most of inflammatory cells were lymphocyte.

acid residue long coiled-coiled domain in the Homer N-terminals may be important for the folding of Homers themselves or involved in interacting with proteins. This short N-terminal coiled-coil domain is present in all Homer-3 proteins except for the Homer-3B. The short domain in Homer-3A is remarkable longer than that in Homer-3C and Homer-3D (5). Homer-3 and mGluR1 (metabotropic glutamate receptor) are expressed predominantly on Purkinje cell dendritic spines (6). Homer-3 is the scaffold protein between mGluR1 and inositol 1,4,5 triphosphate receptors, which regulate the post-synaptic calcium metabolism in Purkinje cells in response to mGLuR1 stimulation (7). Thus, the anti-Homer 3 antibodies might bind Homer-3A, Homer-3C and Homer-3D, especially Homer-3A disturbing the homer 3 function, which could contribute to cerebellar ataxia (1–3, 5). Cerebellar ataxia is also the most common symptom of anti-mGluR1 autoimmunity (8).

In 2007, Zuliani et al. reported a 65-year-old woman with Homer-3 antibodies presenting with subacute cerebellar ataxia. Although the patient received steroids, the cerebellar syndrome had not improved by the last follow-up (2). Guan et al. screened the serum and CSF samples of 750 patients with 'idiopathic' cerebellar ataxia, and Homer-3 antibodies were detected in 6 patients. Interestingly, 2 patients had RBD, a hot cross bun sign, and dysautonomia, which may be considered diagnostic markers for multiple system atrophy of the cerebellar type (MSA-C) (3). Given that there is no effective treatment for MSA-C, immune-mediated cerebellar syndrome can be improved by immunotherapy (3). Homer-3 antibodies are even more rarer, and screening for antibodies in every patient with acute, subacute and insidious cerebellar syndrome is unrealistic. An interesting symptom, "head socking uncontrollably from side to side (**Supplementary Video 1** and **Figure 1A**)," was observed in this patient with Homer-3 antibodies, which might be a characteristic of cerebellar syndrome with Homer-3 antibodies, and was not reported in the previous studies (1–3). Seasonable immunotherapy can contribute to the improvement of cerebellar syndrome, and delayed treatment might lead to unfavorable outcomes in patients with cerebellar ataxia (3). **Figure 1A** was depicted by a female patient with anti-N-methyl-d-aspartate receptor encephalitis in our hospital (9). Immunotherapeutic treatment was not delayed, and the patient had no residual problems (9).

In summary, we report a rare patient with cerebellitis with Homer-3 antibodies who improved after immunotherapeutic treatment. The symptom "head shaking" might lead to cerebellar syndrome associated with Homer-3 antibodies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AM: drafting and revising the manuscript. XW: study concept or design and study supervision. CY, YS, LW, and JG: clinical work. Their contributions helped us to acquire clinical data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2022.837937/full#supplementary-material

Supplementary Video 1 | The patient presented head shaking twice uncontrollably and quickly from side to side, without body shaking.

Supplementary Video 2 | The patient was administered antiviral and immunotherapies and improved.

REFERENCES

1. Höftberger R, Sabater L, Ortega A, Dalmau J, Graus F. Patient with homer-3 antibodies and cerebellitis. *JAMA Neurol.* (2013) 70:506–9. doi: 10.1001/jamaneurol.2013.1955
2. Zuliani L, Sabater L, Saiz A, Baiges JJ, Giometto B, Graus F. Homer 3 autoimmunity in subacute idiopathic cerebellar ataxia. *Neurology.* (2007) 68:239–40. doi: 10.1212/01.wnl.0000251308.79366.f9
3. Liu M, Ren H, Fan S, Zhang W, Xu Y, Zhao W, et al. Neurological autoimmunity associated with homer-3 antibody: a case series from China. *Neurol Neuroimmunol Neuroinflamm.* (2021) 27:8:e1077. doi: 10.1212/NXI.0000000000001077
4. Shiraishi-Yamaguchi Y, Furuichi T. The homer family proteins. *Genome Biol.* (2007) 8:206. doi: 10.1186/gb-2007-8-2-206
5. Soloviev MM, Ciruela F, Chan WY, McIlhinney RA. Molecular characterisation of two structurally distinct groups of human homers, generated by extensive alternative splicing. *J Mol Biol.* (2000) 295:1185–200. doi: 10.1006/jmbi.1999.3436
6. Shiraishi Y, Mizutani A, Yuasa S, Mikoshiba K, Furuichi T. Diffffferential expression of Homer family proteins in the developing mouse brain. *J. Comp. Neurol.* (2004) 473:582–99. doi: 10.1002/cne.20116
7. Mizutani A, Kuroda Y, Futatsugi A, Furuichi T, Mikoshiba K. Phosphorylation of Homer3 by calcium/calmodulin-dependent kinase II regulates a coupling state of its target molecules in Purkinje cells. *J Neurosci.* (2008) 28:5369–82. doi: 10.1523/JNEUROSCI.4738-07.2008
8. Spatola M, Petit Pedrol M, Maudes E, Maudes E, Simabukuro M, Muñiz-Castrillo S, et al. Clinical features, prognostic factors, and antibody effffects in anti-mGluR1 encephalitis. *Neurology.* (2020) 95:e3012–25. doi: 10.1212/WNL.0000000000010854
9. Wang Y, Miao A, Shi Y, Ge J, Wang L, Yu C, et al. Influencing electroclinical features and prognostic factors in patients with anti-NMDAR encephalitis: a cohort follow-up study in Chinese patients. *Sci Rep.* (2020) 10:10753. doi: 10.1038/s41598-020-67485-6

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ORIGINAL RESEARCH
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Long-Term Prognosis of Patients With Anti-N-Methyl-D-Aspartate Receptor Encephalitis Who Underwent Teratoma Removal: An Observational Study

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Objective: This study aimed to evaluate the clinical characteristics and long-term surgical outcomes of patients with anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis with teratoma.

Methods: Patients who were admitted to West China Hospital from June 2012 to June 2019 and diagnosed with anti-NMDAR encephalitis were enrolled in the study. Medical records were reviewed prospectively to gather clinical characteristic data. Patients were followed up at long-term every 3 months.

Results: This study included 192 patients, among whom 21 (10.9%) were detected with having a teratoma. Patients included 20 women, with a mean age of 24.62 ± 7.61 years. Seizure and psychiatric symptoms were the most dominant symptoms in both groups, followed by memory deficits. Central hypoventilation (52.4 vs. 17%, $p < 0.001$) and decreased consciousness (71.4 vs. 31.3%, $p = 0.002$) were significantly more frequent in patients with teratoma than in those without. Moreover, the anti-NMDAR antibody titer was higher ($p = 0.021$) and the baseline modified Rankin scale score was lower ($p = 0.004$) in patients with teratoma than in those without. First-line immunotherapy was performed in 21 (100%) patients with teratoma and 167 (97.7%) patients without teratoma. All patients with teratoma had the tumor removed. During follow-up, two (9.5%) patients with teratoma and 11 (6.4%) patients without teratoma died, whereas 1 (4.8%) patient with teratoma and 37 (21.6%) patients without teratoma had relapses. Overall, 19 (90.5%) patients with teratoma and 151 (88.3%) patients without teratoma achieved favorable clinical outcomes at the final follow-up.

Conclusions: With early detection and removal of teratoma, most patients with anti-NMDAR encephalitis and teratoma achieved a favorable long-term prognosis.

Keywords: anti-NMDAR encephalitis, teratoma, surgery, prognosis, relapse

INTRODUCTION

Anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis is an autoimmune disease that is characterized by psychiatric symptoms, seizures, memory deficits, speech impairment, movement disorders, autonomic instability, central hypoventilation, and decreased consciousness (1–3). The condition is primarily mediated by specific immunoglobulin G (IgG) antibodies against the NR1 subunit of the NMDAR. Tumors (mainly teratomas) containing nerve tissues can induce the production of specific antibodies *via* molecular mimicry and have been identified as a trigger of anti-NMDAR encephalitis (3, 4). Previous studies have reported a prevalence of teratoma at 20.2–45% in patients with anti-NMDAR encephalitis (5, 6). Immunotherapy is the most crucial therapeutic method for anti-NMDAR encephalitis. For patients with a tumor, surgery is an important treatment strategy and is recommended to be performed as soon as possible (3). In this study, we explored the clinical characteristics and long-term prognoses of these patients, following a surgery.

METHODS

Study Design and Participants

The Outcome of anti-NMDAR Encephalitis Study in Western China (ONE-WC) study was registered with the WHO international clinical trial registry platform (registration number: ChiCTR1800019762) and is described in more detail in our previous publications (7–11). Patients were hospitalized patients recruited from the Neurology Department of West China Hospital from October 2011 to June 2019. Inclusion criteria were as follows: (1) rapid onset of at least one of eight major groups of symptoms (psychosis, memory deficits, speech disturbances, seizures, movement disorders, disturbance of consciousness, autonomic dysfunctions, and central hypoventilation) (1); (2) positive for anti-NMDAR antibodies in the cerebrospinal fluid (CSF).

Exclusion criteria were as follows: (1) human immunodeficiency virus infection, meningitis, brain abscess, prior diseases, cerebral malaria, brain tumor, or diagnosis of a non-infectious central nervous system disease, such as acute demyelinating encephalomyelitis; (2) patients with laboratory evidence of infectious encephalitis; (3) patients diagnosed with epilepsy, cerebral trauma, and/or other nervous system diseases prior to the onset of encephalitis; (4) patients with other coexisting positive autoimmune or neurologic paraneoplastic antibodies, such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors-1 and−2, contactin-associated protein-2, leucine-rich glioma-inactivated protein-1, c-aminobutyric acid receptor B1/B2, anti-neuronal nuclear antibody (ANNA)-1, ANNA-2, and Purkinje-cell cytoplasmic autoantibody-1.

Clinical Management

A lumbar puncture was performed in suspected patients with rapid onset of neurological or psychiatric disorders. Samples were assessed using an indirect immunofluorescence assay for the detection of autoimmune neurologic paraneoplastic antibodies. Individuals with confirmed antibodies underwent chest and abdomen CT or abdomen and reproductive system ultrasound to search for potential tumors. Abnormalities were reported by radiologists and reviewed by relevant specialists (e.g., gynecologists for a pelvic mass in women).

Treatments were administered by senior neurologists of West China Hospital, Department of Neurology. Immunotherapies included first-line immunotherapy (intravenous immunoglobulin [IVIg], methylprednisolone, and plasma exchange) and second-line immunotherapies (rituximab, cyclophosphamide, azathioprine, mycophenolate mofetil, and tacrolimus). First-line immunotherapies were administered as follows: .4 g/kg IVIg was administered daily for 5 days as one turn; 1,000 mg intravenous methylprednisolone was administered daily for 3–5 days as one turn, then replaced by daily prednisone. Repeated intravenous immunotherapy was administered to patients with a poor response. Other interventions included anti-epileptic drugs, anti-psychotic drugs, sedative-hypnotic drugs, and other symptomatic/supportive treatments.

Patients with a suspected tumor were evaluated by a multi-disciplinary team to determine whether surgery was recommended and its timing. Operations were performed by surgeons of West China Hospital of West China Second University Hospital. Patients were discharged following a surgery or transferred to a neurological intensive care unit for post-surgical immunotherapy depending on their neurological symptoms. Pathological diagnoses were made, following a surgery.

Data Collection and Definition

Patients' clinical characteristics during hospitalization were extracted from medical records and included epidemiologic data (sex and age), clinical data (date of onset, date of admission, and typical symptoms), biological data (CSF antibody titers, CSF cell count, glucose/protein/IgG synthesis rates, and IgG index), auxiliary examination (MRI/CT results, ultrasound results, electroencephalography [EEG] results), and clinical management data (treatment administered, date of immunotherapy, and date of surgery). All data were collected by clinicians using a standardized form. Follow-up visits were conducted by a clinician every 3 months from clinical onset over the telephone. Patients' neurological and psychiatric sequelae were questioned, and patients who reported worsening or new onset of the eight major groups of symptoms were requested to attend the neurological clinic to evaluate the possibility of relapse and undergo further investigations if necessary.

Outcomes were assessed using the modified Rankin scale (mRS) (12). Evaluations were carried out face-to-face by neurologists during hospitalization and by patients' or guardians' responses over the telephone, following the discharge. Relapse was defined as a worsening or new onset of previous symptoms, occurring after at least 2 months of improvement or stabilization and was confirmed by antibodies detected in the CSF. Clinical improvement was defined as a decrease of 1 or more in mRS score (2). A long-term favorable outcome was defined as an mRS score ≤ 2 (2).

TABLE 1 | Clinical features of patients with and without teratoma.

	Total, n (%)	With teratoma, n (%)	Without teratoma, n (%)	*P*-value
Quantity	192	21	171	-
Age, years (mean ± SD)	29.44 ± 13.01	24.62 ± 7.61	30.03 ± 13.46	0.009*
Sex (female)	107 (55.7)	20 (95.2)	87 (50.9)	< 0.001#
Psychiatric symptoms	175 (91.1)	19 (90.5)	156 (91.2)	0.829#
Seizure	153 (79.7)	18 (85.7)	135 (78.9)	0.660#
Speech impairment	47 (24.5)	8 (38.1)	39 (22.8)	0.124#
Dyskinesias/movement disorders	79 (41.1)	7 (33.3)	72 (42.1)	0.441#
Autonomic instability	92 (47.9)	13 (61.9)	79 (46.2)	0.174#
Memory deficits	157 (81.8)	15 (71.4)	142 (83.0)	0.193#
Decreased consciousness	77 (40.1)	15 (71.4)	62 (36.3)	0.002#
Cognitive disorder	126 (65.6)	13 (61.9)	113 (66.1)	0.650#
Central hypoventilation	40 (20.8)	11 (52.4)	29 (17.0)	< 0.001#
Baseline mRS score	4 (4.5)	5 (4.5)	4 (4.5)	0.004+
Abnormal MRI findings	74/181 (40.9)	7/21 (33.3)	67/160 (41.9)	0.454#
Abnormal EEG findings	141/170 (82.9)	16/18 (88.9)	125/152 (88.2)	0.705#
Antibody titer in cerebrospinal fluid				
1:1–1:10	44	1	43	0.021+
1:10–1:100	115	13	102	
1:100–1:1000	33	7	26	

*Student's t-test.
#chi-squared or Fisher's exact tests.
+Wilcoxon's test.

Statistical Analysis

For all statistical analyses, SPSS 20 (SPSS Inc., Chicago, IL, USA) was used. Quantitative statistics are reported as means ± *SD*s (normally distributed) or medians (interquartile ranges [IQR]). Student's *t*-tests were performed for comparisons of continuous variables. Chi-squared tests or Fisher's exact tests were performed for comparisons of categorical variables. Wilcoxon's test was used to analyze rank variables. A two-tailed $p < 0.05$ was considered significant.

Ethics

This study was approved by the West China Hospital of the Sichuan University Research Ethics Committee. Informed consent was obtained from all patients.

RESULTS

Clinical Characteristics

A total of 192 patients were included in this study, among whom 107 (55.7%) were women. The mean age of patients was 29.44 ± 13.01 years (range 9–78 years, IQR 19–37 years). Furthermore, 21 (10.9%) patients had teratoma, among whom 19 (90.5%) had an ovarian teratoma and two (9.5%) had a mediastinal teratoma. The prevalence of teratoma in women with anti-NMDAR encephalitis was 18.7%. Pathologic subtypes included mature teratoma in 18 (85.7%) patients, immature teratoma in two (9.5%) patients, and mixed germ cell tumor in one (4.8%) patient. The demographic and clinical characteristics are shown in **Table 1**. **Table 2** shows the clinical characteristic of the 21 patients with teratoma.

The mean age of the teratoma cohort was younger than that of patients without teratoma (24.62 vs. 30.03 years, $p = 0.009$). Psychiatric symptoms (91.1%), memory deficits (81.8%), and seizures (79.7%) were the most dominant symptoms. Central hypoventilation (52.4 vs. 17%, $p < 0.001$) and decreased consciousness (71.4 vs. 31.3%, $p = 0.002$) were significantly more frequent in patients with teratoma than in those without. Gynecological symptoms were rare, wherein only one patient complained of a prolonged intermenstrual period, which may be related to the teratoma. One patient had a medical history of teratoma removal, and relapsed teratoma was detected. Patients with teratoma tended to score lower on the mRS during the acute phase than did patients without teratoma ($p = 0.004$). Approximately one-third of patients showed abnormal MRI findings, and over 80% of patients showed abnormal EEG findings. CSF findings suggested higher antibody titer in patients with teratoma ($p = 0.021$).

In-Hospital Management

First-line immunotherapies were administered to all patients with teratoma and 167 (97.7%) patients without teratoma. Patients with teratoma tended to use more turns of first-line immunotherapies ($p = 0.013$). The use of second-line immunotherapies did not significantly differ between the two groups. Among the 21 patients with teratoma, 17 (90%) patients underwent surgery during the acute phase before clinical improvement, and one patient underwent surgery

TABLE 2 | Clinical features of patients with teratoma.

No.	Sex	Age	Prodrome	Initial symptoms	Baseline mRS score	Pathology
Case 1	f	18	headache	seizures	5	mediastinal mature teratoma
Case 2	f	17	fever	seizures	5	ovarian mature teratoma
Case 3	m	25	headache/nausea	psychiatric symptoms	5	mediastinal mixed germ cell tumor (choriocarcinoma and teratoma)
Case 4	f	19	headache/fever	psychiatric symptoms	5	ovarian mature teratoma
Case 5	f	22	-	psychiatric symptoms	3	ovarian mature teratoma
Case 6	f	28	-	psychiatric symptoms	5	ovarian mature teratoma
Case 7	f	31	headache/fever	seizures	5	ovarian mature teratoma
Case 8	f	35	-	psychiatric symptoms	5	ovarian immature teratoma (WHO III)
Case 9	f	40	dizziness	psychiatric symptoms	4	ovarian mature teratoma
Case 10	f	18	headache/fever	seizures	5	ovarian mature teratoma
Case 11	f	20	headache	psychiatric symptoms	5	ovarian mature teratoma
Case 12	f	20	finger numbness	seizures	5	ovarian mature teratoma
Case 13	f	22	sleep disorder	psychiatric symptoms	4	ovarian mature teratoma
Case 14	f	43	-	seizures	4	ovarian immature teratoma (WHO III)
Case 15	f	17	sleep disorder	psychiatric symptoms	4	ovarian mature teratoma
Case 16	f	29	headache/fever	psychiatric symptoms	5	ovarian mature teratoma
Case 17	f	16	upper-respiratory-tract symptoms	psychiatric symptoms	4	ovarian mature teratoma
Case 18	f	26	-	psychiatric symptoms	3	ovarian mature teratoma
Case 19	f	26	headache/fever	psychiatric symptoms	5	ovarian mature teratoma
Case 20	f	19	-	seizures	5	ovarian mature teratoma
Case 21	f	26	-	psychiatric symptoms	5	ovarian mature teratoma

before immunotherapy was administered. **Table 3** shows the management of teratoma patients.

Follow-Up and Outcome

The median follow-up period was 46 months (6–91 months). During the follow-up period, two (9.5%) patients with teratoma and 11 (6.4%) patients without teratoma died. Among the two patients with teratoma who died, one died because of pulmonary metastasis of the tumor (mixed germ cell tumor) and secondary respiratory failure, and the other died because of pancreatitis, pulmonary infection, septic shock, and multiple organ dysfunction syndromes. In total, 19 (90.5%) teratoma patients and 151 (88.3%) patients without teratoma achieved a favorable clinical outcome at the final follow-up. The clinical management and outcomes of patients are shown in **Table 4**.

During the follow-up period, 39 patients experienced 47 relapses. Only one (4.8%) patient with teratoma relapsed at 7 months after initial onset, with recurrent seizures and memory deficits and CSF IgG titer of 1:100. Symptoms were controlled swiftly following the administration of IVIg, and there was no evidence of relapsed teratoma. **Figure 1** shows the Kaplan-Meier curves of anti-NMDAR encephalitis patients with and without teratoma.

DISCUSSION

We found that most patients with teratoma recovered slowly. However, favorable clinical outcomes were achieved over long-term follow-up, although mild sequelae may last several years. Immunotherapy was comparably ineffective in patients with teratoma before surgery, but effectiveness improved following removal surgery. Patients with teratoma presented with a more acute onset, more severe neurological symptoms, and higher IgG titer, than those without teratoma. Therefore, earlier and more immunotherapy turns were recommended for these patients.

The prevalence of teratoma in this study was comparably lower than that in previous studies. Titulaer et al. reported a prevalence of 211/577 patients, who were predominantly Asian and African-American (2). On the other hand, Xu et al. reported a prevalence of 42/143 women patients in a Chinese cohort (13). In our study, the prevalence of teratoma was 21/192 patients and was higher in younger individuals. This is consistent with the findings of Titulaer et al. (2) in which teratoma primarily affects individuals aged between 12 and 45 years, recommending more comprehensive inspections for teratoma in female youth. Anti-NMDAR encephalitis triggered by extra-ovarian teratoma, especially mediastinum teratoma, was detected in 2/21 patients with teratoma in our cohort. Overlooked extra-ovarian teratoma may result in delayed diagnosis in some cases (14). In addition, the teratoma cohort presented with more severe neurological sequelae, with greater disturbance of consciousness and central hypoventilation, and higher anti-NMDAR antibody titer in the CSF, which is similar to the report by Gresa-Arribas et al. (15). Accordingly, wider use of ventilators and intensive care has also been reported in this patients group (16). MRI

TABLE 3 | Management and outcomes of patients with teratoma.

No.	Intravenous immunoglobulin turns	Intravenous methylprednisolone turns	Second-line immunotherapy	Duration from onset to immuontherapy (days)	Duration from onset to removal surgery (days)	mRS score at surgery	mRS score at final follow-up
Case 1	1	1	rituximab	15	165	2	0
Case 2	2	1	-	20	98	3	1
Case 3	2	-	-	7	87	5	6
Case 4	3	1	-	7	50	5	2
Case 5	1	-	-	21	272	0	0
Case 6	2	1	-	10	58	5	0
Case 7	3	3	-	15	221	0	0
Case 8	1	-	-	20	27	5	1
Case 9	1	3	-	15	39	4	0
Case 10	2	-	-	2	23	5	0
Case 11	3	1	-	14	42	5	2
Case 12	2	-	-	20	38	5	0
Case 13	1	1	-	21	34	4	0
Case 14	1	-	-	22	15	4	2
Case 15	2	1	-	10	19	4	0
Case 16	4	1	rituximab	20	125	5	1
Case 17	1	1	cyclophosphamide	5	32	4	1
Case 18	1	1	-	10	27	3	0
Case 19	2	2	rituximab	35	58	5	6
Case 20	2	-	-	20	47	5	1
Case 21	1	2	-	7	22	5	1

TABLE 4 | Management and outcomes of patients with and without teratoma.

	Total, n (%)	With teratoma, n (%)	Without teratoma, n (%)	*P*-values
Intravenous immunoglobulin	172 (89.6)	21 (100)	151 (88.3)	0.136[#]
Intravenous methylprednisolone	122 (63.5)	14 (66.7)	108 (63.2)	0.940[#]
Second-line immunotherapy	16 (8.3)	4 (19)	12 (7)	0.143[#]
Intravenous first-line immunotherapy turns (median, IQR)	2 (1–5)	2 (1–4)	2 (1–5)	0.013[+]
mRS score at final follow up	0 (0–1)	1 (0–1)	0 (0–1)	0.864[+]
Death	13 (6.8)	2 (9.5)	11 (6.4)	0.943[#]
Relapse	38 (19.8)	1 (4.8)	37 (21.6)	0.123[#]

[#]*chi-squared or Fisher's exact tests.*
[+]*Wilcoxon's test.*

and EEG during the acute phase showed non-specific changes, with limited significance for diagnoses. However, the evaluation using positron emission tomography was recommended in several cases.

Immunotherapy is a crucial element of autoimmune encephalitis treatment. The combination of steroids, intravenous immunoglobulins, and plasma exchange is recommended, and second-line therapy should be administered as soon as possible if first-line therapy is unsuccessful (17). In our study, patients with teratoma responded more poorly to immunotherapy and required more turns of immunotherapy than patients without teratoma. Persistent germinal center response in teratoma can produce NR1-IgG continuously (18). Single immunotherapy has a limited effect in patients with teratoma. However, a high proportion of patients with inadequate response to immunotherapy has been found to improve, following a surgery as reflected in the control of seizures and increased consciousness level. Dalmau et al. observed 105 anti-NMDAR encephalitis patients and reported an 80% response rate to first-line immunotherapy plus surgery in patients with teratoma, whereas the response rate in patients without teratoma was 48% (19). Improvement can be dramatic in some patients, and can even occur within a few days of surgery (20).

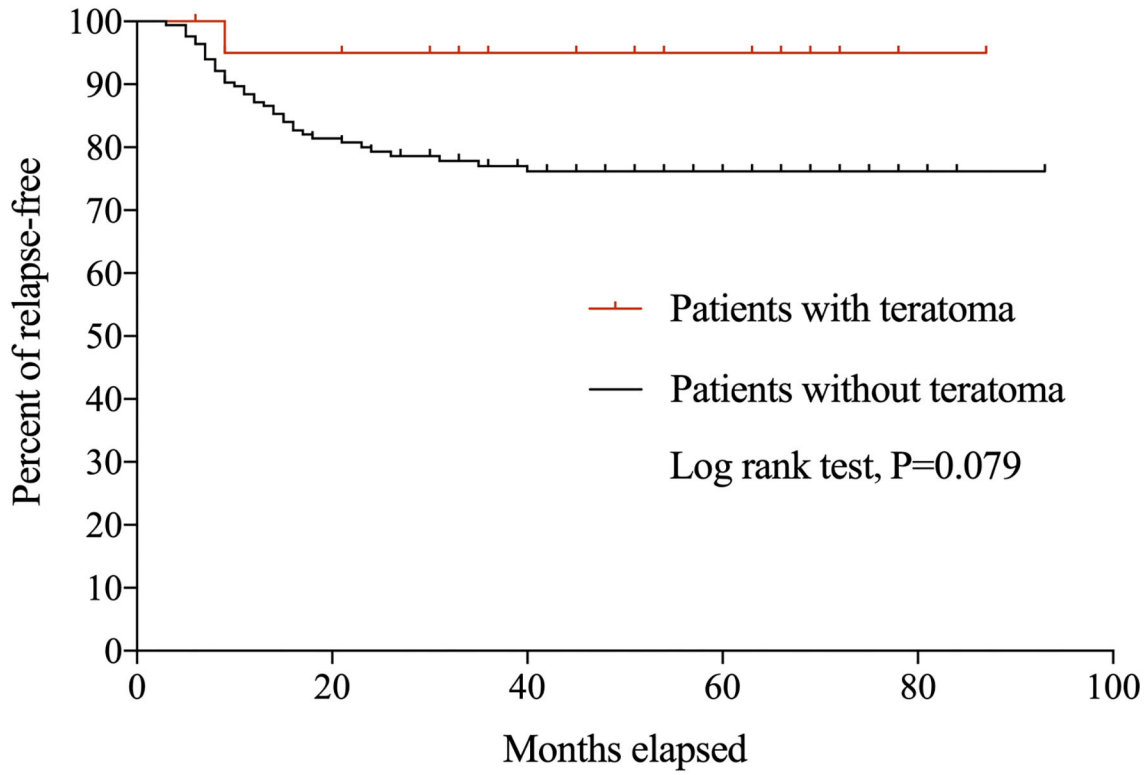

FIGURE 1 | Kaplan-Meier curves for anti-NMDAR encephalitis patients with and without teratoma.

Surgery during the acute phase is strongly recommended for a good long-term prognosis. Lee et al. suggest that delayed surgery is associated with poor improvement over time (21). Moreover, Dalmau et al. reported that patients who do not undergo surgery have a higher mortality rate (20). Furthermore, a previous study found that patients who undergo tumor removal within 4 months have milder neurological deficits than those who undergo a delayed surgery (22). Although the safety of undergoing surgery during the acute phase is a significant concern, consistent with a previous study (16), there were no surgical complications during the perioperative period in our cohort. Two of our patients died because of multiple organ failure due to anti-NMDAR encephalitis; however, there was no evidence to indicate that surgery hastened death in these patients.

The detection of teratoma during the early phase is important for medical management. CT and MRI have higher sensitivity than ultrasound for teratoma screening and thus are recommended for patients with anti-NMDAR encephalitis (23). However, patients with anti-NMDAR encephalitis have smaller teratoma with fewer teeth, less calcification, and a smaller fat-occupied space, which makes the detection of teratoma challenging (24). Indeed, Lee et al. reported that diagnosis of teratoma was missed in 26.1% of patients during initial pelvis CT, even when combined with MRI (21). Thus, continual reassessment for teratoma in patients who show no significant improvement with immunotherapy or those who relapse repeatedly is recommended. Although delayed surgery is criticized by many (21), our study indicated that patients can benefit from surgery, even with a delay of over 6 months.

Patients with anti-NMDAR encephalitis along with teratoma have benign prognoses, with a low relapse rate and mild sequelae. The teratoma group had long-term prognoses similar to patients without teratoma in terms of relapse, mortality, and mRS score, regardless of more dreadful onset. However, a lower relapse rate in patients with teratoma has been reported previously (16). One study reported that relapsed or residual teratoma can induce relapse (25), although we found no evidence of relapsed teratoma in patients who relapsed in this study.

Our study has several limitations. Firstly, our sample size of the single-center study was small. A multicenter study would increase the sample size and reduce selection bias. Secondly, the evaluation of prognoses during the post-surgical follow-up was based primarily on patients' subjective descriptions, and regular anti-NMDAR antibody tests and cranial MRI were not performed for further analysis.

In conclusion, removal surgery to treat anti-NMDAR encephalitis patients with teratoma is effective. Although anti-NMDAR encephalitis patients with teratoma had more serious medical conditions than patients without teratoma, timely removal surgery enabled favorable long-term outcomes.

Comprehensive assessments are required for early tumor detection and timely management, especially in patients who respond poorly to immunotherapy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by West China Hospital of Sichuan University Research Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

HZ: drafting of the manuscript for content, analysis and interpretation of data. WX and WL: revision of the manuscript for content. XL: major role in the acquisition of data. DZ and XW: study concept, design, and revision of the manuscript for content. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Graus F, Titulaer MJ, Balu R, Benseler S, Bien CG, Cellucci T, et al. A Clinical Approach to Diagnosis of Autoimmune Encephalitis. *Lancet Neurol.* (2016) 15:391–404. doi: 10.1016/S1474-4422(15)00401-9
2. Titulaer MJ, McCracken L, Gabilondo I, Armangué T, Glaser C, Iizuka T, et al. Treatment and prognostic factors for long-term outcome in patients with anti-NMDA receptor encephalitis: an observational cohort study. *Lancet Neurol.* (2013) 12:157–65. doi: 10.1016/S1474-4422(12)70310-1
3. Dalmau J, Gleichman AJ, Hughes EG, Rossi JE, Peng X, Lai M, et al. Anti-NMDA-receptor encephalitis: case series and analysis of the effects of antibodies. *Lancet Neurol.* (2008) 7:1091–8. doi: 10.1016/S1474-4422(08)70224-2
4. Jiang XY, Lei S, Zhang L, Liu X, Lin MT, Blumcke I, et al. Co-expression of NMDA-receptor subunits NR1, NR2A, and NR2B in dysplastic neurons of teratomas in patients with paraneoplastic nmda-receptor-encephalitis: a retrospective clinico-pathology study of 159 patients. *Acta Neuropathol Commun.* (2020) 8:130. doi: 10.1186/s40478-020-00999-2
5. Bost C, Chanson E, Picard G, Meyronet D, Mayeur ME, Ducray F, et al. Malignant tumors in autoimmune encephalitis with anti-NMDA receptor antibodies. *J Neurol.* (2018) 265:2190–200. doi: 10.1007/s00415-018-8970-0
6. Florance NR, Davis RL, Lam C, Szperka C, Zhou L, Ahmad S, et al. Anti-N-methyl-D-aspartate Receptor (NMDAR) encephalitis in children and adolescents. *Ann Neurol.* (2009) 66:11–8. doi: 10.1002/ana.21756
7. Liu X, Zhang L, Chen C, Gong X, Lin J, An D, et al. Long-term cognitive and neuropsychiatric outcomes in patients with anti-NMDAR encephalitis. *Acta Neurol Scand.* (2019) 140:414–21. doi: 10.1111/ane.13160
8. Zhang L, Lu Y, Xu L, Liu L, Wu X, Zhang Y, et al. Anti-N-methyl-D-aspartate receptor encephalitis with accompanying ovarian teratoma in female patients from east china: clinical features, treatment, and prognostic outcomes. *Seizure.* (2020) 75:55–62. doi: 10.1016/j.seizure.2019.12.016
9. Wang R, Lai XH, Liu X, Li YJ, Chen C, Li C, et al. brain magnetic resonance-imaging findings of anti-N-methyl-D-aspartate receptor encephalitis: a cohort follow-up study in Chinese patients. *J Neurol.* (2018) 265:362–9. doi: 10.1007/s00415-017-8707-5
10. Liu X, Yan B, Wang R, Li C, Chen C, Zhou D, et al. Seizure outcomes in patients with anti-NMDAR encephalitis: a follow-up study. *Epilepsia.* (2017) 58:2104–11. doi: 10.1111/epi.13929
11. Gong X, Chen C, Liu X, Lin J, Li A, Guo K, et al. Long-term functional outcomes and relapse of anti-NMDA receptor encephalitis: a cohort study in Western China. *Neurol Neuroimmunol Neuroinflamm.* (2021) 8:e958. doi: 10.1212/NXI.0000000000000958
12. van Swieten JC, Koudstaal PJ, Visser MC, Schouten HJ, van Gijn J. Interobserver agreement for the assessment of handicap in stroke patients. *Stroke.* (1988) 19:604–7. doi: 10.1161/01.STR.19.5.604
13. Xu X, Lu Q, Huang Y, Fan S, Zhou L, Yuan J, et al. Anti-NMDAR encephalitis: a single-center, longitudinal study in China. *Neurol Neuroimmunol Neuroinflamm.* (2019) 7:e633. doi: 10.1212/NXI.0000000000000633
14. Kümpfel T, Gerdes LA, Heck C, Prüss H. Delayed diagnosis of extraovarian teratoma in relapsing anti-NMDA receptor encephalitis. *Neurol Neuroimmunol Neuroinflamm.* (2016) 3:e250. doi: 10.1212/NXI.0000000000000250
15. Gresa-Arribas N, Titulaer MJ, Torrents A. Antibody Titres at diagnosis and during follow-up of anti-NMDA receptor encephalitis: a retrospective study. *Lancet Neurol.* (2014) 13:167–77. doi: 10.1016/S1474-4422(13)70282-5
16. Dai Y, Zhang J, Ren H, Zhou X, Chen J, Cui L, et al. Surgical outcomes in patients with anti-N-methyl D-aspartate receptor encephalitis with ovarian teratoma. *Am J Obstet Gynecol.* (2019) 221:485.e1–10. doi: 10.1016/j.ajog.2019.05.026
17. Dalmau J, Armangué T, Planagumà J, Radosevic M, Mannara F, Leypoldt F, et al. An update on anti-NMDA receptor encephalitis for neurologists and psychiatrists: mechanisms and models. *Lancet Neurol.* (2019) 18:1045–57. doi: 10.1016/S1474-4422(19)30244-3
18. Makuch M, Wilson R, Al-Diwani A, Varley J, Kienzler A-K, Taylor J, et al. N-methyl-D-aspartate receptor antibody production from germinal center reactions: therapeutic implications. *Ann Neurol.* (2018) 83:553–61. doi: 10.1002/ana.25173
19. Dalmau J, Lancaster E, Martinez-Hernandez E, Rosenfeld MR, Balice-Gordon R. Clinical experience and laboratory investigations in patients with anti-NMDAR encephalitis. *Lancet Neurol.* (2011) 10:63–74 doi: 10.1016/S1474-4422(10)70253-2
20. Dalmau J, Tüzün E, Wu HY, Masjuan J, Rossi JE, Voloschin A, et al. Paraneoplastic anti-N-methyl-D-aspartate receptor encephalitis associated with ovarian teratoma. *Ann Neurol.* (2007) 61:25–36. doi: 10.1002/ana.21050
21. Lee WJ, Lee ST, Shin YW, Lee HS, Shin HR, Kim DY, et al. Teratoma removal, steroid, IVIG, rituximab and tocilizumab (T-SIRT) in anti-NMDAR encephalitis. *Neurotherapeutics.* (2021) 18:474–87. doi: 10.1007/s13311-020-00921-7
22. Sommeling C, Santens P. Anti-N-methyl-D-aspartate (anti-NMDA) receptor antibody encephalitis in a male adolescent with a large mediastinal teratoma. *J Child Neurol.* (2014) 29:688–90. doi: 10.1177/0883073813520499
23. Saba L, Guerriero S, Sulcis R, Virgilio B, Melis G, Mallarini G. Mature and immature ovarian teratomas: CT, US and MR imaging characteristics. *Eur J Radiol.* (2009) 72:454–63. doi: 10.1016/j.ejrad.2008.07.044
24. Kawaguchi M, Kato H, Noda Y, Suzui N, Miyazaki T, Furui T, et al. CT and MRI Characteristics of ovarian mature teratoma in patients with anti-N-methyl-D-aspartate receptor encephalitis. *Diagn Interv Imaging.* (2021) 102:447–53. doi: 10.1016/j.diii.2021.02.010
25. Uchida Y, Kato D, Yamashita Y, Ozaki Y, Matsukawa N, et al. Failure to improve after ovarian resection could be a marker of recurrent ovarian

teratoma in anti-NMDAR encephalitis: a case report. *Neuropsychiatr Dis Treat.* (2018) 14:339–42. doi: 10.2147/NDT.S156603

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Safety, Adherence and Persistence in a Real-World Cohort of German MS Patients Newly Treated With Ocrelizumab: First Insights From the CONFIDENCE Study

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Background: Real-world relapsing multiple sclerosis (RMS) and primary progressive MS (PPMS) populations may be more diverse than in clinical trials. Here, we present a first analysis of safety, adherence and persistence data from a real-world cohort of patients newly treated with ocrelizumab.

Methods: CONFIDENCE (ML39632, EUPAS22951) is an ongoing multicenter, non-interventional post authorization safety study assessing patients with RMS or PPMS newly treated with ocrelizumab or other disease-modifying therapies for up to 10 years. For this analysis, patients newly treated with ocrelizumab were analyzed in subgroups by MS phenotype and age over a mean ∼1 year of exposure totaling 2,329 patient years [PY]).

Results: At data cutoff (14 October 2020), 1,702 patients with RMS and 398 patients with PPMS were treated with ≥1 dose of ocrelizumab. At baseline, the mean ages (SD) of patients with RMS and PPMS were 41.59 (11.24) and 50.95 (9.88) years and the mean EDSS (Expanded Disability Status Scale) was 3.18 (1.87) and 4.41 (1.59), respectively. The most common adverse events (AEs) and serious AEs across both phenotypes were infections and infestations, with infection SAE rates of 2.8 events/100 PY and 1.5 events/100 PY in patients with RMS and PPMS, respectively. Across all phenotypes, ocrelizumab persistence was 92% at 24 months; median time between doses was ∼6 months.

Conclusions: The ocrelizumab safety profile observed in the CONFIDENCE real-world MS population was consistent to the one observed in pivotal clinical trials. High treatment persistence and adherence were observed.

Trial Registration: ML39632, EUPAS22951

Keywords: neurodegenerative diseases, multiple sclerosis, non-interventional study (NIS), real-world cohort, safety, drug (or treatment) persistence, humanized monoclonal antibody anti-CD20, ocrelizumab

INTRODUCTION

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS) with a complex immunopathogenesis of inflammation and neurodegeneration with two major disease phenotypes: relapsing MS (RMS) and primary progressive MS (PPMS). MS often warrants long-term drug therapy. Thus, the benefits of a disease-modifying therapy (DMT) must outweigh its long-term risks (1).

Ocrelizumab (Ocrevus®) is a monoclonal antibody that specifically binds to CD20, modulating the immunopathogenesis of MS by depleting $CD20^+$ B-cells. As the first anti-CD20 monoclonal antibody approved for the treatment of RMS and PPMS, ocrelizumab remains the only approved treatment for PPMS to date (2, 3). Pivotal trial data shows that treatment with ocrelizumab has significant effects on slowing disease progression, annualized relapse rate and magnetic resonance imagery outcomes, with no signal of a higher rate of serious infections, compared with interferon in patients with RMS and placebo in patients with PPMS (4, 5). Ocrelizumab efficacy was sustained in the open-label extension phases of the pivotal trials, where adverse events (AEs) were generally consistent with those from the controlled periods and no new safety signals emerged with prolonged treatment (6, 7).

Integrated safety analysis of the data from 11 clinical trials and open-label extension periods (up to 7 years of continuous ocrelizumab treatment) demonstrated a favorable and manageable safety profile (8). There was no indication of higher rates of malignancy compared with matched reference MS and general populations over 8 years (8).

Real-world populations may be more diverse than those included in randomized clinical trials (RCTs), comprising patients with more prior MS-specific treatments, a longer duration of disease, more physical disability, older age (9) or more comorbidities that may affect safety of treatment (10).

CONFIDENCE (ML39632, EUPAS22951) is a large, ongoing, non-interventional post-authorization safety study (PASS) that assesses the long-term safety and effectiveness of ocrelizumab and other DMTs in a real-world MS population in Germany (11). As the central study of the ocrelizumab post-marketing safety program, safety data from CONFIDENCE is integrated into the two multi-source, real-world studies VERISMO (EUPAS30752) and MANUSCRIPT (EUPAS28619). Here, we present an analysis of baseline characteristics, safety, adherence and persistence from patients newly treated with ocrelizumab in CONFIDENCE over a mean of ∼1 year of exposure (max 2.5 years).

MATERIALS AND METHODS

Study Design

CONFIDENCE assesses the long-term safety and effectiveness of patients newly treated with ocrelizumab or other selected DMTs (i.e., alemtuzumab, cladribine, dimethyl fumarate, fingolimod, natalizumab, or teriflunomide). Recruitment was initiated in April 2018 with a target enrollment of 3,000 patients with RMS (relapsing remitting MS or relapsing secondary progressive MS) or PPMS newly treated with ocrelizumab and 767 patients with RMS newly treated with other selected DMTs at ∼185 centers in Germany. Full details on the CONFIDENCE study design and inclusion/exclusion have been previously published (11).

The decision to prescribe the treatment must be made prior to and independent of participation in this study; patients are to be treated according to local label. Key inclusion criteria are ≥18 years of age at enrollment and treatment with ocrelizumab or the respective DMT for the first time during the course of MS therapy. Patients participating in an interventional study examining a MS DMT and patients previously treated with rituximab or any other anti-CD20 antibody for MS are excluded.

Overall, patients will be observed with regular ∼6-month follow-up visits for up to 10 years regardless of treatment change. CONFIDENCE completion is expected in 2029. Data are collected by site staff and entered into an electronic case report form (eCRF) based on the MS management system 3D (MSDS3D; MedicalSyn, Stuttgart, Germany) (12, 13). Patient demographics and informed consent are collected at screening. Other baseline characteristics such as MS disease and treatment history, general medical history and comorbidities (previous and current diseases and disorders in the patient's medical history), pregnancy status and history, malignancy risk factors, cancer screening and MS disease activity are documented at the baseline visit (first ocrelizumab administration).

Here, we present a first analysis of safety, adherence and persistence of patients treated with ocrelizumab (data cutoff 14 October 2020). Other DMT cohorts were not included in this analysis due to insufficient patient numbers.

Safety Endpoints

AEs and serious AEs (SAEs) were recorded according to system organ class (SOC) and preferred term (PT) of the Medical Dictionary for Regulatory Activities (MedDRA; version 23.1). As of a protocol amendment in July 2019, infusion-related reactions (IRRs) were only to be recorded if judged as serious or life-threatening. Malignancies were identified using the SOC "Neoplasms benign, malignant and unspecified (incl.

cysts and polyps)" and further defined using the standardized MedDRA queries (SMQ) "Malignant tumor (narrow)". Reasons for discontinuations were reported by the investigator from multiple choices. No specific details were collected for reasons such as "patient wish" or "insufficient efficacy". Adjunct data from the Roche safety database was used to consummate patient narratives.

Trial Registration and Ethics Statement

This study was registered on 06 March 2018 in the EU PAS Register (http://www.encepp.eu/encepp/studiesDatabase.jsp) under the EU PAS Register Number EUPAS22951. The independent ethics committee at the Technical University Dresden has given the first professional advice for this observational study (Ethikkommission an der Technischen Universität Dresden, Germany; 12 February 2018 and 10 April 2019; reference EK 62022018). Obtaining further ethics approvals was the individual responsibility of the participating physicians.

Statistical Analysis

This analysis was based on data prior to the cutoff. Patients who received at least one dose of ocrelizumab were included for analysis of all safety endpoints (safety analysis set). Persistence and adherence endpoints were analyzed in patients in the safety analysis set with at least one documentation after start of the therapy (full analysis set). Persistence was estimated by Kaplan-Meier time-to-treatment discontinuation, in which patients without discontinuation were censored with their last assessment visit date recorded. Adherence was evaluated by median time interval (interquartile range) between dosing. All outcomes were assessed using descriptive statistics. Analysis of patients with PPMS >55 years old at baseline was prespecified in the statistical analysis plan; patients with RMS >55 years old were assessed in a *post-hoc* analysis.

RESULTS

Patient Population and Treatment Exposure

As of the data cutoff, 1,702 patients with RMS and 398 patients with PPMS have been treated with ≥1 dose of ocrelizumab and were included in the safety analysis. The mean exposure time (standard deviation, SD) to ocrelizumab was 1.03 (0.70) years for patients with RMS (range 0.0–2.5 years; totaling 1,877 patient-years [PY]) and 1.06 (0.68) years for patients with PPMS (range 0.0–2.5 years; totaling 452 PY).

Mean age (SD) of patients with RMS was 41.59 (11.24) years, 66.9% were females and 82.7% had ≥1 MS-specific prior therapy. The mean (SD) baseline EDSS (Expanded Disability Status Scale) of patients with RMS was 3.18 (1.87) in the total cohort, and 4.54 (1.64) in patients >55 years old. At baseline, 66.0% of ocrelizumab-treated patients with RMS had comorbidities. The most common comorbidities (PT) of patients with RMS were vitamin D deficiency, hypertension and depression (**Table 1**). In patients with RMS >55 years old, 80.5% had comorbidities, with the most common (PT) being hypertension (**Table 1**).

TABLE 1 | Baseline characteristics (safety set).

Characteristic	Total RMS (*n* = 1,702)	RMS >55 years (*n* = 200)	Total PPMS (*n* = 398)	PPMS >55 years (*n* = 143)
Age, mean (SD), years	41.59 (11.24)	59.9 (4.12)	50.95 (9.88)	60.90 (4.80)
Sex, n (%)				
Female	1,139 (66.9)	118 (59.0)	208 (52.3)	82 (57.3)
Number of prior MS therapies, n (%)				
Treatment-naïve	294 (17.3)	41 (20.5)	268 (67.3)	102 (71.3)
1	410 (24.1)	45 (22.5)	71 (17.8)	22 (15.4)
2	411 (24.1)	49 (24.5)	32 (8.0)	8 (5.6)
≥3	587 (34.5)	65 (32.5)	27 (6.8)	11 (7.7)
Therapy prior to ocrelizumab, n (%)				
Fingolimod	339 (19.9)	38 (19.0)	11 (2.8)	1 (0.7)
Interferon or GA	274 (16.1)	34 (17)	51 (12.8)	13 (9.1)
Natalizumab	246 (14.5)	11 (5.5)	8 (2.0)	4 (2.8)
Dimethyl fumarate	222 (13.0)	17 (8.5)	19 (4.8)	5 (3.5)
Other/none	621 (36.5)	100 (50.0)	309 (77.6)	120 (83.9)
EDSS, mean (SD)	3.18 (1.87)	4.54 (1.64)	4.41 (1.59)	4.73 (1.48)
Duration to baseline since*:				
First symptoms, mean (SD), years	10.79 (8.69)	17.95 (11.71)	8.66 (7.62)	10.63 (9.26)
Diagnosis, mean (SD), years	8.95 (7.81)	14.12 (10.05)	5.60 (6.75)	6.90 (8.39)
Common comorbidities SOC, n (%)				
≥1	1,123 (66.0)	161 (80.5)	296 (74.4)	123 (86.0)
Metabolism and nutrition disorders	430 (25.3)	62 (31.0)	108 (27.1)	41 (28.7)
Nervous system disorders	367 (21.6)	59 (29.5)	90 (22.6)	41 (28.7)
Psychiatric disorders	326 (19.2)	49 (24.5)	71 (17.8)	32 (22.4)
Vascular disorders	235 (13.8)	71 (35.5)	106 (26.6)	56 (39.2)
Endocrine disorders	196 (11.5)	31 (15.5)	46 (11.6)	19 (13.3)
Common comorbidities, PT, n (%)				
Vitamin D deficiency	305 (17.9)	33 (16.5)	57 (14.3)	23 (16.1)
Hypertension	209 (12.3)	59 (29.5)	96 (24.1)	51 (35.7)
Depression	197 (11.6)	30 (15)	40 (10.1)	21 (14.7)

**Data collected retrospectively.*

GA, glatiramer acetate; PPMS, primary progressive multiple sclerosis; RMS, relapsing MS; SD, standard deviation; PT, preferred term; SOC, system organ class. Data were analyzed in the safety set, which included all enrolled patients with at least one dose of ocrelizumab. Common comorbidities listed are those in ≥10% of patients with RMS.

Patients with PPMS were mean (SD) 50.95 (9.88) years old, 52.3% female, and 32.6% had ≥1 MS-specific prior therapy (**Table 1**). Patients with PPMS had a mean (SD) baseline EDSS of 4.41 (1.59) and 4.73 (1.48) in patients >55 years old. At baseline, 74.4% of patients with PPMS had comorbidities, most commonly (PT) hypertension and vitamin D deficiency. In patients with PPMS > 55 years, 86.0% had comorbidities with the most common (PT) being hypertension (**Table 1**).

TABLE 2 | Adverse events (AEs), serious AEs, malignancies, infections and serious infections observed in patients treated with ocrelizumab.

	Total RMS (n = 1,702)				RMS >55 years (n = 200)				Total PPMS (n = 398)				PPMS >55 years (n = 143)			
Exposure in PY	1,877				242				452				162			
	Total AEs		SAEs		Total AEs		SAEs		Total AEs		SAEs		Total AEs		SAEs	
	E*	R**	E*	R**	E*	R**	E*	R**	E*	R**	E*	R**	E*	R**	E*	R**
Any AE	2,186	116	250	13.3	263	109	55	22.7	380	84	37	8.2	169	104	19	11.7
Fatal events	3	0.2	3	0.2	-	-	-	-	2	0.4	2	0.4	2	1.2	2	1.2
Malignancies***	9	0.5	9	0.5	-	-	-	-	3	0.7	3	0.7	1	0.6	1	0.6
Infections																
Nasopharyngitis	155	8.3	-	-	12	5.0	-	-	26	5.8	-	-	11	6.8	-	-
Urinary tract infection	116	6.2	13	0.7	20	8.3	3	1.2	22	4.9	2	0.4	8	4.9	-	-
Upper respiratory tract infection	35	1.9	1	0.05	4	1.7			3	0.7	-	-	2	1.2	-	-
Respiratory tract infection	26	1.4	-	-	3	1.2	-	-	2	0.4	-	-	1	0.6	-	-
Bronchitis	23	1.2	1	0.05	4	1.7	-	-	2	0.4	-	-	2	1.2	-	-
Sinusitis	22	1.2	3	0.2	3	1.2	1	0.4	2	0.4	-	-	-	-	-	-
Gastrointestinal infection	20	1.1	-	-	-	-	-	-	4	0.9	-	-	2	1.2	-	-
Oral herpes	15	0.8	-	-	1	0.4	-	-	2	0.4	-	-	-	-	-	-
Herpes zoster	12	0.6	1	0.05	2	0.8	-	-	3	0.7	-	-	2	1.2	-	-
Pneumonia	9	0.5	6	0.3	1	0.4	-	-	2	0.4	2	0.4	-	-	-	-
COVID-19****	6	0.3	2	0.1	-	-	-	-	-	-	-	-	-	-	-	-
PML	1	0.05	1	0.05												

Total events. **Rate, AEs/100 PY, calculated by dividing total AEs by exposure in 100 PY. ***Malignant tumor (narrow); a full list of the MedDRA SOC "Neoplasms benign, malignant and unspecified (incl cysts and polyps)" is included in* ***Supplementary Table 3**. ****Includes COVID-19 and COVID-19 pneumonia.*

PML, Progressive multifocal leukoencephalopathy. E, Total number of events; PY, patient years; R, rate of events by PY. Infections and serious infections according to MedDRA SOC "Infections and infestations". A list of all "Infections and infestations" is included in ***Supplementary Table 2****. Data were analyzed in the safety set, which included all enrolled patients with at least one dose of ocrelizumab; AEs were classified according to MedDRA version 23.1. Table data includes all infections ≥0.5 events/100 PY in patients with RMS while COVID-19 and PML were included as infections of interest.*

Adverse Events

In this analysis, 721 (42.4%) ocrelizumab-treated patients with RMS experienced 2,182 AEs [116.2 events/100 PY], most commonly categorized as infections and infestations [32 events/100 PY]. The most common AEs were nasopharyngitis [8.3 events/100 PY], urinary tract infections [6.2 events/100 PY] and infusion-related reactions [5.4 events/100PY] (for a list of all SOCs and the three most common AEs, please see **Supplementary Table 1**). Overall, 146 (8.6%) patients experienced 250 SAEs [13.3 events/100 PY], the most common being categorized as infections and infestations (**Table 2**). The most common SAE was urinary tract infection [0.7 events/100 PY]. Of patients with RMS >55 years old (n = 200), 86 patients experienced ≥1 AE, [108.7 events/100 PY], most commonly categorized as infections and infestations [29.3 events/100 PY]. The most common AEs were urinary tract infection [8.3 events/100 PY] and infusion-related reactions [5.4 events/100 PY]. Twenty-three (11.5%) RMS patients >55 years experienced 55 total SAEs [22.7 events/100 PY], most commonly categorized as injury, poisoning and procedural complication [4.1 events/100 PY]. The most common SAEs were urinary tract infection, fall and trigeminal neuralgia [all 1.2 events/100 PY].

Overall, 147 (36.9%) ocrelizumab-treated patients with PPMS experienced 380 AEs [84.1 events/100 PY], which were most often categorized as infections and infestations [19.7 events/100 PY] (**Table 2**). The most common AEs were nasopharyngitis [5.8 events/100 PY] and urinary tract infection [4.9 events/100 PY]. There were 37 SAEs in 26 (6.5%) patients with PPMS [8.2 events/100 PY]. SAEs were most often categorized in the SOC infections and infestations [1.5 events/100 PY]. The most common SAEs were muscle spasticity (3 patients) and fall (3 patients). Of patients with PPMS >55 years old (n = 143), 39.9% experienced 169 AEs [104 events/100 PY], most commonly categorized as infections and infestations [21.6 events/100 PY]. The most common AEs were nasopharyngitis [6.8 events/100 PY] and urinary tract infections [4.9 events/100 PY]. Thirteen (9.1%) >55 year-old patients with PPMS experienced 19 SAEs [11.7 events/100 PY]; no additional patterns in reported SAEs were observed.

Infections and Infestations

Overall, 21.0% of patients with RMS experienced infections [32.2 events/100 PY]. The most common infections were nasopharyngitis [8.3 events/100 PY], urinary tract infections [6.2 events/100 PY] and respiratory tract infections (for a list of all infections, please see **Supplementary Table 2**). Serious infections were experienced by 2.5% of patients with RMS [2.8 events/100 PY] (**Table 2**), including 13 events of serious urinary

tract infections [0.7 events/100 PY; 12 recovered/recovering and one unknown outcome] and six events of serious pneumonia [0.3 events/100 PY; all recovered/recovering] (**Table 2**).

A single case of suspected carry-over progressive multifocal leukoencephalopathy (PML), associated with prior natalizumab therapy, was reported in 2018. The case was assessed by an independent panel of PML experts and was classed as suspected rather than confirmed carry-over PML. The patient had magnetic resonance imaging findings suggestive of PML, but the cerebrospinal fluid was negative for JC virus DNA and no clinical symptoms consistent with PML were reported, therefore, the case did not meet the American Association of Neurology criteria for confirmed PML (14). No further cases of PML have been reported in this study. COVID-19 was recorded for 6 patients with RMS [0.3 events/100 PY], 2 of which were considered SAEs [0.1 events/100 PY]. One 44-year-old female was hospitalized due to COVID-19, and one case of serious COVID-19 was of 'moderate' severity. Both patients recovered. Mini-narratives of SAE infections of interest such as herpes zoster, neuroborreliosis, meningitis, endocarditis, suspected PML and COVID-19 are included in the **Supplementary Material**.

Five patients with RMS who experienced seven serious infections were >55 years old [2.9 events/100 PY]; these included urinary tract infection (3, all recovered/recovering); urosepsis (2, recovered and unknown outcome); sinusitis (1, recovered); and viral pharyngitis (1, recovered).

Overall, 15.8% of patients with PPMS experienced infections [19.7 events/100 PY], including nasopharyngitis [5.8 events/100 PY] and urinary tract infections [4.9 events/100 PY]. Seven patients with PPMS [1.5 events/100 PY] had serious infections and infestations, (2 pneumonia, recovered and recovered with sequelae; 2 urinary tract infections, recovered and unknown outcome; 1 diverticulitis, recovered; 1 encephalitis, recovered; and 1 urosepsis, recovered). Among patients with PPMS and >55 years old, one case of diverticulitis and one case of encephalitis occurred [1.2 events/100 PY] (for further details see **Supplementary Table 4**).

Fatal Events

Three patients with RMS [0.2%; 0.2 events/100 PY; ≤55 years old] and 2 patients with PPMS [0.5%; 0.4 events/100 PY; >55 years old] died. Among the patients with RMS, one event was reported as "death" with no specific cause given; one patient—who had a history of tobacco use—died of bronchial carcinoma, and one patient died of myocarditis. In patients with PPMS, one event was reported as "death" (not further specified), and one patient with PPMS completed suicide (for further details see the **Supplementary Material**).

Malignancies

Seven patients with RMS experienced malignancies, defined as standardized MedDRA queries (SMQ) "Malignant tumor (narrow)" [0.4%; 0.5 events/100 PY; all ≤55 years old]. These included female breast cancer (two cases, 54 and 53 years old at baseline), malignant melanoma (two cases; one including metastases to the mediastinum; 38 and 45 years) and one case each of bronchial carcinoma (54 years), thyroid cancer (52 years) and basal cell carcinoma (42 years). Six of seven patients with a malignancy were female.

Three patients with PPMS experienced a malignancy [0.8%; 0.7 events/100 PY], including malignant melanoma (55 years old at baseline), squamous cell carcinoma of the skin (60 years) and basal cell carcinoma (44 years). Two patients were female, and one was male. Further information on patient history and risk factors can be found in the **Supplementary Material**.

TABLE 3 | Reasons for discontinuation of ocrelizumab as reported by the investigator.

Reason for discontinuation*, n (%)	Total RMS (n = 1,702)	RMS >55 years (n = 200)	Total PPMS (n = 398)	Total PPMS >55 years (n = 143)
Total discontinuation	80 (4.7)	14 (7.0)	19 (4.8)	10 (7.0)
Reasons for discontinuation				
Patient wish	37 (2.2)	7 (3.5)	9 (2.3)	4 (2.8)
Adverse event	13 (0.8)	3 (1.5)	4 (1.0)	3 (2.1)
Insufficient efficacy**	12 (0.7)	3 (1.5)	3 (0.8)	2 (1.4)
Pregnancy wish	6 (0.4)	-	-	-
Pregnancy	4 (0.2)	-	-	-
Other	8 (0.5)	1 (0.5)	3 (0.8)	1 (0.7)

Data were analyzed in the safety set, which included all enrolled patients with at least one dose of ocrelizumab.
**Only one reason was given per patient. **Insufficient efficacy as reported by the investigator, not further specified.*

Discontinuation, Persistence, and Adherence

Of patients with RMS, 80 (4.7%) discontinued ocrelizumab; the most common reasons were "patient wish" (37), AE (13) and "insufficient efficacy" (12). Fourteen patients >55 years old (7.0%) discontinued, most commonly due to "patient wish" (7), AE (3) and "insufficient efficacy" (3) (**Table 3**).

Of patients with PPMS, 19 (4.8%) discontinued ocrelizumab; the most common reasons were "patient wish" (9), AE (4) and "insufficient efficacy" (3). Ten patients >55 years old (7.0%) discontinued, most commonly due to "patient wish" (4), AE (3) and "insufficient efficacy" (2) (**Table 3**). Known AEs that led to discontinuation are listed in **Supplementary Table 5**.

Persistence and adherence were examined in the full analysis set (all patients in the safety set with at least one documentation after start of the therapy; RMS n = 1,510; PPMS n = 363). Kaplan-Meier analysis showed that patients treated with ocrelizumab achieved 96% and 92% persistence at 12 and 24 months, regardless of MS phenotype (**Figures 1A,B**). Patients >55 years old (n = 184) achieved a 95% and 87% persistence at 12 and 24 months. Patients >55 years with PPMS (n = 143) achieved a 95% and 86% persistence at 12 and 24 months.

The median time between infusions was ∼6 months, regardless of age group or MS phenotype; and infusion intervals remained stable throughout the treatment duration (**Table 4**).

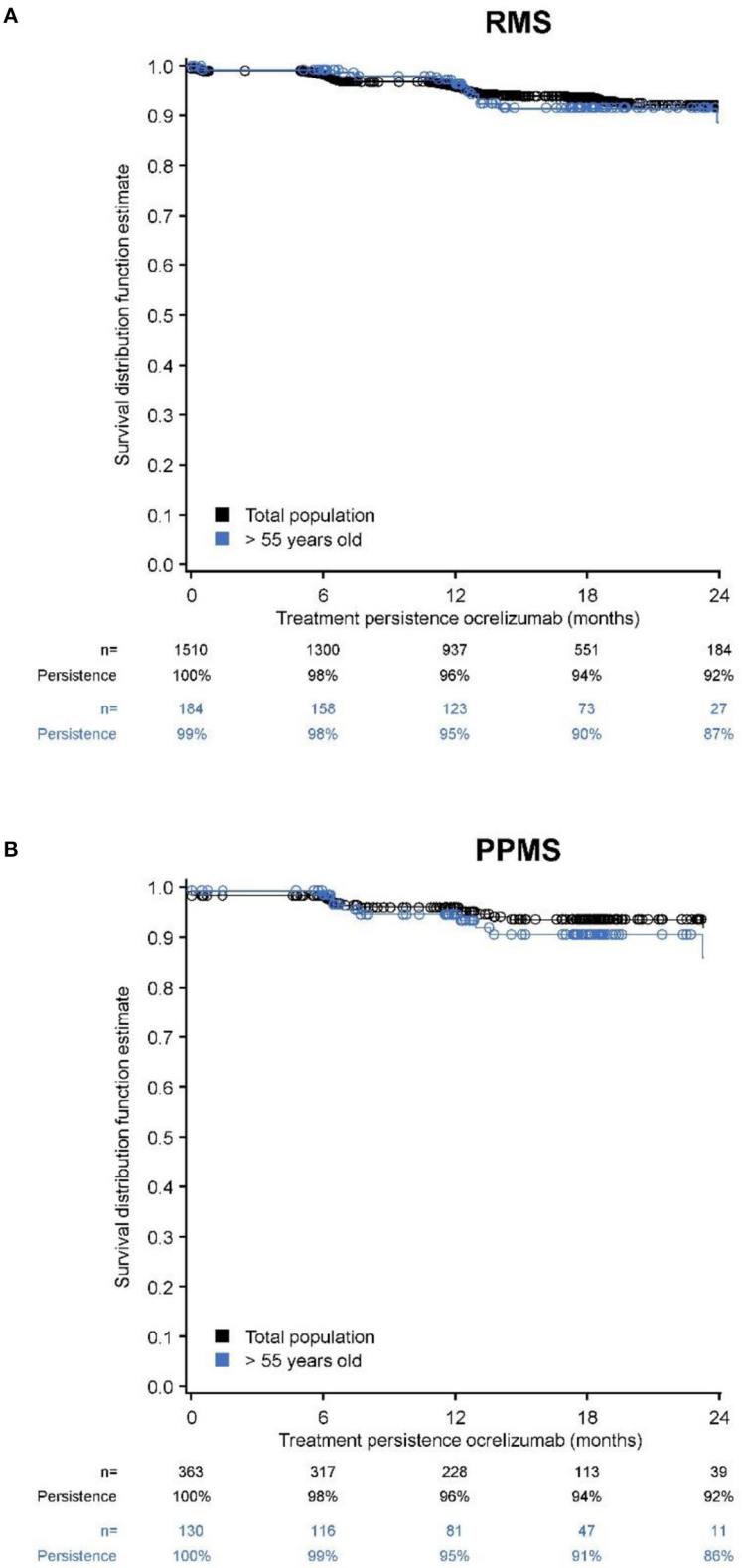

FIGURE 1 | Kaplan-Meier estimates of persistence over 24-months of treatment with ocrelizumab (full analysis set). **(A)** RMS total + >55 years; **(B)** PPMS total + >55 years.

TABLE 4 | Median time interval between ocrelizumab dosing.

Dose interval	Total RMS (n = 1,510)		RMS >55 years (n = 184)		Total PPMS (n = 363)		PPMS >55 years (n = 143)	
	n	Median, mo (25Q; 75Q)		Median, mo (25Q; 75Q)	n	Median, mo (25Q; 75Q)		Median, mo (25Q; 75Q)
2nd−3rd	1361	5.95 (5.59; 6.18)	163	5.98 (5.70; 6.21)	330	5.95 (5.59; 6.18)	119	5.88 (5.55; 6.18)
3rd−4th	1001	5.98 (5.78; 6.21)	127	5.98 (5.77; 6.21)	246	5.98 (5.75; 6.21)	84	5.98 (5.78; 6.21)
4th−5th	622	5.98 (5.75; 6.21)	86	5.98 (5.75; 6.21	149	5.98 (5.91; 6.21)	53	6.01 (5.98; 6.21)
5th−6th	295	5.98 (5.72; 6.11)	38	5.96 (5.75; 6.01)	67	5.98 (5.75; 6.01)	21	5.88 (5.62; 5.98)

Mo, months.

Data were analyzed in the full analysis set (all enrolled patients who received at least one dose of ocrelizumab with at least one documentation after start of the therapy). Q, quartile.

DISCUSSION

Real-world studies provide data to further evaluate the risk/benefit profiles described for new therapies in RCTs. To mitigate potential confounding factors, RCTs include selected patient populations. Real-world studies include diverse patient populations, reflecting features of daily clinical practice. This analysis of the CONFIDENCE study represents a real-world cohort of patients with comorbidities and no limits regarding maximum age or EDSS. According to the later onset of disease, patients with PPMS were on average slightly older and had a higher EDSS than patients with RMS. As ocrelizumab is the only treatment currently available for PPMS, a higher proportion of patients with PPMS were treatment-naïve. Irrespective of MS phenotype, patients >55 years had a higher average baseline EDSS (RMS 4.54; PPMS 4.73) and more comorbidities (RMS 80.5%; PPMS 86.0%) than their respective total phenotypic cohorts.

Although there were no restrictions on the disability status of enrolled patients, average baseline EDSS scores were similar to pivotal trials (4, 5). However, patients in CONFIDENCE were on average older than in pivotal trials, with ∼12% of patients with RMS and ∼36% of patients with PPMS >55 years (a population excluded from pivotal trials). In addition, patients had longer times since diagnosis, and a greater proportion of patients with RMS had prior MS therapy (∼83 vs ∼27% in pivotal trials) (4). Moreover, this study includes patients with comorbid conditions who were excluded from RCTs, such as patients with a history of malignancy or congestive heart failure (4, 5). Comorbid conditions often observed in real-world MS populations such as cardiovascular disorders and mood disorders (15) were also seen in CONFIDENCE.

To date, real-world data from large ocrelizumab cohorts that may reflect treatment patterns are rare. CONFIDENCE is a German study and baseline characteristics are largely comparable to another German real-world cohort of ocrelizumab treated patients (16). Compared to a recent smaller US cohort (17), patients with RMS in CONFIDENCE had similar mean EDSS and fewer patients in CONFIDENCE were treated with first-line ocrelizumab. With respect to ocrelizumab treated patients documented in the global MSBase registry (2), CONFIDENCE populations had similar age profiles. Patients with RMS tended to have a higher EDSS and proportions of patients without prior therapy were similar. However, patients with PPMS had a lower EDSS and were more often treatment-naïve.

In alignment with populations reported in large real-world studies examining other highly effective DMTs, the CONFIDENCE population was largely similar regarding age, types of comorbidities, baseline EDSS scores, and the majority of patients had been treated with ≥1 prior MS therapy (3, 18).

No new safety signals were identified in this analysis, where many patients had comorbidities and many RMS patients had multiple previous therapies (∼35% had ≥3). Patients with PPMS in CONFIDENCE experienced numerically lower rates of both AEs and SAEs than patients with RMS. Patients with PPMS >55 years experienced SAEs at approximately half the rate (SAE/100 PY) of patients with RMS >55 years. Nevertheless, patients with PPMS comprised a smaller population, and patients with PPMS >55 years were less likely to have previous/multiple previous therapies and had shorter disease durations since diagnosis. As expected, patients >55 years (irrespective of MS phenotype) had higher SAE rates than their respective total cohorts.

Compared with the general population, patients with MS experience infections and hospitalizations due to infections at higher rates (19). Accordingly infections were among the most common AEs reported in CONFIDENCE. However, rates of serious infections remained low. Patients with PPMS had a numerically lower rate of infections and serious infections than patients with RMS. Patients >55 years, however, experienced similar rates of serious infections to that of their overall respective populations. Overall, infections most often reported in CONFIDENCE (respiratory infections and urinary tract infections) were consistent with the described ocrelizumab safety profile (8) and the general MS population (19). The single case of suspected PML in a patient with RMS was considered a carry-over from previous natalizumab treatment.

During this analysis (data cutoff 14/Oct/2020), six patients were reported to have COVID-19 or COVID-19 pneumonia (all with RMS). Two cases were considered serious with only one requiring hospitalization. All patients with known COVID-19 outcomes recovered.

Presented data on COVID-19 are from an early time (pre vaccination era) in the COVID-19 pandemic. A recent analysis (May 2021) using the ocrelizumab post-marketing safety database and clinical trial data show that COVID-19 infections in patients treated with ocrelizumab were mostly mild to moderate, and risk factors known to be associated with severe disease course in the general population were associated with severity in ocrelizumab-treated (20). However, a number of real-world studies (21–24) suggest an increased risk of severe COVID-19 in patients with MS treated with anti-CD20 treatments although subject to potential limitations, including biases, confounding, sample size and data completeness (25). Further analyses are required to understand the risk and severity of COVID-19 in ocrelizumab treated patients.

Another important question will be to determine the clinical protection conferred by SARS-CoV-2 vaccines against severe forms of COVID-19. Attenuated humoral immune response has been associated with ocrelizumab treatment to non-live vaccines (26). However, the development of a protective immune response after vaccination involves a variety of mechanisms, of which T and B cells are variably involved (27). In the context of COVID-19, whether antibody production is the appropriate or sole immune correlate of protection is currently unknown and the role of T or B cell-mediated immunity for effective clinical protection requires additional investigations. Available data report impaired humoral response to SARS-CoV-2 infection or vaccines in ocrelizumab treated patients, but induced robust cellular response (28–35), significantly boosted after a third vaccine dose (36), or preserved against SARS-CoV-2 Delta or Omicron variants (37). Despite the impaired humoral immune response to SARS-CoV2 infection or vaccine, no known correlation with clinical severity has been established, as compensatory cellular-mediated immune response could provide protection against serious complications from COVID-19 infection.

Immunoglobulins and B-cell levels are not routinely checked for in clinical practice and neither the assessment nor the collection of corresponding data are mandatory in this real-world study. Available data collected on immunoglobulins and B-cell levels as part of the CONFIDENCE study are limited in time and potentially biased by lack of systematic data collection across participating centers, therefore they do not allow to assess the association between serious infections, duration of treatment and respective laboratory values.

There was no indication of increased malignancy rates in analyses of the overall ocrelizumab clinical program and post-marketing data compared with matched reference MS and general populations (8, 38). In this analysis of CONFIDENCE, rates of malignancy resembled previously published data from RCTs including open-label extension phases, which were within the expected epidemiological ranges (8, 39).

Persistence and adherence to an effective DMT are associated with lower relapse rates, better clinical outcomes, and reductions in the cost of patient care (40, 41) and can be related to treatment satisfaction and safety (42). Data in CONFIDENCE were consistent with US claims data (43), which show that ocrelizumab has a high persistence. Persistence remained high in patients >55 years across all MS phenotypes. Because there were few discontinuations, no major reasons could be identified. Furthermore, discontinuations due to AEs were rare. Adherence was consistent across MS phenotypes and age groups, with median intervals of ∼6 months in between ocrelizumab infusions, in accordance with the regulatory label.

CONFIDENCE is a real-world study and is thus susceptible to the limitations of non-interventional studies (e.g., potential enrollment and channeling biases between cohorts). Efforts to mitigate limitations and biases associated with long-term real-world cohort studies (such as healthy user bias and depletion of susceptibles) included only enrolling patients newly treated with ocrelizumab or selected DMTs. All study sites underwent standardized training and used standardized documentation for the completion of eCRFs at enrollment and for each follow-up assessment, specifically for collecting exposure and outcome variable information. Due to the observational nature of the CONFIDENCE study and spontaneous reporting of AEs, a bias in the reporting of AEs cannot be excluded (e.g., underreporting of non-serious AEs and overrepresentation of SAEs) and information on fatal cases and laboratory values (e.g., Immunoglobulins, B-cell levels) is limited.

Overall, CONFIDENCE represents the use of ocrelizumab in clinical practice and includes patients with physical disability, with comorbid conditions, and patients >55 years. No new safety signals were detected in this analysis, confirming the tolerability and safety of ocrelizumab treatment in a real-world population over a mean of ∼1 year of exposure (max 2.5 years). High adherence and persistence to ocrelizumab were observed in patients with RMS or PPMS, and discontinuations were rare due to AE. Further analyses of this large, real-world study will be conducted on a regular basis to provide continuing safety and effectiveness data for the treatment of patients with ocrelizumab for up to 10 years.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethikkommission an der Technischen Universität Dresden, Germany (12 February 2018 and 10 April 2019; reference EK 62022018). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors analyzed and interpreted the data in study, participated in the writing, and approved the final version of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2022.863105/full#supplementary-material

REFERENCES

1. Jakimovski D, Vaughn CB, Eckert S, Zivadinov R, Weinstock-Guttman B. Long-term drug treatment in multiple sclerosis: safety success and concerns. *Expert Opin Drug Saf.* (2020) 19:1121–42. doi: 10.1080/14740338.2020.1805430
2. Butzkueven H, Spelman T, Ozakbas S, Kalincik T, Boz C, Buzzard K, Skibina O, Alroughani R: Real-world experience with Ocrelizumab in the MSBase Registry. *Presented at ECTRIMS-ACTRIMs.* (2020). doi: 10.1136/bmjno-2021-ANZAN.10
3. Ziemssen T, Lang M, Tackenberg B, Schmidt S, Albrecht H, Klotz L, et al. Clinical and demographic profile of patients receiving fingolimod in clinical practice in germany and the benefit-risk profile of fingolimod after 1 year of treatment: initial results from the observational, noninterventional study PANGAEA. *Neurotherapeutics.* (2018) 15:190–9. doi: 10.1007/s13311-017-0595-y
4. Montalban X, Hauser SL, Kappos L, Arnold DL, Bar-Or A, Comi G, et al. Ocrelizumab versus placebo in primary progressive multiple sclerosis. *N Engl J Med.* (2017) 376:209–20. doi: 10.1056/NEJMoa1606468
5. Hauser SL, Bar-Or A, Comi G, Giovannoni G, Hartung HP, Hemmer B, et al. Ocrelizumab versus interferon beta-1a in relapsing multiple sclerosis. *N Engl J Med.* (2017) 376:221–34. doi: 10.1056/NEJMoa1601277
6. Hauser SL, Kappos L, Arnold DL, Bar-Or A, Brochet B, Naismith RT, et al. Five years of ocrelizumab in relapsing multiple sclerosis: OPERA studies open-label extension. *Neurology.* (2020) 95:e1854–67. doi: 10.1212/WNL.0000000000010376
7. Wolinsky JS, Arnold DL, Brochet B, Hartung HP, Montalban X, Naismith RT, et al. Long-term follow-up from the ORATORIO trial of ocrelizumab for primary progressive multiple sclerosis: a post-hoc analysis from the ongoing open-label extension of the randomised, placebo-controlled, phase 3 trial. *Lancet Neurol.* (2020) 19:998–1009. doi: 10.1016/S1474-4422(20)30342-2
8. Hauser SL, Kappos L, Montalban X, Craveiro L, Chognot C, Hughes R, et al. Safety of ocrelizumab in patients with relapsing and primary progressive multiple sclerosis. *Neurology.* (2021) 10.1212/WNL.0000000000012700. doi: 10.1212/WNL.0000000000012700
9. Ziemssen T, Hillert J, Butzkueven H. The importance of collecting structured clinical information on multiple sclerosis. *BMC Med.* (2016) 14:81. doi: 10.1186/s12916-016-0627-1
10. Magyari M, Sorensen PS. Comorbidity in multiple sclerosis. *Front Neurol.* (2020) 11:851. doi: 10.3389/fneur.2020.00851
11. Dirks P, Zingler V, Leemhuis J, Berthold H, Hieke-Schulz S, Wormser D, et al. Design of a non-interventional post-marketing study to assess the long-term safety and effectiveness of ocrelizumab in German real world multiple sclerosis cohorts - the CONFIDENCE study protocol. *BMC Neurol.* (2020) 20:95. doi: 10.1186/s12883-020-01667-7
12. Ziemssen T, Kern R, Voigt I, Haase R. Data collection in multiple sclerosis: the MSDS approach. *Front Neurol.* (2020) 11:445. doi: 10.3389/fneur.2020.00445
13. Haase R, Wunderlich M, Dillenseger A, Kern R, Akgun K, Ziemssen T. Improving multiple sclerosis management and collecting safety information in the real world: the MSDS3D software approach. *Expert Opin Drug Saf.* (2018) 17:369–78. doi: 10.1080/14740338.2018.1437144
14. Berger JR, Aksamit AJ, Clifford DB, Davis L, Koralnik IJ, Sejvar JJ, et al. PML diagnostic criteria: consensus statement from the AAN Neuroinfectious Disease Section. *Neurology.* (2013) 80:1430–8. doi: 10.1212/WNL.0b013e31828c2fa1
15. Hauer L, Perneczky J, Sellner J. A global view of comorbidity in multiple sclerosis: a systematic review with a focus on regional differences, methodology, and clinical implications. *J Neurol.* (2020). doi: 10.1007/s00415-020-10107-y
16. Ellwardt E, Rolfes L, Klein J, Pape K, Ruck T, Wiendl H, et al. Ocrelizumab initiation in patients with MS: A multicenter observational study. *Neurol Neuroimmunol Neuroinflamm.* (2020) 7. doi: 10.1212/NXI.0000000000000719
17. Smoot K, Chen C, Stuchiner T, Lucas L, Grote L, Cohan S. Clinical outcomes of patients with multiple sclerosis treated with ocrelizumab in a US community MS center: an observational study. *BMJ Neurol Open.* (2021) 3:e000108. doi: 10.1136/bmjno-2020-000108
18. Butzkueven H, Kappos L, Wiendl H, Trojano M, Spelman T, Chang I, et al. Long-term safety and effectiveness of natalizumab treatment in clinical practice: 10 years of real-world data from the Tysabri Observational Program (TOP). *J Neurol Neurosurg Psychiatry.* (2020) 91:660–8. doi: 10.1136/jnnp-2019-322326
19. Wijnands JM, Kingwell E, Zhu F, Zhao Y, Fisk JD, Evans C, et al. Infection-related health care utilization among people with and without multiple sclerosis. *Mult Scler.* (2017) 23:1506–16. doi: 10.1177/1352458516681198
20. Hauser SL, Gold R, Cutter G, Fitovski K, Schneble HM, Whitley L, et al. COVID-19 in people with multiple sclerosis treated with ocrelizumab. *Presented at ECTRIMS congress* 2021. (2021).
21. Sormani MP, De Rossi N, Schiavetti I, Carmisciano L, Cordioli C, Moiola L, et al. Disease-modifying therapies and coronavirus disease (2019). Severity in multiple sclerosis. *Ann Neurol.* (2021) 89:780–9. doi: 10.1002/ana.26028
22. Salter A, Fox RJ, Newsome SD, Halper J, Li DKB, Kanellis P, et al. Outcomes and risk factors associated with SARS-CoV-2 infection in a north american registry of patients with multiple sclerosis. *JAMA Neurol.* (2021) 78:699–708. doi: 10.1001/jamaneurol.2021.0688
23. Simpson-Yap S, De Brouwer E, Kalincik T, Rijke N, Hillert JA, Walton C, et al. Associations of disease-modifying therapies with COVID-19 severity in multiple sclerosis. *Neurology.* (2021) 97:e1870–85. doi: 10.1212/WNL.0000000000012753
24. Reder AT, Centonze D, Naylor ML, Nagpal A, Rajbhandari R, Altincatal A, et al. COVID-19 in patients with multiple sclerosis: associations with disease-modifying therapies. *CNS Drugs.* (2021) 35:317–30. doi: 10.1007/s40263-021-00804-1

25. Hada M, Mosholder AD, Leishear K, Perez-Vilar S. Systematic review of risk of SARS-CoV-2 infection and severity of COVID-19 with therapies approved to treat multiple sclerosis. *Neurol Sci.* (2022) 43:1557–67. doi: 10.1007/s10072-021-05846-3
26. Bar-Or A, Calkwood JC, Chognot C, Evershed J, Fox EJ, Herman A, et al. Effect of ocrelizumab on vaccine responses in patients with multiple sclerosis: The VELOCE study. *Neurology.* (2020) 95:e1999–2008. doi: 10.1212/WNL.0000000000010380
27. Hughes R, Whitley L, Fitovski K, Schneble HM, Muros E, Sauter A, et al. COVID-19 in ocrelizumab-treated people with multiple sclerosis. *Mult Scler Relat Disord.* (2020) 49:102725. doi: 10.1016/j.msard.2020.102192
28. Kister I, Patskovsky Y, Curtin R, Pei J, Perdomo K, Rimler Z, et al. Cellular and humoral immunity to SARS-CoV-2 infection in multiple sclerosis patients on ocrelizumab and other disease-modifying therapies: a multi-ethnic observational study. *Ann Neurol.* (2022). doi: 10.1101/2022.01.10.22268752
29. Habek M, Zeljko C, Savic Mlakar A, Bendelja K, Rogic D, Adamec I, et al. Humoral and cellular immunity in convalescent and vaccinated COVID-19 people with multiple sclerosis: Effects of disease modifying therapies. *Mult Scler Relat Disord.* (2022) 59:103682. doi: 10.1016/j.msard.2022.103682
30. Rauber S, Korsen M, Huntemann N, Rolfes L, Muntefering T, Dobelmann V, et al. Immune response to SARS-CoV-2 vaccination in relation to peripheral immune cell profiles among patients with multiple sclerosis receiving ocrelizumab. *J Neurol Neurosurg Psychiatry.* (2022).
31. Tortorella C, Aiello A, Gasperini C, Agrati C, Castilletti C, Ruggieri S, et al. Humoral- and T-cell-specific immune responses to SARS-CoV-2 mRNA vaccination in patients with MS using different disease-modifying therapies. *Neurology.* (2022) 98:e541–54. doi: 10.1212/WNL.0000000000013108
32. Katz JD, Bouley AJ, Jungquist RM, Douglas EA, O'Shea IL, Lathi ES. Humoral and T-cell responses to SARS-CoV-2 vaccination in multiple sclerosis patients treated with ocrelizumab. *Mult Scler Relat Disord.* (2022) 57:103382. doi: 10.1016/j.msard.2021.103382
33. Brill L, Rechtman A, Zveik O, Haham N, Oiknine-Djian E, Wolf DG, et al. Humoral and T-cell response to SARS-CoV-2 vaccination in patients with multiple sclerosis treated with ocrelizumab. *JAMA Neurol.* (2021) 78:1510–4. doi: 10.1001/jamaneurol.2021.3599
34. Madelon N, Lauper K, Breville G, Sabater Royo I, Goldstein R, Andrey DO, et al. Robust T cell responses in anti-CD20 treated patients following COVID-19 vaccination: a prospective cohort study. *Clin Infect Dis.* (2021). doi: 10.1093/cid/ciab954
35. Apostolidis SA, Kakara M, Painter MM, Goel RR, Mathew D, Lenzi K, et al. Cellular and humoral immune responses following SARS-CoV-2 mRNA vaccination in patients with multiple sclerosis on anti-CD20 therapy. *Nat Med.* (2021) 27:1990–2001. doi: 10.1038/s41591-021-01507-2
36. Brill L, Raposo C, Rechtman A, Zveik O, Levin N, Oiknine-Djian E, et al. Severe acute respiratory syndrome coronavirus 2 third vaccine immune response in multiple sclerosis patients treated with ocrelizumab. *Ann Neurol.* (2022). doi: 10.1002/ana.26343
37. Madelon N, Heikkila N, Sabater Royo I, Fontannaz P, Breville G, Lauper K, et al. Omicron-specific cytotoxic T-cell responses after a third dose of mRNA COVID-19 vaccine among patients with multiple sclerosis treated with ocrelizumab. *JAMA Neurol.* (2022). doi: 10.1001/jamaneurol.2022.0245
38. Norgaard M, Veres K, Didden EM, Wormser D, Magyari M. Multiple sclerosis and cancer incidence: A Danish nationwide cohort study. *Mult Scler Relat Disord.* (2019) 28:81–5. doi: 10.1016/j.msard.2018.12.014
39. Pontieri L, Blinkenberg M, Bramow S, Papp V, Rasmussen PV, Kant M, et al. Ocrelizumab treatment in multiple sclerosis: a Danish population-based cohort study. *Eur J Neurol.* (2022) 29:496–504. doi: 10.1111/ene.15142
40. Lizan L, Comellas M, Paz S, Poveda JL, Meletiche DM, Polanco C. Treatment adherence and other patient-reported outcomes as cost determinants in multiple sclerosis: a review of the literature. *Patient Prefer Adherence.* (2014) 8:1653–64. doi: 10.2147/PPA.S67253
41. McQueen RB, Livingston T, Vollmer T, Corboy J, Buckley B, Allen RR, et al. Increased relapse activity for multiple sclerosis natalizumab users who become nonpersistent: a retrospective study. *J Manag Care Spec Pharm.* (2015). 21:210–218b. doi: 10.18553/jmcp.2015.21.3.210
42. Schriefer D, Haase R, Kullmann JS, Ziemssen T. Health-related quality of life and the relationship to treatment satisfaction in patients with multiple sclerosis: insights from a large observational study. *Patient Prefer Adherence.* (2020) 14:869–80. doi: 10.2147/PPA.S248272
43. Engmann NJ, Sheinson D, Bawa K, Ng CD, Pardo G. Persistence and adherence to ocrelizumab compared with other disease-modifying therapies for multiple sclerosis in U.S. commercial claims data. *J Manag Care Spec Pharm.* (2021) 27:639–49. doi: 10.18553/jmcp.2021.20413

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Models of Care in Multiple Sclerosis: A Survey of Canadian Health Providers

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Objective: Little work has evaluated integrated models of care in multiple sclerosis (MS) and the composition of MS care teams across Canada is largely unknown. We aimed to gather information regarding existing models of MS care across Canada, and to assess the perceptions of health care providers (HCPs) regarding the models of care required to fully meet the needs of the person with MS.

Methods: We conducted an anonymous online survey targeting Canadian HCPs working in MS Clinics, and neurologists delivering MS care whether or not they were based in an MS Clinic. We queried the types of HCPs delivering care within formal MS Clinics, wait times for HCPs, the perceived importance of different types of HCPs for good quality care, assessments conducted, and whether clinic databases were used. We summarized survey responses using descriptive statistics.

Results: Of the 716 HCPs to whom the survey was distributed, 100 (13.9%) people responded. Of the 100 respondents, 85 (85%) indicated that their clinical practice included people with MS and responded to specific questions about clinical care. The most common types of providers within MS Clinics with integrated models of care were neurologists and MS nurses. Of 23 responding MS Clinics, 10 (43.5%) indicated that there were not enough neurologists, and 16 (69.6%) indicated that there were not enough non-neurologist HCPs to provide adequate care. More than 50% of clinics reported wait times exceeding 3 months for physiatrists, physiotherapists, psychiatrists, psychologists, neuropsychologists and urologists; in some clinics wait times for these providers exceeded 1 year. Multiple disciplines were identified as important or very important for delivering good quality MS care. Over 90% of respondents thought it was important for neurologists, nurse practitioners, MS nurses and psychiatrists to be co-located within MS Clinics.

Conclusion: Canadian HCPs viewed the ideal MS service as being multidisciplinary in nature and ideally integrated. Efforts are needed to improve timely access to specialized MS care in Canada, and to evaluate how outcomes are influenced by access to care.

Keywords: multiple sclerosis, models of care, multidisciplinary, Canada, survey

INTRODUCTION

Multiple sclerosis (MS) is a chronic, immuno-inflammatory disease of the central nervous system affecting over 90,000 Canadians, and more than 2.8 million persons worldwide (1, 2). MS is a complex chronic disease characterized by relapses and progression of physical and cognitive impairment over time. Comorbid conditions such as depression and anxiety disorders are also common. MS has a negative effect on employment status (3), health-related quality of life (4–7), and the ability to perform personal and instrumental activities of daily living (ADL).

Comprehensive management of MS typically involves treatment of acute relapses, disease-modifying therapy (DMT) to modify the course of the disease by reducing relapses and disability progression, chronic symptom management, supports in regards to coping and function, and education. The National Collaborating Centre for Chronic Conditions at the Royal College of Physicians (United Kingdom, UK) developed a national clinical guideline for diagnosis and management of multiple sclerosis in primary and secondary care (8). These guidelines included a recommendation that people with MS have access to specialist rehabilitation services to assess complex problems which cannot be evaluated by a single team member and to provide an integrated program of rehabilitation, to monitor change, and to advise other members of the health care team. Integral components of the team were a physician, nurse, physiotherapist, occupational therapist, social worker, speech and language therapist, and clinical psychologists, consistent with recommendations for an MS Care Unit proposed by Sorensen et al. (9). A recent review suggested that multidisciplinary rehabilitation improves activity, participation and quality of life (10).

Multiple models of care exist for the management of chronic diseases such as MS. These include shared care models, primary-care specialist referral models, and specialized multi-disciplinary team-based models. Integrated models of care are those in which multiple health care providers are co-located and collaboratively manage patients, but relatively little work has evaluated integrated models of care in MS (11). In Canada, government-funded, specialized MS Clinics exist in most provinces, in part because government-funded access to MS-specific DMTs often requires assessment by a neurologist with specific expertise in MS. The composition of MS care teams across Canada is largely unknown, including whether the teams involve an integrated care model, and what disciplines are involved. Access to those teams, as assessed using the wait times are for each discipline, are also unknown. This information is important to inform policy development and resource allocations aimed at improving access to care and disease outcomes.

We aimed to gather information regarding existing models of MS care across Canada, and to assess the perceptions of health care providers regarding the models of care required to fully meet the needs of the person with MS. We hypothesized that models of MS care would vary with respect to their components (that is, what health care disciplines are considered to be part of the MS team), and structure (that is, whether team members and services are fully integrated and co-localized, integrated but not co-localized, not integrated or co-localized). We further hypothesized that MS health care providers (HCP) would identify a broad range of disciplines as being needed to support high quality care for persons with MS.

METHODS

We report the design and findings of this study according to the Consensus-Based Checklist for Reporting of Survey Studies (CROSS) (12).

Setting

This study was conducted in Canada, a country with a population of >38 million, distributed over 10 provinces and 3 territories. Health care in Canada is universal, and publicly funded for essential services, including hospitalizations and physician visits. Because health care is organized and delivered at the provincial level, variation exists in the services available and in how they are delivered. Thus, care from non-physician providers such as psychologists and physical therapists is often not covered except through specific disease-oriented programs, such as MS Clinics. Private health insurance plans may be used to obtain coverage for services not paid for by the universal health system.

Design and Population

This was a cross-sectional study utilizing an anonymous online survey. We targeted two populations, both comprised of HCPs practicing in Canada who were currently delivering MS care. The first population was neurologists, whether or not they practiced in the setting of an MS Clinic, given their critical role in diagnosis of MS and their role in access to DMTs. The second population was providers of all disciplines working within MS Clinics. To create the survey distribution list for neurologists, we collated names of health care professionals in Canada from multiple sources including Medical Directors of provincial MS Clinics, the Canadian Network of MS Clinics (a national network of academic and community-based clinics for MS care), provincial college of physician listings, and the American Academy of Neurology member directory. We used the provincial college listings, and the American Academy of Neurology member directory to enhance identification of neurologists practicing in

Canada who might deliver MS care outside formally labeled MS Clinics. Medical Directors of MS Clinics assisted with identification of non-neurologist HCPs working in MS Clinics. The University of Manitoba Health Research Ethics Board and Shared Health approved the study. The survey included a consent statement indicating that completion of the survey implied consent.

Survey

We adapted an existing questionnaire that assessed models of care in inflammatory bowel disease (IBD), another chronic immune-mediated disease that often requires multidisciplinary care (13). The survey assessed characteristics of the respondent, their work settings, types of HCPs delivering care within formal MS Clinics, the perceived importance of different types of HCPs for good quality care, clinic databases and assessments conducted. **Supplementary Appendix I** includes the full questionnaire. The questionnaire was pilot tested by two individuals who were not involved in survey development prior to distribution.

Respondent Characteristics

Respondent characteristics queried included age, gender, discipline, whether they had a particular interest in MS, if they had fellowship training in MS and whether their clinical practice included people living with MS. Disciplines included neurologist, physiatrist, MS Nurse, nurse practitioner, physician assistant, physiotherapist, occupational therapist, social worker, psychologist, psychiatrist/neuropsychiatrist, neuroradiologist/radiologist, dietician, urologist/urogynecologist, general ophthalmologist, neuro-ophthalmologist, speech-language pathologist, pharmacist, neuropsychologist, and other. We did not include primary care providers as they are not integrated within MS Clinics in Canada. Respondents who indicated that their clinical practice did not include people living with MS were not asked any further questions, and were excluded from the primary analysis.

Work Setting

The remaining respondents were asked to provide details regarding their MS-related work including their training, length of time working in the MS field, the setting of their MS practice, what percentage of their clinical work concerns MS, practice size, whether they treated adults or children with MS, whether they worked within a formally labeled MS Clinic (and if so, which one); and whether they considered their MS service to apply an integrated model of care.

Composition of MS Clinics and Timeliness of Care

To limit response burden, questions regarding services available within formally labeled MS Clinics were answered by a single respondent who had been designated to do so in advance of that survey through contact with the Medical Director of the clinic. We asked whether the MS Clinic used an integrated model of care (model in which several HCPs are located at the same site and manage patients collaboratively), and which types of HCPs worked in the clinic. For each provider indicated as working in the clinic, respondents indicated the wait time for a new referral (0–3, 4–6, 7–12, >12 months), as well as the total number of HCPs and total full-time equivalents (FTE) for each type of HCP? For all MS services, whether or not they were formally labeled or integrated, we asked respondents to indicate which publicly funded types of HCPs were accessible outside their MS service, as well as the wait times for a new referral. Respondents also indicated whether the number of neurologists (FTEs), and non-neurologist HCPs at the MS Clinic allowed for provision of optimal care.

Clinical Assessments and Referral Patterns

Given the high prevalence of mood and anxiety disorders among people with MS, we asked if providers routinely asked about stress, anxiety, or depression during their encounters with patients (yes/no). If yes, they were asked if this was by verbally asking questions, using a questionnaire or other means. We also asked about the use of standardized assessments not related to mental health, focusing on those which are widely recognized, accessible and validated for use in MS, including the Timed 25 Foot Walk, Nine Hole Peg Test, a measure of processing speed including the Symbol Digit Modalities Test, the Expanded Disability Status Scale (EDSS) score, a measure of quality of life (specify), and screening measures for depression and anxiety disorders including the Patient Health Questionnaire-9, Hospital Anxiety and Depression Scale, Clinical Epidemiology Studies Depression scale, Beck Depression Inventory, Generalized Anxiety Disorder-7, OASIS, PROMIS Depression and PROMIS Anxiety measures. An "other" option was provided for respondents to specify other assessment measures used.

Respondents reported the approximate percentage of their MS patients they referred to the following health professionals: physiotherapist, occupational therapist, social worker, psychologist, psychiatrist, neuropsychologist, and dietitian. These providers were selected based on the high prevalence of comorbid mental health disorders in people with MS (14), the benefits of multi-disciplinary rehabilitation in MS (10), and recommendations for MS care in the UK (8).

Database Information

Quality improvement requires the ability to measure processes and outcomes. Therefore, we asked the designated responder within MS Clinics "Does your clinic currently collect the following data electronically (clinic database or administrative data) to allow determination of outcomes?", including date of symptom onset, date of first neurologist encounter, date of each MRI after symptom onset, date of first MS Clinic visit, date of first DMT discussion, date of first DMT initiation, date of diagnosis, date DMT insurance effective, dates of each visit, date of each EDSS, dates of each care provider encounter and who provided care, dates of each DMT started and stopped, reason for DMT switch, dates and scores of each cognitive test (and which test), dates of each relapse, referral to MS Clinic date, reason for referral, whether the referral was internal to the institution or external, and the health professional who referred. For each item we asked if the information was collected at the clinical

level or for each physician. If an item was collected, we asked the completeness and accuracy of the information using visual analog scales marked low/medium/high.

Perceived Ideal Models of Care

Respondents indicated "How important are these types of health professionals for good quality MS care?" on Likert-type response scale (not at all important, unimportant, neither important nor unimportant, important, very important). If a health professional was identified as important or very important a follow-up question asked how important it was to good quality care that they work within the MS Clinic using the same Likert-type response scale. The survey closed with two open-ended questions asked respondents to (i) Describe the ideal MS service; and (ii) What resources would be most helpful in improving MS care at your clinic.

Survey Administration

The survey was developed and managed using REDCap electronic data capture tools hosted at the University of Manitoba. REDCap (Research Electronic Data Capture) is a secure, web-based software platform designed to support data capture for research studies (15). The survey was distributed beginning in mid-September 2021 and closed January 31, 2022. Prior to questionnaire distribution, members of the Canadian Network of MS Clinics were advised via email that the survey was going to be distributed. Initially, the individual survey links were distributed directly using the REDCap survey distribution tools. However, it became apparent that email invitations issued via the REDCap server were sometimes being treated as junk/spam emails. To address this problem reminders were manually generated and sent from the institutional email address of a study coordinator at least three times. Two general reminders were also issued through the Canadian Network of MS Clinics listserv.

Analysis

We summarized the responses to survey questions using descriptive statistics including mean [standard deviation (SD)], median [interquartile range (IQR)], and frequency (percent). Missing data were not imputed. Bivariate analyses tested the association between respondent characteristics and models of care using chi-square tests, Fisher's exact tests, and non-parametric measures of association as appropriate. Formal qualitative analysis of the responses to the open-ended questions will be reported separately.

The analysis was conducted using SAS V9.4.2 (SAS Institute Inc., Cary, NC).

RESULTS

Participant Characteristics

Overall, of 716 to whom the survey was distributed, 100 (13.9%) people responded. Of the 100 respondents, 85 (85%) indicated that their clinical practice included people with MS and were presented with specific questions about MS care (**Table 1**). The demographic characteristics of respondents were similar for those whose practices did and did not include people with MS.

TABLE 1 | Characteristics of respondents, stratified according to whether practice includes people with multiple sclerosis.

Characteristic	Practice includes MS		*P*-value*
	No (*N* = 13)	Yes (*N* = 85)	
Age (yrs), mean (SD)	51.4 (13.8)	47.6 (11.4)	0.28
Gender, *n* (%)			
Male	6 (46.2)	34 (40.0)	0.86
Female	7 (53.8)	50 (58.8)	
Prefer not to answer	0 (0)	1 (1.2)	
Discipline, *n* (%)			
Neurologist	12 (92.31)	57 (67.06)	0.76
Physiatrist	0 (0)	2 (2.35)	
MS nurse	0 (0)	7 (8.24)	
Nurse practitioner	0 (0)	4 (4.71)	
Physician assistant	0 (0)	1 (1.18)	
Physiotherapist	0 (0)	3 (3.53)	
Occupational therapist	0 (0)	3 (3.53)	
Social worker	0 (0)	3 (3.53)	
Psychologist	0 (0)	1 (1.18)	
Neuropsychiatrist	0 (0)	2 (2.35)	
Neuropsychologist	0 (0)	1(1.18)	
Other (MS educator, administrator)	1 (7.69)	1 (1.18)	
Particular interest in MS, *n* (%)	3 (23.08)	78 (91.76)	**<0.001**
Fellowship Training in MS, *n* (%)	-	39 (50.0)	0.089
No. years following training involved in MS Care, median (p25–p75)		13 (5–20)	
Province, *n* (%)			
British Columbia	–	12 (14.29)	
Alberta		14 (16.67)	
Saskatchewan		4 (4.76)	
Manitoba		21 (25.0)	
Ontario		22 (26.19)	
Quebec		6 (7.14)	
New Brunswick		2 (2.38)	
Nova Scotia		3 (3.57)	
Work setting[b], *n* (%)			
General hospital		19 (22.9)	
University hospital		57 (68.7)	
Solo private practice		4 (4.8)	
Group private practice		3 (3.6)	
Work in formally labeled MS Clinic[a], *n* (%)		63 (5.0)	
Age of MS population treated, *n* (%)			
Adults	–	5 (89.29)	
Children (≤16 years)	–	2 (32.14)	
Percentage of clinical work that concerns MS, median (p25–p5)		0 (30–90)	
No. MS patients per week, median (p25–p5)		20 (6–30)	
No. MS patients in practice, median (p25–p5)		400 (50–950)	

*[a]missing; [b]indicated other but did not specify. Bold indicates statistical significance. *Comparing No vs. Yes.*

TABLE 2 | Availability of health care providers to multiple sclerosis (MS) Clinics.

Type of provider	Non-integrated ($n = 4$*)	Integrated ($n = 21$)**			
		Outside MS Clinic	Within MS Clinic	# within MS Clinic	# FTEs within MS Clinic
Neurologist	4 (100)	12 (60.0)	20 (95.2)	4 (3.5–5.5)	2.5 (2.0–3.0)
MS nurse	4 (100)	3 (15.0)	20 (95.2)	3 (1–4)	2.0 (1.2–2.5)
Nurse practitioner	1 (25.0)	5 (25.0)	10 (47.6)	1 (1–1)	1 (1–1)
Physician assistant	0 (0)	1 (5.3)	4 (19.1)	4 (1–7)	1 (1–1)
Physiotherapist	2 (50.0)	13 (68.4)	14 (66.7)	1 (1–2)	1 (0.5–1.1)
Occupational therapist*	2 (50.0)	11 (57.9)	12 (60.0)	1 (1–2)	1 (0.5–2.2)
Social worker*	2 (50.0)	7 (36.4)	5 (25.0)	1 (1–2)	1 (0.5–2.2)
Psychologist	1 (25.0)	9 (47.4)	5 (25.0)	2 (1–4)	0.80 (0.45–1.5)
Psychiatrist*	2 (50.0)	10 (52.6)	14 (70.0)	1 (1–2)	0.2 (0.1–1.0)
Radiologist*	2 (50.0)	8 (40.0)	13 (61.9)	3 (4–6)	2.5 (0.8–5.0)
Dietitian	2 (50.0)	9 (47.4)	6 (38.6)	1 (1–2)	0.5 (0.3–2.0)
Urologist	2 (50.0)	12 (63.2)	8 (38.1)	2 (1–3)	2.0 (0.2–3.0)
General ophthalmologist	2 (50.0)	16 (84.2)	2 (9.5)	2.5 (2–3)	1.1 (0.2–2.0)
Neuro-ophthalmologist	2 (50.0)	10 (52.6)	11 (52.4)	2 (2–3)	1.5 (0.4–3.0)
Speech language pathologist	2 (50.0)	13 (68.4)	6 (28.6)	1 (1–2)	0.2 (0.2–0.5)
Physiatrist	2 (50.0)	11 (57.9)	15 (71.4)	2 (1–2)	0.4 (0.2–1.9)
Pharmacist	2 (50.0)	10 (55.6)	6 (28.6)	1 (1–1)	1 (1–1)
Neuropsychologist	2 (50.0)	8 (42.1)	9 (42.9)	1 (1–2)	0.75 (0.15–1)
Orthotist	2 (50.0)	13 (68.4)	3 (14.3)	2.5 (2–3)	2 (1–3)

*$n = 1$ missing; **Within MS Clinic indicates provider is located within the integrated MS Clinic. Outside MS Clinic indicates provider is not integrated within the MS Clinic but available by referral.*

Respondents whose practice included people with MS constitute the study sample used for the remaining analyses.

Among the 85 respondents, most were neurologists ($n = 57$), followed by MS nurses ($n = 7$); slightly over half were female. All other types of HCPs had ≤3 respondents. Eight out of the ten Canadian provinces were represented, and we received responses from 26 (76.5%) of the 34 formally labeled MS Clinics.

Work Setting

Overall, the median (IQR) practice size was 400 (50–950) people with MS, but this varied by discipline. Among neurologists, median (IQR) practice size was 425 (75–800), whereas it was much larger for MS nurses [1,600 (500–4,000), $p = 0.016$] and similar for nurse practitioners [300 (40–500), $p = 0.44$]. The number of respondents for other disciplines limited inference about practice size.

Composition of MS Clinics and Timeliness of Care

Although 63 respondents who reported working in an MS clinic, only 26 (1 per site) were designated respondents for this group of questions. Of these 26, 21 (80.8%) respondents reported that they worked within an integrated model of care, and 1 respondent did not indicate the model of care. The most common types of providers within MS Clinics with integrated models of care were neurologists and MS nurses, with 20 of 21 MS Clinics reporting that they had both types of HCPs. One clinic reported having neither neurologists nor MS nurses. After neurologists and MS nurses, physiatrists, psychologists, and physiotherapists and occupational therapists were the most common HCPs (**Table 2**).

The most common types of providers available outside those clinics by referral were general ophthalmologists, physiotherapists, orthotists, and speech language pathologists. In MS Clinics without integrated models of care, the most common types of providers that comprised those clinics were neurologists and MS nurses (100%). Availability of all other HCPs (outside those clinics) except nurse practitioners was 50%. Of the 19/21 MS Clinics with integrated models of care who responded to this question, 13/19 (68.4%) reported that they hold multi-disciplinary team meetings, whereas only 1/4 (25.0%) of the non-integrated clinics did so.

Of 23 responses, 10 (43.5%) indicated that there were not enough neurologists to provide adequate care, and 16 (69.6%) indicated that there were not enough non-neurologist HCPs to provide adequate care. Wait times for providers within MS Clinics were variable (**Figure 1**). More than 50% of clinics reported wait times exceeding 3 months for physiatrists, physiotherapists, psychiatrists, psychologists, neuropsychologists and urologists; in some clinics wait times for these providers exceeded 1 year. The only providers who were uniformly accessible within 3 months of referral were orthotists, pharmacists, general ophthalmologists, and MS nurses. Generally, wait times were longer for providers located outside MS Clinics (**Figure 2**).

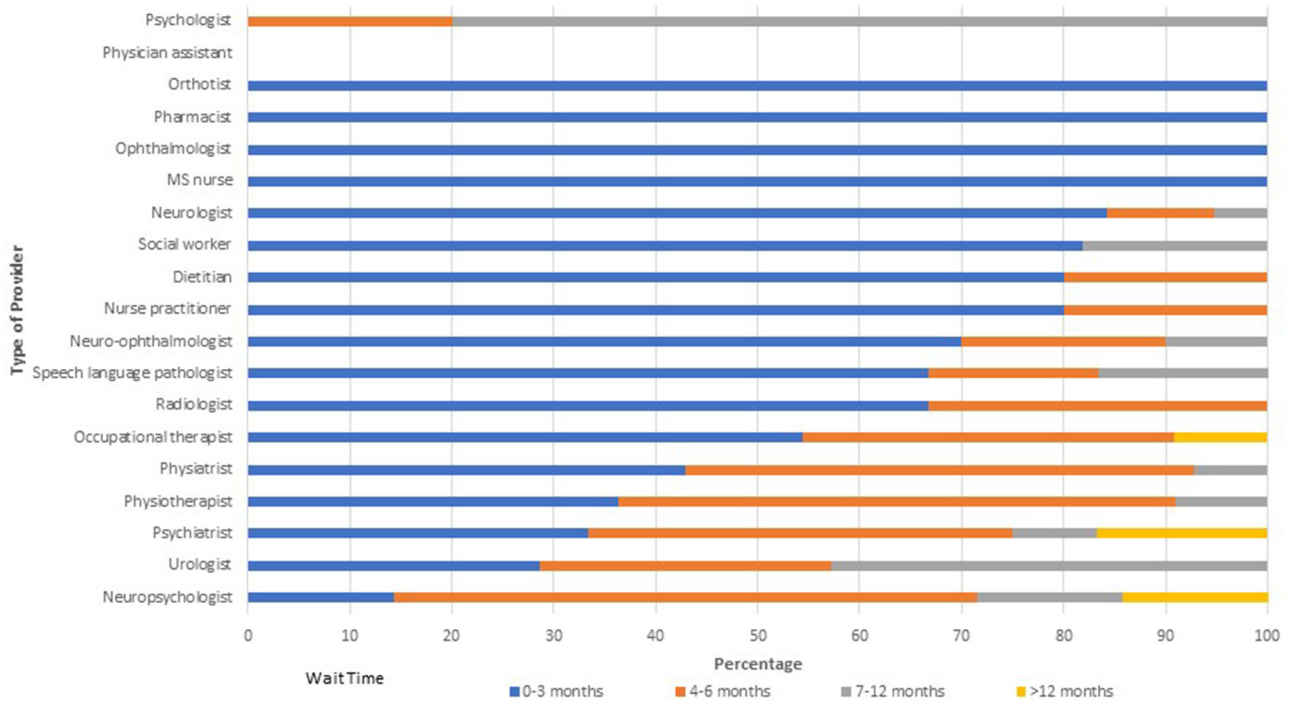

FIGURE 1 | Wait times for providers within multiple sclerosis clinics.

Research and Database-Related Questions

With respect to research, all but one MS Clinic (with an integrated model of care) reported participating in research including 15 (57.69%) clinics reported that they participate in research lead by their team members, and 16 (61.54%) that they participated in research lead by others. Of the 23/26 clinics who responded, 19 (82.6%) indicated that they had a database. The information captured varied across clinics (**Supplementary Appendix II**). The most commonly captured information was the date of the first clinic visit, dates of other clinic visits, and dates related to initiation and switching of DMT. The least commonly captured information was whether referrals to the MS Clinic were internal or external to the institution and dates DMT coverage became effective. Reported completeness and data accuracy for most data elements captured exceeded 80%, but was lower for dates of DMT coverage, dates of each relapse, and dates of each EDSS.

Clinical Assessments and Referral Patterns

All respondents provided information regarding referral patterns, clinical assessments and ideal models of care. Overall, HCPs most often referred to physiotherapists, followed by occupational therapists (**Figure 3**). Although the findings should be interpreted with caution due to small numbers for HCPs other than neurologists, physiatrists ($n = 2$) were more likely to refer to physiotherapy (85 vs. 50%, $p = 0.11$) and occupational therapists (71.5 vs. 40.5%, $p = 0.035$) than neurologists. Occupational therapists ($n = 3$) were similarly more likely to refer to physiotherapists (71%, $p = 0.051$) as well as social workers (67%, $p = 0.043$) than neurologists.

The most common routinely performed assessment was the EDSS whether care was provided within or external to a formally labeled MS Clinic (**Table 3**), followed by the timed 25-foot walk and the nine hole peg test. Assessment with a timed 25-foot walk or nine-hole peg test was statistically significantly more common within an MS Clinic. When we restricted the analysis to neurologists, the differences with respect to the timed 25-foot walk or nine-hole peg test were larger, and screening of cognition with a processing speed test of some kind was more common within MS Clinics. Two providers (neurologist, occupational therapist) reported that they used the Montreal Cognitive Assessment when applicable. Other assessments reported included the BERG Balance Scale (physiotherapist, $n = 1$), grip strength (physiotherapist, $n = 1$), Modified Fatigue Impact Scale (occupational therapist, $n = 1$), Adolescent Adult Sensory Profile (occupational therapist, $n = 1$), measures of visual function (neurologist, $n = 1$), the Godin Leisure Time Activity Questionnaire (neurologist, $n = 1$), and a locally developed questionnaire (neurologist, $n = 1$). Assessment of quality of life was uncommon. Instruments used to assess quality of life included the Health Utilities Index Mark-3 ($n = 2$), the PEDS-QoL ($n = 2$), and MS-specific instruments ($n = 2$).

Overall, 91.0% (71/78) of respondents indicated that they routinely asked about stress, anxiety or depression. This proportion was higher among respondents working within MS Clinics (57/59, 96.6%) than among those who did not (14/19, 73.7%, $p = 0.0082$). When we restricted the analysis to

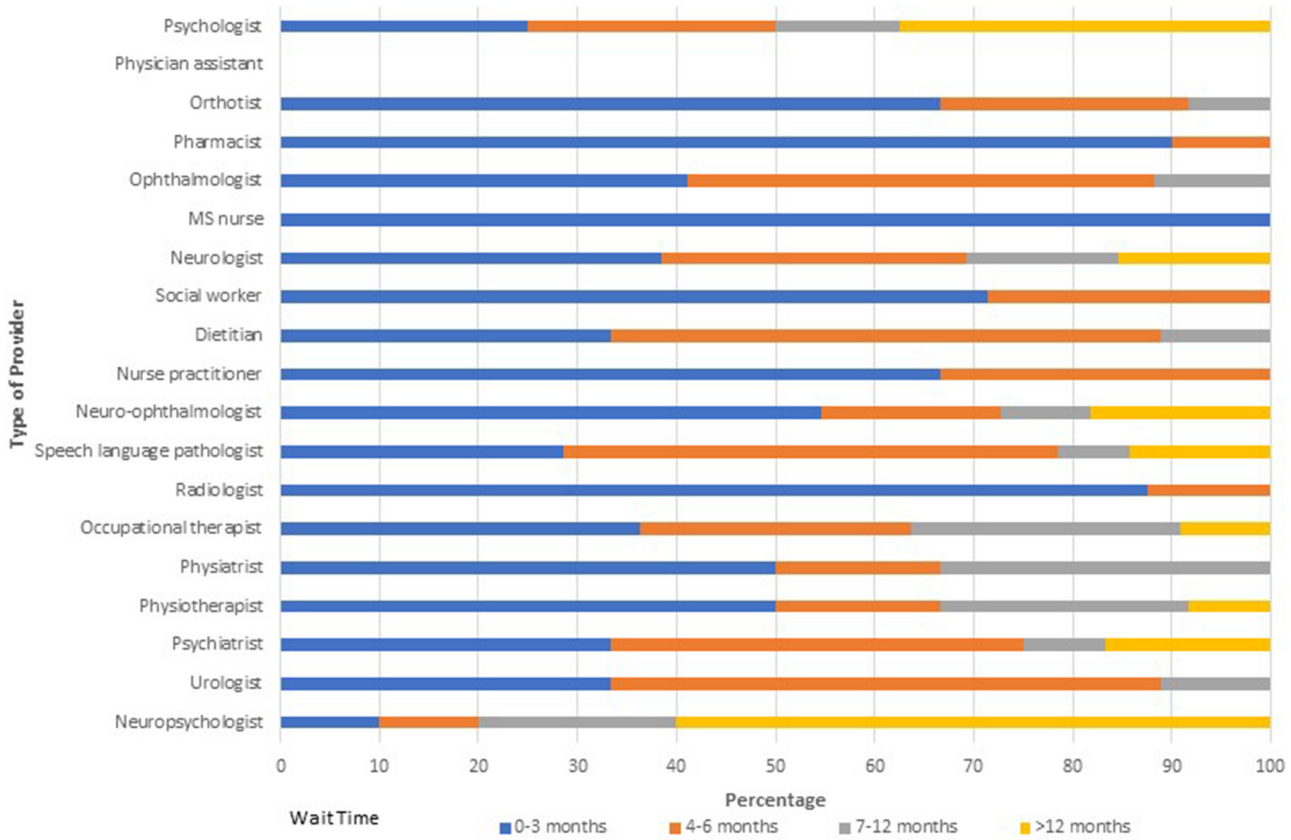

FIGURE 2 | Wait times for providers external to multiple sclerosis clinics.

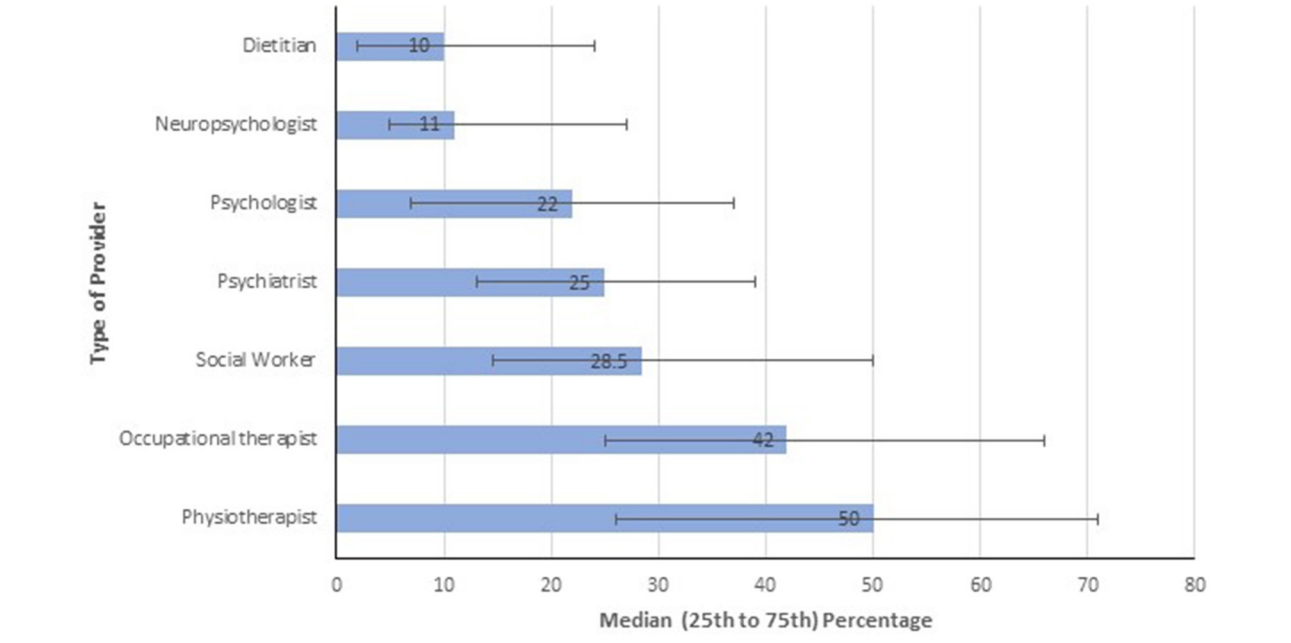

FIGURE 3 | Percentage of patients seen referred to different types of providers.

TABLE 3 | Percentage of assessments routinely performed stratified by whether the health care provider is in an integrated multiple sclerosis clinic or not.

Assessment	Integrated clinic[a]		*P*-value
	No ($n = 21$)	Yes ($n = 63$)	
All providers including neurologists			
Nine hole peg test	1 (4.8)	23 (36.5)	0.0048
Timed 25-foot walk	6 (28.6)	40 (63.5)	0.0056
SDMT or PST	6 (28.6)	30 (47.6)	0.13
EDSS	1 (4.8)	9 (14.3)	0.44
HRQOL	2 (9.5)	6 (9.5)	1
Depression questionnaire	5 (23.8)	13 (20.6)	0.76
Anxiety questionnaire	2 (9.5)	7 (11.1)	1
Neurologists	$n = 16$	$n = 40$	
Nine hole peg test	0 (0)	12 (30.0)	0.012
Timed 25-foot walk	4 (25.0)	26 (65.0)	0.0087
SDMT or PST	3 (18.8)	18 (45.0)	0.078
EDSS	11 (68.8)	5 (90.0)	0.1
HRQOL	1 (6.3)	4 (10.0)	1
Depression questionnaire	3 (18.8)	8 (20.0)	1
Anxiety questionnaire	1 (6.3)	4 (10.0)	1

SDMT, Symbol Digit Modalities Test; PST, processing speed test; EDSS, Expanded Disability Status Scale score; HRQOL, health-related quality of life; a-85 respondents to the question regarding assessments but one did not report whether s/he worked in an integrated clinic.

neurologists, this difference was larger (MS Clinic: 100%, non-MS Clinic: 0%, $p = 0.0008$).

Ideal Models of Care

Multiple disciplines were identified as important or very important for delivering good quality MS care (**Figure 4**). Only speech language pathologists (71.8%), orthotists (69.2%) and pharmacists (66.7%) were considered important or very important by fewer than 80% of respondents. Responses to the follow-up question indicated that it was important or very important for most types of HCPs queried to be working within an MS Clinic (**Figure 5**). Specifically, over 90% of respondents thought it was important for neurologists, nurse practitioners, MS nurses and psychiatrists to work within MS Clinics, and 75–89% thought it was important for occupational therapists, physiotherapists and social workers. In contrast, fewer than one-third of respondents thought that general ophthalmologists, urologists or orthotists needed to work within an MS Clinic. We did not identify any differences in responses across disciplines (all $p > 0.05$).

DISCUSSION

In this cross-sectional study we surveyed health care professionals, primarily neurologists, caring for people with MS across Canada. Over 40% of MS Clinics reported that they did not have enough neurologists to provide adequate care and nearly 70% of clinics reported that they did not have enough non-neurologist professionals to provide adequate care. More than half of MS Clinics reported wait times longer than 3 months for multiple types of providers including physiatrists, physiotherapists, psychiatrists, psychologists, neuropsychologists and urologists. However, multiple disciplines were perceived as important or very important for delivering good quality care. The ideal MS service was described as multidisciplinary, adequately staffed without time constraints for patient care, and systematic assessments of patient outcomes. Routinely performed assessments most often included the EDSS and screening for symptoms of stress, depression and anxiety. Although 81% of the MS Clinics represented reported practicing in an integrated model of care, and nearly all integrated clinics had neurologists and MS nurses, the remaining complement of HCPs was not consistent across clinics.

Individuals living with MS may suffer from a plethora of symptoms including weakness, sensory symptoms, bowel and bladder dysfunction, fatigue, spasticity, pain, and cognitive impairment. This was reflected in the widespread agreement that health care professionals from multiple disciplines are needed to provide good quality care for people living with MS. However, our survey suggests substantial variability with respect to the types of providers that are readily accessible to people living with MS, whether internal to or external to formal MS Clinics. Nearly all MS Clinics had access to neurologists and MS nurses but timely access to providers, as defined by wait times for referrals of <3 months, was more limited including for neurologists. The 2021 Atlas of MS reported that unmet needs for rehabilitation and symptom management were high (16), but was unable to discriminate between availability of providers vs. ability to access them in a timely fashion. Our findings suggest that in a Canadian context, both availability and timely access are a concern. Further, the 2021 Atlas of MS reported that availability of therapy for impaired mobility and spasticity was greater than for fatigue and cognitive impairment, mirroring the more limited access to occupational therapy and neuropsychology that we observed.

A European colloquium did not reach agreement regarding the structural organization of MS care teams and whether they needed to be co-localized (17). The ECTRIMS-EAN guidelines similarly recommend that the full spectrum of DMTs be provided only in centres (e.g., specialized MS Clinics) with adequate expertise and resources to provide appropriate assessments, monitoring and the ability to address adverse effects (18). Our findings demonstrate that there is no standard model of care across Canada, and also highlight a gap between current practice models and perspectives of ideal care models. Respondents indicated that the ideal MS service would be adequately staffed, and multidisciplinary, involving neurologists, nurses, psychiatrists, social workers, physiotherapists and occupational therapists, to provide timely integrated comprehensive care. Routine assessments at regular visits, and adequate time to spend with patients were also described as key components of an ideal MS service. In 2019, Sorensen et al. promoted the need for comprehensive "MS Care Units" to ensure early diagnosis, provide timely access to the full spectrum of interventions for care, including DMT, support shared decision-making, and provide appropriate monitoring and risk mitigation (9). The core of these MS Care Units was proposed to be MS

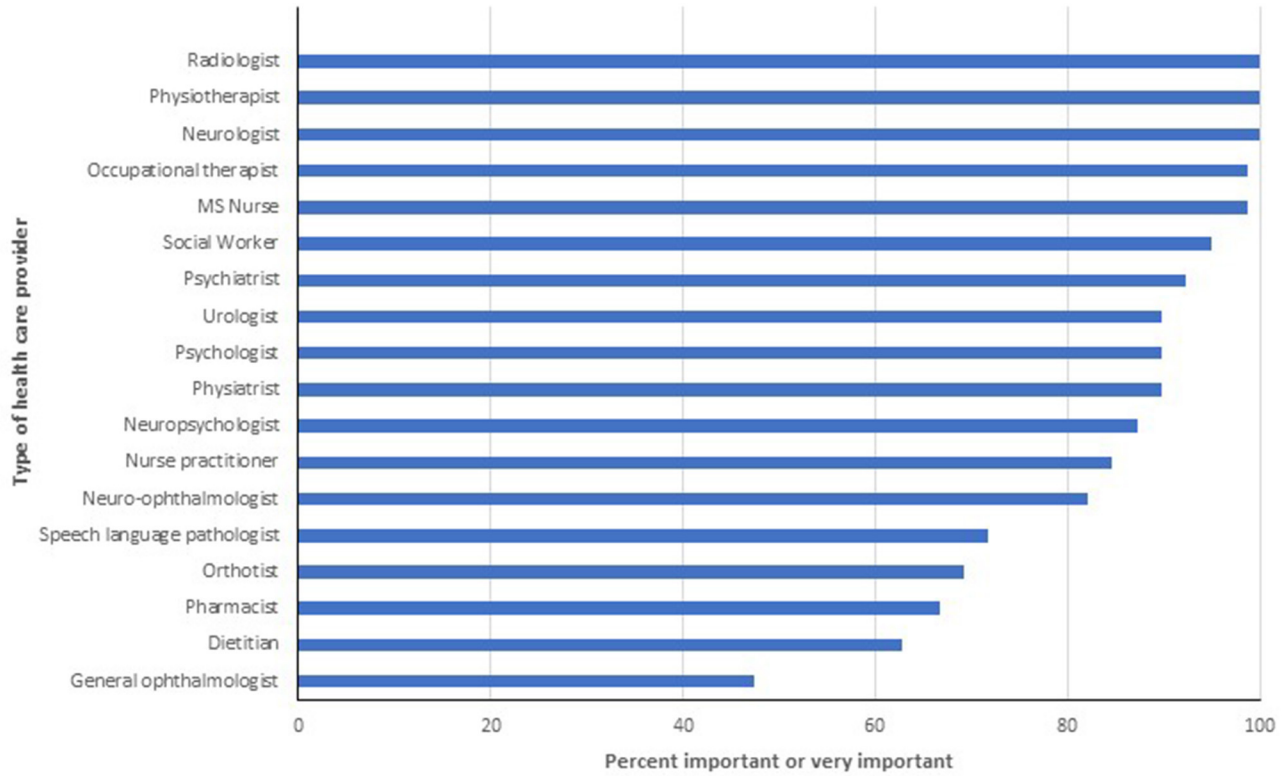

FIGURE 4 | Percentage of respondents indicating provider is important or very important for high quality multiple sclerosis care.

neurologists and nurses, at least three of neuropsychologists, clinical psychologists, physiotherapists, occupational therapists, speech therapists, social workers as well as specialist services related to diet, management of spasticity, incontinence and pain.

Findings in this survey regarding the ideal MS service, and poor access to the full range of providers expressed by HCPs are concordant with concerns expressed by people living with MS in other studies. A survey of 324 Canadians with MS found that two-thirds reported that their neurologist was their main source of MS care, but had difficulty accessing their neurologist as often as they wished (19). Occupational therapists, mental health providers and physiotherapists were the top HCPs whom participants needed to see but could not access. Encountering providers who lacked knowledge about MS and understood their situations was also a significant concern (19), echoed in a related qualitative study (20) and in a second qualitative study among moderately to severely affected individuals with MS in Germany (21). A recent survey of 1,190 persons, 75% of whom had MS, identified the influence of multidisciplinary teams on health outcomes and experiences as one of the top five research priorities (22). A study involving 707 patients from 81 centers in Italy found that patient satisfaction was lower in larger centres, and higher when a centre provided access to psychotherapy, suggesting a widespread need for mental health supports (23).

Our findings should be interpreted in light of limitations. We did not include a random sample of all clinicians delivering care to persons with MS. Further, the response rate was low despite the use of multiple reminders as recommended (24), potentially causing selection bias. The low response was likely influenced by several factors. First, response rates to electronic surveys have declined over time (25). Second, physicians who constituted the largest proportion of professionals in the sampling frame are known to have low response rates. We intentionally tried to capture neurologists who might deliver MS care outside MS clinics to gather a range of perspectives but found that very few neurologists who did not deliver MS care responded to the survey. This is consistent with prior observations that potential respondents are more likely to complete a survey when it is of high interest to them (24). Third, the survey was distributed during a period when a wave of the COVID-19 pandemic was placing substantial demands on Canadian health care professionals, some of whom who were assigned additional or alternative clinical responsibilities, which may have further reduced response rates. Third, we learned that some institutional spam filters were classifying the invitations as junk or blocking them altogether; it is not known how many invitations were adversely affected by this issue. We sought to mitigate this issue by subsequently issuing each email reminder manually (26). However, the response rate for MS Clinics regarding their models of care, including composition of clinics, timelines of care, and database practices was 80.8%. It is unknown if our findings would generalize to other health systems. We investigated the

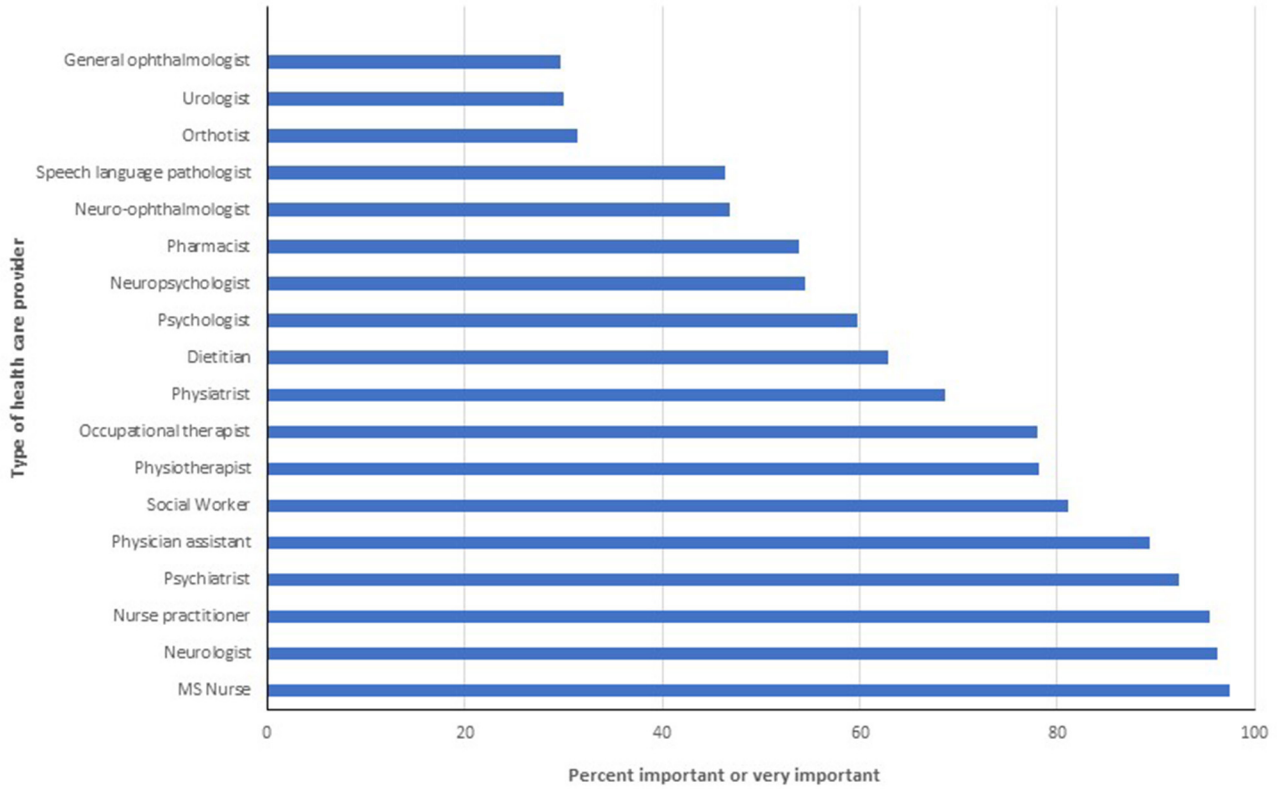

FIGURE 5 | Percentage of respondents indicating it is important or very important that the provider be located within a multiple sclerosis clinic.

existence of multidisciplinary models of care, but did not assess the existence of integrated care pathways, which are designed to provide a clear pathway for timely delivery of multidisciplinary care for a specific symptom or condition. Responses regarding wait times were informed by available wait time data in some but not all MS clinics, which may have affected accuracy of those responses. Comparisons between integrated and non-integrated clinics should be viewed cautiously, given the small number of non-integrated clinics/services that responded. We captured the perspectives of HCPs regarding the ideal MS service which may be influenced by the types of providers and models of care to which they have been exposed. Our list of potential providers within MS services did not include all possible providers, such as those offering palliative care. In 2020, the European Academy of Neurology proposed that home-based palliative care be offered to individuals living with severe progressive MS, although the quality of evidence supporting this statement was weak (27). In the United States inpatient palliative care remains uncommon, with only 6.1% of hospitalized people with MS receiving it in 2014 (28). A relatively low proportion of Canadians (<15%) receive palliative care in their last year of life, even among those receiving long-term care (22%) (29). Future studies should evaluate the role and integration of palliative care providers in MS Clinics. We also did not address the role of primary care providers because they are not usually integrated within MS Clinics, but they are key members of the larger care team. Finally, we did not capture the perspectives of people living with MS or their caregivers who report unmet needs (30), but prior studies in the Canadian setting are available.

CONCLUSION

Canadian HCPs viewed the ideal MS service as being multidisciplinary in nature, ideally integrated, with timely access to care. This is concordant with needs identified by people living with MS, which highlights the importance and urgency of ensuring availability of these models of care. Substantial variability existed in the types of providers situated within MS Clinics, and in the types of providers who are accessible outside MS Clinics. Wait times for were also highly variable but exceeded 3 months in many centres for multiple types of providers. Efforts are needed to improve access to specialized MS care in Canada, and to evaluate how outcomes are influenced by access to care.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the survey consent statement did not indicate data would be shared beyond study investigators. Requests to access the datasets should be directed to rmarrie@hsc.mb.ca.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Manitoba Health Research Ethics Board and Shared Health. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

RM: conceptualization, methodology, data curation, formal analysis, writing—original draft, writing—review and editing, and supervision. SD and JP: conceptualization, methodology, and writing—review and editing. DJ, JO, LM, PS, VD, OH, and SM: conceptualization and writing—review and editing. All authors reviewed the draft, provided feedback, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2022.904757/full#supplementary-material

REFERENCES

1. Gilmour H, Ramage-Morin PL, Wong SL. Multiple sclerosis: prevalence and impact. *Ottawa*. (2018) 82–003-X. doi: 10.4992/pacjpa.82.0_SL-003
2. King R. *Atlas Of MS 3rd Edition: Mapping Multiple Sclerosis Around The World.* Multiple Sclerosis International Federation (2020).
3. The Canadian Burden of Illness Study Group. Burden of illness of multiple sclerosis: part ii: quality of life. *Can J Neurol Sci*. (1998) 25:31–8. doi: 10.1017/S0317167100033448
4. Hermann B, Vickrey B, Hays RD, Cramer J, Devinsky O, Meador K, et al. A comparison of health-related quality of life in patients with epilepsy, diabetes and multiple sclerosis. *Epilepsy Res.* (1996) 25:113–8. doi: 10.1016/0920-1211(96)00024-1
5. Nortvedt MW, Riise T, Myhr K-M, Nyland HI. Quality of life in multiple sclerosis: measuring the disease effects more broadly. *Neurology.* (1999) 53:1098–103. doi: 10.1212/WNL.53.5.1098
6. Rudick RA, Miller DM, Clough JD, Gragg LA, Farmer RG. Quality of life in multiple sclerosis: comparison with inflammatory bowel disease and rheumatoid arthritis. *Arch Neurol.* (1992) 49:1237–42. doi: 10.1001/archneur.1992.00530360035014
7. Lankhorst GJ, Jelles F, Smits RC, Polman CH, Kuik DJ, Pfennings LE, et al. Quality of life in multiple sclerosis: the disability and impact profile (DIP). *J Neurol.* (1996) 243:469–74. doi: 10.1007/BF00900502
8. *The National Collaborating Centre for Chronic Conditions. Multiple Sclerosis: National Clinical Guideline for Diagnosis and Management in Primary and Secondary Care*. London: Royal college of physicians of London (2004).
9. Soelberg Sorensen P, Giovannoni G, Montalban X, Thalheim C, Zaratin P, Comi G. The multiple sclerosis care unit. *Multi Scler J.* (2019) 25:627–36. doi: 10.1177/1352458518807082
10. Khan F, Turner-Stokes L, Ng L, Kilpatrick T. Multidisciplinary rehabilitation for adults with multiple sclerosis (Review). *Cochrane Database Syst Rev.* (2007) 2007:CD006036. doi: 10.1002/14651858.CD006036
11. Jansen DEMC, Krol B, Groothoff JW, Post D. Integrated care for MS patients. *Disabil Rehabil.* (2007) 29:597–603. doi: 10.1080/09638280600845555
12. Sharma A, Minh Duc NT, Luu Lam Thang T, Nam NH, Ng SJ, Abbas KS, et al. A consensus-based checklist for reporting of survey studies (CROSS). *J Gen Intern Med.* (2021) 36:3179–87. doi: 10.1007/s11606-021-06737-1
13. Mikocka-Walus A, Andrews JM, Rampton D, Goodhand J, van der Woude J, Bernstein CN. How can we improve models of care in inflammatory bowel disease? An international survey of IBD health professionals. *J Crohns Colitis.* (2014) 8:1668–74. doi: 10.1016/j.crohns.2014.07.009
14. Marrie R, Reider N, Cohen J, Stuve O, Trojano M, Sorensen PS, et al. The incidence and prevalence of psychiatric disorders in multiple sclerosis: a systematic review. *Mult Scler J.* (2015) 21:305–17. doi: 10.1177/1352458514564487
15. Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap)—A metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform.* (2009) 42:377–81. doi: 10.1016/j.jbi.2008.08.010
16. *Multiple Sclerosis International Federation. Atlas of MS 3rd Edition, Part 2: Clinical Management of Multiple Sclerosis Around the World.* Multiple Sclerosis International Federation (2021).
17. Feys P, Giovannoni G, Dijsselbloem N, Centonze D, Eelen P, Lykke Andersen S. The importance of a multi-disciplinary perspective and patient activation programmes in MS management. *Multi Scler J.* (2016) 22:34–46. doi: 10.1177/1352458516650741
18. Montalban X, Gold R, Thompson AJ, Otero-Romero S, Amato MP, Chandraratna D, et al. ECTRIMS/EAN guideline on the pharmacological treatment of people with multiple sclerosis. *Multi Scler J.* (2018) 24:96–120. doi: 10.1177/1352458517751049
19. Pétrin J, McColl MA, Donnelly C, French S, Finlayson M. Prioritizing the healthcare access concerns of Canadians with MS. *Multi Scler J Exp Transl Clin.* (2021) 7:20552173211029672. doi: 10.1177/20552173211029672
20. Petrin J, Finlayson M, Donnelly C, McColl MA. Healthcare access experiences of persons with MS explored through the candidacy framework. *Health Soc Care Community.* (2021) 29:789–99. doi: 10.1111/hsc.13320
21. Galushko M, Golla H, Strupp J, Karbach U, Kaiser C, Ernstmann N, et al. Unmet needs of patients feeling severely affected by multiple sclerosis in Germany: a qualitative study. *J Palliat Med.* (2014) 17:274–81. doi: 10.1089/jpm.2013.0497
22. Celani MG, Nonino F, Mahan K, Orso M, Ridley B, Baldin E, et al. Identifying unanswered questions and setting the agenda for future systematic research in multiple sclerosis. A worldwide multi-stakeholder priority setting project. *Mult Scler Relat Disord.* (2022) 60:103688. doi: 10.1016/j.msard.2022.103688
23. Mattarozzi K, Baldin E, Zenesini C, Solari A, Amato MP, Leone M, et al. Effect of organizational features on patient satisfaction with care in Italian multiple sclerosis centres. *Eur J Neurol.* (2017) 24:631–7. doi: 10.1111/ene.13263
24. Fan W, Yan Z. Factors affecting response rates of the web survey: a systematic review. *Comput Hum Behav.* (2010) 26:132–9. doi: 10.1016/j.chb.2009.10.015
25. Sheehan KB. E-mail survey response rates: a review. *JCMC.* (2001) 6. doi: 10.1111/j.1083-6101.2001.tb00117.x
26. Ison DC. Mitigating nonresponse error in online surveys through research-based design and delivery. *Surv Pract.* (2017) 10:1–10. doi: 10.29115/SP-2017-0012
27. Solari A, Giordano A, Sastre-Garriga J, Köpke S, Rahn AC, Kleiter I, et al. EAN guideline on palliative care of people with severe, progressive multiple sclerosis. *Eur J Neurol.* (2020) 27:1510–29. doi: 10.1111/ene.14248

28. Lee Y-J, Yoo JW, Hua L, Kim PC, Kim SJ, Shen JJ. Ten-year trends of palliative care utilization associated with multiple sclerosis patients in the United States from 2005 to 2014. *J Clin Neurosci.* (2018) 58:13–9. doi: 10.1016/j.jocn.2018.10.082
29. *Access to Palliative Care in Canada*. Ottawa, ON: Canadian Institute for Health Information (2018).
30. Ponzio M, Tacchino A, Zaratin P, Vaccaro C, Battaglia MA. Unmet care needs of people with a neurological chronic disease: a cross-sectional study in Italy on multiple sclerosis. *Eur J Public Health.* (2015) 25:775–80. doi: 10.1093/eurpub/ckv065

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Making Every Step Count: Minute-by-Minute Characterization of Step Counts Augments Remote Activity Monitoring in People With Multiple Sclerosis

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Background: Ambulatory disability is common in people with multiple sclerosis (MS). Remote monitoring using average daily step count (STEPS) can assess physical activity (activity) and disability in MS. STEPS correlates with conventional metrics such as the expanded disability status scale (Expanded Disability Status Scale; EDSS), Timed-25 Foot walk (T25FW) and timed up and go (TUG). However, while STEPS as a summative measure characterizes the number of steps taken over a day, it does not reflect variability and intensity of activity.

Objectives: Novel analytical methods were developed to describe how individuals spends time in various activity levels (e.g., continuous low versus short bouts of high) and the proportion of time spent at each activity level.

Methods: 94 people with MS spanning the range of ambulatory impairment (unaffected to requiring bilateral assistance) were recruited into FITriMS study and asked to wear a Fitbit continuously for 1-year. Parametric distributions were fit to minute-by-minute step data. Adjusted R^2 values for regressions between distributional fit parameters and STEPS with EDSS, TUG, T25FW and the patient-reported 12-item MS Walking scale (MSWS-12) were calculated over the first 4-weeks, adjusting for sex, age and disease duration.

Results: Distributional fits determined that the best statistically-valid model across all subjects was a 3-compartment Gaussian Mixture Model (GMM) that characterizes the step behavior within 3 levels of activity: high, moderate and low. The correlation of GMM parameters for baseline step count measures with clinical assessments was improved when compared with STEPS (adjusted R^2 values GMM vs. STEPS: TUG: 0.536 vs. 0.419, T25FW: 0.489 vs. 0.402, MSWS-12: 0.383 vs. 0.378, EDSS: 0.557 vs. 0.465). The GMM correlated more strongly (Kruskal-Wallis: $p = 0.0001$) than STEPS and gave further information not included in STEPS.

Conclusions: Individuals' step distributions follow a 3-compartment GMM that better correlates with clinic-based performance measures compared with STEPS. These data support the existence of high-moderate-low levels of activity. GMM provides an interpretable framework to better understand the association between different levels of activity and clinical metrics and allows further analysis of walking behavior that takes step distribution and proportion of time at three levels of intensity into account.

Keywords: multiple sclerosis, Fitbit, remote monitoring, activity level, accelerometry, minute-by-minute steps

INTRODUCTION

Ambulatory disability is one of the most common, bothersome and limiting symptoms for people living with multiple sclerosis (MS) and greatly decreasing quality of life (1, 2). Walking *capacity* is measured in the clinic using a variety of validated outcomes (e.g. Timed 25-Foot Walk [T25FW] test), however, measurement and evaluation of walking *performance* (i.e. what they actually do in daily life) may be more important to the patient and reflect actual function (3, 4).

Efforts by several groups focused on remote (real-world) monitoring of ambulatory function mostly using average daily step count (STEPS), obtained from research-based and commercially available accelerometers (5–12). In the Fitbit remote monitoring in MS (FITriMS) study, daily step counts were collected continuously for over 1-year (5, 6). The STEPS averaged over the first 30 days correlated with disability (Expanded Disability Status Score [EDSS]), clinic-based metrics (T25FW, Timed-Up and Go Test [TUG], 2-min walk test [2MWT]) and patient reported outcomes (i.e. 12-item MS Walking Scale [MSWS-12]) (5) Longitudinal analysis over 1 year demonstrated a change in STEPS over time, even when conventional measures remained stable (6). These findings suggest remote physical activity monitoring provides additional sensitivity when capturing change in performance in people with MS.

Physical activity (activity) is quantified in different ways. The STEPS summarizes the total number of steps taken during an allotted epoch (usually 1 day) but does not reflect how different ambulatory behavior results in unique or distinctive step distributions, nor does it provide information or understanding of variability and intensity of the activity. Minute-by-minute (M-M) step count data can provide more granular information on the intensity, duration and frequency of ambulatory behavior. The aims for this analysis were to: determine the best probabilistic model using M-M step data to characterize activity distribution in people with MS with a range of ambulatory disability, evaluate the statistical validity of this new outcome, and compare with STEPS and conventional disability correlates at baseline.

METHODS

Study Procedures

The FITriMS study methods were described previously (5, 6). Briefly, adults (>18 years old) with either progressive or relapsing MS (13) were prospectively recruited from a single MS Center (University of California San Francisco; UCSF) into the FITriMS study between July 2015 and April 2016. For inclusion, participants were able walk continuously for at least 2 min, had WiFi access, experienced no relapse for the last 30 days, and were free from any musculoskeletal or cardiovascular comorbidities affecting ambulatory function (in the opinion of the study physical therapist). A range of ambulatory disability levels were block recruited to ensure a wide representation of ambulatory participants. MS disability was evaluated at study entry in the clinic using the EDSS (14), walking speed via the T25FW, (15), mobility and balance via the TUG (15), and endurance via a 2MWT (16, 17). Patient-reported impact of MS on walking, MSWS-12 questionnaires, was completed online using secure REDCap email link at study entry (18). Study personnel provided training on the maintenance and use of a Fitbit Flex for each participant. Participants were asked to wear the Fitbit as much as possible on their non-dominant wrist. Aggregated

TABLE 1 | Demographic and clinical characteristics.

	Mean (SD)	Min	Max	IQR
Age (years)	55.5 (13.7)	28	80	24.3
Number of Valid Days (Out of 28)	25.0 (5.46)	5	28	3
Step Counts (per minute)	26.5 (25.1)	1	293	25
Disease Duration (years)	19.6 (11.9)	5	55	16
TUG (seconds)	11.9 (11.3)	4.3	88.7	5.9
T25FW (seconds)	7.4 (5.6)	2.8	44.2	2.9
2MWT (meters)	133.5 (50.2)	16.5	237.6	78.1
	Median	Min	Max	IQR
EDSS	4.0	0.0	6.5	3.5
MSWS-12 (score 12–60)	41	12	60	25.5
Sex	*N* (%)	-	-	-
Male	36 (23.7)	-	-	-
Female	58 (76.3)	-	-	-
MS subtype	*N* (%)	-	-	-
Relapsing	59 (62.8)	-	-	-
Progressive	35 (37.2)	-	-	-

EDSS, Expanded Disability Status Score; TUG, Timed-Up-and Go Test (Greater times indicate worse balance and walking ability, and higher fall risk); T25FW, Timed-25 Foot Walk test (Greater times indicate slower walking speed and greater disability); 2MWT, 2-min Walk Test (Shorter distances indicate less endurance); MSWS-12, 12-item MS Walking Scale (higher scores reflect greater self-reported impact of MS on walking). MS, multiple sclerosis; Step count, After cleaning the data; this is the average Step count per minute during active time (>0 steps/ min on valid days) - averaged over 4 weeks.

daily, and granular M-M, step count data from the Fitbit were uploaded and stored on the UCSF Eureka platform (https://info.eurekaplatform.org/). In this data set, "physical activity" refers to outcome derived from step count (daily or minute-by-minute). The study protocol was approved by the institutional review board at UCSF, and all participants provided informed consent.

Quality Control (QC) and Data Cleaning

From the date of study entry, the first 4 week (or 28 days = "baseline") of M-M step count data were gathered for each individual. The "baseline" was chosen for comparison with previous STEPS analysis (5). To ensure only valid days were analyzed, any day that had a total sum of < 128 steps/day was removed. In M-M data, data points with > 300 steps per minute were excluded. Days with fewer than 128 steps were previously reported as non-valid, non-wear days (5) Weeks with < 3 valid days were also excluded. Data cleaning was based on MS literature and our previous work on this data set where: 1) no clear pattern of reactivity (i.e., temporary increase in activity after initial donning -due to the knowledge of being monitored – followed by a drop in activity when novelty wears off) was observed, 2) higher correlation was found using 13 days or more of monitoring, and 3) lower reliability with monitoring epochs of 3 days (5, 19). Night-time sleep data from Fitbit has not been validated in people MS and the majority of our patients only wore the device during the day. Long epochs of zero data were indicative of non-use or sleep, therefore only non-zero M-M data was used for subsequent analysis.

Analysis

After quality control, the cohort data were combined to include all valid participants. To determine the best probabilistic model and statistical validation, multiple statistical distributions were fit to the data and evaluated on an individual and group level. (**Supplementary Table 1**).

Previous visual observation of the step distribution revealed distinct 'clustering' of steps; therefore, mixture distributions (Gaussians) were included.

A single Gaussian distribution is characterized by two parameters, $\boldsymbol{\mu}$ (the mean) and $\boldsymbol{\sigma}$ (the variance) that control the location and spread of the distribution, respectively. In a 3-component Gaussian Mixture Model (GMM), consists of several Gaussian distributions where each Gaussian is assigned a proportion (π) parameter, a mean ($\boldsymbol{\mu}$) parameter and a variance ($\boldsymbol{\sigma}$) parameter. The proportion (π) describes how much each Gaussian contributes to the overall model.

Linear regression was used to compare the chosen model with clinical and patient-reported outcomes. The inverse of TUG and T25FW was used to transform the data and allow for normally distributed residuals for the linear regressions. Next,

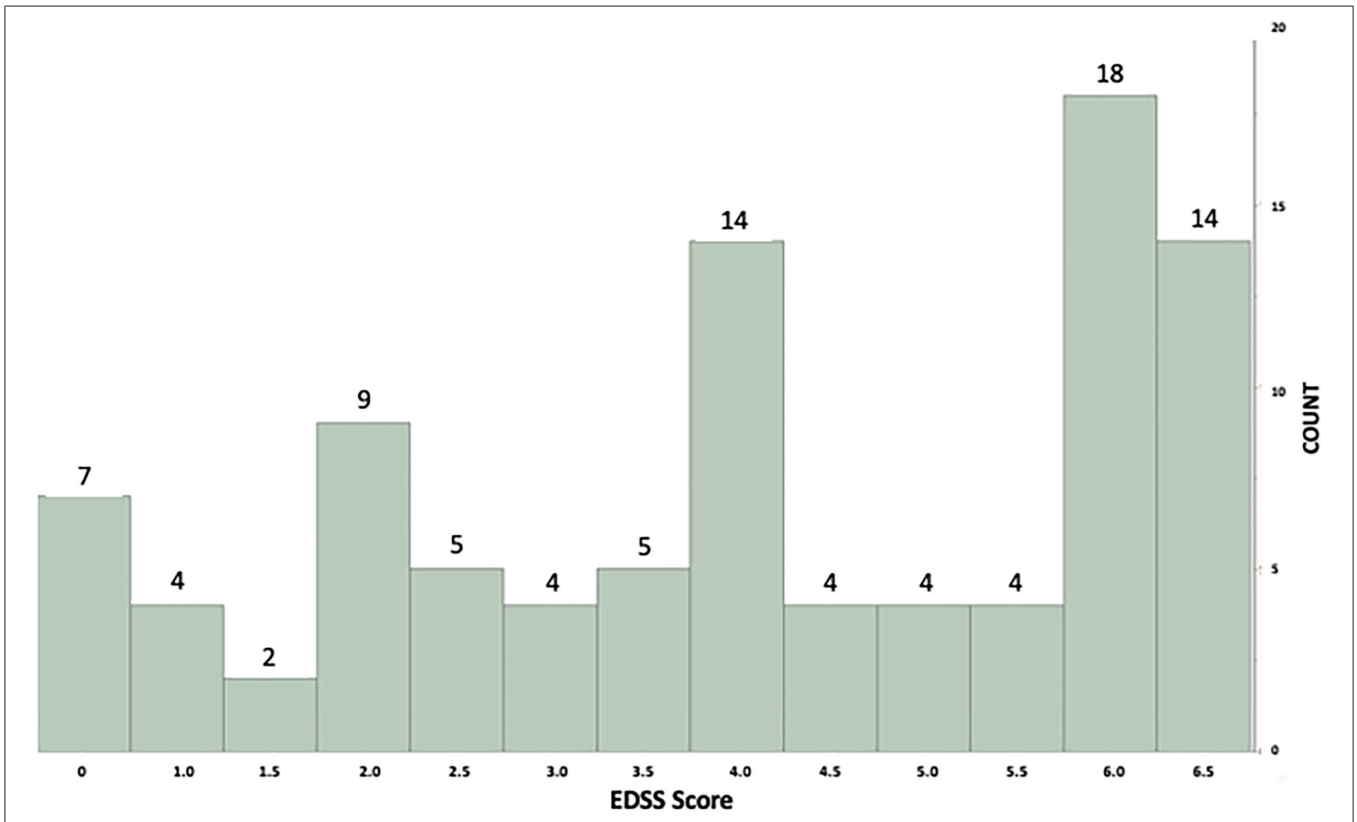

FIGURE 1 | Histogram showing individual example of minute-by-minute step count distribution with the GMM model fit. X axis = Expanded Disability Status Score (EDSS) scores. Y axis = Number of subjects in each EDSS level.

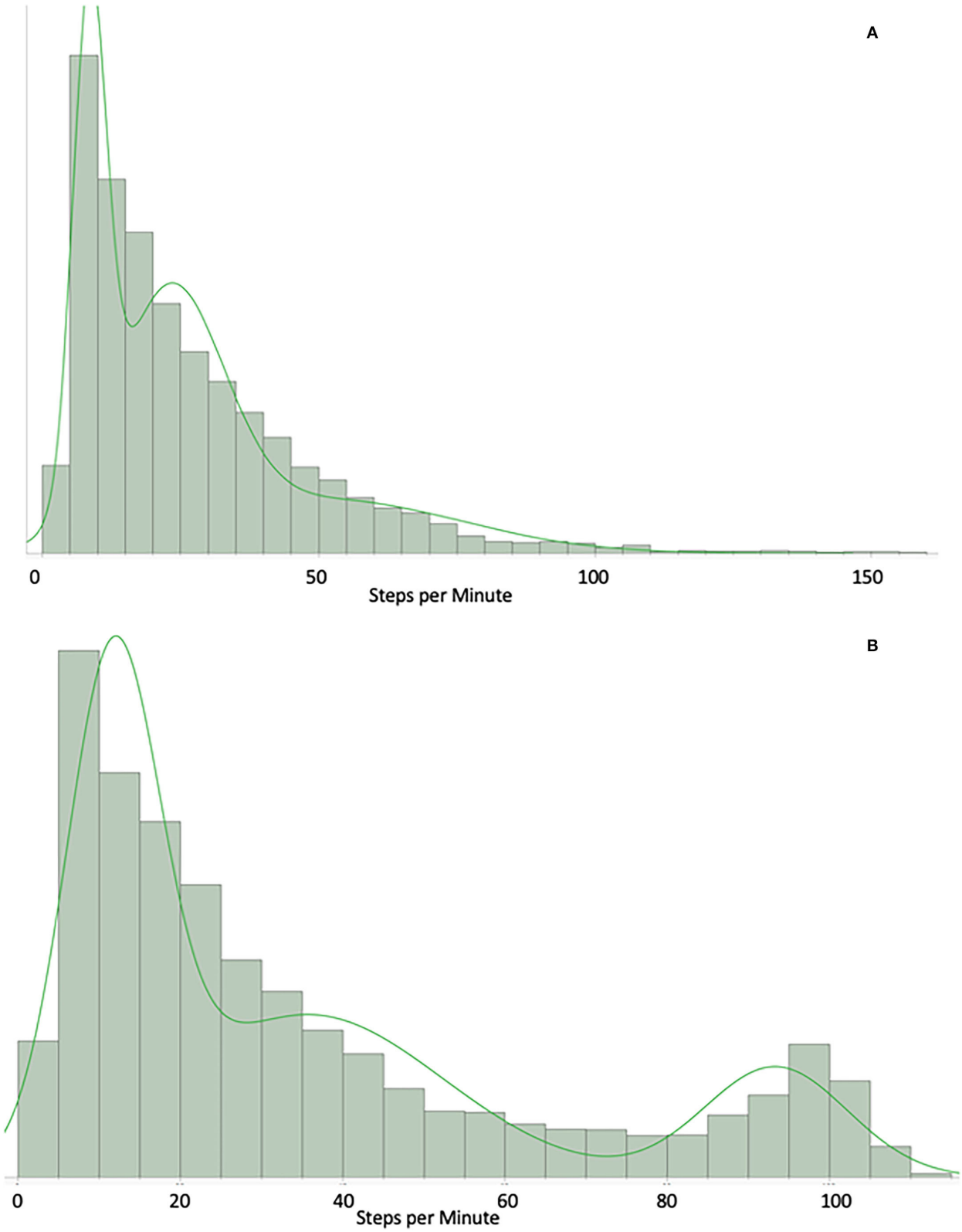

FIGURE 2 | (A) Example of a participant who spends most of the time in low levels of physical activity. GMM: X axis = count of steps per minute over 1 month. Example of a participant who spend most of their time in low levels of physical activity (10–30 steps/min). **(B)** Example of a participant who performs some higher physical activity. GMM: X axis = count of steps per minute over 1 month. Example of a participant who do some level of higher physical activity (bump around the 100–130 steps/min).

TABLE 2 | Comparison of GMM model and STEPS with conventional clinic-based and patient-reported outcomes.

Adjusted R^2	EDSS	TUG	T25FW	2MWT	MSWS−12
GMM	0.557	0.536	0.489	0.560	0.383
STEPS	0.465	0.419	0.402	0.432	0.378
GMM + STEPS	0.631	0.541	0.503	0.542	0.446
GMM (Adjusted)	0.675	0.587	0.546	0.538	0.512
STEPS (Adjusted)	0.569	0.525	0.453	0.439	0.461
GMM + STEPS (Adjusted)	0.710	0.583	0.544	0.548	0.533

EDSS, Expanded Disability Status Score; TUG, Timed-Up-and Go Test; T25FW, Timed-25 Foot Walk test; 2MWT, 2-min Walk Test; MSWS-12, 12-item MS Walking Scale. GMM - Normal 3 Mixture; STEPS, Average daily step count over the first 4 weeks (or 28 days). (Adjusted for = sex; age; and disease duration into the model).

linear regression on the same Gaussian parameters including age, sex, MS subtype and STEPS with clinical and patient-reported metrics was performed. JMP, version Pro 16 (20) was used for the analysis and figure generation.

RESULTS

From the 104 patients recruited into FITriMS, 10 did not have M-M data - due to sporadic syncing resulting in only daily step count data rather than M-M which requires weekly syncing. Of 94 participants used for this analysis, 63.5% carried a relapsing MS diagnosis (the remaining had progressive forms of MS) and more than two-thirds (76.3%) were women. The mean (SD) age was 55.5 years (13.7), median disease duration was 19.6 (IQR: 16) years, and median EDSS 4.0 (IQR: 3.5). All participant characteristics are summarized in **Table 1** and EDSS distribution in **Figure 1**.

The GMM was found to be the best fit for individual subject data (see **Supplemental Table 1** for full comparison of distributions). GMM fits a greater variability in activity distribution and provides more flexibility in generalizable representation of MS activity. For example, **Figure 2A** shows a participant who spend most of their time in low levels of activity and **Figure 2B** depicts the GMM fit for a participant who perform some higher activity over the day (100–130 steps per min).

All Gaussian parameters (μ, σ, and π) except for $\pi 3$ (since $\pi 1$, $\pi 2$ and $\pi 3$ are perfectly collinear [$\pi 1 + \pi 2 + \pi 3 = 1$]) correlated with clinical metrics (EDSS, TUG, MSWS12, 2MWT, T25FW). Using individual participant data, GMM was fit to M-M step data.

We propose that the 3 Gaussians correspond to 3 activity levels for each patient (low activity, medium activity, high activity – as ordered by their $\boldsymbol{\mu}$ [means]): each $\boldsymbol{\mu}$ represents the average step count we would expect from each step activity level, as characterized by its corresponding Gaussian; each $\boldsymbol{\sigma}$ represents the variability we would expect for each activity level; and each π represents the proportion of activities we would expect from each activity region.

Linear regression was used to show moderate to high correlation between GMM acquired over the study's baseline first 4 weeks of monitoring, and both clinic-based and patient-reported outcome measures. The results using GMM were consistently stronger than results obtained using only STEPS. Adding STEPS to GMM (GMM + STEPS) consistently outperforms either measure its own. Adjusting for sex, age and disease duration improved all models (**Table 2**).

Table 3A shows the centers for each Gaussian for different EDSS groupings. EDSS groups with lower levels of disability had consistently higher Gaussian centers. Further, Gaussians at higher activity levels had higher variances than those at lower levels.

DISCUSSION

These results provide preliminary evidence for the use of a GMM probabilistic model to characterize activity distribution using granular M-M step count in people with MS. This model performs better (stronger correlations and adjusted R^2) than previous methods using crude STEPS.

The GMM model was able to generalize over a range of activity profiles. More specifically, it was able to capture the step distributions of those in the cohort where a significant percentage of steps come from high activity levels. Previous work from our group illustrated the wide variability in activity levels in people with MS, within and between all ambulatory disability levels (5). Therefore, the ability to generalize M-M modeling to highly variable distributions using the 3 compartment GMM has clinical appeal. In addition, the GMM dovetails well with existing literature regarding activity levels classified into three levels: low, moderate/moderate to vigorous, and high physical activity (21–23).

The GMM representation of activity outperforms other statistical models and performs better than STEPS as compared to conventional disability correlates (5). The GMM may provide an interpretable framework to better understand the association between different levels of activity and clinical metrics. It also allows further analysis of walking performance and behavior by taking step distribution and proportion of time at each intensity into account. GMM and STEPS are complimentary; STEPS provides a mean, whereas GMM presents information regarding intensity, variance, and proportional step distribution. The model including STEPS and GMM generated high correlation with the conventional outcomes, suggesting that the overall mean (STEPS) is a useful metric in combination with the more descriptive GMM.

This analysis has important limitations. Although this cohort was well-phenotyped, larger studies in more heterogeneous populations are needed to provide additional evidence of

TABLE 3A | Mean physical activity level distribution per disability (EDSS) group (distribution of μ).

EDSS group [*N*]	Disability level	Low activity μ_1 (SD)	Moderate activity μ_2 (SD)	High activity μ_3 (SD)
0.0–3.5 [36]	No "walking" disability	10.96 (1.22)	32.70 (5.52)	82.37 (21.59)
4.0–5.5 [26]	Walking disability present	10.46 (1.74)	30.10 (7.65)	75.63 (27.56)
6.0 [18]	Needs a cane to ambulate	9.68 (1.20)	24.89 (4.28)	58.17 (12.94)
6.5 [14]	Needs 2 canes or a walker to ambulate	8.44 (0.94)	20.52 (2.98)	53.28 (11.51)

Each participant was fit individually, and the mean physical activity was extracted for the group distribution. People with lower levels of disability (i.e. EDSS = 0.0–3.5) tended to have greater mean physical activity in each level (low: $\boldsymbol{\mu_1}$, moderate: $\boldsymbol{\mu_2}$ and high: $\boldsymbol{\mu_3}$).

TABLE 3B | Variance of physical activity level distribution per disability (EDSS) group (distribution of σ).

EDSS group [*N*]	Disability level	Low variance σ_1 (SD)	Moderate variance σ_2 (SD)	High variance σ_3 (SD)
0.0–3.5 [36]	No "walking" disability	27.18 (9.99)	178.74 (78.72)	394.38 (191.20)
4.0–5.5 [26]	Walking disability present	23.67 (13.06)	152.10 (122.27)	487.65 (772.72)
6.0 [18]	Needs a cane to ambulate	17.31 (8.25)	83.20 (43.58)	362.86 (159.58)
6.5 [14]	Needs 2 canes or a walker to ambulate	10.47 (4.26)	58.32 (21.10)	294.96 (196.31)

Each participant was fit individually, and the variance of each physical activity type was extracted for the group distribution. Greater levels of disability demonstrated smaller variance when compared with people characterized with lower disability scores.

generalizability and replicability. Due to the limited availability of M-M, longitudinal (>7 days) datasets in people with MS, we were not able to perform a replication analysis. In addition, analysis of larger datasets collected in randomly recruited cohorts (rather than block enrolled) will be required. Data processed with the same granularity (minute-by-minute steps) from healthy age-matched controls would provide a better understanding about the proportions of time spent in each activity level.

A GMM based model is also relatively inflexible when approximating activity distributions that are not Gaussian in nature. A possible solution to overcome this representational limitation is to use an autoencoder (24), a type of neural network, to compress the distribution into a flexible lower dimensional representation with greater generalizability.

Without access to a platform that automatically pulls the M-M data, retrieving these detailed metrics would be more burdensome than simply downloading daily step count from the Fitbit.com website. Although we were fortunate to be able to use an in-house platform (Eureka: https://info.eurekaplatform.org/), there are fee-based companies that offer such services. In addition, the M-M data and GMM model provide improved correlations with conventional measures and provide insight into how a patient spends their time in different activity levels. For instance, a larger "$\boldsymbol{\mu 3}$" represents greater *average* step count; a smaller "$\boldsymbol{\sigma 1}$" denotes less variability; and "$\boldsymbol{\pi 2}$" denotes greater proportion of activities in the moderate range. These granular information combined with the level of disability (**Tables 3A–C**), provide a potential avenue for predictive algorithms and later, individualized rehabilitation plans.

Moderate-to-Vigorous activity equates with our $\boldsymbol{\pi 2}$, and has frequently been cited as the benchmark for determining optimal physical activity in MS and the general population. People needing double support to ambulate (EDSS = 6.5) tended to spend a larger proportion their activity in $\boldsymbol{\pi_1}$ (corresponding to low levels of activity or sedentarism). On the other hand, people with lower disability (EDSS = 0.0–3.5) presented with a greater proportion in $\boldsymbol{\pi_2}$ and $\boldsymbol{\pi_3}$ (corresponding with more moderate and higher activity levels). Therefore, it may be more clinically useful to evaluate $\boldsymbol{\pi 1}$, and $\boldsymbol{\pi 3}$ (the time spent in low levels and very high levels of activity) when assessing an individual patient's activity level and subsequent risk factors or rehabilitation needs. For example, understanding fluctuations in activity over the day to better personalize when to focus rehabilitation interventions and on what (intensity and duration of activity). Research awareness already shifted toward investigating the effect of

TABLE 3C | Proportion of physical activity level distribution per disability (EDSS) group (distribution of π).

EDSS group [*N*]	Disability level	Low π_1 (SD)	Moderate π_2 (SD)	High π_3 (SD)
0.0–3.5 [36]	No "walking" disability	0.47 (0.07)	0.37 (0.04)	0.15 (0.06)
4.0–5.5 [26]	Walking disability present	0.51 (0.05)	0.36 (0.03)	0.13 (0.05)
6.0 [18]	Needs a cane to ambulate	0.55 (0.05)	0.34 (0.04)	0.11 (0.04)
6.5 [14]	Needs 2 canes or a walker to ambulate	0.61 (0.06)	0.31 (0.07)	0.09 (0.03)

Each participant was fit individually, and the proportion of physical activity was extracted for the group distribution. The greater the level of disability (i.e. EDSS = 6.5) the higher proportion of lower levels of physical activity ($\boldsymbol{\pi}_1$) recorded, and the lower disability (i.e. EDSS = 0.0–3.5) the greater proportion of moderate ($\boldsymbol{\pi}_2$) and high physical activity ($\boldsymbol{\pi}_3$) levels recorded (Kruskal-Wallis Test: $p = 0.0001$).

EDSS, Expanded Disability Status Score; N, sample size in each group; SD, standard deviation; $\mu 1$, mean steps - low physical activity; $\mu 2$, mean steps - moderate physical activity; $\mu 3$, mean steps - "high" (relatively) physical activity, Low variance, $\boldsymbol{\sigma_1}$; moderate variance, σ_2 and high variance, $\boldsymbol{\sigma_3}$, $\boldsymbol{\pi}_1$, Low proportion of physical activity; $\boldsymbol{\pi}_2$, moderate proportion of physical activity; π_3, high proportion of physical activity.

sedentary time on health and disease (25–27). Higher physical activity has been associated with beneficial changes in the brain and spinal cord, as well with decreased levels of disability (28, 29). However, this area of investigation is in its infancy and the underlying mechanism of action is not yet understood. Methods presented in this paper could build on and support these lines of investigation – with aims at promoting greater overall wellness, and potentially delay disease progression in people with MS (29–32).

This model will be used as a framework to predict disease progression over the longer term (>2 years) and to develop further descriptive metrics for activity. How time spent at various activity levels is associated with MS disability over time – i.e., temporal validation of the Gaussian parameters for prediction on disability progression – remains to be determined. Models including associations of fall-risk prediction in people with MS would also be highly clinically valuable.

CONCLUSION

Results from this analysis favor a 3 compartment GMM as the best probabilistic model to characterize dynamic ambulatory activity in people with MS with a wide range of disability. As compared to STEPS as a sole outcome, this method demonstrated stronger associations with conventional clinic-based and patient-reported outcomes. To unearth the full potential of this method, additional longitudinal exploration of predictive value is required.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by UCSF Human Research Protection Program, Box 1288 490 Illinois Street, Floor 6 San Francisco, CA 94143. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

VB and MW: design and conceptualized study, analyzed the data, and drafted the manuscript for intellectual content. ZX and AA: analyzed the data and drafted the manuscript for intellectual content. RB, MP, GM, JO, and BC: interpreted the data and revised the manuscript for intellectual content. JG: design and conceptualized study, interpreted the data, and revised the manuscript for intellectual content. RH: design and conceptualized study, analyzed and interpreted the data, and revised the manuscript for intellectual content. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2022.860008/full#supplementary-material

REFERENCES

1. Compston A, Coles A. Multiple sclerosis. *Lancet (London, England).* (2008) 372:1502–17. doi: 10.1016/S0140-6736(08)61620-7
2. Gelfand JM. Multiple sclerosis: diagnosis, differential diagnosis, clinical presentation. *Handb Clin Neurol.* (2014) 122:269–90. doi: 10.1016/B978-0-444-52001-2.00011-X
3. Mate KKV, Mayo NE. Clinically assessed walking capacity versus real-world walking performance in people with multiple sclerosis. *Int J MS Care.* (2020) 22:143–50. doi: 10.7224/1537-2073.2019-047
4. Ehling R, Bsteh G, Muehlbacher A, Hermann K, Brenneis C. Ecological validity of walking capacity tests following rehabilitation in people with multiple sclerosis. *PLoS ONE.* (2019) 14:e0220613. doi: 10.1371/journal.pone.0220613
5. Block VJ, Lizée A, Crabtree-Hartman E, Bevan CJ, Graves JS, Bove R. Continuous daily assessment of multiple sclerosis disability using remote step count monitoring. *J neurol.* (2016) 2016:1-11. doi: 10.1007/s00415-016-8334-6
6. Block VJ, Bove R, Zhao C, et al. Association of continuous assessment of step count by remote monitoring with disability progression among adults with multiple sclerosis. *JAMA Network Open.* (2019) 2:e190570–e190570. doi: 10.1001/jamanetworkopen.2019.0570
7. Block VJ, Bove R, Gelfand JM, Cree BAC. Effects of cOVID-19 "Sheltering in place" on activity in people with multiple sclerosis. *Neurol: Clin Pract.* 2020:10.1212/CPJ.0000000000000982. doi: 10.1212/CPJ.0000000000000982
8. Norris M, Anderson R, Motl RW, Hayes S, Coote S. Minimum number of days required for a reliable estimate of daily step count and energy expenditure, in people with MS who walk unaided. *Gait and posture.* (2017) 53:201–6. doi: 10.1016/j.gaitpost.2017.02.005
9. Kruger T, Behrens JR, Grobelny A, Otte K, Mansow-Model S, Kayser B, et al. Subjective and objective assessment of physical activity in multiple sclerosis and their relation to health-related quality of life. *BMC Neurol.* (2017) 17:10. doi: 10.1186/s12883-016-0783-0
10. Cavanaugh JT, Gappmaier VO, Dibble LE, Gappmaier E. Ambulatory activity in individuals with multiple sclerosis. *J Neurol Phys Ther.* (2011) 35:26–33. doi: 10.1097/NPT.0b013e3182097190
11. Sosnoff JJ, Goldman MD, Motl RW. Real-life walking impairment in multiple sclerosis: preliminary comparison of four methods for processing accelerometry data. *Multiple sclerosis (Houndmills, Basingstoke, England).* (2010) 16:868–77. doi: 10.1177/1352458510373111
12. Motl RW, Dlugonski D, Suh Y, Weikert M, Fernhall B, Goldman Accelerometry M. and its association with objective markers of walking limitations in ambulatory adults with multiple sclerosis. *Arch Phys Med Rehabil.* (2010) 91:1942–7. doi: 10.1016/j.apmr.2010.08.011
13. Polman CH, Reingold SC, Banwell B, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the MCdonald criteria. *Ann Neurol.* (2011) 69:292–302. doi: 10.1002/ana.22366
14. Kurtzke JF. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale. (EDSS) *Neurol.* (1983) 33:44. doi: 10.1212/WNL.33.11.1444
15. Kieseier BC, Pozzilli C. Assessing walking disability in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England).* (2012) 18:914–24. doi: 10.1177/1352458512444498

16. Rossier P, Wade D. Validity and reliability comparison of 4 mobility measures in patients presenting with neurologic impairment. *Arch Phys Med Rehabil.* (2001) 82:9–13. doi: 10.1053/apmr.2001.9396
17. Andersen LK, Knak KL, Witting N. Vissing 2 and 6-min walk tests assess walking capability equally in neuromuscular diseases. *Neurol.* (2016) 86:442–5. doi: 10.1212/WNL.0000000000002332
18. Hobart JC, Riazi A, Lamping DL, Fitzpatrick R, Thompson AJ. Measuring the impact of MS on walking ability: the 12-Item mS walking scale. (MSWS-12) *Neurol*. (2003) 60:31–6. doi: 10.1212/WNL.60.1.31
19. Motl RW. Physical activity and its measurement and determinants in multiple sclerosis. *Minerva Med.* (2008) 99:157–65.
20. JMP®, Version Pro 15. Cary, NC: SAS Institute Inc., 1989–2021.
21. Ainsworth BE, Haskell WL, Herrmann SD, Meckes N, Bassett DR Jr, Tudor-Locke C, et al. Compendium of physical activities: a second update of codes and MET values. *Med Sci Sports Exerc*. (2011) 43:1575–81. doi: 10.1249/MSS.0b013e31821ece12
22. Saint-Maurice PF, Troiano RP, Matthews CE, Kraus WE. Moderate-to-vigorous physical activity and all-cause mortality: do bouts matter? *J Am Heart Assoc*. (2018) 7:e007678. doi: 10.1161/JAHA.117.007678
23. Klaren RE, Sasaki JE, McAuley E, Motl R. Patterns and predictors of change in moderate-to-vigorous physical activity over time in multiple sclerosis. *J Phys Act Health*. (2016) 4:1–19. doi: 10.1123/jpah.2016-0335
24. Dor Bank Noam Koenigstein Giryes R. *Autoencoders*. Available online at: https://arxiv.org/abs/2003.05991 (accessed August 20, 2003).
25. Hubbard EA, Motl RW. Sedentary behavior is associated with disability status and walking performance, but not cognitive function, in multiple sclerosis. *Appl Physiol Nutr Metab.* (2014) 40:203–6. doi: 10.1139/apnm-2014-0271
26. Veldhuijzen van Zanten JJ, Pilutti LA, Duda JL, Motl RW. Sedentary behaviour in people with multiple sclerosis: Is it time to stand up against MS? *Mult. Scler. J.* (2016) 22:1250–6. doi: 10.1177/1352458516644340
27. Sjöros T, Vähä-Ypyä H, Laine S, et al. Both sedentary time and physical activity are associated with cardiometabolic health in overweight adults in a 1 month accelerometer measurement. *Sci Rep.* (2020) 10:20578. doi: 10.1038/s41598-020-77637-3
28. Dalgas U. Exercise therapy in multiple sclerosis and its effects on function and the brain. *Neurodegener Dis Manag.* (2017) 7:35–40. doi: 10.2217/nmt-2017-0040
29. Dalgas U, Stenager Exercise E. and disease progression in multiple sclerosis: can exercise slow down the progression of multiple sclerosis? *Ther Adv Neurol Disord.* (2012) 5:81–95. doi: 10.1177/1756285611430719
30. Keller JL, Tian F, Fitzgerald KC. Using real-world accelerometry-derived diurnal patterns of physical activity to evaluate disability in multiple sclerosis. *J Rehabil Assist Technol Eng.* (2022) 9:20556683211067362. doi: 10.1177/20556832110 67362
31. Kratz AL, Fritz NE, Braley TJ, Scott EL, Foxen-Craft E, Murphy SL. Daily temporal associations between physical activity and symptoms in multiple sclerosis. *Ann Behav Med.* (2019) 53:98–108. doi: 10.1093/abm/kay018
32. Motl RW, Sandroff BM, Kwakkel G, Dalgas U, Feinstein A, Heesen C, et al. Exercise in patients with multiple sclerosis. *Lancet Neurol.* (2017) 16:848–56. doi: 10.1016/S1474-4422(17)30281-8

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Effects of Vascular Comorbidity on Cognition in Multiple Sclerosis Are Partially Mediated by Changes in Brain Structure

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Objective: Vascular comorbidities are associated with reduced cognitive performance and with changes in brain structure in people with multiple sclerosis (MS). Understanding causal pathways is necessary to support the design of interventions to mitigate the impacts of comorbidities, and to monitor their effectiveness. We assessed the inter-relationships among vascular comorbidity, cognition and brain structure in people with MS.

Methods: Adults with neurologist-confirmed MS reported comorbidities, and underwent assessment of their blood pressure, HbA1c, and cognitive functioning (i.e., Symbol Digit Modalities Test, California Verbal Learning Test, Brief Visuospatial Memory Test-Revised, and verbal fluency). Test scores were converted to age-, sex-, and education-adjusted z-scores. Whole brain magnetic resonance imaging (MRI) was completed, from which measures of thalamic and hippocampal volumes, and mean diffusivity of gray matter and normal-appearing white matter were converted to age and sex-adjusted z-scores. Canonical correlation analysis was used to identify linear combinations of cognitive measures (cognitive variate) and MRI measures (MRI variate) that accounted for the most correlation between the cognitive and MRI measures. Regression analyses were used to test whether MRI measures mediated the relationships between the number of vascular comorbidities and cognition measures.

Results: Of 105 participants, most were women (84.8%) with a mean (SD) age of 51.8 (12.8) years and age of symptom onset of 29.4 (10.5) years. Vascular comorbidity

was common, with 35.2% of participants reporting one, 15.2% reporting two, and 8.6% reporting three or more. Canonical correlation analysis of the cognitive and MRI variables identified one pair of variates (Pillai's trace = 0.45, $p = 0.0035$). The biggest contributors to the cognitive variate were the SDMT and CVLT-II, and to the MRI variate were gray matter MD and thalamic volume. The correlation between cognitive and MRI variates was 0.50; these variates were used in regression analyses. On regression analysis, vascular comorbidity was associated with the MRI variate, and with the cognitive variate. After adjusting for the MRI variate, vascular comorbidity was not associated with the cognitive variate.

Conclusion: Vascular comorbidity is associated with lower cognitive function in people with MS and this association is partially mediated via changes in brain macrostructure and microstructure.

Keywords: multiple sclerosis, MRI, cognition, diabetes, hypertension

INTRODUCTION

Multiple sclerosis (MS) is a central nervous system disease characterized by multiple signs and symptoms, including cognitive impairment. Over 40% of individuals with MS struggle with cognitive impairment (1, 2) and its adverse effects on daily function (3). MS is characterized by demyelination and axonal injury, therefore it is associated with macrostructural changes in the brain such as atrophy, as well as microstructural changes in normal appearing white matter (NAWM). Lower whole brain and regional gray matter volumes, particularly thalamic volumes (4) are associated with cognitive dysfunction (5–7). Microstructural abnormalities, as measured using diffusion tensor imaging (DTI) appear to provide even stronger prediction of cognitive impairment than macrostructural abnormalities (8–10).

Comorbid conditions are highly prevalent among individuals with MS (11). The vascular comorbidities of hypertension and hyperlipidemia are among the most common comorbidities with MS, and increase in prevalence with age. They are associated with outcomes such as relapses, disability progression and lower quality of life (12, 13). More recent studies suggest that hypertension and diabetes are also associated with reduced cognitive function in domains such as processing speed, verbal learning and visual memory for persons with MS (14–16). However, findings have varied across studies, possibly reflecting differences in study populations, comorbidity measurement and cognitive tests employed. Although findings are inconsistent as to the magnitude of the effect and the specific comorbidities involved (14, 17–19), vascular comorbidities have been associated with macrostructural brain changes such as lower brain volumes in people with MS. In the general population widespread changes in white matter microstructure are known to be associated with vascular risk factors; mean diffusivity (MD) appears to be more sensitive to these effects than FA or mean kurtosis (20, 21). The association of vascular comorbidity and brain microstructure has not been explored in people with MS.

Depression and anxiety disorders are other common comorbidities associated with lower cognitive performance in people with MS (22). Depression has also been associated with lower brain volumes, specifically affecting the temporal lobes and hippocampus (23–25), and has also been associated with altered microstructure in the form of higher MD in NAWM and gray matter in the left temporal lobe in persons with MS (26). Therefore these comorbidities need to be accounted for when the effects of vascular comorbidities on brain structure and cognition are assessed.

Better understanding of the relationships among comorbidities, brain structural changes, and outcomes such as cognitive functioning is needed for persons with MS. Understanding causal pathways is necessary to support the design of interventions to mitigate the impacts of comorbidities, and to monitor their effectiveness. For example, if the effects of vascular comorbidity on cognition were mediated by changes in brain structure, intervention studies aimed at treating vascular comorbidity to improve cognition could use MRI measures as intermediate outcomes to enable shorter, smaller studies. We aimed to extend our prior work examining relations between comorbidity and cognition (15) and hypothesized that the effects of vascular comorbidity on cognition would be mediated by changes in brain structure in people with MS.

METHODS

Study Population

As described previously (15), our study sample was drawn from a subgroup of adults with MS participating in a longitudinal study regarding psychiatric comorbidity in immune-mediated inflammatory diseases (the "IMID" study). This subgroup included persons aged ≥18 years with definite MS (27), as confirmed by a neurologist and medical records review. Exclusion criteria included comorbid brain tumors, neurodegenerative disorders, or contraindications to MRI. We did not exclude any other comorbidities because comorbidities

(predominantly vascular and psychiatric) were the focus of the sub-study.

We also enrolled healthy controls who have been described in detail elsewhere (28). Briefly, healthy controls were aged 18 years or older. Exclusion criteria for this group included any chronic medical condition including vascular comorbidities, cognitive impairment, a positive response to the Structured Clinical Interview for DSM-IV (SCID-IV) screening questions for depressive or anxiety disorders, head injury associated with loss of consciousness or amnesia, or chronic medication use (29). Hypertension, as measured during the study visit, was also an exclusion criterion. For this analysis, they predominantly served to allow us to develop regression-based norms for cognitive and MRI measures.

All participants in the sub-study underwent standardized assessments of physical, cognitive, and mental health functioning, which they completed the same day. They also had a brain MRI, which was completed within a maximum of 4 weeks of the study visit in which they completed their standardized assessments (30). All participants provided written informed consent. The University of Manitoba Health Research Ethics Board approved the study. Study data were collected and managed using REDCap electronic data capture tools (31) hosted at the University of Manitoba.

Sociodemographic Information

Participants reported gender, date of birth, race and ethnicity, highest level of education attained, annual household income, and marital status using self-administered questionnaires. Race and ethnicity were assessed using response options from Statistics Canada; race was categorized as white vs. non-white because the number of non-white participants was too small to further subdivide. We categorized level of education as high school or less, vs. more than high school (including college, university, technical/trade).

Clinical Characteristics

Age at MS symptom onset, clinical course (relapsing remitting, secondary progressive, primary progressive), relapses in the last 12 months, and current disease-modifying therapy (DMT) were determined based on patient report and medical records review. The Expanded Disability Status Scale (EDSS) was assessed by a certified neurologist (RAM/JJM) (32).

Comorbidity and Health Behaviors

Participants reported their lifetime history of comorbidities (including hypertension, diabetes, hyperlipidemia, and heart disease) using a validated questionnaire (33), including the year of diagnosis and whether the condition was currently treated. This information was complemented by medical records review and other assessments (15). During the study visit, we recorded blood pressure in the seated position using an automatic blood pressure machine. Participants were classified as currently having hypertension if they reported physician-diagnosed hypertension, or had an elevated blood pressure of at least 140/90 mm Hg, and/or used anti-hypertensive medications. Participants were classified as currently having diabetes if they self-reported physician-diagnosed diabetes, used medications for diabetes and/or had a hemoglobin A1c measured at the study visit >6.5% (34). We did not discriminate between type 1 and type 2 diabetes. Participants were classified as currently having heart disease if they self-reported physician-diagnosed heart disease. We classified current smoking status as yes/no. We calculated body mass index (BMI, kg/m^2) based on measured height and weight.

Given prior findings in the literature indicating that psychiatric comorbidity affects cognition in MS including our prior work (22, 35), current major depression and anxiety disorders were assessed for inclusion as covariates using the Structured Clinical Interview for DSM-IV (SCID-IV) (36), which was administered by trained study staff, as described elsewhere (30). We classified each condition as present or absent.

Cognitive Function

As delineated elsewhere, we chose validated neuropsychological assessments included in the Brief International Cognitive Assessment for Multiple Sclerosis (BICAMS) (37), and which tested most cognitive domains addressed via the Minimal Assessment of Cognitive Function in MS (MACFIMS) (38). BICAMS uses the Symbol Digit Modalities Test (SDMT) (39), the California Verbal Learning Test (CVLT-II; Trial 1–5 total recall score) (40), and the Brief Visuospatial Memory Test-Revised (BVMT-R; summed recall score for all three learning trials) (41). The MACFIMS includes all of the tests from BICAMS, the Controlled Oral Word Association Test (fluency) as well as the Paced Auditory Serial Addition Test (processing speed and working memory), Delis-Kaplan Executive Function System Sorting Test (executive function), and Judgement of Line Orientation Test (spatial processing). Specifically, we used the SDMT (39) to assess information processing speed, the CVLT-II; Trial 1–5 total recall score (40) to assess verbal learning and memory, the BVMT-R (summed recall score for all three learning trials) (41) to assess visual learning and memory, and tests of verbal fluency (letter and animal categories) (42) to assess language and executive abilities. We converted raw test scores to age-, sex- and education-adjusted z-scores using local regression-based norms because we previously demonstrated that these performed better in our population than other published regression-based norms (28). *Z*-scores of $\leq$-1.5 were classified as impaired. To characterize the sample we also included the Wechsler Test of Adult Reading (WTAR) (43) that provided an age-, sex-, education-, and ethnicity-adjusted Full Scale IQ estimate of premorbid intelligence. Test administration was completed by trained study staff, overseen by a registered clinical neuropsychologist.

Magnetic Resonance Imaging

Acquisition

As described previously (18), all participants underwent a 3 Tesla brain MRI (Siemens TIM Trio, software version VB17a, Siemens Healthcare, Erlangen, Germany; Siemens 32-channel receive-only head coil), within 4 weeks of their study visit. The images acquired included a high-resolution T1-weighted (T1w) whole brain 3D magnetization prepared rapid gradient

echo (MPRAGE), dual-echo proton density-weighted (PDw), T2-weighted (T2w), fluid attenuated inversion recovery (FLAIR) images, and two 55-direction high angular resolution diffusion imaging (HARDI) scans that had phase encoding in opposite directions (see **Supplementary Table e1** for scan parameters). Gadolinium was not administered. A radiologist reviewed the MRIs to screen for any clinically relevant findings unrelated to MS. All images were visually reviewed to assess for bulk motion or other artifacts.

T1-Weighted Images

We used FSL's FLIRT, and FNIRT (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FLIRT) to linearly and non-linearly warp the T1w brain images to the MNI152 template (44, 45). We created lesion masks from FLAIR and T1w images using the Lesion Segmentation Tool (LST) for SPM (46), and FSL's automated Brain Intensity AbNormality Classification Algorithm (BIANCA; https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/BIANCA) (47). We created final lesion masks for each participant as a binary cluster overlap of the BIANCA and LST maps, as we have found that LST is more specific but less sensitive and BIANCA is more sensitive but less specific. This allowed us to eliminate spurious small clusters identified by only one technique, reducing false positives. Lesions were filled using the lesion filling command in FSL by inputting each participant's: (1) cluster-overlapped T1w_final_lesion_map, (2) binary WM tissue map, and (3) bias-corrected T1w_brain (48). We estimated whole brain volume and gray matter volume from lesion-filled T1w images using FSL's SIENA (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/SIENA) (49, 50). Volume estimates for the thalamus (total of right and left) and hippocampus (total of right and left) were obtained using FSL FIRST (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FIRST). All volumes were normalized relative to intracranial volume for each participant.

Diffusion-Weighted Images

Artifact Correction

Diffusion-weighted images were processed using SPM12 Artifact Correction in Diffusion MRI Toolbox (ACID; version beta 02; http://diffusiontools.com). This included simultaneous motion and eddy current correction (51), and EPI distortion correction based on the opposite polarity DWI images using the Hyperelastic Susceptibility artifact Correction (HySCo) algorithm (52, 53).

Tensor Model Fitting

We used the Fit Diffusion Tensor module to generate fractional anisotropy (FA), mean diffusivity (MD), radial diffusivity (RD) and axial diffusivity (AD) maps. The robust least-squares fitting algorithm was used to down-weight potential outliers in the diffusion signal (54).

Registration

We non-linearly warped each participant's high resolution, lesion-filled T1w image to the MNI52 Template (using the geodesic shooting method in the Computational Anatomy Toolbox for SPM12 (CAT12 version r1318; http://www.neuro.uni-jena.de/cat/)), then co-registered each of the diffusion maps to the participant's T1w image (by co-registering the b0 image and applying the same transformations). Then we spatially normalized the diffusion maps to the MNI152_T1_1mm template using subject-specific deformation fields generated previously using CAT12. We extracted mean values of these four DTI metrics for whole brain white matter (WM) as well as gray matter (GM) using each participant's CAT12 tissue segmentations, and calculated mean values for NAWM by removing voxels within each participant's lesion mask from their CAT12 WM segmentation.

Choice of Diffusion Metric

It is increasingly recognized that a large proportion of white matter fiber tracts have complex architecture including crossing fibers such that variations in DTI measures do not necessarily reflect variations in structural integrity of myelin or axons (55, 56). Of the four DTI measures, FA, AD, and RD are most affected by this and therefore we focused our analyses on MD (57).

Regression-Based Norms

Using a healthy control population which was enrolled concurrently and underwent the same study procedures, we developed regression-based norms for each MRI measure that incorporated age and gender, similar to the approach used to develop norms for cognitive tests in this population (28). This allowed us to convert each MRI measure to a z-score, enhancing their comparability despite the differences in their value ranges, and normalizing them for subsequent regression analyses. Because this was a healthy control population this means that negative z-scores for a brain volume, for example, indicated that the brain volume is lower than in a healthy person.

Analyses

Descriptive

We described the study population using means (standard deviation [SD]), medians (interquartile range [IQR]), and frequencies (percent). We observed strong Spearman correlations between several of the MRI measures (**Supplementary Figure e1** and **Supplementary Table e2**).

Summarizing MRI Measures

We selected 4 measures for our analyses which captured brain macrostructure and microstructure [thalamic and hippocampal volumes, MD of NAWM and of gray matter (GM)] based on as their established associations with cognition in the MS literature. These measures also met the statistical criteria of no multicollinearity amongst them (**Supplementary Table e3**), and met the assumption of multivariate normality required for our subsequent analyses (**Supplementary Table e4**).

Summarizing Vascular Comorbidity

Given the high degree of overlap between vascular comorbidities, and our limited sample size, we summarized the four vascular comorbidities (diabetes, hypertension, hyperlipidemia, heart disease) as a count (0, 1, 2, 3+). We used an unweighted count for consistency with a prior study showing a dose-response association between an unweighted vascular comorbidity count and brain volumes, and with performance-based measures

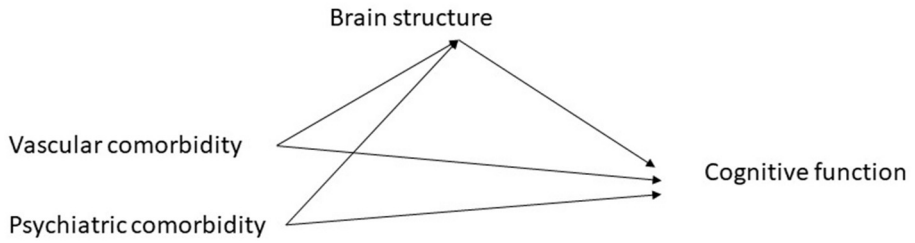

FIGURE 1 | Hypothesized pathways between comorbidity and cognitive function.

including a cognitive test of processing speed (17). Moreover, comorbidity counts are readily understood measures that have been associated with multiple outcomes in MS (58, 59). The prior study did not include smoking or BMI in the vascular comorbidity count. Although we included smoking in the vascular count in a complementary analysis as described further below, we did not include higher BMI (i.e., being overweight or obese) in the count. Seventy-five percent of the cohort was overweight or obese. We had previously observed that higher BMI was associated with better cognitive performance (15), an effect opposite to those anticipated for other vascular comorbidities of interest on cognition, and an effect opposite to that expected on MRI outcomes. Studies in the general population suggest that higher BMI may be protective of cognition (60–62) and that this effect may be non-linear. The assumption of using an unweighted comorbidity count is that the effects of comorbidities are additive with the effects in the same direction.

Primary Analyses

Our goal was to understand the relationship between vascular comorbidity and cognition, and whether this was mediated via brain structure (**Figure 1**). First, a multivariate approach was used, due to the large number of variables assessing each of cognition and MRI, the size of our sample, and to minimize the number of comparisons made. Specifically, our primary analysis began with canonical correlation analysis to model the association between cognition and MRI; (63) vascular comorbidity was not evaluated in this step. Canonical correlation analysis has been used in other studies of cognition in MS (64). In this situation we view the cognitive variables as assessing a common underlying latent construct, and the MRI variables as assessing the underlying latent construct of brain structural integrity. In canonical correlation analysis, weighted linear combinations of variables ("variates") are created within each dataset that account for the most correlation between the two datasets. The first pair of variates has the highest possible correlation, and successive pairs of variates are orthogonal and independent of other variates. Variable loadings measure the correlation between the original variable and the variate, indicating the relative contribution of the variable to the variate. This analytic approach is more powerful and reduces the number of comparisons. We assessed the assumptions of multicollinearity using correlations, multivariate normality using the Doornik-Hansen test, and linearity (**Supplementary Figures e1, e2**). We report the redundancy index (amount of variance explained).

Second, we constructed a series of linear regression models. In the first model, we tested the association between the count of vascular comorbidities and the cognitive variate (dependent variable). In the second model we changed the dependent variable to the MRI variate. In the third model, we tested the association between the count of vascular comorbidities and the cognitive variate (dependent variable), adjusting for the MRI variate. The count of vascular comorbidities was included as indicator variables. These regression analyses did not include age or gender since these were captured in the z-scores for the cognitive and MRI measures. In all models, covariates included current depressive disorder, current anxiety disorder and use of disease-modifying therapy (yes/no). We included use of disease-modifying therapy as a covariate because of literature suggesting that vascular comorbidity is associated with initiation (or not) of disease-modifying therapy (65), and the association of disease-modifying therapy with cognition (66). Regression analyses were bootstrapped 1,000 times, and we report bias-corrected 95% confidence intervals (95%CI). We assessed the proportion of the direct effect of comorbidity on cognition mediated by MRI as described for multi-level categorical variables (67).

Secondary Analyses

Third, we performed exploratory secondary analyses using multivariate regression models. These analyses aimed to provide insight into the relationships between vascular comorbidity, cognitive and MRI measures at a more granular level. However, these analyses need to be interpreted cautiously given the number of comparisons (68). We used the same three model approach as described using the canonical variates but we included the z-scores for each of the cognitive measures as dependent variables rather than the single cognitive variate, and included all of the z-scores for the MRI measures as independent variables rather than the single MRI variate. If a statistically significant global association was identified between an independent variable of interest and cognition, we explored this further using linear models which included only one cognitive z-score as the dependent variable. Non-significant global associations were not examined further.

Complementary Analyses

We performed complementary analyses to test the sensitivity of our findings to changes in sub-population or inclusion of other variables. First, we limited the primary analyses to women. Second, we included a history of ever smoking in the count of vascular comorbidities, and repeated the regression analyses that tested the association between the count of vascular comorbidities and the cognitive variate (dependent variable), adjusting for current depressive disorder, current anxiety disorder and use of disease-modifying therapy (yes/no) and for the MRI variate. Third, we repeated the primary analyses after limiting the study population to participants who were overweight or obese since the overlap between overweight/obesity and vascular comorbidity was too substantial to include it as a covariate.

Statistical analyses used SAS V9.4 (SAS Institute Inc., Cary, NC) and STATA 17.0 (Statacorp LLC, College Station, TX).

RESULTS

We included 105 participants. Most participants were women, and most had a moderate level of disability (**Table 1**). Vascular comorbidity was common, affecting 62 (59.0%). Just over half of participants had hypertension (50.5%), whereas only 11.4% had diabetes. Overlap between comorbidities was common. All 12 participants with diabetes had hypertension, while 10 (90.9%) were overweight or obese and 9 (75%) had hyperlipidemia. Twelve of the 53 participants with hypertension had diabetes (22.6%), while 47 (90.4%) were overweight or obese. Of six participants with heart disease, 5 (83.3%) had hypertension, and one-third had diabetes. Nearly 10% of participants currently had an anxiety disorder, of whom 8 (80%) were currently using a psychotropic medication. Fifteen percent of participants currently had a depressive disorder, of whom 13 (81.2%) were currently using a psychotropic medication. Based on average (standard error) z-scores determined using regression-based norms, cognitive performance was lowest for the SDMT (−0.76 [0.12]), followed by verbal fluency (animals, −0.61 [0.11]), verbal fluency (letter, −0.25 [0.10]), BVMT-R (−0.064 [0.11]), and the CVLT-II (0.031 [0.12]). Overall, 28 (26.7%) participants were classified as cognitively impaired based on the SDMT. In contrast, 11 (10.5%) were impaired on the CVLT-II, 13 (12.4%) on the BVMT-R, 12 (11.4%) on verbal fluency (averaging fluency for animals and letters).

The number of vascular comorbidities correlated with MD of NAWM ($r = -0.27$; 95%CI: −0.44, −0.086) but not with MD of GM (0.18; −0.014, 0.36), nor with thalamic (−0.084; 95%CI: −0.27, 0.11) or hippocampal ($r = 0.001$; −0.19, 0.19) volumes. Age at MS symptom onset was not correlated with the number of vascular comorbidities after accounting for age at assessment ($r = 0.12$, $p = 0.21$). Similarly, disease duration was not correlated with the number of vascular comorbidities after accounting for age at assessment ($r = -0.12$, $p = 0.21$).

TABLE 1 | Cohort demographic and clinical characteristics.

Characteristic	Value
N	105
Age, year mean (SD)	51.8 (12.8)
Female gender, *n* (%)	89 (84.8)
Education, *n* (%)	
≤High School/GED	33 (32.0)
Post-secondary	70 (68.0)
MS characteristics	
Age at MS onset, years, mean (SD)	29.4 (10.5)
Age at MS diagnosis, years, mean (SD)	35.1 (10.2)
Disease duration, years, mean (SD)	22.4 (12.3)
Current course, *n* (%)	
Relapsing remitting	85 (81.7)
Secondary progressive	13 (12.5)
Primary progressive	6 (5.8)
EDSS, median (p25–p75)	3.5 (3.0–5.0)
Any relapses in last 12 months, *n* (%)	3 (2.9)
Any disease-modifying therapy, *n* (%)	58 (55.2)
Any psychotropic medication, *n* (%)	65 (61.9)
Comorbidity & health behaviors	
SCID Current anxiety disorder, *n* (%)	10 (9.5)
SCID Current depressive disorder, *n* (%)	16 (15.2)
Hypertension (self-reported physician diagnosis), *n* (%)	28 (26.7)
Hypertension (self-reported physician diagnosis, measured blood pressure and medication use), *n* (%)	53 (50.5)
Hyperlipidemia (self-reported physician diagnosis), *n* (%)	24 (22.9)
Hyperlipidemia (self-reported physician diagnosis and medications), *n* (%)	25 (23.8)
Diabetes (self-reported physician diagnosis), *n* (%)	11 (10.5)
Diabetes (self-reported physician diagnosis, medications and HbA1c), *n* (%)	12 (11.4)
Heart disease (self-reported physician diagnosis), *n* (%)	6 (5.7)
No. vascular comorbidities, *n* (%)	
0	43 (41.0)
1	37 (35.2)
2	16 (15.2)
3+	9 (8.6)
Ever smoker, *n* (%)	62 (59.1)
Current smoker, *n* (%)	15 (14.3)
BMI (kg/m^2), mean (SD)	29.1 (6.4)

EDSS, Expanded Disability Status Scale.

Canonical Correlation Analysis

The canonical correlation analysis identified one statistically significant pair of variates (Pillai's trace = 0.45, $p = 0.0035$), which had a correlation of 0.50 (**Supplementary Figure e3**). Based on variable loadings, the biggest contributor to the cognitive variate was the SDMT (0.88), followed by verbal fluency (letter, 0.76), visual memory (0.52), verbal fluency (animals, 0.49); the CVLT-II verbal learning score was the smallest contributor (0.20) (**Supplementary Figure e4**). The biggest contributors to the MRI variate were gray matter MD (−0.79) and thalamic volume (0.63), followed by hippocampal volume (0.26) and NAWM MD (0.20) (**Supplementary Figure e5**). The canonical redundancy index for the cognitive variate (i.e., the total fraction of variance accounted for by the

MRI variables) was 9.7%. The canonical redundancy index for the MRI variate was 7.2%. Age at MS symptom onset was not correlated with the cognitive variate ($r = -0.13$, $p = 0.20$).

TABLE 2 | Association of comorbidity with cognitive variate and magnetic resonance imaging (MRI) variate.

	MRI variate β (95% CI)*	Cognitive variate[b] β (SE)*	Cognitive variate[c] β (SE)*
Vascular comorbidity[a]			
1	−0.54 (−0.94, −0.057)	−0.38 (−0.87, 0.11)	−0.096 (−0.58, 0.37)
	$p = 0.015$	$p = 0.12$	$p = 0.68$
2	−0.76 (−1.35, −0.18)	−0.65 (−1.19, −0.040)	−0.32 (−0.84, 0.17)
	$p = 0.013$	**$p = 0.025$**	$p = 0.23$
≥3	−1.24 (−1.83, −0.50)	−0.91 (−1.52, −0.24)	−0.38 (−1.10, 0.38)
	$p = 0.0001$	**$p = 0.05$**	$p = 0.32$
Anxiety	0.036 (−0.56, 0.68),	0.48 (−0.17, 1.21)	0.43 (−0.18, 1.21)
	$p = 0.91$	$p = 0.16$	$p = 0.21$
Depression	0.60 (0.091, 1.18)	0.14 (−0.51, 0.77)	−0.12 (−0.73, 0.41)
	$p = 0.03$	$p = 0.66$	$p = 0.67$
Disease-modifying therapy	−0.082 (−0.45, 0.29)	−0.050 (−0.47, 0.34)	−0.029 (−0.40, 0.34)
	$p = 0.68$	$p = 0.80$	$p = 0.88$
MRI variate			0.46 (0.28, 0.64)
			$p = 0.0001$
Adjusted R^2	0.12	0.045	0.23

**Based on 1,000 bootstrap replications; a-reference group = 0; b-without adjustment for MRI variate; c-with adjustment for MRI variate. Bold indicates $p < 0.05$.*

After adjusting for disease-modifying therapy, vascular comorbidity was associated with the MRI variate (**Table 2**, global test $\chi^2 = 16.88$, $p = 0.0007$). We observed that the higher the number of vascular comorbidities, the lower the value of (i.e., the more abnormal) the MRI variate. Similarly, vascular comorbidity was associated with the cognitive variate (**Table 2**, global test $\chi^2 = 9.78$, $p = 0.021$) and we observed that the higher the number of vascular comorbidities the lower the value of the cognitive variate. After we added the MRI variate to the model, vascular comorbidity was no longer associated with the cognitive variate (global test $\chi^2 = 2.0$, $p = 0.57$) but the MRI variate was associated with the cognitive variate ($\chi^2 = 22.98$, $p = <0.0001$). Over one-third (37%) of the effect of vascular comorbidities on the cognitive variate was mediated by the MRI variate.

Multivariate Regression Analyses

In the multivariate regression analysis which included all cognitive variables as dependent variables, vascular comorbidity remained associated with cognition in the global test ($\chi^2 = 26.9$, $p = 0.03$). In the follow-up individual regression analyses, vascular comorbidity was associated with lower performance on the SDMT, CVLT-II, and verbal fluency (animal) (**Table 3**).

In the multivariate regression analysis which included all MRI variables as dependent variables, the number of vascular comorbidities was associated with the MRI variables overall ($\chi^2 = 39.7$, $p = 0.0001$). In the follow-up individual regression analyses, the number of vascular comorbidities was associated was not associated with individual MRI measures (**Table 4**), indicating it was important to consider them in aggregate.

Therefore, subsequent analyses focused on the association of vascular comorbidity with the SDMT, CVLT-II, and verbal (animal) fluency. After addition of the MRI variables to the model, vascular comorbidity was no longer associated with the

TABLE 3 | Association of vascular comorbidity with individual cognitive tests.

	SDMT β (SE)*	CVLT-II β (SE)*	BVMTR β (SE)*	COWAT-FAS β (SE)*	COWAT- Animals β (SE)*
Vascular comorbidity[a]					
1	−0.48 (−1.02, 0.030)	−0.63 (−1.13, −0.11)	−0.38 (−0.90, 0.14)	−0.30 (−0.82, 0.24)	−0.73 (−1.18, −0.29)
	$p = 0.079$	**$p = 0.018$**	$p = 0.15$	$p = 0.26$	**$p = 0.001$**
2	−0.61 (1–0.33, 0.13)	−0.52 (−1.23, 0.26)	−0.80 (−1.45, −0.11)	−0.47 (−1.06, 0.098)	−0.94 (−1.64, −0.35)
	$p = 0.119$	$p = 0.20$	**$p = 0.026$**	$p = 0.12$	**$p = 0.004$**
≥3	−0.94 (−1.74, −0.12)	−0.39 (−1.57, 0.51)	−0.55 (−1.45, 0.22)	−0.77 (−1.62, 0.037)	−0.87 (−1.65, 0.0055)
	$p = 0.023$	$p = 0.45$	$P = 0.20$	$p = 0.078$	**$p = 0.037$**
Global test vascular comorbidity	$\chi^2 = 20.3$, **$p = 0.042$**	$\chi^2 = 22.9$, **$p = 0.018$**	$\chi^2 = 19.5$, $p = 0.052$	$\chi^2 = 19.6$, $p = 0.051$	$\chi^2 = 22.2$, **$p = 0.023$**
Anxiety	0.45 (−0.42, 1.37)	0.071 (−1.05, 1.04)	0.43 (−0.73, 1.42)	0.049 (−0.69, 0.69)	(0.51, 1.78)
	$p = 0.99$	$p = 0.90$	$P = 0.44$	$p = 0.89$	**$p <0.0001$**
Depression	−0.13 (−1.13, 0.76)	0.052 (−0.91, 0.81)	0.33 (−0.55, 1.23)	0.38 (−0.16, 0.88)	0.032 (−0.58, 0.17)
	$p = 0.79$	$p = 0.90$	$P = 0.46$	$p = 0.16$	$p = 0.91$
Disease-modifying therapy	−0.11 (−0.61, 0.32)	−0.27 (−0.032, 0.98)	−0.063 (−0.49, 0.46)	−0.12 (−0.63, 0.32)	−0.037 (−0.46, 0.35)
	$p = 0.65$	$p = 0.29$	$P = 0.80$	$p = 0.63$	$p = 0.85$

**Based on 1,000 bootstrap replications; a- reference group = 0; SDMT, Symbol Digit Modalities Test; CVLT-II, California Verbal Learning Test-II; BVMT-R, Brief Visuospatial Memory Test-Revised; COWAT, Controlled Oral Word Association Test.*
Bold indicates $p < 0.05$.

TABLE 4 | Association of vascular comorbidity with individual magnetic resonance imaging measures.

	Thalamic volume β (SE)*	Hippocampal volume β (SE)*	GM MD β (SE)*	NAWM MD β (SE)*
Vascular comorbidity[a]				
1	−0.32 (0.39)	0.089 (0.27)	0.49 (0.32)	−0.48 (0.43)
	$p = 0.41$	$p = 0.74$	$p = 0.13$	$p = 0.26$
2	−0.52 (0.61)	0.23 (0.50)	0.70 (0.41)	−0.56 (0.60)
	$p = 0.39$	$p = 0.65$	$p = 0.087$	$p = 0.35$
≥3	−0.91 (0.59)	−0.28 (0.54)	0.86 (0.66)	−1.71 (0.70)
	$p = 0.12$	$P = 0.60$	$p = 0.19$	**$p = 0.015$**
Global test vascular comorbidity	1.15	0.64	0.49	3.34,
	$p = 0.56$	$p = 0.72$	$p = 0.78$	$p = 0.19$
Anxiety	−0.50 (0.43)	−0.23 (0.39)	0.049 (0.52)	0.53 (0.47)
	$P = 0.24$	$P = 0.78$	$p = 0.92$	$p = 0.27$
Depression	0.20 (0.50)	−0.013 (0.40)	−0.45 (0.44)	0.89 (0.57)
	0.69	$P = 0.98$	$p = 0.31$	$p = 0.27$
Disease-modifying therapy	−0.75 (0.36)	−0.079 (0.299)	0.35 (0.28)	0.99 (0.37)
	$P = 0.039$	0.78	$p = 0.21$	**$p = 0.007$**

**Based on 1,000 bootstrap replications; a- reference group = 0; GM, gray matter; MD, mean diffusivity; NAWM, normal-appearing white matter.*
Bold indicates $p < 0.05$.

cognitive variate in the global test ($\chi^2 = 18.9$, $p = 0.22$), nor with the individual cognitive variables SDMT ($\chi^2 = 12.0$, $p = 0.36$), CVLT-II ($\chi^2 = 14.4$, $p = 0.21$) or animal fluency ($\chi^2 = 13.5$, $p = 0.26$). Collectively, the MRI variables were associated with cognition in a global test ($\chi^2 = 45.4$, $p = 0.001$), and specifically with the SDMT ($\chi^2 = 45.4$, $p = 0.001$), the CVLT-II ($\chi^2 = 28.5$, $p = 0.028$) and animal fluency ($\chi^2 = 28.3$, $p = 0.029$).

Complementary Analyses

After we limited our primary analyses to women, our findings were similar. Vascular comorbidity was associated with the cognitive variate in the model that did not include the MRI variate ($\chi^2 = 18.8$, $p = 0.0003$) but not when the MRI variate was added to the model ($\chi^2 = 1.15$, $p = 0.76$). After we included ever smoking in the count of vascular comorbidities, and repeated the primary analyses our findings were similar (**Supplementary Table e5**). When we limited the analysis to participants who were overweight or obese, our findings were similar (**Supplementary Table e6**).

DISCUSSION

In this cross-sectional study we assessed the inter-relationships between vascular comorbidity, brain structure as measured by MRI, and cognition among 105 individuals with MS enrolled from a population-based MS Clinic. We found that a higher number of vascular comorbidities was associated with lower cognitive function overall, and specifically with measures of processing speed, verbal learning and memory, and oral fluency. These associations were fully attenuated after we accounted for MRI measures of thalamic and hippocampal volume, and mean diffusivity of gray matter and NAWM, consistent with our hypothesis that the impacts of vascular comorbidity on cognition in people with MS are mediated by differences in brain structure (as depicted in **Figure 1**). This suggests that future intervention studies targeted at treating vascular comorbidity to improve cognition could use MRI measures as intermediate outcomes. It also highlights the complexity of relationships between comorbidity and outcomes in MS. Impacts of vascular comorbidity on brain health, including brain structure and cognition, may reflect increased peripheral inflammation, endothelial injury, and alterations in blood vessel function, cerebral blood flow and metabolism (69–72).

Some prior studies have reported an association between vascular comorbidities and brain volumes in persons with MS. The largest cross-sectional study to date, which included 6,409 from the MS-PATHS study, found that the presence of two or more vascular comorbidities was associated with lower whole brain and gray matter volumes (17). However, another MS-PATHS study including some of these participants, but based at a single center, found that while depression was associated with lower whole brain and gray matter volumes, hyperlipidemia was associated with higher whole brain volumes for unclear reasons (14). In the general population vascular comorbidities are also reportedly associated with differences in brain structure. A recent study including 9,722 participants from the UK Biobank found that the higher the total number of vascular risk factors the lower the brain volume and the greater the changes in brain microstructure (73). To our knowledge, prior studies in the MS population have not examined the association between vascular comorbidity and DTI measures. In the general population, vascular comorbidity is associated with differences in FA and MD in the NAWM. Higher systolic blood pressure and higher glucose in midlife are reportedly associated with worse white matter microstructure as measured using FA and MD (20, 74).

A handful of studies have examined the association between vascular comorbidity and cognition in people with MS. A study involving 11,506 individuals in the MS-PATHS study found that those with two or more vascular comorbidities, including diabetes, hypertension and hyperlipidemia, had lower correct scores on a test of processing speed, though no other cognitive domains were examined (17). A retrospective study involving 69 persons with MS found that a one-point increase in the Framingham risk score was associated with lower CVLT-II scores, and this appeared to be driven by male sex and higher lipid levels, though they did not observe any associations with the SDMT and BVMT-R (16). Even without overt cerebrovascular disease, hypertension, hypercholesterolemia, and diabetes are associated with cognitive impairment, an increased risk of dementia (75–77). However, a systematic review of several studies that included individuals without dementia reported that diabetes and hypertension were associated with reduced cognitive function (78). A 10-point increment in diastolic blood pressure (BP) is associated with increased odds of cognitive impairment (7%; 1–14%) in a North American sample even after

controlling for numerous other factors (79). Thus, the findings reported for studies of MS samples appear consistent with these adverse impacts of vascular comorbidity, including diabetes and hypertension, on cognition in the general population.

Limitations to our study include our modest sample size, though we were careful to take several steps to reduce the number of variables examined and the number of comparisons performed in our primary analysis. Nonetheless, our findings should be replicated in other, larger populations. Most of our participants were women, consistent with the general female predominance of MS, thus our findings may not generalize as well to men with MS. While we did not comprehensively measure all cognitive domains, we assessed those most often affected in people with MS, those included in the BICAMS, and those affected by the comorbidities investigated here. Like other studies to date we were unable to account for the severity of vascular and other comorbidities or their treatments, and could not discriminate the effects of individual vascular comorbidities or behavioral factors such as smoking; this warrants further investigation. Prior studies have reported that depression and anxiety disorders are associated with lower cognitive performance and alterations in brain structure, and we did incorporate these variables into all of our regression models as covariates (80–82). Use of psychotropic medications may adversely influence cognition, however, their use overlapped substantially with the depression and anxiety disorders in our cohort, precluding an assessment of their effects (including whether they were mediated by changes in brain structure as illustrated in **Figure 1** or via other pathways). Psychotropic medications may improve cognition as the psychiatric disorder remits, or worsen cognition (83). These effects on cognitive function may vary by drug class and possibly by specific agent, mandating the use of large samples to elucidate their effects. However, though we used the gold standard structured interview to identify these conditions, the small number of individuals affected precluded more detailed analysis. Our MRI protocol did not include gadolinium so it is possible that we included participants with focal inflammatory activity, which might have affected cognitive performance (84, 85). Other studies have suggested that vascular comorbidities, such as hyperlipidemia, are associated with an increase in gadolinium-enhancing lesions. Therefore, it is possible that a larger proportion of cognitive performance might have been mediated by MRI measures if we had been able to capture gadolinium-enhancing lesions. However, the proportion of participants with a relapse in the prior year was quite low. We used a small number of MRI measures to address multicollinearity and meet assumptions of our analyses. We focused on a subset of readily available MRI measures. Use of more advanced imaging measures, and incorporating other measures such as lesion volume might have increased the proportion of the vascular comorbidity effect on cognition mediated by MRI measures. That is, using a more limited set of measures may have biased our findings toward the null. Moreover, targeting more focal hypotheses may provide greater insight into the mechanisms evaluated herein. Although our study suggests that changes in MRI measures mediate the effects of vascular comorbidity on cognition, we cannot determine whether the changes in MRI measures solely reflect vascular effects similar to those in the general population, or whether the vascular comorbidities lead to increases in MS-specific pathologic changes. Finally, the cross-sectional nature of the study design limits causal inference. Nonetheless, cross-sectional studies that use mediation analyses can provide strong theoretical frameworks to guide future research, and more appropriately account for variables that lie in the same causal pathway than other approaches, as illustrated in the chronic pain literature (86). Future studies should examine these relationships longitudinally.

Our findings demonstrate that vascular comorbidity is associated with lower cognitive function in people with MS and this association is mediated, at least in part, via measurable changes in brain macrostructure and microstructure. This underscores the importance of preventing and treating vascular comorbidity effectively in persons with MS to mitigate their impacts on cognition and brain structure. Our findings highlight the importance of ensuring that etiologies other than MS, such as vascular comorbidity, are considered when evaluating individuals experiencing cognitive impairment. Our findings also suggest that additional MRI measures, such as DTI, may be considered useful methods of assessing the efficacy of interventions aimed at vascular comorbidities affecting persons with MS in the future, potentially warranting future consensus efforts (87).

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because some participants did not agree to data sharing. Components of the datasets may be made accessible to qualified investigators with the appropriate ethical approvals and data use agreements upon request. Requests to access the datasets should be directed to RM, rmarrie@hsc.mb.ca.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Manitoba Health Research Ethics Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RM: conceptualization, project administration, supervision, funding acquisition, and writing—original draft. JF: conceptualization, project administration, supervision, funding acquisition, and writing—review and editing. RP: conceptualization and writing—review and editing. CF: conceptualization, funding acquisition, methodology, and writing—review and editing. JK, JB, EM, JM, CB, and LG: conceptualization, funding acquisition, and writing—review and editing. CH: methodology, analysis, resources, and writing—review and editing. MU: methodology and writing—review

and editing. TF: analysis and writing—review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2022.910014/full#supplementary-material

REFERENCES

1. Rao SM, Leo GJ, Bernardin L, Unverzagt F. Cognitive dysfunction in multiple sclerosis I: frequency, patterns, and predictions. *Neurology.* (1991) 41:685–91. doi: 10.1212/WNL.41.5.685
2. Rocca MA, Amato MP, De Stefano N, Enzinger C, Geurts JJ, Penner I-K, et al. Clinical and imaging assessment of cognitive dysfunction in multiple sclerosis. *Lancet Neurol.* (2015) 14:302–17. doi: 10.1016/S1474-4422(14)70250-9
3. Goverover Y, Strober L, Chiaravalloti N, DeLuca J. Factors that moderate activity limitation and participation restriction in people with multiple sclerosis. *Am J Occup Ther.* (2015) 69:6902260020p1–9. doi: 10.5014/ajot.2015.014332
4. Houtchens MK, Benedict RH, Killiany R, Sharma J, Jaisani Z, Singh B, et al. Thalamic atrophy and cognition in multiple sclerosis. *Neurology.* (2007) 69:1213–23. doi: 10.1212/01.wnl.0000276992.17011.b5
5. Zivadinov R, Sepcic J, Nasuelli D, De Masi R, Bragadin LM, Tommasi MA, et al. A longitudinal study of brain atrophy and cognitive disturbances in the early phase of relapsing-remitting multiple sclerosis. *J Neurol Neurosurg Psychiatry.* (2001) 70:773–80. doi: 10.1136/jnnp.70.6.773
6. Comi G, Filippi M, Martinelli V, Sirabian G, Visciani A, Campi A, et al. Brain magnetic resonance imaging correlates of cognitive impairment in multiple sclerosis. *J Neurol Sci.* (1993) 115:S66–S73. doi: 10.1016/0022-510X(93)90212-H
7. Berg D, Maurer M, Warmuth-Metz M, Rieckmann P, Becker G. The correlation between ventricular diameter measured by transcranial sonography and clinical disability and cognitive dysfunction in patients with multiple sclerosis. *Arch Neurol.* (2000) 57:1289–92. doi: 10.1001/archneur.57.9.1289
8. Filippi M, Tortorella C, Rovaris M, Bozzali M, Possa F, Sormani MP, et al. Changes in the normal appearing brain tissue and cognitive impairment in multiple sclerosis. *J Neurol Neurosurg Psychiatry.* (2000) 68:157–61. doi: 10.1136/jnnp.68.2.157
9. Hulst HE, Steenwijk MD, Versteeg A, Pouwels PJW, Vrenken H, Uitdehaag BMJ, et al. Cognitive impairment in MS: Impact of white matter integrity, gray matter volume, and lesions. *Neurology.* (2013) 80:1025–32. doi: 10.1212/WNL.0b013e31828726cc
10. Rovaris M, Filippi M, Minicucci L, Iannucci G, Santuccio G, Possa F, et al. Cortical/subcortical disease burden and cognitive impairment in patients with multiple sclerosis. *Am J Neuroradiol.* (2000) 21:402–8.
11. Marrie RA, Reider N, Cohen J, Stuve O, Trojano M, Sorensen PS, et al. A systematic review of the incidence and prevalence of comorbidity in multiple sclerosis: overview. *Mult Scler.* (2015) 21:263–81. doi: 10.1177/1352458514564491
12. Salter A, Kowalec K, Fitzgerald KC, Cutter G, Marrie RA. Comorbidity is associated with disease activity in MS: findings from the CombiRx trial. *Neurology.* (2020) 95:e446–e56. doi: 10.1212/WNL.0000000000010024
13. McKay KA, Tremlett H, Fisk JD, Zhang T, Patten SB, Kastrukoff L, et al. Psychiatric comorbidity is associated with disability progression in multiple sclerosis. *Neurology.* (2018) 90:e1316–23. doi: 10.1212/WNL.0000000000005302
14. Abbatemarco JR, Ontaneda D, Nakamura K, Husak S, Wang Z, Alshehri E, et al. Comorbidity effect on processing speed test and MRI measures in multiple sclerosis patients. *Mult Scler Relat Disord.* (2020) 46:102593. doi: 10.1016/j.msard.2020.102593
15. Marrie RA, Patel R, Figley CR, Kornelsen J, Bolton JM, Graff L, et al. Diabetes and anxiety adversely affect cognition in multiple sclerosis. *Mult Scler Relat Disord.* (2019) 27:164–70. doi: 10.1016/j.msard.2018.10.018
16. Reia A, Petruzzo M, Falco F, Costabile T, Conenna M, Carotenuto A, et al. A retrospective exploratory analysis on cardiovascular risk and cognitive dysfunction in multiple sclerosis. *Brain Sci.* (2021) 11:502. doi: 10.3390/brainsci11040502
17. Fitzgerald KC, Damian A, Conway D, Mowry EM. Vascular comorbidity is associated with lower brain volumes and lower neuroperformance in a large multiple sclerosis cohort. *Mult Scler.* (2021) 27:1914–23. doi: 10.1177/1352458520984746
18. Marrie RA, Patel R, Figley CR, Kornelsen J, Bolton JM, Graff LA, et al. Higher framingham risk scores are associated with greater loss of brain volume over time in multiple sclerosis. *Mult Scler Relat Disord.* (2021) 54:103088. doi: 10.1016/j.msard.2021.103088
19. Lorefice L, Frau J, Coghe G, Pitzalis R, Gessa I, Contu F, et al. Assessing the burden of vascular risk factors on brain atrophy in multiple sclerosis: a case- control MRI study. *Mult Scler Relat Disord.* (2018) 27:74–8. doi: 10.1016/j.msard.2018.10.011
20. Fuhrmann D, Nesbitt D, Shafto M, Rowe JB, Price D, Gadie A, et al. Strong and specific associations between cardiovascular risk factors and white matter micro- and macrostructure in healthy aging. *Neurobiol Aging.* (2019) 74:46–55. doi: 10.1016/j.neurobiolaging.2018.10.005
21. Maniega SM, Valdés Hernández MC, Clayden JD, Royle NA, Murray C, Morris Z, et al. White matter hyperintensities and normal-appearing white matter integrity in the aging brain. *Neurobiol Aging.* (2015) 36:909–18. doi: 10.1016/j.neurobiolaging.2014.07.048
22. Arnett PA. Longitudinal consistency of the relationship between depression symptoms and cognitive functioning in multiple sclerosis. *CNS Spectr.* (2005) 10:372–82. doi: 10.1017/S1092852900022744
23. Gold SM, O'Connor M-F, Gill R, Kern KC, Shi Y, Henry RG, et al. Detection of altered hippocampal morphology in multiple sclerosis-associated depression using automated surface mesh modeling. *Hum Brain Mapp.* (2014) 35:30–7. doi: 10.1002/hbm.22154
24. Gold SM, Kern KC, O'Connor M-F, Montag MJ, Kim A, Yoo YS, et al. Smaller cornu ammonis 2–3/dentate gyrus volumes and elevated cortisol in multiple sclerosis patients with depressive symptoms. *Biol Psychiatry.* (2010) 68:553–9. doi: 10.1016/j.biopsych.2010.04.025
25. Zorzon M, Zivadinov R, Nasuelli D, Ukmar M, Bratina A, Tommasi MA, et al. Depressive symptoms and MRI changes in multiple sclerosis. *Eur J Neurol.* (2002) 9:491–6. doi: 10.1046/j.1468-1331.2002.00442.x
26. Feinstein A, O'Connor P, Akbar N, Moradzadeh L, Scott C, Lobaugh N. Diffusion tensor imaging abnormalities in depressed multiple sclerosis patients. *Mult Scler.* (2010) 16:189–96. doi: 10.1177/1352458509355461
27. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol.* (2011) 69:292–302. doi: 10.1002/ana.22366

28. Marrie RA, Whitehouse CE, Patel R, Figley CR, Kornelsen J, Bolton JM, et al. Performance of regression-based norms for cognitive functioning of persons with multiple sclerosis in an independent sample. *Front Neurol.* (2021) 11:621010. doi: 10.3389/fneur.2020.621010
29. Mazziotta JC, Woods R, Iacoboni M, Sicotte N, Yaden K, Tran M, et al. The myth of the normal, average human brain—The ICBM experience: (1) Subject screening and eligibility. *Neuroimage.* (2009) 44:914–22. doi: 10.1016/j.neuroimage.2008.07.062
30. Marrie RA, Graff LA, Walker JR, Fisk JD, Patten SB, Hitchon CA, et al. A prospective study of the effects of psychiatric comorbidity in immune-mediated inflammatory disease: rationale, protocol and participation. *JMIR Res Protoc.* (2018) 7:e15. doi: 10.2196/resprot.8794
31. Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap)—A metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform.* (2009) 42:377–81. doi: 10.1016/j.jbi.2008.08.010
32. Kurtzke JF. Rating neurologic impairment in multiple sclerosis: an Expanded Disability Status Scale (EDSS). *Neurology.* (1983) 33:1444-52. doi: 10.1212/WNL.33.11.1444
33. Horton M, Rudick RA, Hara-Cleaver C, Marrie RA. Validation of a self-report comorbidity questionnaire for multiple sclerosis. *Neuroepidemiology.* (2010) 35:83–90. doi: 10.1159/000311013
34. American Diabetes Association. Standards of medical care in diabetes—2011. *Diabetes Care.* (2011) 34:S11–61. doi: 10.2337/dc11-S011
35. Whitehouse CE, Fisk JD, Bernstein CN, Berrigan LI, Bolton JM, Graff LA, et al. Comorbid anxiety, depression, and cognition in MS and other immune-mediated disorders. *Neurology.* (2019) 92:e406–e17. doi: 10.1212/WNL.0000000000006854
36. First M, Gibbon M, Spitzer R, Williams J. *User's Guide for the Structured Clinical Interview for DSM-IV-TR Axis I Disorders - Research Version - (SCID-I for DSM-IV-TR, November 2002 Revision).* New York, New York Biometrics Research Department, New York State Psychiatric Institute (2002).
37. Langdon DW, Amato MP, Boringa J, Brochet B, Foley F, Fredrikson S, et al. Recommendations for a Brief International Cognitive Assessment for Multiple Sclerosis (BICAMS). *Mult Scler.* (2012) 18:891–8. doi: 10.1177/1352458511431076
38. Benedict RHB, Fischer JS, Archibald CJ, Arnett PA, Beatty WW, Bobholz J, et al. Minimal neuropsychological assessment of MS patients: a consensus approach. *Clin Neuropsychol.* (2002) 16:381–97. doi: 10.1076/clin.16.3.381.13859
39. Smith A. *Symbol Digit Modalities Test*. 9th ed. Torrance, California: Western Psychological Services (2002).
40. Delis DC, Kramer JH, Kaplan E, Ober BA. *California Verbal Learning Test Second Edition Adult Version Manual*. San Antonio, TX: The Psychological Corporation (2000).
41. Benedict RHB, Hopkins BJ. *Verbal Learning Test-Revised/Brief Visuospatial Memory Test-Revised Professional Manual Supplement*. Odessa, FL: Psychological Assessment Resources. (2001).
42. Strauus E, Sherman E, Spreen O. *A Compendium of Neuropsychological Tests: Administration, Norms, and Commentary*. *3rd Edn.* New York, NY: Oxford University Press (2006).
43. The Psychological Corporation. *The Wechsler Test of Adult Reading (WTAR)* (2001).
44. Jenkinson M, Smith S. A global optimisation method for robust affine registration of brain images. *Med Image Anal.* (2001) 5:143–56. doi: 10.1016/S1361-8415(01)00036-6
45. Jenkinson M, Bannister P, Brady M, Smith S. Improved optimization for the robust and accurate linear registration and motion correction of brain images. *Neuroimage.* (2002) 17:825–41. doi: 10.1006/nimg.2002.1132
46. Schmidt P, Gaser C, Arsic M, Buck D, Förschler A, Berthele A, et al. An automated tool for detection of FLAIR-hyperintense white-matter lesions in Multiple Sclerosis. *Neuroimage.* (2012) 59:3774–83. doi: 10.1016/j.neuroimage.2011.11.032
47. Griffanti L, Zamboni G, Khan A, Li L, Bonifacio G, Sundaresan V, et al. BIANCA (Brain Intensity AbNormality Classification Algorithm): a new tool for automated segmentation of white matter hyperintensities. *Neuroimage.* (2016) 141:191–205. doi: 10.1016/j.neuroimage.2016.07.018
48. Battaglini M, Jenkinson M, De Stefano N. Evaluating and reducing the impact of white matter lesions on brain volume measurements. *Hum Brain Mapp.* (2012) 33:2062–71. doi: 10.1002/hbm.21344
49. Smith SM, Zhang Y, Jenkinson M, Chen J, Matthews PM, Federico A, et al. Accurate, robust, and automated longitudinal and cross-sectional brain change analysis. *Neuroimage.* (2002) 17:479–89. doi: 10.1006/nimg.2002.1040
50. Smith SM, Jenkinson M, Woolrich MW, Beckmann CF, Behrens TE, Johansen-Berg H, et al. Advances in functional and structural MR image analysis and implementation as FSL. *Neuroimage.* (2004) 23:S208–19. doi: 10.1016/j.neuroimage.2004.07.051
51. Mohammadi S, Möller HE, Kugel H, Müller DK, Deppe M. Correcting eddy current and motion effects by affine whole-brain registrations: evaluation of three-dimensional distortions and comparison with slicewise correction. *Magn Reson Med.* (2010) 64:1047–56. doi: 10.1002/mrm.22501
52. Ruthotto L, Kugel H, Olesch J, Fischer B, Modersitzki J, Burger M, et al. Diffeomorphic susceptibility artifact correction of diffusion-weighted magnetic resonance images. *Phys Med Biol.* (2012) 57:5715–31. doi: 10.1088/0031-9155/57/18/5715
53. Ruthotto L, Mohammadi S, Heck C, Modersitzki J, Weiskopf N. Hyperelastic susceptibility artifact correction of DTI in SPM. In: Meinzer H-P, Deserno TM, Handels H, Tolxdorff T, editors. *Bildverarbeitung für die Medizin 2013*. Heidelberg: Springer. (2013) p. 344–9. doi: 10.1007/978-3-642-36480-8_60
54. Mohammadi S, Hutton C, Nagy Z, Josephs O, Weiskopf N. Retrospective correction of physiological noise in DTI using an extended tensor model and peripheral measurements. *Magn Reson Med.* (2013) 70:358–69. doi: 10.1002/mrm.24467
55. Jeurissen B, Leemans A, Tournier JD, Jones DK, Sijbers J. Investigating the prevalence of complex fiber configurations in white matter tissue with diffusion magnetic resonance imaging. *Hum Brain Mapp.* (2013) 34:2747–66. doi: 10.1002/hbm.22099
56. Figley CR, Uddin MN, Wong K, Kornelsen J, Puig J, Figley TD. Potential pitfalls of using fractional anisotropy, axial diffusivity, and radial diffusivity as biomarkers of cerebral white matter microstructure. *Front Neurosci.* (2021) 15:799576. doi: 10.3389/fnins.2021.799576
57. Soares J, Marques P, Alves V, Sousa N. A hitchhiker's guide to diffusion tensor imaging. *Front Neurosci.* (2013) 7:31. doi: 10.3389/fnins.2013.00031
58. Marrie RA, Elliott L, Marriott J, Cossoy M, Tennakoon A, Yu N. Comorbidity increases the risk of hospitalizations in multiple sclerosis. *Neurology.* (2015) 84:350–8. doi: 10.1212/WNL.0000000000001187
59. Marrie RA, Horwitz R, Cutter G, Tyry T. Cumulative impact of comorbidity on quality of life in MS. *Acta Neurol Scand.* (2012) 125:180–6. doi: 10.1111/j.1600-0404.2011.01526.x
60. Kim S, Kim Y, Park SM. Body mass index and decline of cognitive function. *PLoS ONE.* (2016) 11:e0148908. doi: 10.1371/journal.pone.0148908
61. Suemoto CK, Gilsanz P, Mayeda ER, Glymour MM. Body mass index and cognitive function: the potential for reverse causation. *Int J Obes.* (2015) 39:1383–9. doi: 10.1038/ijo.2015.83
62. Xiang X, An R. Body weight status and onset of cognitive impairment among U.S. middle-aged and older adults. *Arch Gerontol Geriatr.* (2015) 60:394–400. doi: 10.1016/j.archger.2015.02.008
63. Sherry A, Henson RK. Conducting and interpreting canonical correlation analysis in personality research: a user-friendly primer. *J Pers Assess.* (2005) 84:37–48. doi: 10.1207/s15327752jpa8401_09
64. Lin SJ, Lam J, Beveridge S, Vavasour I, Traboulsee A, Li DKB, et al. Cognitive performance in subjects with multiple sclerosis is robustly influenced by gender in canonical-correlation analysis. *J Neuropsychiatry Clin Neurosci.* (2017) 29:119–27. doi: 10.1176/appi.neuropsych.16040083
65. Zhang T, Tremlett H, Leung S, Zhu F, Kingwell E, Fisk JD, et al. Examining the effects of comorbidities on disease-modifying therapy use in multiple sclerosis. *Neurology.* (2016) 86:1287–95. doi: 10.1212/WNL.0000000000002543
66. Landmeyer N, Burkner P-C, Wiendl H, Ruck T, Hartung H-P, Holling H, et al. Disease-modifying therapies and cognition in relapsing remitting MS - a meta-analysis. *Neurology.* (2020) 94:e2373–e83. doi: 10.1212/WNL.0000000000009522
67. Hayes AF, Preacher KJ. Statistical mediation analysis with a multicategorical independent variable. *Br J Math Stat Psychol.* (2014) 67:451–70. doi: 10.1111/bmsp.12028

68. Goodman SN. Multiple comparisons, explained. *Am J Epidemiol.* (1998) 147:807–12; discussion 15. doi: 10.1093/oxfordjournals.aje.a009531
69. Jefferson AL, Massaro JM, Wolf PA, Seshadri S, Au R, Vasan RS, et al. Inflammatory biomarkers are associated with total brain volume: the Framingham Heart Study. *Neurology.* (2007) 68:1032–8. doi: 10.1212/01.wnl.0000257815.20548.df
70. Klein JP, Waxman SG. The brain in diabetes: molecular changes in neurons and their implications for end-organ damage. *Lancet Neurol.* (2003) 2:548–54. doi: 10.1016/S1474-4422(03)00503-9
71. Launer LJ. Diabetes and brain aging: epidemiologic evidence. *Curr Diab Rep.* (2005) 5:59–63. doi: 10.1007/s11892-005-0069-1
72. Waldstein SR. The relation of hypertension to cognitive function. *Curr Dir Psychol Sci.* (2003) 12:9–12. doi: 10.1111/1467-8721.01212
73. Cox SR, Lyall DM, Ritchie SJ, Bastin ME, Harris MA, Buchanan CR, et al. Associations between vascular risk factors and brain MRI indices in UK Biobank. *Eur Heart J.* (2019) 40:2290–300. doi: 10.1093/eurheartj/ehz100
74. Power MC, Tingle JV, Reid RI, Huang J, Sharrett AR, Coresh J, et al. Midlife and late-life vascular risk factors and white matter microstructural integrity: the atherosclerosis risk in communities neurocognitive study. *J Am Heart Assoc.* (2017) 6:e005608.doi: 10.1161/JAHA.117.005608
75. Elkins JS, O'Meara ES, Longstreth WT Jr, Carlson MC, Manolio TA, Johnston SC, et al. Stroke risk factors and loss of high cognitive function. *Neurology.* (2004) 63:793–9. doi: 10.1212/01.WNL.0000137014.36689.7F
76. Pavlik VN, Hyman DJ, Doody R. Cardiovascular risk factors and cognitive function in adults 30-59 years of age (NHANES III). *Neuroepidemiology.* (2005) 24:42–50. doi: 10.1159/000081049
77. Spinelli C, De Caro MF, Schirosi G, Mezzapesa D, De Benedittis L, Chiapparino C, et al. Impaired cognitive executive dysfunction in adult treated hypertensives with a confirmed diagnosis of poorly controlled blood pressure. *Int J Med Sci.* (2014) 11:771–8. doi: 10.7150/ijms.8147
78. van den Berg E, Kloppenborg RP, Kessels RPC, Kappelle LJ, Biessels GJ. Type 2 diabetes mellitus, hypertension, dyslipidemia and obesity: a systematic comparison of their impact on cognition. *Biochim Biophys Acta.* (2009) 1792:470–81. doi: 10.1016/j.bbadis.2008.09.004
79. Tsivgoulis G, Alexandrov AV, Wadley VG, Unverzagt FW, Go RCP, Moy CS, et al. Association of higher diastolic blood pressure levels with cognitive impairment. *Neurology.* (2009) 73:589–95. doi: 10.1212/WNL.0b013e3181b38969
80. Morrow SA, Rosehart H, Pantazopoulos K. Anxiety and depressive symptoms are associated with worse performance on objective cognitive tests in MS. *J Neuropsychiatry Clin Neurosci.* (2016) 28:118–23. doi: 10.1176/appi.neuropsych.15070167
81. Leavitt VM, Brandstadter R, Fabian M, Katz Sand I, Klineova S, Krieger S, et al. Dissociable cognitive patterns related to depression and anxiety in multiple sclerosis. *Mult scler.* (2019) 26:1247–55. doi: 10.1177/1352458519860319
82. Ribbons K, Lea R, Schofield PW, Lechner-Scott J. Anxiety levels are independently associated with cognitive performance in an australian multiple sclerosis patient cohort. *J Neuropsychiatry Clin Neurosci.* (2017) 29:128–34. doi: 10.1176/appi.neuropsych.16050085
83. Brooks JO, Hoblyn JC. Neurocognitive costs and benefits of psychotropic medications in older adults. *J Geriatr Psychiatry Neurol.* (2007) 20:199–214. doi: 10.1177/0891988707308803
84. Pardini M, Uccelli A, Grafman J, Yaldizli Ö, Mancardi G, Roccatagliata L. Isolated cognitive relapses in multiple sclerosis. *J Neurol Neurosurg Psychiatry.* (2014) 85:1035–7. doi: 10.1136/jnnp-2013-307275
85. Fenu G, Arru M, Lorefice L, Frau J, Coghe G, Fronza M, et al. Does focal inflammation have an impact on cognition in multiple sclerosis? An MRI study. *Mult Scler Relat Disord.* (2018) 23:83–7. doi: 10.1016/j.msard.2018.05.012
86. Rudy TE, Kerns RD, Turk DC. Chronic pain and depression: toward a cognitive-behavioral mediation model. *Pain.* (1988) 35:129–40. doi: 10.1016/0304-3959(88)90220-5
87. Wattjes MP, Ciccarelli O, Reich DS, Banwell B, de Stefano N, Enzinger C, et al. 2021 MAGNIMS-CMSC-NAIMS consensus recommendations on the use of MRI in patients with multiple sclerosis. *Lancet Neurol.* (2021) 20:653–70. doi: 10.1016/S1474-4422(21)00095-8

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BRIEF RESEARCH REPORT
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Investigating Serum sHLA-G Cooperation With MRI Activity and Disease-Modifying Treatment Outcome in Relapsing-Remitting Multiple Sclerosis

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Relapsing-remitting multiple sclerosis (RRMS) is a demyelinating disease in which pathogenesis T cells have a major role. Despite the unknown etiology, several risk factors have been described, including a strong association with human leukocyte antigen (HLA) genes. Recent findings showed that HLA class I-G (HLA-G) may be tolerogenic in MS, but further insights are required. To deepen the HLA-G role in MS inflammation, we measured soluble HLA-G (sHLA-G) and cytokines serum level in 27 patients with RRMS at baseline and after 12 and 24 months of natalizumab (NTZ) treatment. Patients were divided into high (sHLA-G>20 ng/ml), medium (sHLA-G between 10 and 20 ng/ml), and low (sHLA-G<10 ng/ml) producers. Results showed a heterogeneous distribution of genotypes among producers, with no significant differences between groups. A significant decrease of sHLA-G was found after 24 months of NTZ in low producers carrying the +3142 C/G genotype. Finally, 83.3% of high and 100% of medium producers were MRI-activity free after 24 months of treatment, compared to 63.5% of low producers. Of note, we did not find any correlation of sHLA-G with peripheral cell counts or cytokines level. These findings suggest that serum sHLA-G level may partly depend on genotype rather than peripheral inflammation, and that may have impacted on MRI activity of patients over treatment.

Keywords: multiple sclerosis, natalizumab, serum sHLA-G, cytokines, disease activity

INTRODUCTION

Multiple sclerosis (MS) is a heterogeneous, autoimmune, and inflammatory disease of the central nervous system (CNS), characterized by the disruption of myelin. An interplay of immune mediators contributes to MS pathogenesis, with a crucial role of T lymphocytes (1). About 2.8 million people worldwide are affected with MS (2) and 85% of all patients show a relapsing-remitting pattern of MS (RRMS), characterized by relapses interspersed with periods of

partial or complete recovery (3). The etiology of MS is currently unknown, but several factors have been attributed to a higher risk to develop the disease. The association with human leukocyte antigens (HLA) genes was widely demonstrated (4, 5), as the haplotype HLA-DRB1*1501 in North Europe, the USA, and continental Italy, and DR3 and DR4 in the island of Sardinia (6).

Recently, the non-classical HLA histocompatibility antigen G (HLA-G) has been linked with MS susceptibility, particularly with the RRMS form, in a study performed within the Italian population (7, 8). HLA-G is a non-canonical HLA class I molecule, consisting of a heavy and a light chain, that may exist as membrane-bound or soluble isoforms (9). HLA-G is expressed within a limited variety of tissues, e.g., the thymus and pancreas (10, 11). Furthermore, it was identified on placental trophoblast cells, where it seems to exert a protective role in sustaining immune tolerance between the fetus and the mother during pregnancy (8, 12, 13). Soluble forms of HLA-G (sHLA-G) may exert a regulative and protective role in both normal conditions (14) and disease. sHLA-G was found able to trigger apoptosis of cytotoxic CD8 cells (15, 16) and to shape T-cell phenotype toward regulatory phenotypes (17–19). Promising studies suggested sHLA-G as tolerogenic in MS pathogenesis: higher levels of sHLA-G in the cerebrospinal fluid (CSF) of patients with MS were positively correlated with less inflammation and no disease activity at MRI detection (7, 20, 21), whereas lower serum sHLA-G levels were found in patients with MS having clinically active disease (22).

The human leukocyte antigens G (HLA-G) gene is located on chromosome 6 in the major histocompatibility complex (MHC) locus (23). The expression of HLA-G protein is inherently affected by the genetic polymorphism characterizing the gene locus. The main polymorphisms that regulate the HLA-G production are: (i) a deletion/insertion of 14 base pairs (14 bp) and (ii) a single-nucleotide polymorphism (SNP) where a cytosine substitutes guanine (C>G) at the position +3142 bp in the untranslated region at the 3' of the gene (3' UTR) (23, 24), as demonstrated by Cree and co-authors (24). These polymorphisms impact on mRNA stability *in vivo*: it was demonstrated that the genotypes 14 bp insertion/insertion (ins/ins) and +3142 G/G determine a low production of HLA-G compared to 14 bp insertion/deletion (ins/del) or to the genotypes deletion/deletion (del/del) and +3142 C/G or C/C (20, 25–27). Of note, these functional polymorphisms are associated with MS susceptibility in the Tunisian population (8). Furthermore, the presence of the 14 bpI affects mRNA stability and protein production (27) and is associated with pregnancy pathologies and autoimmune diseases (28, 29). On the other hand, the +3142 G allele binding to 3 microRNAs (miRNAs) miR-148a, miR-148b, and miR-15 is predicted to be more stable than binding to the +3142 C allele, resulting in lower protein production (25). We have previously demonstrated that the highest and the lowest plasma sHLA-G values were identified in patients with MS having +3142 C/C and 14 bp D/D and +3142 G/G and 14 bp I/I genotypes, respectively (30). These findings raised the issue of whether sHLA-G may potentiate the immunomodulant action of MS treatments. A recent study reports a higher serum sHLA-G level in patients with MS under interferon-β compared to healthy individuals, although no differences were found between the overall MS cohort and healthy people (31).

Despite these interesting findings, our knowledge about the role of different HLA-G genotypes in MS pathogenesis and in MS treatment outcomes is still very limited. With the aim to shed light on this topic, we investigated sHLA-G and its genotypes in the serum of patients with RRMS before and after 12 and 24 months of treatment with natalizumab (NTZ), a monoclonal antibody that blocks the alpha4-integrin, or very late antigen-4 (VLA-4), expressed on the surface of T lymphocytes, preventing their migration into the CNS. NTZ is an effective second-line immunomodulant treatment for RRMS, usually applied when first-line treatments fail. NTZ is known to impact immune cell populations, especially leading to a reversible increase of peripheral cell counts due to its mechanism of action (32, 33). With this study, we showed that patients with RRMS are distinguishable in different subgroups based on their serum sHLA-G concentration. Furthermore, when under NTZ, most high and medium sHLA-G producers were free from MRI activity after 24 months of treatment.

MATERIALS AND METHODS

Patients Enrollment: Characteristics and Inclusion Criteria

A total of 27 patients were enrolled at the Department of Neurology II at Careggi University Hospital, Florence, Italy. Patients were diagnosed with RRMS according to McDonald criteria (34) and shared the following characteristics: age between 18 and 60 years; Expanded Disability Status Scale (EDSS) score between 0 and 5.5. Inclusion and exclusion criteria are described in detail in SURPASS study (ClinicalTrials.gov Identifier: NCT01058005). Patient characteristics are reported in **Table 1**. Patients with RRMS included in the study showed

TABLE 1 | Patients' characteristics.

No. of patients	27
F:M ratio	20:7
Age (years) (median, range)	36 (20–52)
Disease duration (months) (average, range)	87.53 (2–188)
EDSS at baseline (T0) (median, range)	2 (1–4)
EDSS at T12 of NTZ (median, range)	1.5 (0–6)
EDSS at T24 of NTZ (median, range)	1.8 (1–6)
ARR[A] last 2 years	2 (0–4)
ARR last year median (range)	1 (0–4)
Treatments pre-NTZ	
IFN[B] (n. of administration) (average, range)	55.75 (5–158)
Cop[C] (n. of administration) (average, range)	39.67 (9–94)
AZA[D] (n. of administration) (average, range)	55.22 (9–94)

[A]ARR, analyzed relapse rate; [B]IFN, interferon-β 1a; [C]Cop, Copaxone; [D]AZA, Azathioprine. In this study, before NTZ 23 patients were treated with IFN, 5 with Cop, 13 with AZA and 4 did not receive any treatment.

highly active disease before NTZ treatment, e.g., failed the first-line treatments or presented rapidly evolving MS with 2 or more relapses in 1 year and 1 or more Gd+ lesions with a significant increment of T2 lesions. At the baseline sample, patients were not receiving any disease-modifying treatments (DMTs).

Study Approval and Patient Consents

The study was performed according to the Declaration of Helsinki. Written informed consent was signed by all patients involved in the study, and approved by the Local Ethical Committee (#CEAVC12745).

Patients' Clinical Follow-Up

During NTZ, patients with RRMS underwent clinical follow-up every 6 months and at least yearly brain MRI, according to clinical practice. All patients with RRMS were evaluated for the presence of anti-JCV and anti-NTZ antibodies in the serum. Clinical MRI and laboratory data were retrospectively collected; occurrence of relapses and MRI activity (new T2 lesions and/or Gd+ lesions) were recorded. Disability was assessed by EDSS score. Additional clinical evaluations were performed upon patients' request in the event of new neurological symptoms or any other neurological issues.

Peripheral Blood and Serum Collection

For each patient, the whole peripheral blood (PB) was collected in heparin-containing tubes. Peripheral blood mononuclear cells (PBMCs) were collected by density gradient centrifugation by Pancoll (density: 1.077 g/ml, PAN-Biotech) at 1,500 rpm, RT, for 30 min within 2 h from PB collection. To collect serum, 10 ml of blood were drawn in serum-separated tubes (SSTs) according to the same time schedule as performed for PB. Serum was then separated from blood by centrifugation at 3,000 rpm, RT, for 10 min, then aliquoted and stored in −80°C freezer until used.

Patients Immunophenotype

Immunophenotype of patients with RRMS during NTZ was analyzed as routine from fresh whole blood upon erythrocyte lysis (BD Lysis buffer, BD Bioscences) by labeling cells with the following panel of fluorescent monoclonal antibodies: anti-CD3 FITC (clone: clone SK7), anti-CD16 PE clone B73.1 and anti-CD56 PE (clone NCAM16.2), anti-CD45 PerCP-Cy5.5 (clone 2D1), anti-CD4 PE-Cy7 (clone: SK3), anti-CD19 APC (clone: SJ25C1), and anti-CD8 APC-Cy7 (clone: SK1). Data acquisition was performed using a 3-laser 8-color flow cytometer (FACSCanto II, BD Biosciences); data were analyzed using FACSDiva software version 8.0.1. (BD Biosciences).

Serum Soluble HLA-G Analysis

The concentration of sHLA-G in serum samples was measured in triplicate by ELISA as previously described (30, 35, 36) by using the capture monoclonal antibody (MoAb) MEM-59 (Exbio, Praha, Czech Republic), which recognizes the β2-microglobulin-associated form of HLA-G. The intra- and inter-assay coefficient of variation (CV) was 1.4 and 4%, respectively. The sensitivity limit was 1.0 pg/ml.

HLA-G Polymorphism Typing

Genomic DNA (gDNA) was isolated from PB by the Nucleon Bacc 3 kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's protocol. The HLA-G polymorphism 14 bp ins/del was genotyped by PCR as previously described (30). Briefly, 100 ng of gDNA were amplified in a 25 μl reaction together with 10 pmol of each primer (GE14H LAG, RHG4). The HLA-G polymorphism +3142 C>G was genotyped by the 7300 Real-Time PCR System (Applied Biosystems) using a forward primer 3142 for (50-CCTTTAATTAACCCAT-CAATCTCTCTTG-30), a reverse primer 3142 rev (50-TGTCTCCGTCTCTGTCTCAAATTT-3), and 2 probes for the identification of the 3142 C (0-VIC-TAAGTTATAGCTCAGTGGAC-30; 3142CFVIC) or the 3142G (50-FAM-TAAGTTA-TAGCTCAGTGCAC-30; 3142GFAM) allele, respectively.

Cytokines and Chemokines Measurement

Serum from each patient was analyzed by Bioplex device (Biorad) using Milliplex assay (Merck Millipore) following the manufacturer's protocol for the determination of the following 17 cytokines and chemokines: IL1α, IL1β, IL2, IL4, IL6, IL8, IL10, IL12p40, IL12p70, IL17, IL23, IFNγ, TNFα, GM-CSF, CXCL10, CXCL13, and MMP9. Cytokines and chemokines concentrations were reported in pg/ml. The sensitivity limit was 1.0 pg/ml.

Statistics

Statistical analyses were performed by IBM SPSS statistic 20 (IBM Corp) and by GraphPad Prism v.6 (GraphPad Software, Inc.). One-way ANOVA followed by *post-hoc* Tukey's test was used to compare patient subgroups (high, medium, and low sHLA-G producers) for peripheral absolute cell counts, serum cytokines level, HLA-G genotype distribution, and sHLA-G level over 24 months of NTZ treatment. Non-parametric Kaplan–Meier estimator or chi-square test were used to compare patient subgroups for MRI disease activity by evaluating the cumulative proportion of survival percentage. Pearson correlation coefficient (r) was calculated to evaluate the correlation between variables. Statistical significance was considered when p-value < 0.05.

RESULTS

Absolute Peripheral Cell Counts of RRMS Cohort Increase, Along With Serum Level of IL2 and TNFα, Over Natalizumab Treatment

Immunophenotypes were analyzed by flow cytometry on fresh blood samples of patients. Phenotypic analyses were performed before starting the treatment (T0) and at 6, 12, and 24 months of NTZ treatment (T6, T12, and T24) and included the evaluation of the percentage and absolute cell count of total T, B, natural killer (NK), lymphokine-activated killer (LAK) cells, and CD4/CD8 ratio. Flow cytometry gating strategy and analysis of cell subsets are reported in **Supplementary Figure 1**. Accordingly to the mechanism of action of NTZ, which blocks immune cells in the periphery, we found an increase in absolute cell counts over time

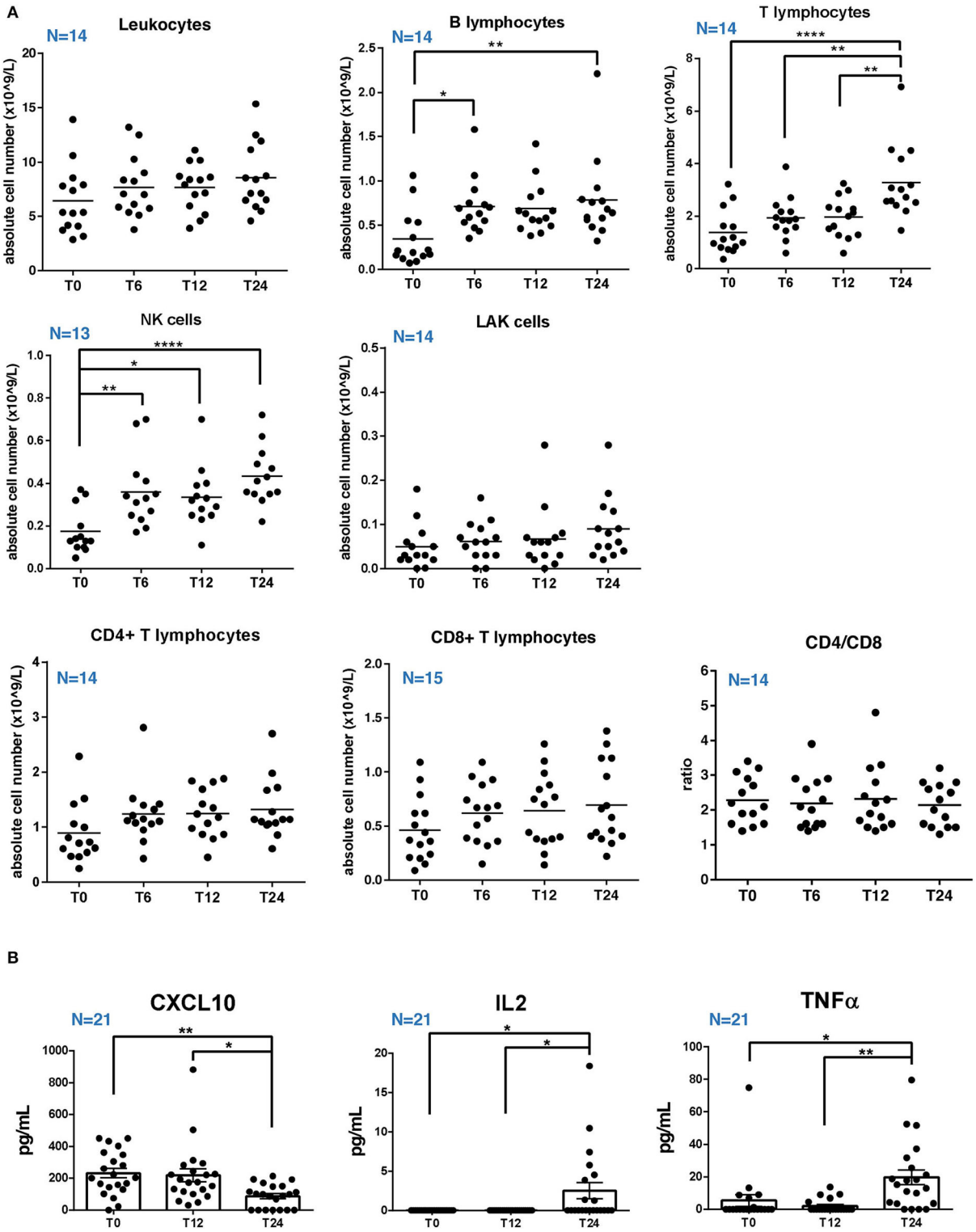

FIGURE 1 | Absolute cell counts of circulating lymphocytes and serum cytokines level in RRMS patients under natalizumab. **(A)** Percentage and absolute cell count of total T, B, natural killer (NK), lymphokine-activated killer (LAK) cells, and the ratio between CD4 and CD8 T cells (CD4/CD8) at baseline (T0) and after 6, 12, or 24 (T6, T12, and T24) months of natalizumab (NTZ) treatment. Each dot represents a patient. Patients' number is shown in blue for each graph. Mean ± SEM is reported. Statistical significance was determined by one-way ANOVA followed by *post-hoc* Tukey test (*$p < 0.05$; **$p < 0.01$; and ****$p < 0.0001$). **(B)** Concentration (pg/ml) of CXCL10, TNFα, and IL2 in serum of patients with RRMS at T0 or at T12 and T24 of NTZ treatment. Each dot represents a patient. Patients' number is shown in blue for each graph. Mean ± SEM is reported. Statistical significance was determined by one-way ANOVA followed by *post-hoc* Tukey test (*$p < 0.05$; **$p < 0.01$).

(**Figure 1A**). Such increase is significant in B lymphocytes at T6 (p-value = 0.04) and T24 (p-value = 0.01) compared to T0; in T lymphocytes at T24 compared to T0 (p-value < 0.0001), T6 (p-value = 0.003), and T12 (p-value = 0.004); and in NK cells at T6 (p-value = 0.007), T12 and T24 (p-value < 0.0001) compared to T0. The increase of total leukocytes and of CD4 and CD8 absolute counts over time was not statistically significant. CD4/CD8 ratio was not altered. On serum of patients with RRMS, we determined the level of 17 cytokines and chemokines at T0, T12, and T24 of NTZ, to evaluate fluctuations in the peripheral inflammatory profile of patients over treatment. In our analysis, IL1α, IL1β, IL2, IL17, IFNγ, IL10, and GM-CSF were below the detection limit (1.0 pg/ml) at all time points, therefore excluded from the analysis. CXCL10, TNFα, and IL2 showed significant variation over time: specifically, we found a significant decrease of CXCL10 at T24 compared to T0 (p-value = 0.004) and T12 (p-value = 0.01); a significant increase of TNFα at T24 compared to T0 (p value = 0.01) and T12 (p-value = 0.001) and a significantly higher level of IL2 at T24 compared to T0 and T12 (p-value = 0.01) (**Figure 1B**). To sum up, T, B, and NK cells significantly augmented over 24 months of NTZ treatment, along with serum levels of TNFα and IL2.

RRMS Patients Under Natalizumab Are Distinguishable Into High, Medium, and Low sHLA-G Producers

We evaluated the levels of sHLA-G in serum samples of 27 patients with RRMS at baseline (T0) and at T12 and T24 of treatment with NTZ. Based on sHLA-G concentration (ng/ml), we considered patients as low producers with a serum sHLA-G level below 10 ng/ml at T0 and as high producers with a serum sHLA-G level up to 20 ng/ml at T0. With an sHLA-G level between 10 and 20 ng/ml, patients were classified as medium producers. Therefore, the 27 patients with RRMS were subdivided into 6 high sHLA-G producers, 7 medium producers, and 14 low producers.

We found that the serum sHLA-G level is significantly (p-value < 0.0001) higher in high producers compared to low and medium producers at baseline (T0) (**Figure 2A**). At T12, sHLA-G is significantly (p-value = 0.006) increased in high producers compared to low producers. No significant differences were detected between groups at T24, and across timepoints within each group (high producers: mean ± SEM at T0 = 38.65 ± 7.24 ng/ml; mean ± SEM at T12 = 38.06 ± 15.68 ng/ml; mean ± SEM at T24 = 26.86 ± 12.25 ng/ml; medium producers: mean ± SEM at T0 = 13.49 ± 0.88 ng/ml; mean ± SEM at T12 = 19.74 ± 9.21 ng/ml; mean ± SEM at T24 = 21.08 ± 14.23 ng/ml; low producers: mean ± SEM at T0 = 3.36 ± 1.07 ng/ml; mean ± SEM at T12 = 1.86 ± 0.79 ng/ml; and mean ± SEM at T24 = 2.61 ± 0.91 ng/ml).

14 bp and +3142 Genotypes Are Differentially Distributed Among High, Low, and Medium sHLA-G Producers

We next genotyped patients with RRMS and divided them according to the results. The group including the 14 bp HLA-G genotype was subdivided based on the polymorphism into ins/ins (I/I), ins/del (I/D), and del/del (D/D) subgroups. The group including the +3142 genotype was subdivided based on the SNP C>G in C/C, C/G, and G/G subgroups.

Globally, we did not find any significant difference in polymorphism distribution among high, medium, and low sHLA-G producers. High producers ($N = 6$) are equally divided between patients carrying the 14 bp I/D and D/D polymorphism. Medium producers ($N = 7$) are mainly represented by the 57.14% of patients carrying the 14 bp I/D genotype, followed by the 28.57% with the D/D genotype and the remaining 14.29% with the I/I one. On the other hand, the 50% of low producers ($N = 14$) were characterized by the 14 bp I/I genotype, whereas the 21.43% and the 28.57% with the 14 bp I/D and D/D genotypes, respectively (**Figure 2B**). Concerning +3142 genotypes (**Figure 2C**), half of the high producers carry the G/G genotype (50%), whereas the other half is divided between C/G (33.33%) and C/C (16.67%) genotypes. Medium producers were typed as C/G (42.86%) or G/G (57.14%); none of them was typed as C/C. Finally, low producers are equally distributed between C/G and G/G genotypes (35.71%), with 28.57% carrying the C/C genotype.

In summary, genotype polymorphism distribution does not significantly differ among sHLA-G producers; of note, 14 bp I/I genotype is not present among high producers, and +3142 C/C among medium producers.

Serum sHLA-G Variation in RRMS Patients Over 24 Months of Natalizumab

To investigate a possible correlation between treatment/genotype and sHLA-G serum level, we evaluated the sHLA-G production during NTZ treatment and the associated genotype distribution.

We did not find any significant variation in sHLA-G production over NTZ treatment among high and medium producers based on 14 bp (**Figures 3A,B**, left panels) or +3142 (**Figures 3A,B**, right panels) polymorphisms. Of note, sHLA-G production is quite stable among +14 bp I/D high producers over 24 months of treatment (mean ± SEM at T0 = 42.65 ± 8.46 ng/ml; mean ± SEM at T12 = 44.80 ± 31.82 ng/ml; and mean ± SEM at T24 = 45.64 ± 18.87 ng/ml) (**Figure 3A**, left graph). On the other hand, sHLA-G production is globally variable among medium producers (**Figure 3B**). Concerning low producers, we observed a significant ($p = 0.032$) decrease of sHLA-G at T24 compared to T0 in patients carrying the +3142 C/G genotype and a significant ($p = 0.038$) decrease at T12 of +3142 G/G patients compared to C/G at T0 (**Figure 3C**). Of note, we did not find any positive correlation, calculated as Pearson coefficient, between sHLA-G levels and peripheral absolute cell counts of patients or with respect to serum cytokines (data not shown).

The Majority of High and Medium Producers Are Free From MRI Activity After 24 Months of Treatment

The occurrence of relapses is rare during NTZ treatment (37). In our sample, among high producers, 2/6 (33.3%) experienced a relapse; among medium producers, 1/7 was lost from follow-up at 17 months and no one of the other 6 patients experienced

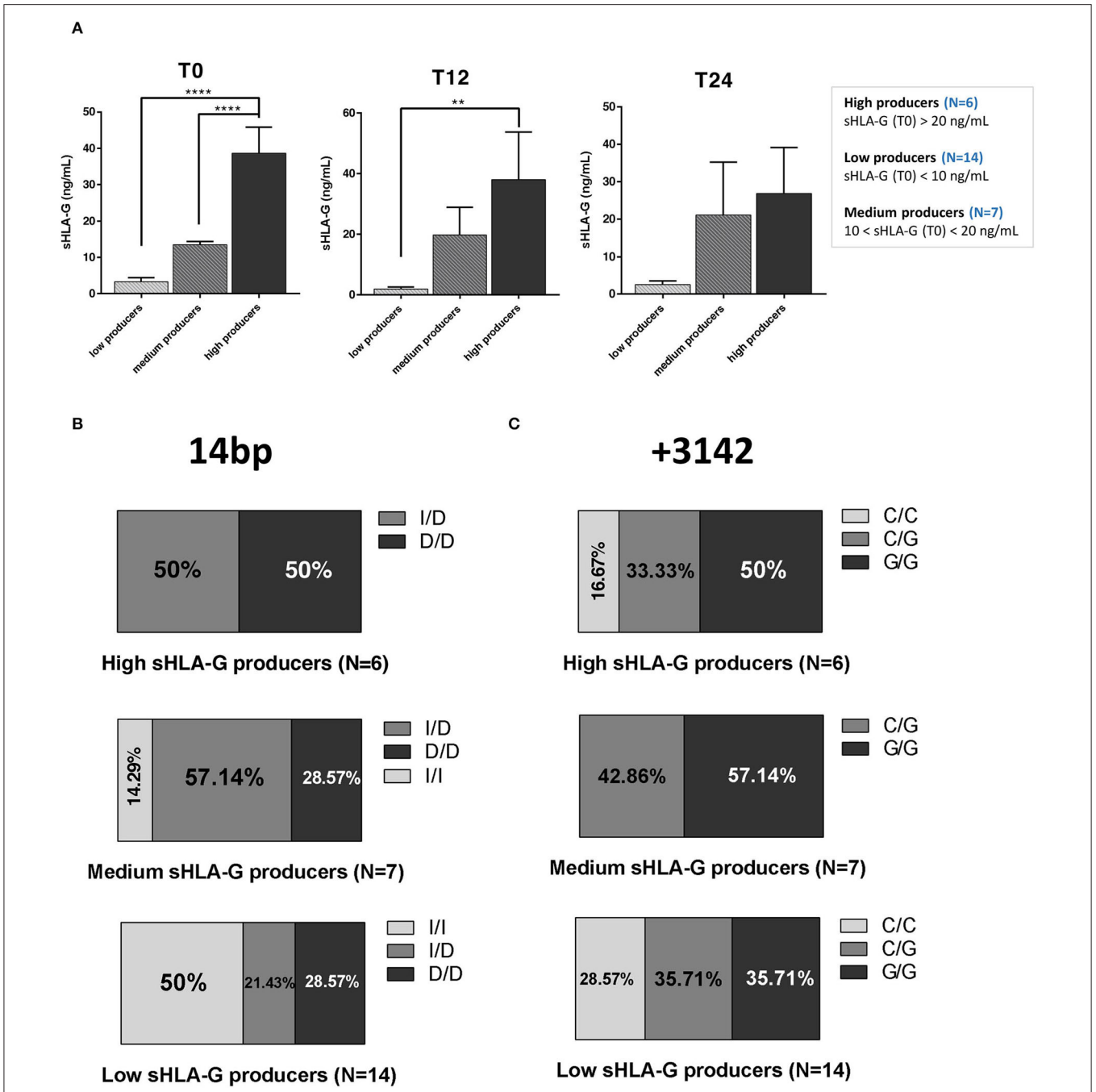

FIGURE 2 | RRMS patients under natalizumab are divided into high, medium, and low sHLA-G producers, who are characterized by different 14 bp and +3142 genotypes distribution. **(A)** sHLA-G production (ng/ml) in low, medium, and high producers at baseline (T0; left graph), at T12 (middle graph), and at T24 (right graph) of NTZ treatment. A total of 27 RRMS were divided into 3 groups (14 low producers, 7 medium producers, and 6 high producers) based on serum sHLA-G concentration, as reported in the legend. Mean ± SEM is reported (one-way ANOVA followed by *post-hoc* Tukey's test; $^{**}p < 0.01$; $^{****}p < 0.0001$). **(B)** Percentage of 3 different genotypes of the 14 bp polymorphism of the HLA-G gene in high, medium, and low sHLA-G producers within the RRMS cohort: insertion/insertion (I/I), insertion/deletion (I/D), and deletion/deletion (D/D). **(C)** Percentage of 3 different genotypes of the +3142 C>G polymorphism (C/C, C/G, and G/G) of the HLA-G gene in high, medium, and low sHLA-G producers within the RRMS cohort. Chi-square test was used.

a relapse. On the other hand, 2/13 (1/14 was lost from follow-up at 17 months) low producers relapsed (15.4%) (**Figure 4A**). Patient MRI shows that 5/13 low producers had new T2 lesions and, among them, 2/5 had Gd-enhancing lesions at follow-up scan. Among these, 2 had a relapse. Concerning other groups, only 1 high producer showed Gd-enhancing lesions or T2 new lesions (**Figure 4B**). Differences among producers were not statistically significant.

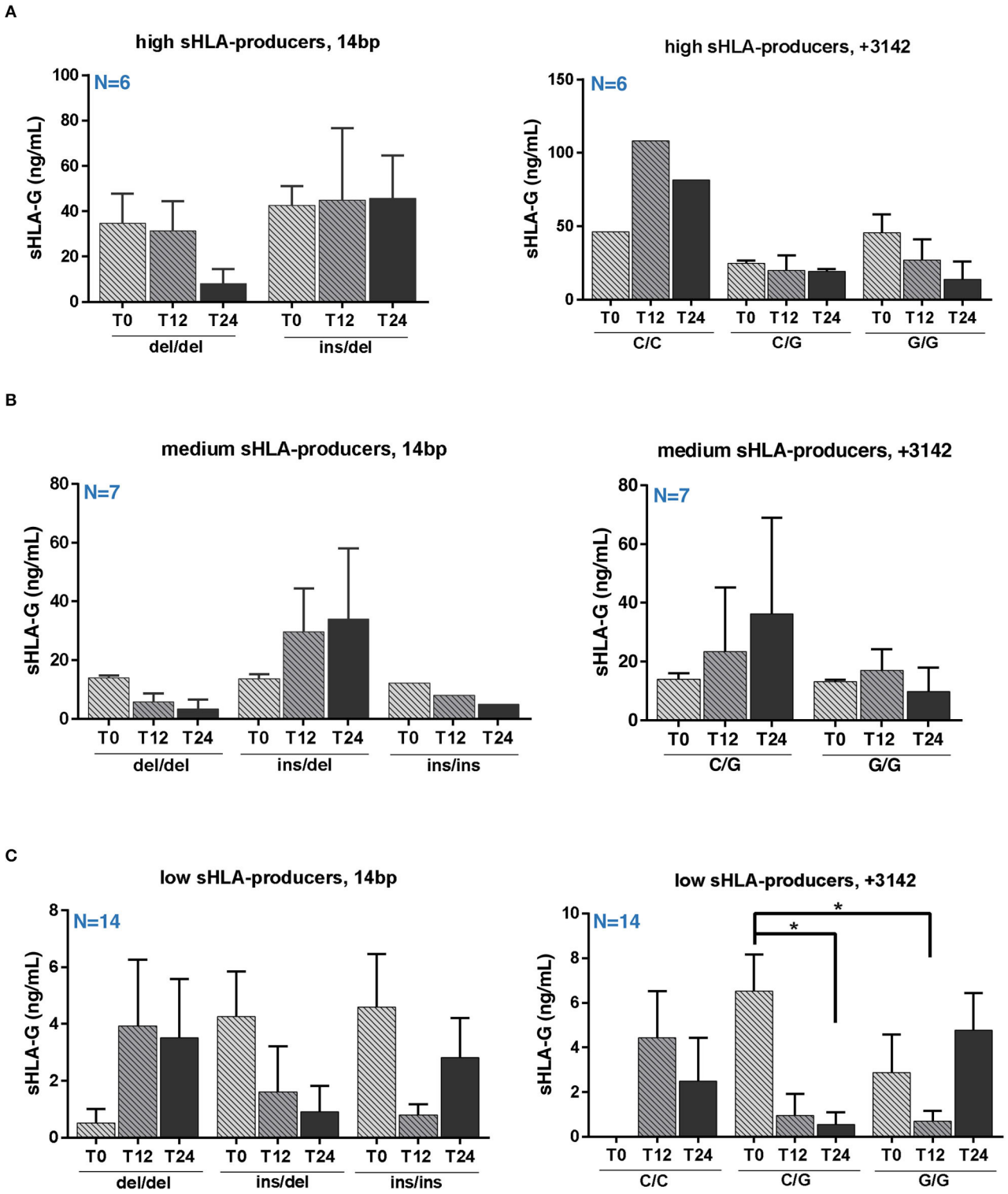

FIGURE 3 | Serum sHLA-G variation in RRMS patients during natalizumab is partially genotype-dependent. Serum sHLA-G production (ng/ml) in RRMS patients at T0, T12, and T24 of NTZ treatment divided patients into high **(A)**, medium **(B)**, and low **(C)** sHLA-G producers. Each group of patients is furtherly distinguished based on their HLA-G genotype (14 bp, left graphs; +3142 bp, right graphs) and polymorphisms. Patients' number is shown in blue for each graph. Mean ± SEM is reported; only the mean is shown for groups including a single observation. Statistical significance was determined by one-way ANOVA followed by *post-hoc* Tukey test (*$p < 0.05$).

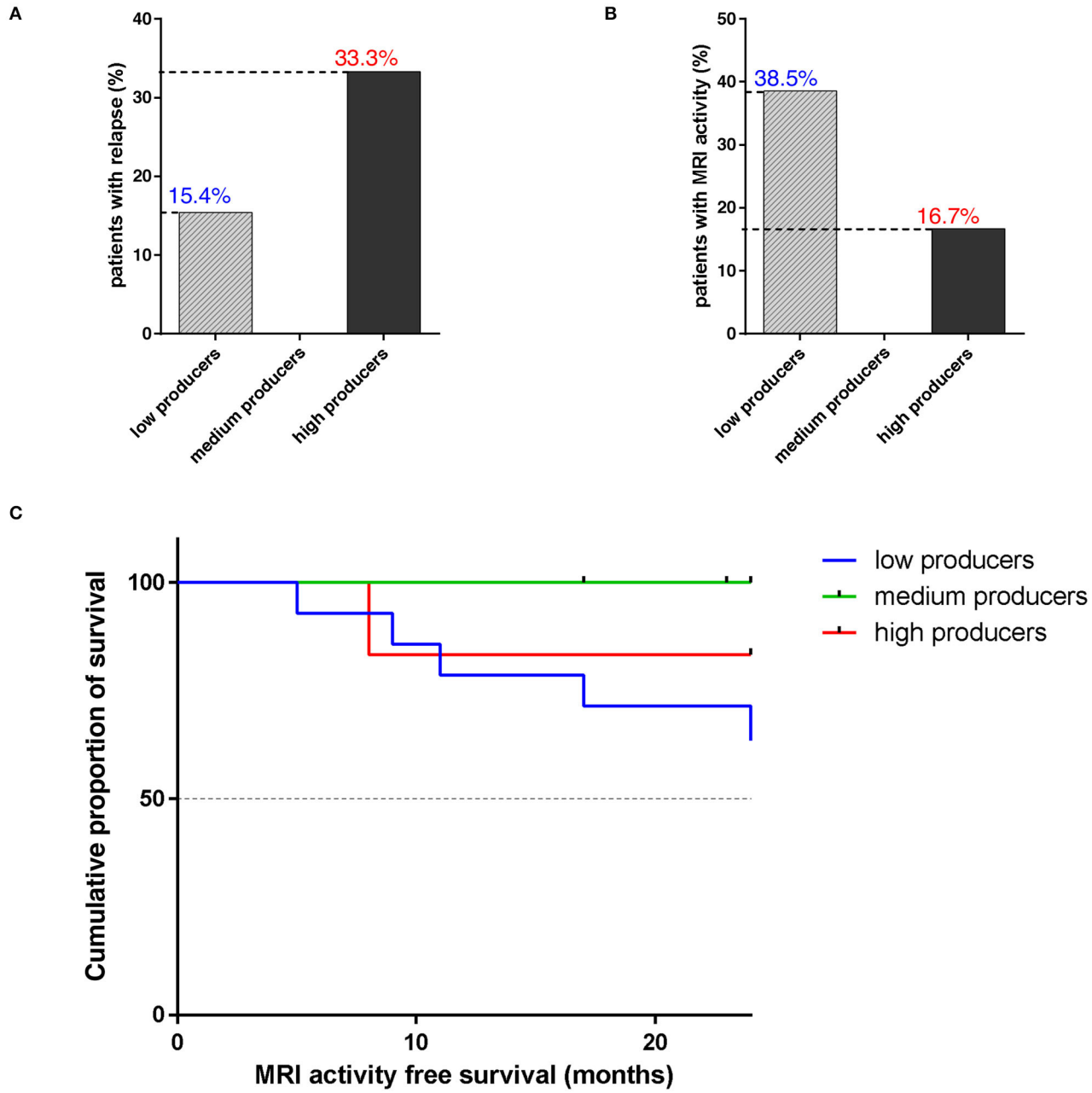

FIGURE 4 | The majority of high and medium sHLA-G producers are free from disease relapse and MRI activity after 24 months of natalizumab. **(A)** Percentage of patients with RRMS, divided into low ($N = 13$; 1/14 was lost from follow-up at 17 months), medium ($N = 6$; 1/7 was lost from follow-up at 17 months), and high ($N = 6$) sHLA-G producers, who experienced a disease relapse during NTZ treatment. Chi-square test was used. **(B)** Percentage of patients with RRMS, divided into low, medium, and high sHLA-G producers, who showed MRI disease activity during treatment. **(C)** Cumulative proportion of survival (percentage) from MRI activity of patients with RRMS over NTZ treatment (time from 0 to 24 months is reported on x-axis) for low (blue line), medium (green line), and high (red line) sHLA-G producers. Chi-square test was used.

Magnetic resonance imaging (MRI) activity-free survival was 83.3% in high sHLA-G producers, 63.5% in low producers, and 100% in medium producers; such difference was not statistically significant (p-value = 0.211) (**Figure 4C**).

DISCUSSION

In this work, we evaluated the sHLA-G production and genotype in patients with RRMS during NTZ treatment. We measured serum sHLA-G level and correlated this feature with treatment

outcome in terms of disease relapses and MRI activity. Our data suggest that low sHLA-G producers are more at risk of showing disease activity during treatment compared to high and medium producers.

Soluble forms of HLA-G (sHLA-G) molecule is known for contributing to maintaining the immune tolerance in both health and disease (15) and has been correlated to a better disease outcome when high in CSF of patients with MS (6). Here, we were able to divide our RRMS cohort into 3 groups, low, medium, and high producers, based on their serum sHLA-G level. Within each group, we did not observe any significant variation in sHLA-G level; instead, we found that sHLA-G concentration is significantly higher in high producers compared to low and medium producers at baseline and with respect to low producers at T12 (**Figure 2A**).

The characterization of patients by sHLA-G genotype revealed that I/D and D/D genotypes are mostly represented in high and medium producers, while I/I genotype is not present among high producers (**Figure 2B**), in accordance with the role of this genotype in controlling HLA-G production (30, 31). In fact, the 14 bp I allele affects mRNA stability and protein production, with the consequent lower secretion of sHLA-G.

When investigating sHLA-G concentration across patients based on their genotypes, we did not observe any significant variation among high and medium producers (**Figures 3A,B**), whereas +3142 C/G low producers reported a significant decrease of sHLA-G after 24 months of NTZ compared to baseline (**Figure 3C**).

The analysis of serum cytokines and chemokines in the RRMS cohort showed a significant decrease in CXCL10 at T24 and a significant increase in TNFα and IL2 at T24 (**Figure 1B**). The increase in cytokines level is expected during NTZ, since the treatment leads to a peripheral enrichment of T cells, including potential pathogenic clones, which is also in agreement with the immunophenotype of patients showing a significant increase in the absolute cell counts over treatment (**Figure 1A**). Comparing these results with sHLA-G variation over time, we did not observe any association between sHLA-G changes and either cytokines or absolute cell counts variations; therefore, we suggest that sHLA-G production does not depend on the numerousness or function of T cells in peripheral blood.

We then evaluated the association between serum sHLA-G production and patients' outcomes to NTZ treatment in terms of relapse rate and MRI disease activity over 24 months (**Figure 4**). Results showed that 33.3% of high, 15.4% of low, and none among medium producers experienced a relapse (**Figure 4A**). On the other hand, 38.5% of low sHLA-G producers, compared to medium (0%) and high (16.7%) showed MRI disease activity (**Figure 4B**). Finally, 83.3% of high and 100% of medium producers were MRI-activity free at T24, with respect to 63.5% of low producers (**Figure 4C**).

Study Limitations and Conclusions

To sum up, we found that sHLA-G significantly decreases over NTZ treatment in low sHLA-G producers carrying the +3142 C/G genotype (**Figure 3C**) and that serum sHLA-G concentration does not correlate with peripheral cell counts or peripheral inflammatory profile. Our findings also showed a variable distribution of HLA-G polymorphisms among producers (**Figures 2B,C**) and, finally, 83.3% of high and 100% of medium producers are free from MRI activity over 24 months of treatment, with respect to 63.5% of low producers (**Figure 4C**). Although interesting, these differences between groups are not statistically significant: the narrowness of our 27-RRMS patient cohort is in fact the main limitation of our study and may have impacted on significance. However, in light of numerous previous findings pointing out sHLA-G's role in modulating immunotolerance in MS (6, 21, 22, 24, 30, 35, 36), we suggest that sHLA-G contributes to NTZ treatment outcome in patients with RRMS and that such contribution would be particularly noticeable when considering MRI activity; therefore, we stress the need for further confirmation by larger cohort size.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study was performed in accordance with the Declaration of Helsinki, reviewed and approved by the Local Ethics Committee of the Tuscany region, Centre Area (#CEAVC12745). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RA experiments (PBMCs and serum collection, DNA isolation, and cytokines assay), manuscript writing, and data analysis. RR, DB, and VG experiments (patients HLA-G typization and sHLA-G quantification) and data analysis. AM manuscript writing and clinical data analysis. EB and AA PBMCs and serum collection, cytokines assay, and data analysis. AC data analysis. BP flow cytometry analysis of patients' immunophenotype. AR clinical data collection. LM critical discussion on clinical data. EF MRI data collection. CB study conceptualization and manuscript writing revision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REFERENCES

1. Jelcic I, Al Nimer F, Wang J, Lentsch V, Planas R, Jelcic I, et al. Memory B cells activate brain-homing, autoreactive CD4+ T cells in multiple sclerosis. *Cell.* (2018) 175:85–100.e23. doi: 10.1016/j.cell.2018.08.011
2. Walton C, King R, Rechtman L, Kaye W, Leray E, Marrie RA, et al. Rising prevalence of multiple sclerosis worldwide: insights from the Atlas of MS, third edition. *Mult Scler Houndmills Basingstoke Engl.* (2020) 26:1816–21. doi: 10.1177/1352458520970841
3. Milo R, Kahana E. Multiple sclerosis: geoepidemiology, genetics and the environment. *Autoimmun Rev.* (2010) 9:A387–94. doi: 10.1016/j.autrev.2009.11.010
4. Ballerini C. HLA–multiple sclerosis association in Continental Italy and correlation with disease prevalence in Europe. *J Neuroimmunol.* (2004) 150:178–85. doi: 10.1016/j.jneuroim.2004.01.015
5. Barcellos LF, Oksenberg JR, Green AJ, Bucher P, Rimmler JB, Schmidt S, et al. Genetic basis for clinical expression in multiple sclerosis. *Brain J Neurol.* (2002) 125:150–8. doi: 10.1093/brain/awf009
6. Cocco E, Sardu C, Pieroni E, Valentini M, Murru R, Costa G, et al. HLA-DRB1-DQB1 haplotypes confer susceptibility and resistance to multiple sclerosis in sardinia. *PLoS ONE.* (2012) 7:e33972. doi: 10.1371/journal.pone.0033972
7. Fainardi E, Rizzo R, Castellazzi M, Stignani M, Granieri E, Baricordi OR. Potential role of soluble human leukocyte antigen-G molecules in multiple sclerosis. *Hum Immunol.* (2009) 70:981–7. doi: 10.1016/j.humimm.2009.07.014
8. Ben Fredj N, Sakly K, Bortolotti D, Aissi M, Frih-Ayed M, Rotola A, et al. The association between functional HLA-G 14bp insertion/deletion and +3142 C>G polymorphisms and susceptibility to multiple sclerosis. *Immunol Lett.* (2016) 180:24–30. doi: 10.1016/j.imlet.2016.10.006
9. Paul P, Cabestre FA, Ibrahim EC, Lefebvre S, Khalil-Daher I, Vazeux G, et al. Identification of HLA-G7 as a new splice variant of the HLA-G mRNA and expression of soluble HLA-G5, -G6, and -G7 transcripts in human transfected cells. *Hum Immunol.* (2000) 61:1138–49. doi: 10.1016/S0198-8859(00)00197-X
10. Cirulli V, Zalatan J, McMaster M, Prinsen R, Salomon DR, Ricordi C, et al. The class I HLA repertoire of pancreatic islets comprises the nonclassical class Ib antigen HLA-G. *Diabetes.* (2006) 55:1214–22. doi: 10.2337/db05-0731
11. Mallet V, Blaschitz A, Crisa L, Schmitt C, Fournel S, King A, et al. HLA-G in the human thymus: a subpopulation of medullary epithelial but not CD83(+) dendritic cells expresses HLA-G as a membrane-bound and soluble protein. *Int Immunol.* (1999) 11:889–98. doi: 10.1093/intimm/11.6.889
12. Ellis SA, Palmer MS, McMichael AJ. Human trophoblast and the choriocarcinoma cell line BeWo express a truncated HLA Class I molecule. *J Immunol.* (1990) 144:731–5.
13. Rizzo R, Melchiorri L, Stignani M, Baricordi OR. HLA-G expression is a fundamental prerequisite to pregnancy. *Hum Immunol.* (2007) 68:244–50. doi: 10.1016/j.humimm.2006.10.012
14. Rebmann V, Pfeiffer K, Pässler M, Ferrone S, Maier S, Weiss E, et al. Detection of soluble HLA-G molecules in plasma and amniotic fluid. *Tissue Antigens.* (1999) 53:14–22. doi: 10.1034/j.1399-0039.1999.530102.x
15. Fournel S, Aguerre-Girr M, Huc X, Lenfant F, Alam A, Toubert A, et al. Cutting edge: soluble HLA-G1 triggers CD95/CD95 ligand-mediated apoptosis in activated CD8+ cells by interacting with CD8. *J Immunol.* (2000) 164:6100–4. doi: 10.4049/jimmunol.164.12.6100
16. Horuzsko A, Lenfant F, Munn DH, Mellor AL. Maturation of antigen-presenting cells is compromised in HLA-G transgenic mice. *Int Immunol.* (2001) 13:385–94. doi: 10.1093/intimm/13.3.385
17. Kanai T. Soluble HLA-G influences the release of cytokines from allogeneic peripheral blood mononuclear cells in culture. *Mol Hum Reprod.* (2001) 7:195–200. doi: 10.1093/molehr/7.2.195
18. LeMaoult J, Caumartin J, Daouya M, Favier B, Le Rond S, Gonzalez A, et al. Immune regulation by pretenders: cell-to-cell transfers of HLA-G make effector T cells act as regulatory cells. *Blood.* (2007) 109:2040–8. doi: 10.1182/blood-2006-05-024547
19. Rodi M, Dimisianos N, de Lastic AL, Sakellaraki P, Deraos G, Matsoukas J, et al. Regulatory cell populations in Relapsing-Remitting Multiple Sclerosis (RRMS) patients: effect of disease activity and treatment regimens. *Int J Mol Sci.* (2016) 17:1398. doi: 10.3390/ijms17091398
20. Hviid TVF, Rizzo R, Christiansen OB, Melchiorri L, Lindhard A, Baricordi OR, et al. HLA-G and IL-10 in serum in relation to HLA-G genotype and polymorphisms. *Immunogenetics.* (2004) 56:135–41. doi: 10.1007/s00251-004-0673-2
21. Fainardi E, Rizzo R, Melchiorri L, Castellazzi M, Paolino E, Tola MR, et al. Intrathecal synthesis of soluble HLA-G and HLA-I molecules are reciprocally associated to clinical and MRI activity in patients with multiple sclerosis. *Mult Scler.* (2006) 12:2–12. doi: 10.1191/1352458506ms1241oa
22. Fainardi E, Granieri E, Tola MR, Melchiorri L, Vaghi L, Rizzo R, et al. Clinical and MRI disease activity in multiple sclerosis are associated with reciprocal fluctuations in serum and cerebrospinal fluid levels of soluble HLA class I molecules. *J Neuroimmunol.* (2002) 133:151–9. doi: 10.1016/S0165-5728(02)00348-X
23. Carosella ED, Moreau P, Lemaoult J, Rouas-Freiss N. HLA-G from biology to clinical benefits. *Trends Immunol.* (2008) 29:125–32. doi: 10.1016/j.it.2007.11.005
24. Cree BA, Rioux JD, McCauley JL, Gourraud PA, Goyette P, McElroy J, et al. A major histocompatibility Class I locus contributes to multiple sclerosis susceptibility independently from HLA-DRB1*15:01. *PLoS ONE.* (2010) 5:e11296. doi: 10.1371/journal.pone.0011296
25. Veit TD, Chies JB. Tolerance versus immune response – microRNAs as important elements in the regulation of the HLA-G gene expression. *Transpl Immunol.* (2009) 20:229–31. doi: 10.1016/j.trim.2008.11.001
26. Consiglio CR, Veit TD, Monticielo OA, Mucenic T, Xavier RM, Brenol JC, et al. Association of the HLA-G gene +3142C>G polymorphism with systemic lupus erythematosus. *Tissue Antigens.* (2011) 77:540–5. doi: 10.1111/j.1399-0039.2011.01635.x
27. Tan Z, Randall G, Fan J, Camoretti-Mercado B, Brockman-Schneider R, Pan L, et al. Allele-specific targeting of microRNAs to HLA-G and risk of asthma. *Am J Hum Genet.* (2007) 81:829–34. doi: 10.1086/521200
28. Yan WH, Lin A, Chen XJ Dai MZ, Gan LH, Zhou MY, et al. Association of the maternal 14-bp insertion polymorphism in the HLA-G gene in women with recurrent spontaneous abortions. *Tissue Antigens.* (2006) 68:521–3. doi: 10.1111/j.1399-0039.2006.00723.x
29. Hviid TVF, Christiansen OB. Linkage disequilibrium between human leukocyte antigen (HLA) class II and HLA-G–possible implications for human reproduction and autoimmune disease. *Hum Immunol.* (2005) 66:688–99. doi: 10.1016/j.humimm.2005.03.003
30. Rizzo R, Bortolotti D, Fredj NB, Rotola A, Cura F, Castellazzi M, et al. Role of HLA-G 14bp deletion/insertion and +3142C>G polymorphisms in the production of sHLA-G molecules in relapsing-remitting multiple sclerosis. *Hum Immunol.* (2012) 73:1140–6. doi: 10.1016/j.humimm.2012.08.005
31. Jahanbani-Ardakani H, Alsahebfosoul F, Etemadifar M, Salehi R, Eskandari N, Abtahi SH, et al. HLA-G gene polymorphism and soluble HLA-G serum level in patients with multiple sclerosis. *APMIS.* (2018) 126:538–9. doi: 10.1111/apm.12846
32. Plavina T, Muralidharan KK, Kuesters G, Mikol D, Evans K, Subramanyam M, et al. Reversibility of the effects of natalizumab on peripheral immune cell dynamics in MS patients. *Neurology.* (2017) 89:1584–93. doi: 10.1212/WNL.0000000000004485
33. Saraste M, Penttilä TL, Airas L. Natalizumab treatment leads to an increase in circulating CXCR3-expressing B cells. *Neurol Neuroimmunol Neuroinflamm.* (2016) 3:e292. doi: 10.1212/NXI.0000000000000292
34. Carroll WM. 2017 McDonald MS diagnostic criteria: evidence-based revisions. *Mult Scler.* (2018) 24:92–5. doi: 10.1177/1352458517751861
35. Fainardi E, Rizzo R, Melchiorri L, Stignani M, Castellazzi M, Tamborino C, et al. CSF levels of soluble HLA-G and Fas molecules are inversely associated to MRI evidence of disease activity in patients with relapsing-remitting multiple sclerosis. *Mult Scler.* (2008) 14:446–54. doi: 10.1177/1352458507085137
36. Fainardi E, Rizzo R, Melchiorri L, Stignani M, Castellazzi M, Caniatti ML, et al. Soluble HLA-G molecules are released as HLA-G5 and not as soluble HLA-G1 isoforms in CSF of patients with relapsing-remitting multiple sclerosis. *J Neuroimmunol.* (2007) 192:219–25. doi: 10.1016/j.jneuroim.2007.10.002
37. Perumal J, Fox RJ, Balabanov R, Balcer LJ, Galetta S, Makh S, et al. Outcomes of natalizumab treatment within 3 years of relapsing-remitting

multiple sclerosis diagnosis: a prespecified 2-year interim analysis of STRIVE. *BMC Neurol.* (2019) 19:116. doi: 10.1186/s12883-019-1337-z

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BRIEF RESEARCH REPORT
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Metabolomics of Cerebrospinal Fluid in Multiple Sclerosis Compared With Healthy Controls: A Pilot Study

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Background: Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS) leading to the loss of myelin and axons. Diagnosis is based on clinical findings, MRI, and analysis of cerebrospinal fluid (CSF). CSF is an ultrafiltrate of plasma and reflects inflammatory processes in the CNS. The aim of this study was to perform metabolomics analysis of CSF in patients after the first attack of MS and healthy controls and try to find new specific analytes for MS including those potentially predicting disease activities at the onset.

Methods: We collected CSF from 19 patients (16 females, aged 19–55 years) after the first attack of clinical symptoms who fulfilled revised McDonald criteria of MS and CSF of 19 controls (16 females, aged 19–50 years). Analyses of CSF samples were provided using the high-performance liquid chromatography system coupled with a mass spectrometer with a high-resolution detector (TripleTOF 5600, AB Sciex, Canada).

Results: Approximately 130 selected analytes were identified, and 30 of them were verified. During the targeted analysis, a significant decrease in arginine and histidine and a less significant decrease in the levels of asparagine, leucine/isoleucine, and tryptophan, together with a significant increase of palmitic acid in the patient group, were found.

Conclusion: We observed significant differences in amino and fatty acids in the CSF of newly diagnosed patients with MS in comparison with controls. The most significant changes were observed in levels of arginine, histidine, and palmitic acid that may predict inflammatory disease activity. Further studies are necessary to support these findings as potential biomarkers of MS.

Keywords: multiple sclerosis, CSF, metabolomics, biomarker, fatty acids, amino acids, arginine

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INTRODUCTION

The increasing incidence of autoimmune diseases in the population poses a serious problem to contemporary medicine. One such condition is multiple sclerosis (MS), a severe autoimmune inflammatory disease of the central nervous system (CNS) that primarily affects the young, working age population. The disease is chronic and progressive in nature and leads to a disability due to multifocal demyelination and axonal loss. The CNS white matter is predominantly involved (1).

The exact cause of MS is still unknown. It is believed that various pathogenic mechanisms may play a role in disease development, including genetic and environmental factors. The pathology of MS is characterized by demyelination of the axons in the CNS as a result of malfunctioning autoimmune processes. Initially, the myelin loss is reversed by the oligodendrocytes. However, repetitive loss and repair of the myelin, microglial activation, and leukocyte infiltration due to increased permeability of the blood-brain barrier (BBB) along with decreased oligodendrocyte efficiency result in long-term axonal degeneration, neuronal death, and plaque formation. The subsequent accumulation of neurological damage and progression of neurodegenerative processes ultimately lead to irreversible neurological disability (2). Diagnosis of MS is based on a combination of clinical (3) and magnetic resonance imaging (MRI) observations, supported by findings in cerebrospinal fluid (CSF) (oligoclonal bands or increased intrathecal immunoglobulin production) (4).

Cerebrospinal fluid analysis remains a valuable, supporting diagnostic test for MS (5). It reflects the inflammatory processes occurring in the CNS in detail. Metabolomics is the systematic study of unique chemical fingerprints that specific cellular processes leave behind. It targets metabolites—small molecular substrates and products of metabolism. This metabolic profiling can help to gain insight into the current state of cellular metabolism.

The desire for early treatment of MS and to assign each patient to the most suitable therapy is hampered by the lack of useful prognostic biomarkers that could predict disease progression, severity, and responses to treatment. In recent years, several studies have investigated the metabolomics of CSF in MS. The reported results suggested significant differences between patients with MS and healthy controls. The significant changes were found in amino acid and lipid groups (6–11), where they reported decreased levels of tyrosine, leucine, and phenylalanine. Other studies (12, 13) found significant differences in CSF fatty acids between the groups. One of the most notable findings was an increase in glutamate levels in patients with MS with active lesions observed in CSF (14, 15) and in serum (16, 17). Several contradicting reports on myoinositol and choline results have been published. Some authors (9, 18, 19) found increased levels of choline and myoinositol, whereas others have observed a decreased value (20).

The aim of this study was to analyze the metabolomics of CSF in patients with MS and compare them with healthy controls and establish a statistically significant list of differences in CSF of patients with MS and potentially identify MS-specific analytes. We have performed an untargeted approach, but based on our results, we focused on the groups of amino acids and fatty acids, as they play important roles in the pathophysiology of MS. Fatty acids form a part of myelin, and amino acids have a close connection to immunological processes building elements of proteins and functioning as neurotransmitters.

MATERIALS AND METHODS

Recruitment of Patients

Recruitment of suspected patients with MS was conducted using the database of the Centre for Multiple Sclerosis, Third Faculty of Medicine, Charles University and University Hospital Královské Vinohrady (FNKV). We have already selected 19 patients (16 females, 3 males, mean age of 36 years) after the first attack of clinical signs and symptoms, who fulfilled revised McDonald criteria for MS from 2017 (5) (onset of the disease, without specific Disease Modifying Drugs (DMD) treatment, with MS-specific MRI finding, and meet CSF criteria for diagnosis of MS) and 19 controls (16 females 3 males, mean age of 35 years) with normal neurological status, normal CSF findings, and no anamnesis of autoimmune disorders. Subjects chosen to be part of the control group mostly suffer from sensory disturbances, dizziness, polymorphic complaints, or headache but with negative objective clinical or paraclinical findings to define a specific neurological disease. This group was defined as symptomatic controls [according to already published data (21)] aged between 18 and 55 years, with no use of psychopharmacological drugs (which can alter CSF composition) and no history of other autoimmune disorders.

All subjects participating in this study provided consent and received a full explanation about the entire study. All subjects underwent baseline serum and CSF assessment and clinical and brain and/or spinal cord MRI examination according to their clinical signs and symptoms. In the case of the patient group with MS, the CSF was on average collected on the 15th day from the beginning of clinical signs and symptoms; two exceptions (patients no. 12 and 18) had their CSF collected 4 months from the onset of symptoms, and a further two patients (no. 11 and 19) saw their symptoms last for years before CSF collection.

CSF Sample Preparation

Cerebrospinal fluid samples collected by lumbar puncture were carefully thawed on ice. After thawing, the samples for the first analysis (untargeted metabolomics) were vortexed, and 100 μl was transferred into a precooled Eppendorf tube. An immediate addition of 400 μl of ice-cold ACN:MeOH mixture (1:1, v/v) was used to maintain the MeOH:ACN:H_2O (2:2:1, v/v) ratio. The samples were vortexed for 30 s and consequently incubated for 1 h at −20°C. Incubation was followed by 10 min of centrifugation at 13,000 rpm at 4°C for 20 min. The supernatant was transferred and evaporated to dryness by speedVAc. The dry extract was reconstituted in 100 μl of H_2O:MeOH (1:1, v/v) and sonicated for 10 min. The insoluble debris was removed by centrifugation (13,000 rpm for 10 min at 4°C), and the supernatant was transferred into a vial and directly analyzed by liquid chromatography–mass spectrometry (LC–MS).

Samples for targeted analysis of amino acids were prepared the same way as is described above. Samples for analysis of fatty acids were extracted according to the modified Bligh and Dyer method (Bligh and Dyer, 1959). Notably, 100 μl of thawed CSF was mixed with 400 μl of chloroform-methanol 50:50 (v/v) with added internal standard and placed in an ultrasound bath for 5 min. Extraction was performed for 2 h at 4°C, followed by

the addition of 100 μl of mili-Q water, 15 s of vortexing, and centrifugation at 4,000 rpm for 10 min at 4°C. The upper and lower phases divided by thin protein discs were pooled together and dried under a nitrogen stream. Samples were reconstituted with 100 μl of MeOH/IPA/H_2O 65:35:5 (v/v/v), vortexed for 10 s, and sonicated for 5 min before injection.

High-Performance Liquid Chromatography With Tandem Mass Spectrometry (HPLC-MS/MS) Analysis

All analyses were performed on a Thermo Ultimate 3000 coupled with a high-resolution AB Sciex TripleTOF 5600 mass spectrometer. During the first round of analyses, untargeted metabolomics analyses were all the collected samples, together with quality control samples and blank samples, injected in both positive and negative (ESI+, ESI–) modes using the information-dependent acquisition (IDA) method. Samples were analyzed *via* separation on a Phenomenex high-performance liquid chromatography (HPLC) Kinetex C18 2.6 μm 150 × 3 mm column. The column temperature was maintained constant at 30°C. The mobile phase was composed of A = 0.1% formic acid in water and B = 0.1% formic acid in 100% MeOH for both positive and negative modes, the linear elution gradient from 5% B (0–2 min) to 100% B (18–23 min) was applied, the initial gradient conditions were restored within 2 min (23–25 min), and the last 5 min of the HPLC method (25–30 min) were applied to maintain the beginning conditions. The flow rates were 220 μl min^{-1}, and the sample injection volume was 5 μl. Samples were held in an autosampler at 4°C, and each sample was injected twice for each mass/charge (*m*/*z*) range (50–500 Da, 500–1,200 Da). The ESI source conditions were set as follows: ion source gas 1 (GS1) 35 psi, ion source gas 2 (GS2) 30 psi, curtain gas (CUR) 25 psi, ion spray voltage 4,000 V, and source temperature 300°C.

The method that targeted the determination of small metabolites including amino acids was slightly modified from the previous one. The separation was achieved on the Phenomenex Kinetex C18 2.6 μm 150 × 3 mm HPLC column with the following eluent system: A = 0.1% HCOOH, 2.5 mM NFPA in water, and B = 0.1% HCOOH in MeOH. A linear gradient (5–100% B) from 2nd to 12th min was used, with a flow rate of 0.22 ml min^{-1}. The injection volume was set to 5 μl. Samples were held in an autosampler at 4°C, and each sample was injected twice for an *m*/*z* range of 50–500 Da. The ESI source conditions were set as follows: ion GS1 35 psi, ion GS2 30 psi, CUR 25 psi, ion spray voltage 4,500 V, and source temperature 400°C.

Separation of targeted fatty acids (palmitic, stearic, oleic, and arachidonic) was performed with C18 reverse-phase column – Phenomenex Kinetex C18, 2.6 μm, 150 × 3 mm column at 45°C. Mobile phase A consisted of 1% 1 M NH_4Ac and 0.1% acetic acid in water and mobile phase B consisted of acetonitrile/isopropanol 7:3 (v/v) with 1% 1 M NH_4Ac and 0.1% acetic acid, with an injection volume of 5 μl. The following gradient was applied: 1 min — 50% of B; 3 min – linear gradient from 50% B to 80% B; 8 min – linear gradient from 80% B to 90% B; 13 min – linear gradient from 90% B to 100% B; 15 min — 100% B; 17 min — 50% B; 20 min — 50% B; with a constant flow rate of the mobile phase of 300 μl/min. Data were acquired in TOF MS full scan and IDA in both ESI+ and ESI- modes. The source parameters were set as follows: GAS1: 50 psi; GAS2: 45 psi; CUR: 30 psi; TEM: 300°C; ISVF: 5,500 V in positive mode and −4,500 V in negative mode, respectively.

Data Processing

The liquid chromatography with tandem mass spectrometry (LC-MS/MS) data was processed using Sciex OS software (version 1.3 with Formula Finder plug-in, AB SCIEX, Canada), which offers the evaluation of the retention time (RT) and *m*/*z* variability of the experiment. MarkerView software (version 1.3.1, AB SCIEX, Canada) was used in the second step to process raw LC-HRMS data (peak detection, alignment, data filtering, and determining the m/z ratio, RT, and ion peak area for each sample). Data mining was performed by the program algorithm—the peak intensity cutoff was set at 100 cps. Peak settings were achieved using RT and *m*/*z* tolerances of 0.1 min and 0.005 Da, respectively. Monoisotopic peaks alone were considered to reduce mass redundancy and enhance the selection of a true molecular feature. Finally, mass signals differentially expressed by the control and case study samples (Sclerosis Multiplex) were identified by applying an additional filtering procedure with fold change (<1.5) and *t*-test ($p > 0.05$). This whole procedure is necessary for the elimination of the background and contaminants and preserved the true biological mass signals from the LC-HRMS data. The following steps were carried out using the MetaboAnalyst 5.0 Web Server. Acquired and filtered data from MetaboAnalyst 5.0 were, in the following step, verified with previously acquired data from targeted analyses (analyses of ∼80 standards include amino acids, fatty acids, and other small metabolites already set into the spectral library using Sciex OS software).

Statistical Data Analysis

The Student's *t*-test was used for the comparison between the control group and sclerosis multiplex group, followed by the application of the Benjamini-Hochberg false discovery rate (FDR) correction for multiple comparisons to minimize false positives.

RESULTS

The demographic and clinical data of the suspected patients with MS are summarized in **Supplementary Table S1**. All recruited patients with MS had hyperintensive lesions on MRI that meet revised McDonald criteria (5). All the patients with MS presented brain MRI lesions in the supratentorial region, and 11 of them also had hyperintensive lesions in the spinal cord MRI. All suspected patients with MS had positive IgG oligoclonal bands (OCB) in CSF with a median of 8 IgG OCB in the whole spectrum (ranging from 1 to 17), negative aquaporin 4 antibodies, and negative myelin oligodendrocyte glycoprotein antibodies in serum and CSF. More detailed information about the CSF findings and OCB is presented in **Supplementary Table S2**. Patients with MS and control group subjects have the normal level of proteins in CSF. Some of the patients with MS (no. 1, 3, 6,

9, 10, 12, and 16) presented increased numbers of mononuclear cells in CSF, but without any signs of neuroinfection or other kinds of neuroinflammation other than that caused by MS.

Subjects from both groups (suspected patients with MS and control group) had no history of psychopharmacological drug use. Four patients with MS (no. 6, 11, 14, and 18) had a history of hormonal anticonception use, and another three patients with MS (no. 6, 11, and 12) had a history of using levothyroxinum at a daily dose of 50 μg.

In the metabolic pathways of fatty acids and amino acids, significant differences were found in the CSF between the two mentioned groups. The most significant differences were observed in arginine, histidine, and palmitic acid. Statistically, the most significant results were found in the level of arginine (p-value: 0.007), where a lower level of arginine was observed in patients with MS (mean responses 2.91909) and then in the control group (mean responses 3.91752) (**Figure 1**). In histidine (p-value: 0.012), we found a significantly lower level in patients with MS (mean responses 5.31349) than in the control group (mean responses 7.27157) (**Figure 2**). The level of palmitic acid (p-value: 0.039) was significantly higher in patients with MS (mean responses 1.28959) than in the control group (mean responses 1.02343) (**Figure 3**). In contrast, no statistically significant changes were observed in asparagine (p-value: 0.1135), leucine/isoleucine (p-value: 0.1325), and tryptophan (p-value: 0.1384), whereby the levels of these analytes were lower in the patients with MS than in the control group, respectively (for more details, refer to **Table 1**).

The quality control (QC) sample represents an analytical approach employing a sample produced and utilized by the operators to guarantee the quality of the measured data and results. Due to the number of collected and analyzed samples (including QC samples), the analyses required a runtime of ∼120 h. Random injections of QC samples throughout the long runtime ensured no signal changes while performing the experiment. The analytical system provided uniform profiles and yielded excellent reproducibility even after the entire analytical runtime.

DISCUSSION

In this study, we showed statistically significant differences in the CSF metabolomics of patients after the first attack of clinical signs of symptoms fulfilling revised McDonald criteria for MS in comparison to healthy controls. The most significant differences were found in the groups of amino acids and fatty acids, especially with decreased levels of arginine, histidine, asparagine, leucine/isoleucine, and tryptophan and an increased level of palmitic acid. These CSF metabolomics results could become new potential markers in the early stage of MS and can potentially be used in the prediction of the disease severity in the future. In the future, the specificity of these potentially MS-specific analytes needs to be verified.

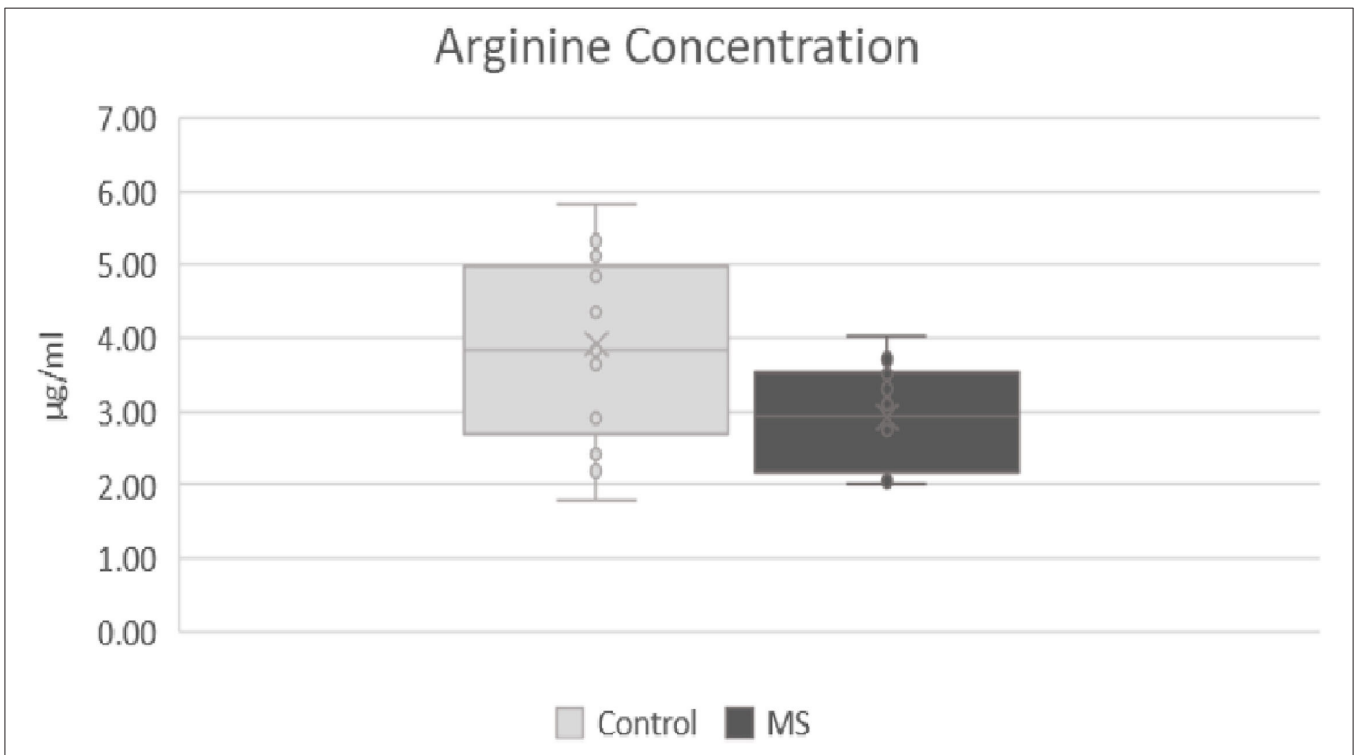

FIGURE 1 | Comparison of levels of arginine in patients after first attack of clinical symptoms fulfilling revised McDonald criteria and control group. MS, patients after first attack of clinical symptoms fulfilling revised McDonald criteria; control, control group.

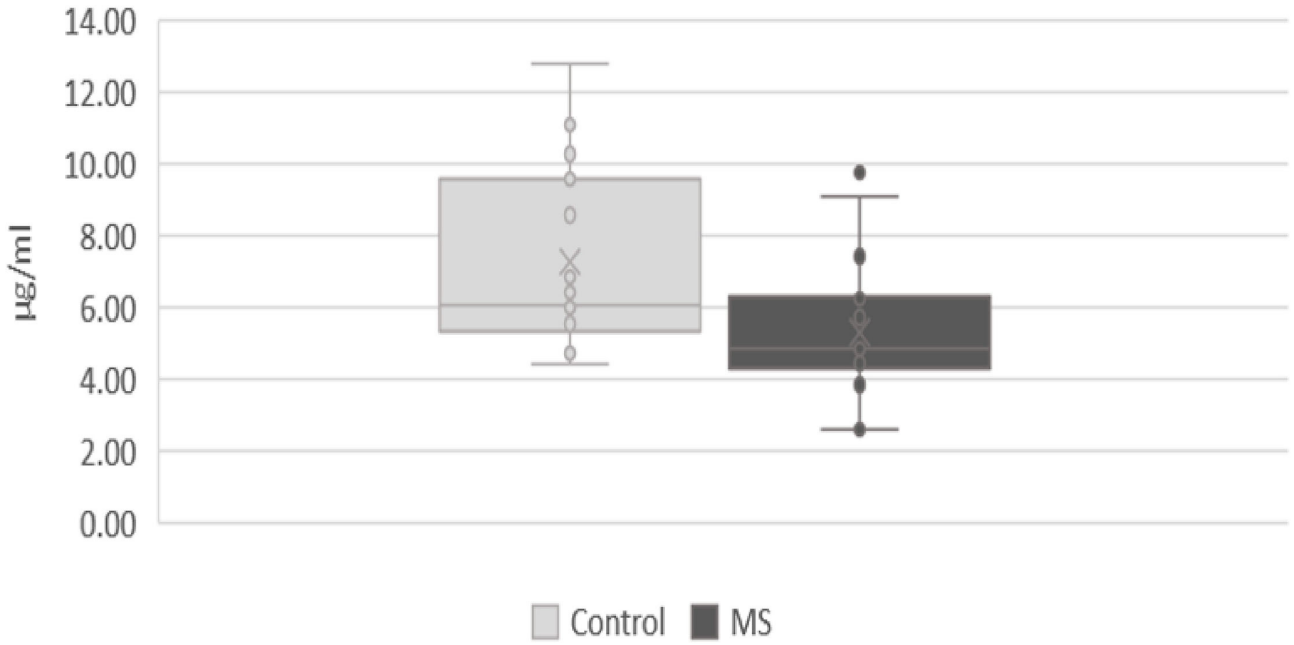

FIGURE 2 | Comparison of levels of histidine in patients after first attack of clinical symptoms fulfilling revised McDonald criteria and control group. MS, patients after first attack of clinical symptoms fulfilling revised McDonald criteria; control, control group.

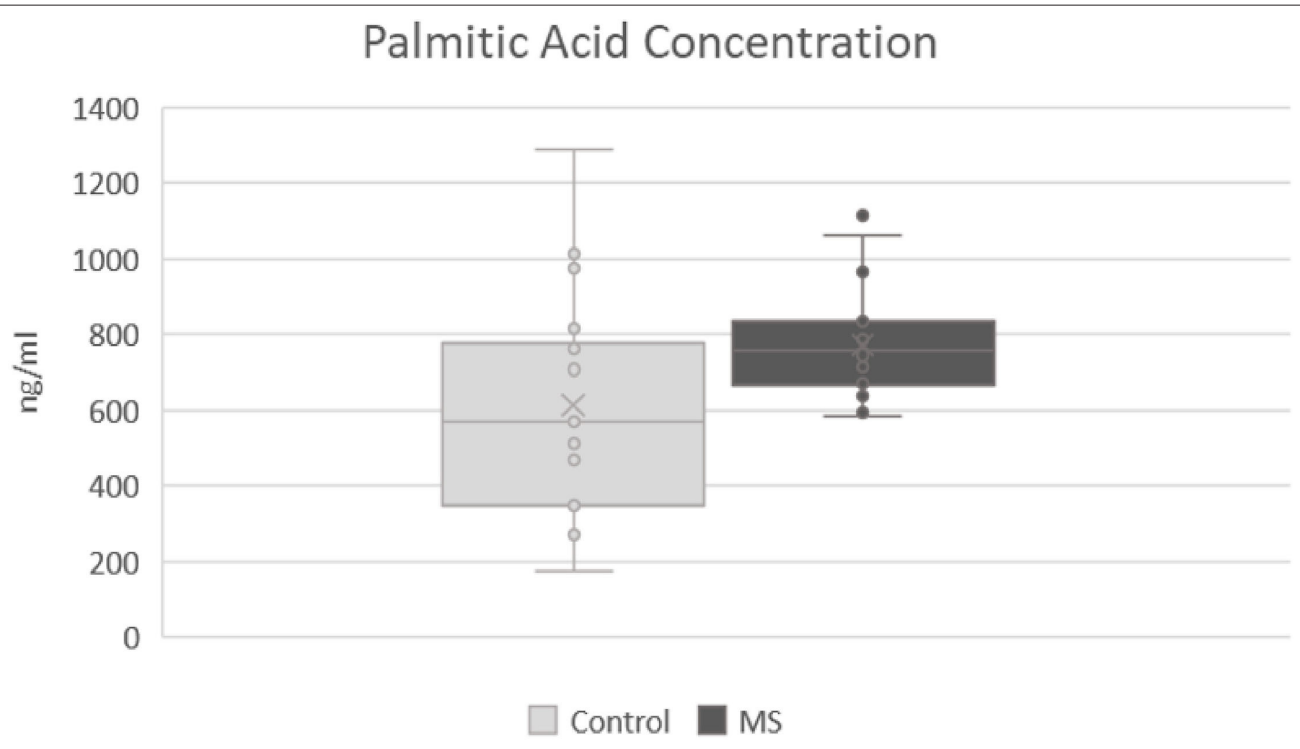

FIGURE 3 | Comparison of levels of palmitic acid in patients after first attack of clinical symptoms fulfilling revised McDonald criteria and control group. MS, patients after first attack of clinical symptoms fulfilling revised McDonald criteria; control, control group.

TABLE 1 | Detailed results of cerebrospinal fluid (CSF) analysis.

Peak name	m/z	*t*-value	*p*-value	Mean of response in MSp	Mean of response in controls	Standard deviation in MSp	Standard deviation in controls
Arginine	175.1194	−2.11990	0.00704	2.91909916	3.9175231	0.6900299	1.2255753
Histidine	156.0772	−2.07235	0.01201	5.31349736	7.2715724	2.0317250	2.5291640
Palmitic acid	257.2494	1.745883	0.03968	1.28959022	1.0234377	0.2596009	0.4869935
Asparagine	133.0797	−1.62355	0.11358	11.5585353	14.179993	4.1724773	5.8151468
Leucine/isoleucine	132.1017	−1.55221	0.13252	0.04613981	0.1116484	0.0757918	0.1719756
Tryptophane	205.0977	−1.51469	0.13841	0.79384388	1.1638709	0.7753553	0.7488412
Lysine	147.113	−1.36629	01806	5.45858649	6.4206882	1.8475467	2.5147676
Cystein	122.043	−1.36132	0.18933	0.0014778	0.1835504	0.0064416	0.5980968
Threonine	120.0662	1.315061	0.19673	0.40598326	0.2515651	0.3813894	0.3502126
Phenylalanine	166.086	−1.32739	0.19894	0.00881132	0.0509445	0.0273785	0.1391445
Glutamate	148.0604	1.193139	0.24041	0.263029	0.294388	0.209961	0.241989
Oleic acid	283.2638	0.868702	0.39148	0.47934379	0.243562	0.9783302	0.6825356
Arachidonic acid	305.2454	−0.94153	0.35309	0.35530687	0.523647	0.4516842	0.6516032
Stearic acid	285.2803	−0.1646	0.87019	0.21131172	0.2398059	0.4726169	0.6035298

MSp, patients after first attack of clinical symptoms fulfilling revised McDonald criteria; m/z, mass spectrum.

Cerebrospinal fluid is the most important biological sample that can help us to understand the pathology of MS. CSF can be used for measurements of various soluble markers and cell populations. It is also considered the "gold standard" matrix in MS diagnostics. However, CSF collection is an invasive procedure and is, therefore, only collected on rare occasions. The majority of proteins found in CSF are blood-derived. These proteins cross the BBB and reach the CSF compartment *via* passive diffusion. CSF is a better medium to identify potential biomarkers of MS due to the lower amount of different proteins. It reflects the actual state of CNS through possible inflammatory processes.

Several authors have already published the results of CSF metabolomics in patients with MS (e.g., 5, 6, 9, and 10); however, they collected samples from patients with MS in various stages of the disease with different DMD treatments and specific pharmacological history. In our study, we have clearly homogenous MS and control patient groups. In the case of MS, we focused on patients after their first attack of clinical signs and symptoms, without a history of psychopharmacological drug use and fulfilling revised McDonald criteria for MS, without any kind of specific medication such as DMDs or high-dosage corticosteroid pulse. Some other authors recruited people in the control group from different types of neurological diseases, even inflammatory CNS diseases such as meningoencephalitis. We used symptomatic controls (21), meaning patients with non-specific complaints, suffering mostly from sensory disturbances, dizziness, or headache, with negative objective clinical or paraclinical findings.

Few reports from last year even focused on the metabolomics of blood samples of patients with MS (10, 22) and found decreased levels in amino acids, more specifically phenylalanine, tyrosine, and tryptophan (10) and modified asparagine and carnitine (22). In our study, we focused solely on CSF; therefore, we cannot relevantly compare our findings.

Amino acids play an important role in the CNS and in the immune system, not only as a "building material" for proteins but also as precursors of neurotransmitters, which have important roles in inflammatory processes and pathogenesis of MS (23). Some authors have published a decreased level of arginine in patients with MS (6–9). This observation can be explained by its mechanism in the metabolism of nitrite oxide (NO). L-arginine is a precursor of NO, which is a neurotransmitter with a potential role in the pathogenesis of MS (24). NO is synthesized from arginine in the regular way by endothelial NO synthase (eNOS) and neuronal NO synthase (nNOS). These forms produce low concentrations of NO in a calcium-dependent way. At sites of inflammation, acute/active lesions in MS, another form of enzyme starts to produce NO. This form of enzyme produces high concentrations of NO and is not dependent on calcium concentrations (25, 26). In MS, the concentrations of NO were found to be increased, especially in locations of active lesions (24, 27). In this study, we did not study NO; however, we found a significantly lower level of arginine in our patients with MS after their first clinical attack. We can speculate that arginine can be a suitable analyte of disease activity at the early onset of MS, but we cannot confirm its specificity for MS. In the next steps, it should be compared with other types of inflammations in the CNS.

Histidine, which was also found to be decreased in MS (6–9), is a precursor of neurotransmitter histamine, synthesized in histaminergic neurons of the tuberomammillary nucleus in the posterior third of the hypothalamus. Histamine plays an important role in the inflammatory processes as well as in the pathogenesis of MS, but its role is not yet certainly established (28). Several studies found increased levels of histamine in the CSF of patients with MS (29–31), which can potentially explain the lower levels of histidine, its precursor. In our study, we have found decreased levels of histidine in patients with MS; therefore, our results are in agreement with others.

In already published reports, leucine/isoleucine and branched-chained amino acid have been found to be decreased in MS (6–9). Leucine/isoleucine has an important role in protein synthesis, as a key nitrogen donor, and in cell growth

and proliferation. During inflammation, there is an increased synthesis of many different proteins and increased immune cell growth, which means increased demand for branched-chained amino acids resulting in a decrease of leucine/isoleucine. In our study, we found a slightly decreased level of leucine/isoleucine, but not a statistically significant one.

Some authors have been studying glutamate and found its high level in the CSF of patients with MS (14, 15). In this study, we did not find significant differences in glutamate in MS compared with the control group. One explanation of this result could be that we have investigated subjects in the early stage of MS, where the acute inflammatory process of demyelination is supposed to be at the beginning of the disease. Other authors have studied patients in different stages of MS, meaning that the most dominant process of axonal destruction followed by an increase in extracellular glutamate may occur in the later phase of MS (32).

We also observed a significantly increased level of palmitic acid that was in agreement with several studies (13, 33). During an acute attack of MS, there is a loss of myelin, consisting mostly of lipids. Myelin plays an important role in normal nerve transmission. In demyelination, an increase in fatty acids as the basic components of lipids and myelin (34) can be found.

Contrary to our results, some authors have reported a decreased level of palmitic acid in MS (12, 13), probably due to the heterogeneity of patients with MS with early and/or late stages of the disease with remyelination or demyelination. The decrease of palmitic acid might be explained by the process of remyelination, in which myelin is being recreated by oligodendrocytes and thereby consuming fatty acids as basic components of myelin (34). In this study, we recruited patients with MS in the early stage of disease with an ongoing first attack of the disease, meaning that the process of demyelination might be the dominant mechanism and, therefore, with increased levels of palmitic acid.

CONCLUSION

In this study, we concluded that arginine, histidine, and palmitic acid may be used as analytes potentially specific for the early stages of MS. Their specificity need yet to be verified, but they still may be used in verification of ongoing inflammation or active lesion in CNS in the early stages of MS.

Decreased levels of arginine and histidine can be explained by their role as precursors of neurotransmitters (arginine as a precursor of NO and histidine as a precursor of histamine), which are significantly increased in the inflammatory processes of MS, and therefore, precursor consumption is increased.

Palmitic acids, as the basic component of fatty acids, are involved in the demyelination process, and therefore, their increased levels may be found in the early stages of MS.

The potential use and specificity of these analytes (arginine, histidine, and palmitic acid) need to be more thoroughly examined in a larger group of patients with MS to establish their role in disease progression and compare them to other inflammatory diseases of CNS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of University Hospital Kralovske Vinohrady under number EK-VP/64/0/20. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MŽ contributed to manuscript writing, collecting and analyzing the data. IŠ was a major contributor in manuscript writing and final approval. MŽ, DK, and KV analyzed and interpreted the patient data. MŽ, ZS, and DZ performed clinical investigation and data collection. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2022.874121/full#supplementary-material

REFERENCES

1. Weiner HL. Multiple sclerosis is an inflammatory T-cell-mediated autoimmune disease. *Arch Neurol.* (2004) 61:1613–5. doi: 10.1001/archneur.61.10.1613
2. Reich DS, Lucchinetti CF, Calabresi PA. Multiple sclerosis. *N Engl J Med.* (2018) 378:169–80. doi: 10.1056/NEJMra1401483
3. Kurtzke JF. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology.* (1983) 33:1444–52. doi: 10.1212/WNL.33.11.1444

4. Hauser SL, Chan JR, Oksenberg JR. Multiple sclerosis: prospects and promise: multiple sclerosis. *Ann Neurol.* (2013) 74:317–27. doi: 10.1002/ana.24009
5. Thompson AJ, Banwell BL, Barkhof F, Carroll WM, Coetzee T, Comi G, et al. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *Lancet Neurol.* (2018) 17:162–73. doi: 10.1016/S1474-4422(17)30470-2
6. Sylvestre DA, Slupsky CM, Aviv RI, Swardfager W, Taha AY. Untargeted metabolomic analysis of plasma from relapsing-remitting multiple sclerosis patients reveals changes in metabolites associated with structural changes in brain. *Brain Res.* (2020) 1732:146589. doi: 10.1016/j.brainres.2019.146589
7. Park SJ, Jeong IH, Kong BS, Lee J-E, Kim KH, Lee DY, et al. Disease type- and status-specific alteration of CSF metabolome coordinated with clinical parameters in inflammatory demyelinating diseases of CNS. *PLoS ONE.* (2016) 11:e0166277. doi: 10.1371/journal.pone.0166277
8. Kasakin MF, Rogachev AD, Predtechenskaya EV, Zaigraev VJ, Koval VV, Pokrovsky AG. Targeted metabolomics approach for identification of relapsing-remitting multiple sclerosis markers and evaluation of diagnostic models. *Medchemcomm.* (2019) 10:1803–9. doi: 10.1039/C9MD00253G
9. Reinke SN, Broadhurst DL, Sykes BD, Baker GB, Catz I, Warren KG, et al. Metabolomic profiling in multiple sclerosis: insights into biomarkers and pathogenesis. *Mult Scler.* (2014) 20:1396–400. doi: 10.1177/1352458513516528
10. Fitzgerald KC, Smith MD, Kim S, Sotirchos ES, Kornberg MD, Douglas M, et al. Multi-omic evaluation of metabolic alterations in multiple sclerosis identifies shifts in aromatic amino acid metabolism. *Cell Rep Med.* (2021) 2:100424. doi: 10.1016/j.xcrm.2021.100424
11. Nogueras L, Gonzalo H, Jové M, Sol J, Gil-Sanchez A, Hervás JV, et al. Lipid profile of cerebrospinal fluid in multiple sclerosis patients: a potential tool for diagnosis. *Sci Rep.* (2019) 9:11313. doi: 10.1038/s41598-019-47906-x
12. Gonzalo H, Brieva L, Tatzber F, Jové M, Cacabelos D, Cassanyé A, et al. Lipidome analysis in multiple sclerosis reveals protein lipoxidative damage as a potential pathogenic mechanism. *J Neurochem.* (2012) 123:622–34. doi: 10.1111/j.1471-4159.2012.07934.x
13. Oliveira EML de, Montani DA, Oliveira-Silva D, Rodrigues-Oliveira AF, Matas SL de A, Fernandes GBP, et al. Multiple sclerosis has a distinct lipid signature in plasma and cerebrospinal fluid. *Arq Neuropsiquiatr.* (2019) 77:696–704. doi: 10.1590/0004-282x20190122
14. Pieragostino D, D'Alessandro M, di Ioia M, Rossi C, Zucchelli M, Urbani A, et al. An integrated metabolomics approach for the research of new cerebrospinal fluid biomarkers of multiple sclerosis. *Mol Biosyst.* (2015) 11:1563–72. doi: 10.1039/C4MB00700J
15. Sinclair AJ, Viant MR, Ball AK, Burdon MA, Walker EA, Stewart PM, et al. NMR-based metabolomic analysis of cerebrospinal fluid and serum in neurological diseases - a diagnostic tool? *NMR Biomed.* (2009) 23:123–32. doi: 10.1002/nbm.1428
16. Sarchielli P, Greco L, Floridi A, Floridi A, Gallai V. Excitatory amino acids and multiple sclerosis: evidence from cerebrospinal fluid: evidence from cerebrospinal fluid. *Arch Neurol.* (2003) 60:1082–8. doi: 10.1001/archneur.60.8.1082
17. Poddighe S, Murgia F, Lorefice L, Liggi S, Cocco E, Marrosu MG, et al. Metabolomic analysis identifies altered metabolic pathways in multiple sclerosis. *Int J Biochem Cell Biol.* (2017) 93:148–55. doi: 10.1016/j.biocel.2017.07.004
18. Srinivasan R, Sailasuta N, Hurd R, Nelson S, Pelletier D. Evidence of elevated glutamate in multiple sclerosis using magnetic resonance spectroscopy at 3 T. *Brain.* (2005) 128:1016–25. doi: 10.1093/brain/awh467
19. Cocco E, Murgia F, Lorefice L, Barberini L, Poddighe S, Frau J, et al. H-NMR analysis provides a metabolomic profile of patients with multiple sclerosis. *Neurol Neuroimmunol Neuroinflamm.* (2016) 3:e185. doi: 10.1212/NXI.0000000000000185
20. Podlecka-Pietowska A, Kacka A, Zakrzewska-Pniewska B, Nojszewska M, Zieminska E, Chalimoniuk M, et al. Altered cerebrospinal fluid concentrations of hydrophobic and hydrophilic compounds in early stages of multiple sclerosis-metabolic profile analyses. *J Mol Neurosci.* (2019) 69:94–105. doi: 10.1007/s12031-019-01336-6
21. Teunissen C, Menge T, Altintas A, Álvarez-Cermeño JC, Bertolotto A, Berven FS, et al. Consensus definitions and application guidelines for control groups in cerebrospinal fluid biomarker studies in multiple sclerosis. *Mult Scler.* (2013) 19:1802–9. doi: 10.1177/1352458513488232
22. Levi I, Gurevich M, Perlman G, Magalashvili D, Menascu S, Bar N, et al. Potential role of indolelactate and butyrate in multiple sclerosis revealed by integrated microbiome-metabolome analysis. *Cell Rep Med.* (2021) 2:100246. doi: 10.1016/j.xcrm.2021.100246
23. Li P, Yin Y-L, Li D, Woo Kim S, Wu G. Amino acids and immune function. *Br J Nutr.* (2007) 98:237–52. doi: 10.1017/S000711450769936X
24. Smith KJ, Lassmann H. The role of nitric oxide in multiple sclerosis. *Lancet Neurol.* (2002) 1:232–41. doi: 10.1016/S1474-4422(02)00102-3
25. Koprowski H, Zheng YM, Heber-Katz E, Fraser N, Rorke L, Fu ZF, et al. In vivo expression of inducible nitric oxide synthase in experimentally induced neurologic diseases. *Proc Natl Acad Sci USA.* (1993) 90:3024–7. doi: 10.1073/pnas.90.7.3024
26. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. *Biochem J.* (2001) 357:593. doi: 10.1042/bj3570593
27. Encinas JM, Manganas L, Enikolopov G. Nitric oxide and multiple sclerosis. *Curr Neurol Neurosci Rep.* (2005) 5:232–8. doi: 10.1007/s11910-005-0051-y
28. Jadidi-Niaragh F, Mirshafiey A. Histamine and histamine receptors in pathogenesis and treatment of multiple sclerosis. *Neuropharmacology.* (2010) 59:180–9. doi: 10.1016/j.neuropharm.2010.05.005
29. Molnár G, Moldován J. Histamine content of the cerebrospinal fluid in multiple sclerosis. A preliminary communication. *Acta Med Acad Sci Hung.* (1966) 22:271–4.
30. Tuomisto L, Kilpeläinen H, Riekkinen P. Histamine and histamine-N-methyltransferase in the CSF of patients with multiple sclerosis. *Agents Actions.* (1983) 13:255–7. doi: 10.1007/BF01967346
31. Kallweit U, Aritake K, Bassetti CL, Blumenthal S, Hayaishi O, Linnebank M, et al. Elevated CSF histamine levels in multiple sclerosis patients. *Fluids Barriers CNS.* (2013) 10:19. doi: 10.1186/2045-8118-10-19
32. Hinzman JM, Thomas TC, Burmeister JJ, Quintero JE, Huettl P, Pomerleau F, et al. Diffuse brain injury elevates tonic glutamate levels and potassium-evoked glutamate release in discrete brain regions at two days post-injury: an enzyme-based microelectrode array study. *J Neurotrauma.* (2010) 27:889–99. doi: 10.1089/neu.2009.1238
33. Andersen SL, Briggs FBS, Winnike JH, Natanzon Y, Maichle S, Knagge KJ, et al. Metabolome-based signature of disease pathology in MS. *Mult Scler Relat Disord.* (2019) 31:12–21. doi: 10.1016/j.msard.2019.03.006
34. Morell P, Quarles RH. *The Myelin Sheath*. Philadelphia, PA: Lippincott-Raven (1999).

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BRIEF RESEARCH REPORT
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Cognitive and Mood Profiles Among Patients With Stiff Person Syndrome Spectrum Disorders

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An emerging body of evidence suggests that changes in cognitive and emotional function are common aspects of stiff person spectrum disorders (SPSD). We sought to examine the pattern of cognitive impairment and psychiatric symptoms in SPSD.

Methods: A retrospective review of medical records was conducted for patients seen at the Johns Hopkins Stiff Person Syndrome (SPS) center from 1997 to January 1st, 2020. Individuals who had received formal cognitive testing as part of routine clinical care for patient-reported cognitive changes were included. Demographics, prevalence of cognitive impairment, psychoactive medication use, and clinically significant psychiatric symptoms were described.

Results: Out of 205 patients screened, 20 completed cognitive testing (75% female, mean age 47.4 years). The most common domains of impairment were verbal learning and recall memory (n = 14, 70%), verbal fluency (n = 10, 50%), processing speed (n = 8, 40%), and attention (n = 8, 40%). 9/11 patients assessed for depression reported clinically significant symptoms, and 4/9 patients assessed for anxiety reported clinically significant symptoms.

Conclusions: Screening for cognitive impairment in SPSD should utilize testing that assesses verbal learning and recall, phonemic verbal fluency, attention, and processing speed. Moreover, it is important to evaluate for co-existing depression and anxiety symptoms, as these are common in SPSD.

Keywords: stiff person syndrome, cognition, attention, verbal fluency, depression, anxiety

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INTRODUCTION

Stiff person spectrum disorders (SPSD) are immune-mediated disorders most often characterized by rigidity, unpredictable and painful spasms, and heightened sensitivity to external stimuli (1). Anti-glutamic acid decarboxylase 65 (anti-GAD65) antibodies are thought to play a role in the GABAergic dysfunction in SPSD. While it is classified as a neurologic disorder, research is limited regarding the effects of stiff person syndrome (SPS) on cognitive and emotional function (1, 2).

SPSD has been associated with lower than expected performance on cognitive testing relative to estimated premorbid intelligence (3). Furthermore, the presence of anti-GAD65 antibody has been associated with cognitive impairment in patients with neurological conditions (4), type 2 diabetes (5), and in animal models (6). In addition to cognitive dysfunction, patients with SPSD are also more likely than the general population to report anxiety and depressive symptoms, and to regularly use prescription benzodiazepines and muscle relaxants (7), all of which may contribute to poor performance on cognitive testing (8–10). To our knowledge, only two prior studies have assessed cognitive symptoms in patients with SPSD (3, 11). While one also included measures of psychiatric symptoms (3), neither study reported on psychiatric symptoms or patterns of medication use in the context of cognitive performance.

The aims of this case series were to: (1) describe the pattern of cognitive impairment in patients with SPSD who reported concerns of cognitive impairment and participated in cognitive testing as part of routine clinical care; and (2) examine the frequency of mood symptoms and use of benzodiazepines and muscle relaxants in the most commonly impaired cognitive domains.

METHODS

A retrospective review of medical records was conducted for patients seen at the Johns Hopkins SPS center from 1997 to January 1st, 2020. All patients had provided informed consent to participate in a longitudinal observational study of clinical characteristics in SPS, approved by the Johns Hopkins Institutional Review Board.

Medical records were reviewed for formal cognitive testing, performed by either a licensed psychologist or a speech and language pathologist, as part of routine clinical care for patient-reported cognitive changes. Information on demographics, clinical characteristics, medical comorbidities, and medications at the time of cognitive testing were extracted. Patients with limbic encephalitis, co-existing intractable epilepsy, and/or other neurological conditions known to affect cognitive performance (e.g., Alzheimer's disease, multiple sclerosis, etc.) were excluded.

As a retrospective review of cognitive testing performed as part of routine clinical care, cognitive testing batteries used were determined at the discretion of the provider and therefore not standardized. Details of cognitive testing reports were extracted; results were interpreted as "impaired" if records included descriptive labels of "abnormal", "extremely low", or "weak". If no descriptive interpretation was offered, an adjusted percentile score of <2 or z-score of <-2 (e.g., more than 2 standard deviations below mean) was interpreted as "impaired" (12). If standardized instruments of psychological symptoms (e.g., depression and/or anxiety) were administered, the scores and descriptive labels (e.g., "clinically significant") were extracted.

Demographic and clinical characteristics were evaluated using descriptive statistics, *t*-test for continuous variables and chi-squared test for dichotomous variables using R Studio Version 1.2.5033 (13). Significance was set at $p < 0.05$. Frequency of domain-specific cognitive impairment across individuals with cognitive testing was examined. For the 4 most commonly impaired cognitive domains, frequency of prescription antidepressants (e.g., selective serotonin reuptake inhibitors, serotonin and norepinephrine reuptake inhibitors), benzodiazepines (e.g., lorazepam, diazepam, clonazepam) and non-benzodiazepine muscle relaxants (e.g., cyclobenzaprine, baclofen, dantrolene), and clinically significant depression and anxiety were assessed.

RESULTS

Out of 205 patients, 66 reported cognitive concerns, of which 20 completed cognitive testing (**Table 1**). There was no statistically significant difference in gender, age, or duration of illness in individuals included in this case series vs. the remainder of the cohort, or between those included in the case series vs. those who reported cognitive concerns but did not have cognitive testing (all $p > 0.05$). Three participants completed testing with a speech and language pathologist using the Repeatable Battery for the Assessment of Neuropsychological Status [RBANS; (20)], and 17 completed testing with a psychologist using a wide array of instruments (**Supplementary Table 1**). Our cohort was mostly female ($n = 15$, 75%), had a mean age at time of cognitive testing of 47.4 years (SD = 12.4), and mean duration of illness of 10.1 years (SD = 7.6). Most had anti-GAD65 antibodies (17/20, 75%), and classic SPS phenotype (15/20, 75%). Three (15%) had a history of seizures, none of which were intractable or poorly controlled. Common classes of medications prescribed included benzodiazepines ($n = 14$, 70%), antidepressants ($n = 13$, 65%), non-benzodiazepine muscle relaxants ($n = 10$, 50%), and opioids ($n = 4$, 20%). Nine out of eleven (82%) patients assessed for depression reported clinically significant symptoms, and 4 out of 9 (44%) patients assessed for anxiety reported clinically significant symptoms.

Of the 20 patients who completed cognitive testing, 19 performed in the "impaired" range in at least one cognitive domain. The most common domains of impairment were verbal learning and recall memory ($n = 14$, 70%), verbal fluency ($n = 11$, 55%), processing speed ($n = 8$, 40%), attention ($n = 8$, 40%), motor speed ($n = 7$, 35%), semantic verbal fluency ($n = 6$, 30%), visual learning and recall memory ($n = 5$, 25%), set-shifting ($n = 5$, 25%), inhibition control ($n = 3$, 15%), and visuospatial processing ($n = 3$, 15%).

Patterns of medication use and clinically significant depressive and anxiety symptoms are described in **Figure 1**.

DISCUSSION

To our knowledge, this is the first detailed examination of cognitive and mood profiles in patients with SPSD who present with cognitive concerns. The most common cognitive domains exhibiting impairment were verbal recall, processing speed, attention, and phonemic verbal fluency. Additionally, results suggest an overlap of cognitive impairment with use of SPSD medications and presence of mood and anxiety symptoms.

TABLE 1 | Clinical and laboratory features of patients with stiff person syndrome spectrum disorders who received formal cognitive testing as part of routine clinical care for patient-reported cognitive changes.

Patient number	Baseline characteristics							Cognitive testing results	
	Age at testing	Years with SPS[f]	SPS phenotypes	Anti GAD-65 titer	Relevant medical comorbidities	Psychiatric comorbidities[a]	Psychoactive and immune-based medications	Areas of impairment	Psychiatric symptoms [g]
1	59	2	GAD+SPS Cerebellar predominant	63,525 IU/mL	Vitiligo B12 deficiency Remote Intestinal Ca Remote Testicular Ca	None	Clonazepam	Processing speed Verbal phonemic fluency	GDS-15: 8/15 NPI-Q: agitation, depression, apathy, irritability, nighttime behaviors, appetitive changes
2	39	<1	GAD -SPS	39 U/mL [e]	T2DM B12 deficiency Vit D deficiency Narcolepsy Small fiber neuropathy Mild OSA	Depression Anxiety PTSD ADHD	Oxymorphone Oxycodone Pregabalin Metaxalone Baclofen Clonazepam Alprazolam Armodafinil Certirizine	No areas of impairment	PHQ-9 = 15 (moderately severe depressive symptoms)
3	74	8	GAD+SPS Cerebellar predominant	6.3 U/mL	Coronary artery disease	Depression	IVIG Duloxetine	Verbal learning and recall Motor speed Executive function (Set-shifting) Processing speed Verbal phonemic fluency	BAI 8 (minimal anxiety) PHQ-9 = 0 (no symptoms)
4	22	2	GAD+SPS	30 U/mL	Hypothyroidism Sickle cell anemia Asthma CVA partial seizures	Generalized anxiety disorder [b] Major depressive disorder, recurrent, moderate [b] Adjustment disorder due to medical condition [b]	Baclofen Diazepam Benzonatate Diphenhydramine	Executive functioning (inhibition) Attention Verbal phonemic fluency Verbal recall Motor speed	PAI: severe depressive symptoms
5	60	8	GAD-Possible SPS	Not available	B12 deficiency Vit D deficiency Ankylosing spondylitis Hypertension OSA	None	Clonazepam Methotrexate Bupropion Tramadol	Verbal recall	Not assessed
6	29	17	GAD+SPS	250 IU/mL	Hypothyroidism Primary Immune deficiency Orthostatic hypotension Crohn's disease Chiari Malformation	None	Adalimumab Tacrolimus Clonidine Duloxetine Modafinil Prednisone Topriamate	Verbal learning and recall Executive functioning (set shifting) Attention Verbal phonemic fluency Verbal semantic fluency Working memory	Not assessed

(Continued)

TABLE 1 | Continued

Patient number	Baseline characteristics							Cognitive testing results	
	Age at testing	Years with SPS[f]	SPS phenotypes	Anti GAD-65 titer	Relevant medical comorbidities	Psychiatric comorbidities[a]	Psychoactive and immune-based medications	Areas of impairment	Psychiatric symptoms [g]
7	54	12	GAD+SPS	21,888 U/mL	SLE	Depression Anxiety	Baclofen Diazepam Clonazepam IVIG Duloxetine Buspirone Doxylamine Melatonin	Verbal phonemic fluency Visual recall Executive function (set shifting) Attention Processing speed	BDI: 29 (moderate depression) PAI: significant depression and anxiety
8	43	6	GAD+SPS Plus	25,000 U/mL	Insulin dependent diabetes Epilepsy, sickle cell trait, migraines	None	Clonazepam Cyclobenzaprine Lacosamide Levitracetam Oxycodone	Language (verbal and reading comprehension, naming, spelling) Visual learning and recall	PAI: significant anxiety
9	36	4	GAD+SPS	320 IU/mL	Neuropathy Migraine	Anxiety Depression PTSD	Baclofen IVIG Clonazepam Diazepam Gabapentin Paroxetine	Verbal phonemic fluency Verbal semantic fluency Verbal recall Motor speed	PAI: significant anxiety, depression, anxiety related to past trauma and stress
10	59	20	GAD-Possible SPS	Not available	Cervical stenosis Migraines	None	Carbamazepine Tizanidine	Verbal recall [c]	Not assessed
11	49	12	GAD+SPS	117 IU/mL	None	Major Depressive Disorder, recurrent [b]	Baclofen Bupropion Buspirone Clonazepam Diazepam IVIG Rituximab	Processing speed Attention Verbal recall Visuospatial judgement	Not assessed
12	59	3	GAD+SPS	615 nmol/L	None	Paranoid schizophrenia[b]	Fluoxetine Levitracitam Diazepam Olanzapine Memantine	Verbal learning and recall [c] Visual learning and recall Language (expression) Verbal phonemic fluency Motor speed Executive function (inhibition) Processing speed	BDI and BAI within normal limits (score not reported)
13	49	24	GAD+SPS	207,650 U/mL	Diabetes Mellitus Epilepsy (s/p temporal lobectomy) Hypothyroidism Pernicious anemia SLE	None	Baclofen Diazepam Lacosamide Levetiracetam Pregabalin	Processing speed Executive function (Set shifting) Verbal learning and recall Language (naming) Verbal phonemic fluency	PHQ-9 = 7 (mild depression) GAD-7 = 12 (moderate anxiety)

(Continued)

TABLE 1 | Continued

Patient number	Baseline characteristics							Cognitive testing results	
	Age at testing	Years with SPS[f]	SPS phenotypes	Anti GAD-65 titer	Relevant medical comorbidities	Psychiatric comorbidities[a]	Psychoactive and immune-based medications	Areas of impairment	Psychiatric symptoms [g]
14	54	22	GAD+SPS	6.6 U/mL	Insomnia Postural orthostatic tachycardia syndrome Migraines	Generalized anxiety disorder[b] Major depressive disorder [b]	Doxepin SCIG Pregabalin	Processing speed[c] Verbal semantic fluency	PHQ-9 = 27 (severe depression) GAD-7 = 19 (severe anxiety)
15	45	9	GAD+SPS	213 IU/mL	Tublerculosis (1 yo) Coronary artery disease Dyslipidemia HTN Hypothyroidism	None	Clonazepam Diazepam Hydralazine IVIG	Verbal recall Verbal semantic fluency Executive functioning (Set-shifting) Processing speed Motor speed	Not assessed
16	45	23	GAD+SPS	174.2 U/mL	Anemia Anticardiolipin antibody positive T1DM Hepatitis Rheumatoid arthritis SLE	Major depressive disorder Anxiety	IVIG Baclofen Clonazepam Escitalopram Prednisone	Attention[d] Verbal learning and recall Visual learning and recall	Not assessed
17	41	9	GAD+SPS	174.2 U/mL	Dysautonomia Idiopathic small fiber sensory neuropathy T1DM	None	Baclofen Clonazepam Diazepam Pregabalin Modafinil Oxycodone Roxicodone Paroxetine IVIG Tizanidine	Verbal phonemic fluency Motor speed	GAD-7 = 1 PHQ-9 = 19
18	41	3	GAD+SPS	53,650 U/mL	Anemia (iron deficiency) Eczema Asthma Seizures	Anxiety	Baclofen Diazepam Mirtazapine	Attention[d] Visuospatial skills Verbal phonemic fluency Verbal learning and recall	Not assessed
19	33	7	GAD+SPS	34 IU/mL	Seizures Ataxia Nystagmus	None	Baclofen Escitalopram IVIG Levetiracetam Rituximab	Attention[d] Verbal phonemic fluency Verbal recall Visual recall Visuospatial skills	Not assessed

(Continued)

TABLE 1 | Continued

Patient number	Baseline characteristics							Cognitive testing results	
	Age at testing	Years with SPS[f]	SPS phenotypes	Anti GAD-65 titer	Relevant medical comorbidities	Psychiatric comorbidities[a]	Psychoactive and immune-based medications	Areas of impairment	Psychiatric symptoms [g]
20	56	1	GAD+SPS	Not available	SLE Psoriatic arthritis T1DM B12 deficiency Autoimmune thyroiditis Hypertension Sjogren's syndrome	Depression	Amlodipine Apremilast Diazepam Escitalopram Estradiol Clonazepam	Attention Verbal phonemic fluency Verbal recall Motor speed	Not assessed

[a]Psychiatric diagnoses are based only on patient report unless noted otherwise; [b]psychiatric diagnoses confirmed by psychiatric or psychologist notes; [c]no raw score or percentiles from neuropsychological batteries were reported for these patients. Domains of impairment were only based on testing interpretation summary; [d]only results of Repeatable Battery for Assessment of Neuropsychological Status (RBANS) completed by a Speech and Language Pathologist available; [e]While this patient had one positive anti-GAD65 antibody test, at the time closest to neuropsychological testing, prior and subsequent tests have been negative, thus categorization of phenotype is GAD-; [f]duration of illness calculated from time of symptom onset to time of cognitive testing; [g]Psychiatric symptoms were considered clinically significant based on previously established cut-offs of >9 for the Generalized Anxiety Disorder Assessment (GAD-7) (14) and >4 for the Patient Health Questionnaire-9 (PHQ-9) (15), >9 for Beck Depression Inventory (BDI) (16), >7 for the Beck Anxiety Inventory (BAI) (17). For individuals whose psychiatric symptoms were only assessed using the PAI, clinically significant symptoms were identified based on interpretation in the neuropsychiatric report. GAD -, anti-glutamic acid decarboxylase antibody negative; GAD +, anti-glutamic acid decarboxylase antibody positive; SLE, systemic lupus erythematosus; T1DM, type 1 diabetes; OSA, Obstructive Sleep Apnea; PTSD, Post-traumatic stress disorder; ADHD, Attention Deficit Hyperactivity Disorder; CVA, Cerebrovascular accident; Ca, Cancer; s/p, status-post; IVIG= Intravenous immunoglobulin; SCIG= Subcutaneous immunoglobulin; NPI-Q, Neuropsychiatric Inventory – Questionnaire (18); PHQ-9, Patient health questionnaire-9 (15); GAD-7, Generalized anxiety disorder-7 (14); PAI, Personality assessment inventory (19); BDI, Beck depression inventory (16); BAI, Beck anxiety inventory (17).

Reduced GABA levels have been associated with anxiety and depression (21), as well as cognitive impairment in schizophrenia (22), multiple sclerosis (23), and Alzheimer's disease (24). Metabolic abnormalities in the frontal cortex, temporal cortex, thalamus, and cingulate cortex (25) have been reported in classic SPS, regions that have previously been associated with psychiatric symptoms in cognitive disorders (26). Thus, there is a biological plausibility that cognitive impairment and mood and anxiety disorders are intrinsic to the disease process.

Our results expand on previously published work by Budhram et al. (11). Though cognitive findings specific to SPS phenotype were not reported separately, they found that 18% ($n = 38$) of their cohort with various anti-GAD65 associated neurological disorders had cognitive impairment as diagnosed by the Kokmen short test of mental status (11, 27). Consistent with our findings, the predominant cognitive domains impacted were verbal learning and recall memory (29/38, 76%), followed by working memory/attention (6/38, 16%), and verbal fluency/language processing (3/38, 8%). Similarly, another study of cognitive profiles in 21 patients with anti-GAD65-positive diabetes (without a co-existing neurological condition, severe psychiatric disorders or use of psychotropic medications) reported that performance on recall memory and phonemic verbal fluency tasks were significantly lower in anti-GAD65-positive individuals than in the control group (5). Psychiatric symptoms, however, were not evaluated in either study in relation to cognition.

Among the 20 patients included in our case series, 65% were prescribed antidepressants, and approximately half of those assessed for depression and anxiety reported clinically significant symptoms. This is consistent with prior studies (3, 28, 29), and a recent systematic review which found that the relative risk of psychiatric comorbidity in SPS was higher than that of the general population (7). Mood and anxiety disorders are associated with deficits in learning and memory, executive function, and attention—areas also impaired in SPSD and anti-GAD65 associated diseases (8, 9). Although the present findings are observational and cannot confirm causation, bidirectional pathways of mood and cognition have been established in longitudinal studies of other patient populations (30, 31).

Both benzodiazepines and muscle relaxants have been associated with increased risk of cognitive impairment (32–34). In particular, long-term benzodiazepine use has been associated with deficits in visuospatial processing, processing speed, and verbal learning (10). While we observed a high prevalence of these medications in individuals with cognitive impairment, future studies on the potential effects of these medications on cognition in SPSD are needed to establish causality. At a minimum, there should be increased consideration for their long-term use given the potentially harmful effects.

These findings should be interpreted within the context of their limitations. This was a convenience, retrospective sample of individuals who had completed cognitive testing following referral based on reported cognitive concerns. Testing was conducted at different sites and by different providers, without standardization of test selection or interpretation. Moreover, as previously noted, certain medications that are used in SPSD can influence cognitive function. Despite the aforementioned

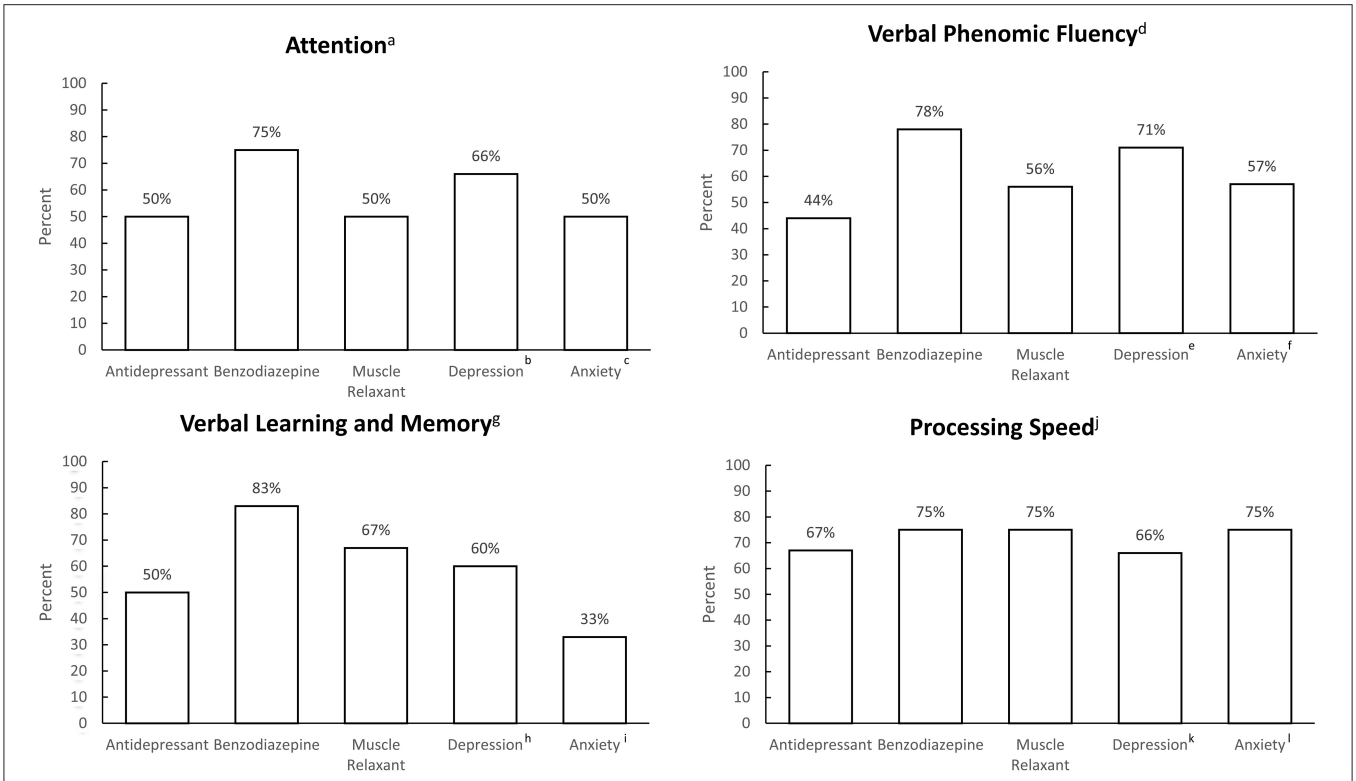

FIGURE 1 | Frequency of antidepressant use, benzodiazepine use, non-benzodiazepine muscle relaxant use, and clinically significant depression and anxiety symptoms, grouped by most commonly impaired cognitive domains. [a]$n = 8$, [b]$n = 3$, [c]$n = 2$; [d]$n = 11$, [e]$n = 7$, [f]$n = 7$; [g]$n = 14$, [h]$n = 11$, [i]$n = 6$; [j]$n = 8$, [k]$n = 6$; [l]$n = 4$.

limitations, our present findings contribute to the limited literature on cognitive and mood profiles in patients with SPSD by identifying common domains of cognitive impairment and potential overlap of cognitive impairment with mood symptoms and medication use.

In summary, assessment of cognitive impairment in SPSD should include testing of verbal learning and recall, phonemic verbal fluency, attention, and processing speed. Cognitive screening tools that examine these domains, such as the Montreal Cognitive Test (MoCA), could be used in the clinical setting to help identify patients who may need additional cognitive evaluation. Psychiatric symptoms and use of medications that may affect cognition are common, and should be considered when evaluating cognitive impairment in this population. Further studies are needed to replicate these findings using longitudinal prospective study designs with consistent cognitive assessment tools and interpretive standards to further clarify the scope of neuropsychiatric disturbance in SPSD and their underlying mechanisms.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Johns Hopkins Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CC: conceptualization of the study, data analysis, data interpretation, drafting, and revision of manuscript. DP, YW, and DO: data acquisition, data interpretation, and revision of manuscript. AH: data interpretation and revision of manuscript. SN: conceptualization of the study, data acquisition, data interpretation, study supervision, and revision of manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2022.865462/full#supplementary-material

REFERENCES

1. Hadavi S, Noyce AJ, Leslie RD, Giovannoni G. Stiff person syndrome. *Pract Neurol.* (2011) 11:272–82. doi: 10.1136/practneurol-2011-000071
2. Dalakas MC. Stiff person syndrome: advances in pathogenesis and therapeutic interventions. *Curr Treat Options Neurol.* (2009) 11:102–10. doi: 10.1007/s11940-009-0013-9
3. Ameli R, Snow J, Rakocevic G, Dalakas MC. A neuropsychological assessment of phobias in patients with stiff person syndrome. *Neurology.* (2005) 64:1961–3. doi: 10.1212/01.WNL.0000163984.71993.FE
4. Lohmann T, Hawa M, Leslie RD, Lane R, Picard J, Londei M. Immune reactivity to glutamic acid decarboxylase 65 in Stiffman syndrome and type 1 diabetes mellitus. *Lancet.* (2000) 356:31–5. doi: 10.1016/S0140-6736(00)02431-4
5. Takagi M, Ishigaki Y, Uno K, Sawada S, Imai J, Kaneko K, et al. Cognitive dysfunction associated with anti-glutamic acid decarboxylase autoimmunity: a case-control study. *BMC Neurol.* (2013) 13:76. doi: 10.1186/1471-2377-13-76
6. Hampe CS, Petrosini L, De Bartolo P, Caporali P, Cutuli D, Laricchiuta D, et al. Monoclonal antibodies to 65kDa glutamate decarboxylase induce epitope specific effects on motor and cognitive functions in rats. *Orphanet J Rare Dis.* (2013) 8:82. doi: 10.1186/1750-1172-8-82
7. Caffrey D, Finn CT, Song SM, Burton F, Arsan C. Stiff-person syndrome and psychiatric comorbidities: a systematic review. *J Acad Consult Liaison Psychiatry.* (2021) 62:3–13. doi: 10.1016/j.psym.2020.08.005
8. Airaksinen E, Larsson M, Forsell Y. Neuropsychological functions in anxiety disorders in population-based samples: evidence of episodic memory dysfunction. *J Psychiatr Res.* (2005) 39:207–14. doi: 10.1016/j.jpsychires.2004.06.001
9. Rock P, Roiser J, Riedel W, Blackwell A. Rock PL, Roiser JP, Riedel WJ, Blackwell AD. Cognitive impairment in depression: a systematic review and meta-analysis. *Psychol Med.* (2013) 44: 2029–40. doi: 10.1017/S0033291713002535
10. Stewart SA. The effects of benzodiazepines on cognition. *J Clin Psychiatry.* (2005) 66(Suppl 2):9—13.
11. Budhram A, Sechi E, Flanagan EP, Dubey D, Zekeridou A, Shah SS, et al. Clinical spectrum of high-titre GAD65 antibodies. *J Neurol Neurosurg Psychiatry.* (2021) 92:645–54. doi: 10.1136/jnnp-2020-325275
12. Ingraham LJ, Aiken CB. An empirical approach to determining criteria for abnormality in test batteries with multiple measures. *Neuropsychology.* (1996) 10:120–4. doi: 10.1037/0894-4105.10.1.120
13. RStudio Team. *RStudio: Integrated Development Environment for R.* Boston, MA: RStudio, PBC (2020). Available online at: http://www.rstudio.com/
14. Spitzer RL, Kroenke K, Williams JB, Löwe B. A brief measure for assessing generalized anxiety disorder: the GAD-7. *Arch Internal Med.* (2006) 166:1092–7. doi: 10.1001/archinte.166.10.1092
15. Kroenke K, Spitzer RL, Williams JB. The PHQ-9: validity of a brief depression severity measure. *J Gen Internal Med.* (2001) 16:606–13. doi: 10.1046/j.1525-1497.2001.016009606.x
16. Beck AT, Steer RA, Brown GK. *Manual for the Beck Depression Inventory-II.* San Antonio, TX: Psychological Corporation (1996).
17. Steer RA, Beck AT. Beck anxiety inventory. In: Zalaquett CP, Wood RJ, editors. *Evaluating Stress: A Book of Resources*. Scarecrow Education (1997). p. 23–40.
18. Kaufer DI, Cummings JL, Ketchel P, Smith V, MacMillan A, Shelley T, et al. Validation of the NPI-Q, a brief clinical form of the neuropsychiatric inventory. *J Neuropsychiatry Clin Neurosci.* (2000) 12:233–9. doi: 10.1176/jnp.12.2.233
19. Morey LC, Lowmaster SE. Personality assessment inventory. In: Irving B, Weiner W, editors. *The Corsini Encyclopedia of Psychology.* Hoboken, NJ: Edward Craighead John Wiley & Sons Inc; American Cancer Society (2010). p. 1–4. doi: 10.1002/9780470479216.corpsy0663
20. Randolph C, Tierney MC, Mohr E, Chase TN. The repeatable battery for the assessment of neuropsychological status (RBANS): preliminary clinical validity. *J Clin Exp Neuropsychol.* (1998) 20:310–9. doi: 10.1076/jcen.20.3.310.823
21. Kalueff AV, Nutt DJ. Role of GABA in anxiety and depression. *Depress Anxiety.* (2007) 24:495–517. doi: 10.1002/da.20262
22. Xu M, Wong AHC. GABAergic inhibitory neurons as therapeutic targets for cognitive impairment in schizophrenia. *Acta Pharmacol Sin.* (2018) 39:733–53. doi: 10.1038/aps.2017.172
23. Cao G, Edden RAE, Gao F, Li H, Gong T, Chen W, et al. Reduced GABA levels correlate with cognitive impairment in patients with relapsing-remitting multiple sclerosis. *Eur Radiol.* (2018) 28:1140–8. doi: 10.1007/s00330-017-5064-9
24. Solas M, Puerta E, Ramirez JM. Treatment options in Alzheimer's disease: the GABA story. *Curr Pharmaceut Design.* (2015) 21:4960–71. doi: 10.2174/1381612821666150914121149
25. Wang Y, Sadaghiani MS, Tian F, Fitzgerald KC, Solnes L, Newsome SD. Brain and muscle metabolic changes by FDG-PET in stiff person syndrome spectrum disorders. *Front Neurol.* (2021) 12:1479. doi: 10.3389/fneur.2021.692240
26. Chen Y, Dang M, Zhang Z. Brain mechanisms underlying neuropsychiatric symptoms in Alzheimer's disease: a systematic review of symptom-general and -specific lesion patterns. *Mol Neurodegen.* (2021) 16:38. doi: 10.1186/s13024-021-00456-1
27. Kokmen E, Naessens JM, Offord KP. A short test of mental status: description and preliminary results. *Mayo Clin Proc.* (1987) 62:281–8. doi: 10.1016/S0025-6196(12)61905-3
28. Gerschlager W, Schrag A, Brown P. Quality of life in stiff person syndrome. *Movement Disord.* (2002) 17:1064–7. doi: 10.1002/mds.10235
29. Newsome SD. *Other Proven and Putative Autoimmune Disorders of the CNS: Anti-GAD Associated Neurological Disorders-Stiff-Person Syndrome, Cerebellar Ataxia, Progressive Encephalopathy with Rigidity and Myoclonus, and Encephalitis*. New York, NY: Oxford University Press (2016).
30. Chruzander C, Johansson S, Gottberg K, Einarsson U, Fredrikson S, Holmqvist LW, et al. A 10-year follow-up of a population-based study of people with multiple sclerosis in Stockholm, Sweden: changes in disability and the value of different factors in predicting disability and mortality. *J Neurol Sci.* (2013) 332:121–7. doi: 10.1016/j.jns.2013.07.003
31. Petkus AJ, Gomez ME, Filoteo JV, Schiehser DM, Petzinger G. Worse cognitive performance predicts increased anxiety and depressive symptoms in patients with Parkinson's disease: a bidirectional analysis. *Neuropsychology.* (2018) 33:35–46. doi: 10.1037/neu0000498
32. Mancuso CE, Tanzi MG, Gabay M. Paradoxical reactions to benzodiazepines: literature review and treatment options. *Pharmacotherapy.* (2004) 24:1177–85. doi: 10.1592/phco.24.13.1177.38089
33. Picton JD, Marino AB, Nealy KL. Benzodiazepine use and cognitive decline in the elderly. *Am J Health Syst Pharm.* (2018) 75:e6–12. doi: 10.2146/ajhp160381
34. Coupland CAC, Hill T, Dening T, Morriss R, Moore M, Hippisley-Cox J. Anticholinergic drug exposure and the risk of dementia: a nested case-control study. *JAMA Intern Med.* (2019) 179:1084. doi: 10.1001/jamainternmed.2019.0677

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Current Status and Future Opportunities in Modeling Clinical Characteristics of Multiple Sclerosis

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Development of effective treatments requires understanding of disease mechanisms. For diseases of the central nervous system (CNS), such as multiple sclerosis (MS), human pathology studies and animal models tend to identify candidate disease mechanisms. However, these studies cannot easily link the identified processes to clinical outcomes, such as MS severity, required for causality assessment of candidate mechanisms. Technological advances now allow the generation of thousands of biomarkers in living human subjects, derived from genes, transcripts, medical images, and proteins or metabolites in biological fluids. These biomarkers can be assembled into computational models of clinical value, provided such models are generalizable. Reproducibility of models increases with the technical rigor of the study design, such as blinding, control implementation, the use of large cohorts that encompass the entire spectrum of disease phenotypes and, most importantly, model validation in independent cohort(s). To facilitate the growth of this important research area, we performed a meta-analysis of publications ($n = 302$) that model MS clinical outcomes extracting effect sizes, while also scoring the technical quality of the study design using predefined criteria. Finally, we generated a Shiny-App-based website that allows dynamic exploration of the data by selective filtering. On average, the published studies fulfilled only one of the seven criteria of study design rigor. Only 15.2% of the studies used any validation strategy, and only 8% used the gold standard of independent cohort validation. Many studies also used small cohorts, e.g., for magnetic resonance imaging (MRI) and blood biomarker predictors, the median sample size was <100 subjects. We observed inverse relationships between reported effect sizes and the number of study design criteria fulfilled, expanding analogous reports from non-MS fields, that studies that fail to limit bias overestimate effect sizes. In conclusion, the presented meta-analysis represents a useful tool for researchers, reviewers, and funders to improve the design of future modeling studies in MS and to easily compare new studies with the published literature. We expect that this will accelerate research in this important area, leading to the development of robust models with proven clinical value.

Keywords: multiple sclerosis (MS), predictive models, machine learning, clinical outcomes, MS disability, MS severity, technical quality, reproducibility

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INTRODUCTION

Multiple sclerosis (MS) is a polygenic, immune-mediated, demyelinating disease of the central nervous system (CNS) that causes substantial personal and societal burden. Understanding the pathophysiology of the initial stages of MS revealed that focal influx of immune cells into CNS tissue can be non-invasively monitored by contrast-enhancing lesions (CELs) on brain magnetic resonance imaging (MRI) (1). CELs, as surrogates of focal inflammation, allowed rapid screening of therapeutic agents (2), identifying many treatments that effectively block the formation of MS lesions.

However, these treatments are not curative, and their efficacy decreases with advancing age at treatment initiation. Indeed, after the age of approximately 54 years, no net benefit on disability progression can be demonstrated in Phase III clinical trials (3). This is partially due to inflammation becoming compartmentalized to CNS tissue during MS evolution (4, 5), making it largely inaccessible to systemically administered treatments. However, neurodegenerative mechanisms (6, 7) likely contribute to the decreasing efficacy of immunomodulatory treatments. To develop effective treatments of MS beyond inhibiting the formation of focal lesions, the MS field must expand its earlier success in gaining pathophysiological insights from early to late disease mechanisms.

Therefore, future therapeutic progress in MS requires the identification and validation of biomarkers that reflect the mechanisms that cause the development of clinical disability in later stages of MS or in patients who no longer form MS lesions thanks to current immunomodulatory treatments. Due to the complexity of these later pathophysiological mechanisms, it is unlikely that a single biomarker can replicate the success of CELs. Indeed, the ability of a single biomarker to reflect key patient-specific outcomes, namely, clinical disability and the rate of its development [as measured by MS severity outcomes (8)] is extremely limited. Consequently, investigators use simple or complex statistical techniques (including machine learning [ML]) to aggregate biomarkers into models with enhanced predictive power.

To our best knowledge, no review exists that summarizes state-of-the-art modeling strategies in MS. The goal of this paper is to present such a critical meta-analysis, to help the MS community, including funders, to identify gaps and opportunities in this important research. We performed a systematic assessment of the technical quality of the reviewed studies, such as sample size, blinding, adjustment for covariates, adjustment for multiple comparisons, integration of healthy volunteer (HV) data to differentiate physiological processes such as aging and gender effects from MS-driven pathologies and, most importantly, we evaluated the level of model validation. Because it has been repeatedly demonstrated that low technical quality (9, 10) and small sample sizes (11–13) overestimate effect sizes and lower the likelihood of reproducible results (14, 15), the attributes we summarize are essential determinants of the generalizability of published models. The broad domain of knowledge included in this work can be utilized as a reference for MS researchers, funders, and reviewers.

METHODS

Search Method

We conducted a literature search to identify studies that generated statistical models to predict clinical outcomes among patients with MS. This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. PubMed searches were performed using keywords related to MS, predictive models, and outcomes. Five PubMed searches were performed to identify relevant MRI studies using various combinations of the following keywords: "multiple sclerosis," "disability," "correlate," "MRI," "machine learning," "predict," "AI," "artificial intelligence," and "neuroimaging." Two searches were performed to identify other relevant studies reporting on statistical modeling in MS with the following PubMed search criteria: "[(Multiple Sclerosis [Title/Abstract]) AND (Prediction) AND (Outcome) AND (Model OR Machine Learning)]" on 24 May 2021 and "(((Multiple Sclerosis [Title/Abstract]) AND (Prediction [Title/Abstract]) AND (Outcome))" on 16 August 2021.

Exclusion Criteria

Two reviewers (JL and EK) independently screened the studies that reported effect sizes for image-, clinical-, or biomarker-based models predicting a clinical outcome. We excluded studies with no predictive models, studies with no imaging, clinical, or biomarker predictors, studies with no clinical outcomes, non-human studies, non-MS studies, and studies with no full text available.

Information Extraction

The following features were extracted from the methods and results of these studies: (1) types of predictors used for modeling (i.e., clinical, MRI, blood biomarkers, CSF biomarkers, and genes); (2) clinical outcome(s) modeled (e.g., expanded disability status scale (EDSS), secondary-progressive MS (SPMS) conversion); (3) cohort sample size; (4) all reported effect sizes (e.g., for modeling continuous outcomes: R^2 [i.e., coefficient of determination; a statistical measure of how well the regression prediction approximate the measured data], Spearman's ρ [a non-parametric correlation coefficient that measures the strength of association between two variables], Pearson's R [a parametric correlation coefficient that measures the strength of association between two variables; should be used only with normally distributed data as it is very sensitive to the effect of outliers]; for dichotomized outcomes such as progression or non-progression: hazard ratios [HR: i.e., an estimate of the ratio of the hazard rate such as disability progression in one vs. other groups: e.g., in treated vs. untreated patients], odds ratios [OR; i.e., the cumulative measure of association between events A and B; with OR $= 1$ signifying independence between A and B, while OR > 1 signifies that A and B are positively associated while OR $<$ 1 means that A and B are negatively associated] and finally, p-value [i.e., the probability of obtaining results at least as extreme as observed if the null hypothesis was correct; please note that because p-value depends not only on effect size but also on

variance and cohort size, it is an extremely poor indicator of effect size alone].

We also extracted seven dichotomized/categorical factors used to assess the quality of the study design (see Section Assessment of the Quality of Study Design in the Reviewed Models). We will refer to these as indicators of the "technical quality" of the study.

Assessment of the Quality of Study Design in the Reviewed Models

Seven technical quality indicators were extracted from the methods and results sections of each paper, with the following justifications: (1) presence and type of model validation (i.e., (A) independent validation cohort (16) [gold standard] or (B) out-of-bag (OOB)/cross-validation of the training cohort (17). ML algorithms are so powerful that, contrary to expectations, developing models that have surprisingly high effect sizes in the training data set is common and easy. Without a validation strategy, it is not possible to determine the utility of such models, as most artificially and greatly inflate the true effect sizes (15). Thus, the presence and type of model validation are the most important indicators of model reproducibility. The next four attributes of methodological study rigor safeguard against bias. Their pre-specification (e.g., in the protocol) before performing the analysis ensures that the analysis is not modified to increase the likelihood of obtaining the desired results. These include: (2) described process of dealing with outliers to prevent bias (yes/no); useful models should be generally applicable and therefore their effect size should not depend on highly influential observations. Such observations should be identified (and excluded) prior to unblinding by a predefined outlier analysis. If such an analysis is not predefined and the description of methods does not specify how many outliers were excluded and based on what criteria, then the model might be biased (18). (3) Described process of dealing with data missingness (19) to prevent bias (yes/no); another way of modeling results may be biased by excluding observations that do not fit the model post-analysis (e.g., with a justification that these observations were technically inadequate) or by not detecting that some observations were systematically omitted (e.g., measurements were not performed on the sickest patients). Finally, a large amount of missingness that is not disclosed in the paper can also falsely overestimate the generalizability and clinical utility of the model. (4) Adjusting for covariates (yes/no); another way to introduce bias is by failing to detect and adjust for effects of confounding factors that influence the model predictors independently of the outcome (such as age, gender, application of treatments, and different socioeconomic status). For example, such confounders may explain up to 60% of the variance in volumetric brain MRI data (20), which may be mistakenly attributed to the model(s) of neurodegenerative diseases, especially if the patient groups are not carefully matched. (5) Blinding (yes/no); the most effective way to prevent bias during the generation of predictors or during data analysis is to blind the investigators who generate the data, and to perform the aforementioned data cleaning steps before unblinding the data analyst (21). Although randomization is also an essential bias-preventing attribute of methodological design, it is mostly applicable to interventional studies, not to modeling studies. (6) The number of comparisons made (i.e., the number of predictors multiplied by the number of outcomes) and whether p-values were adjusted for multiple comparisons (yes/no); this attribute affects the strength of the statistical evidence with which the null hypothesis is rejected. The p-value represents the probability of obtaining results at least as extreme as the presented results if the null hypothesis was valid. We would like to present an analogy that provides a reader without statistical knowledge with a practical intuition of how to judge p-values in the contexts of performing multiple comparisons: let us imagine we have 20 cards numbered from1 to 20 and we are assessing the ability of a blinded person (i.e., a model) to select the card with the number 1 on it. If this person pulls the card #1 on the first attempt, we may be tempted to conclude that the person knows how to select card #1, as there is only a 5% chance ($p = 0.05$) that she/he will select card #1 on the first attempt randomly. Although we eagerly accept the p-value of 0.05 to rule out the null hypothesis in scientific applications related to human health, it is likely that most people would demand stronger evidence that the person can reliably select card #1 in this example. Most people would ask the person to repeat the experiment before they would accept this "model" as valuable. If the person repeats the experiment and selects the card #1 again, then our confidence that she/he knows how to select card #1 will increase to $p = 0.025$ (0.05/2). Now, what happens if the person says that she/he knows how to correctly select the card with a specific number on it: you suggest 19 different numbers and each time the person fails to select the correct one. On the last attempt, you suggest card #1 and the person correctly selects card #1. Will you still conclude that the person represents a good model for selecting card #1? We intuitively understand that if we ignore the previous failed attempts, we reach the wrong conclusion. Yet, when the same is done in reported biomedical research (e.g., the researchers correlated 20 different predictors with the measured outcome and only one of them correlates with $p = 0.05$), we readily accept such a result to reject the null hypothesis. The science of when and how to adjust for multiple comparisons is more complicated (22), but the principle is that we must consider how many comparisons the investigators performed and whether they appropriately adjusted the p-values to make a correct inference. (7) Controls utilized (yes/no); this final attribute of the technical rigor deals with the specificity of the model and thus its clinical value: e.g., a model claims to differentiate relapsing-remitting MS (RRMS) from progressive MS. However, when applied to HVs, the model also differentiates two groups of people: younger and older. Clearly, this is not biologically valuable model of MS progression. Or a model claims to be a diagnostic test of MS, buts its accuracy is tested only by differentiating MS from HV, instead of including appropriate controls such as people with non-MS white matter lesions and focal neurological deficits.

Depending on how many of these criteria study fulfilled, the quality of the study design ranged from 0 to 7. Although it is not necessary for a study to fulfill all seven criteria to be reproducible, the score assesses methodological rigor between studies and identifies areas for improvement.

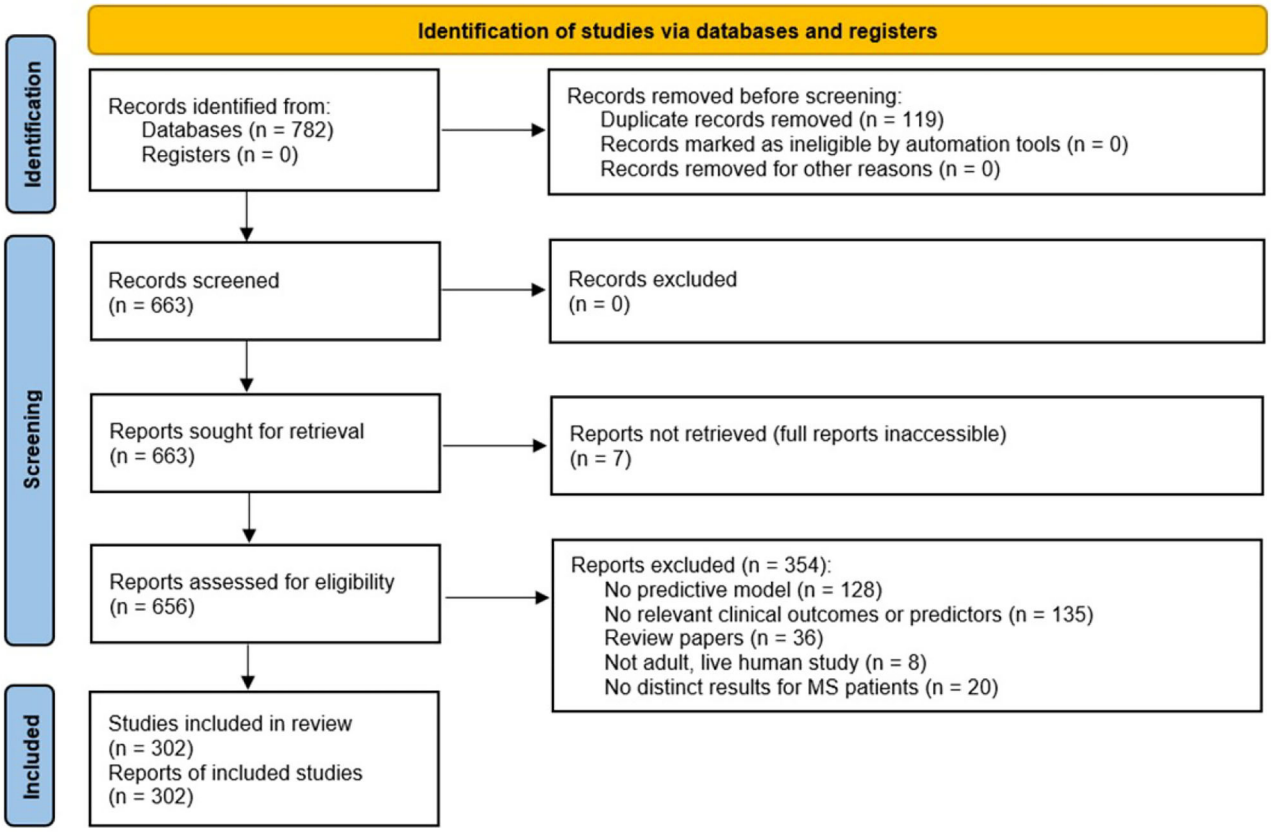

FIGURE 1 | Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) chart summarizing the disposition of records identified from PubMed searches. The searches identified 782 records, of which 663 were unique. After several exclusion criteria defined in the figure, 302 unique records were included in the review.

The Master worksheet containing all these extracted data as well as PubMedIDentifiers (PMID) of individual papers is provided as **Supplementary Table S1**.

Validation of Published Inverse Relationships Between Study Design Quality and Reported Effect Sizes

Previous studies on non-MS fields showed that (1) small cohort studies; (2) studies of low experimental quality; and (3) studies performed only in the training cohort, all significantly overestimate true effect sizes (10, 11, 13, 14). To assess whether the same can be observed in the MS field we investigated the relationships between the technical quality of studies (including cohort sizes and comparisons of training vs. cross-validation vs. independent validation cohorts) and reported effect sizes.

In addition to univariate analyses, we also classified groups of studies based on the combination of cohort size and technical quality criteria: studies were considered high quality if they reached 1 standard deviation (SD) above the mean for both factors, whereas low quality were 1 SD below the mean for both. To compare all identified low- and high-quality studies (two-sample Wilcoxon [Mann–Whitney] test), we normalized the different metrics of effect sizes to yield common metrics ranging from 0 to 1.

Public Database Exploration Tool

To allow readers to independently explore the data beyond the relationships described in this paper, we developed a Shiny App in R version 3.6.1. This application includes selection tools that allow user to select all predictor or specific types, all clinical or specific outcomes and all or specific effect size statistic tools and then generates a set of two-dimensional plots that visualize the relationships between the extracted features. The user can also rapidly identify the PMID for a specific study by clicking a specific point in the two-dimensional plots.

RESULTS

Clinical Outcomes

A total of 663 studies were screened, excluding duplicate records (**Figure 1**; PRISMA diagram). After applying the exclusion criteria, 302 studies were included in the review. A total of 189

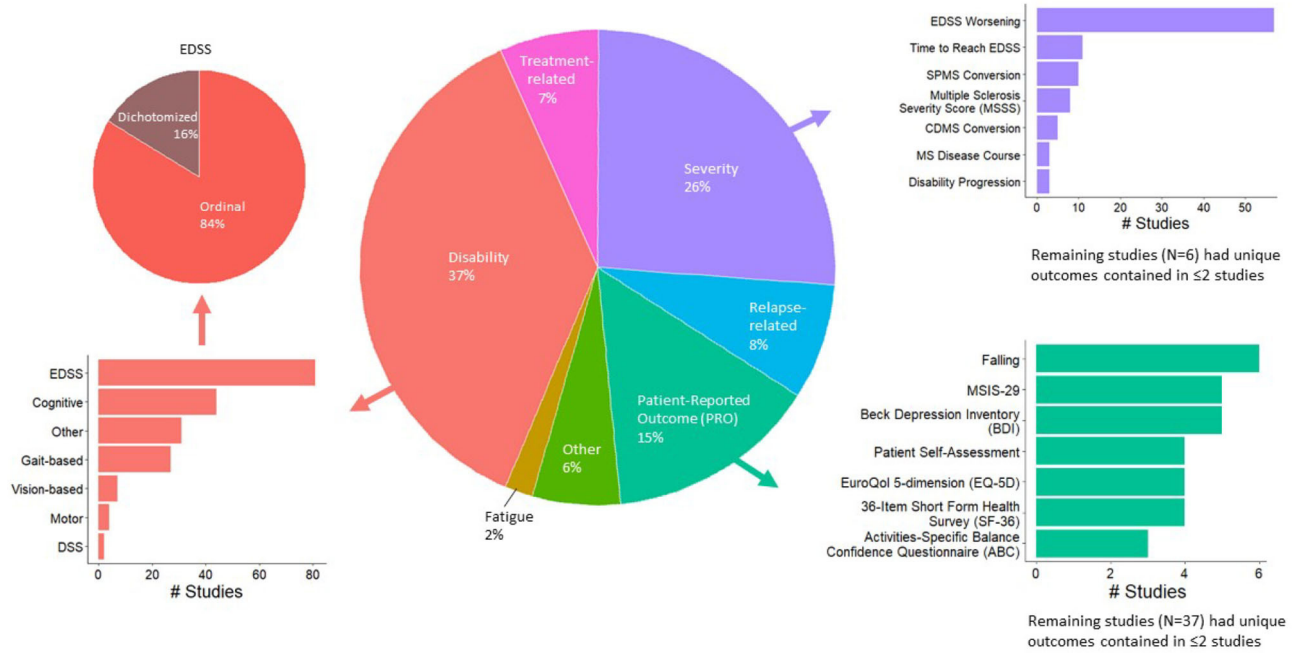

FIGURE 2 | Distribution of modeled clinical outcomes. The large pie chart in the center of the figure shows the distribution of categories of modeled outcomes. The three most frequent outcome categories are disability (37%, red), severity (26%, purple), and patient-reported outcomes (PROs; 15%, teal). The surrounding bar plots show the breakdown of each of these three categories.

clinical outcomes were predicted in the 302 included studies. The breakdown of outcomes by category is shown in **Figure 2**.

The largest category of clinical outcomes was *MS progression as measured by traditional disability outcomes* (**Figure 2**, red color; 37% of the studies reviewed). Of these, the most prevalent outcomes were EDSS-based ($n = 81$ studies), such as predicting EDSS on an ordinal scale, followed by the prediction of EDSS as a dichotomous variable. Cognitive disability outcomes constituted the second largest subcategory ($n = 44$). These included the Paced Auditory Serial Addition Test (PASAT), the Stroop test, the Symbol Digit Modalities Test (SDMT), etc. The third most prevalent progression outcomes were gait-based ($n = 27$), which included the timed 25-foot walk (T25FW), Hauser ambulation index, 6-min walk test, Timed Up and Go [TUG], dynamic gait index, etc.

Following MS progression/disability outcomes, the next largest category of outcomes was *MS severity outcomes*, which were modeled by 26% of the studies reviewed (**Figure 2**, purple color). A total of 69 studies predicted changes in EDSS over time, including EDSS worsening and time to reach a specific EDSS score. A total of 10 studies predicted the conversion to SPMS, eight predicted EDSS-based MS Severity Score (MSSS), five predicted conversions to clinically definite MS, and the remaining outcomes were studied by fewer than five studies.

Finally, *patient-reported outcomes* (PROs; **Figure 2**, teal color) were modeled by 15% of the studies reviewed. This category was fractionated, with falls predicted in six studies, the MS Impact Scale (MSIS-29) and Beck Depression Inventory (BDI) by five studies each. The remaining outcomes were studied by fewer than five studies.

Predictor Variables

Five categories of predictor variables were used in these models, namely, clinical ($n = 166$ studies), MRI ($n = 103$), genes ($n = 13$), blood biomarkers ($n = 20$), and CSF biomarkers ($n = 9$) (**Figure 3A**).

We hypothesized, and confirmed, that the sample sizes would be the largest for models using clinical predictors because they are the easiest to collect. Using similar reasoning, we expected the smallest sample sizes for CSF predictors due to an invasive nature of lumbar punctures. Instead, we observed the smallest sample sizes for models utilizing MRI predictors and blood biomarkers, where most studies had sample sizes of <100 patients, with some as low as 10 patients (**Figure 3B**).

Technical Quality

In addition to recording cohort sizes for each study reviewed, we collected seven study design factors aimed to minimize bias (see Section Methods for details) and therefore maximize the probability that the reported results would be generalizable (**Figure 3C**). These were: (1) blinded analyses; (2) pre-defined/described missing data; (3) pre-defined/described methodology for outlier identification and removal to minimize bias; (4) adjustment for covariates; (5) presence of controls, such

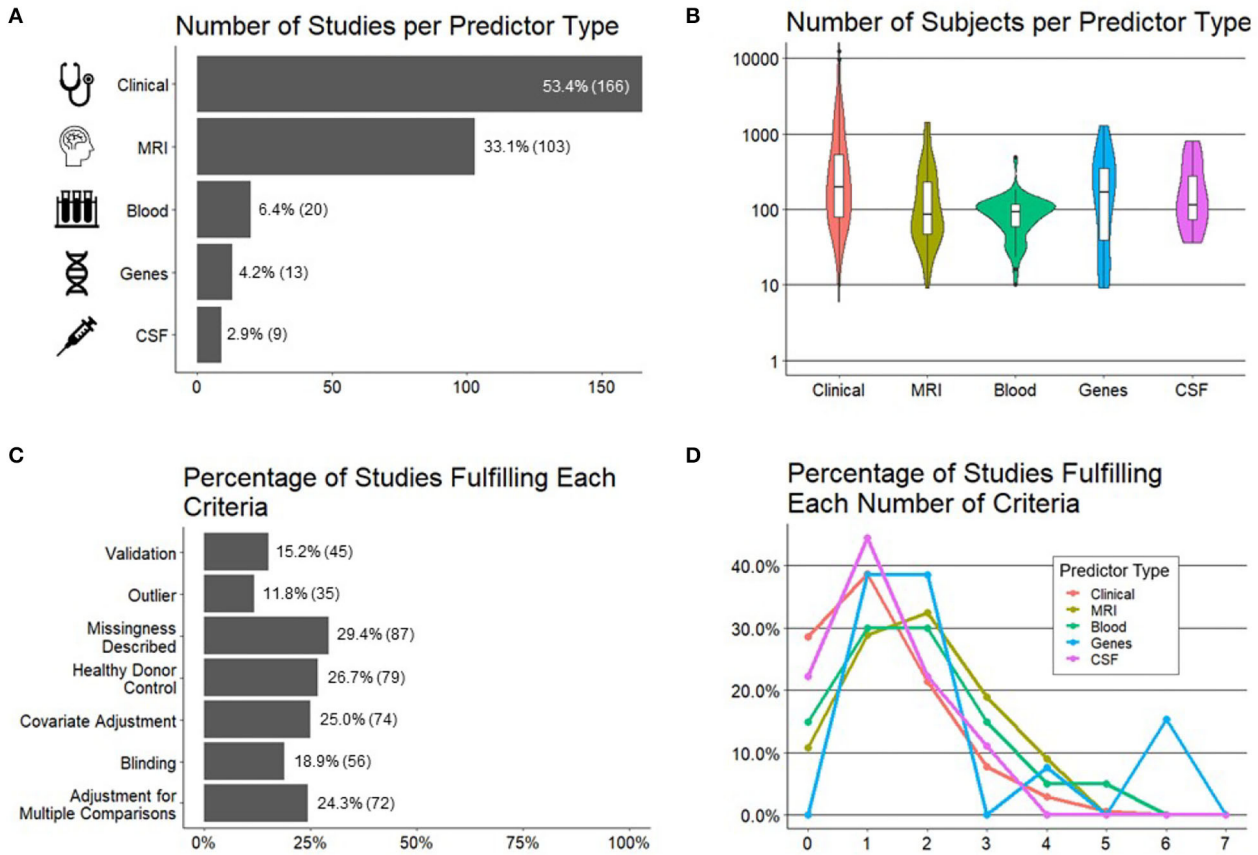

FIGURE 3 | Important characteristics of the studies reviewed. **(A)** Number of studies (*x*-axis) per predictor type (*y*-axis). **(B)** Number of subjects (*y*-axis) by predictor type (*x*-axis). **(C)** Percentage of studies (*x*-axis; number of studies in parentheses) fulfilling each preselected criteria of experimental design/technical quality of the study (*y*-axis). **(D)** Percentage of studies (*y*-axis) per each predictor type fulfilling a number of technical criteria (*x*-axis).

as HVs, to differentiate physiological processes, such as aging or gender effects, from MS-related processes; (6) the number of comparisons performed and whether investigators employed any strategy to adjust significance thresholds if the number of comparisons was high; and finally (7) the level of model validation (if any), differentiating cross-validation methods that reuse training cohort samples from true independent cohort validation, considered the gold standard.

Although no study needs to fulfill all seven criteria to yield reliable results, it was unexpected to observe that majority of the studies fulfilled one or fewer criteria and only 1% of the studies fulfilled more than four. When comparing the technical quality of studies based on different predictors (**Figure 3D**), we observed the highest technical quality of the studies that used genes, followed by MRIs and blood biomarkers. Astonishingly, more than 20% of the studies that used clinical or CSF biomarker predictors fulfilled zero technical quality criteria.

Finally, because current modeling algorithms are highly susceptible to overfitting, an essential determinant of model's generalizability is the level of its validation. Overfitting is caused by the ability of ML algorithms to find and amplify subtle changes in the data, including noise, to achieve fit that is much stronger than biologically plausible. Consequently, when the model is applied to a new set of samples/patients, it will have a much lower fit or may not validate at all. There are two types of validation: the first reuses training cohort data, in various manners that are beyond the scope of this review. It is often called "cross-validation" or "OOB data." We will use term "cross-validation" to signify any validation strategy that reuses training cohort data. To what degree cross-validation faithfully predicts the generalizability of the model depends on the details of how it was performed. Cross-validation may be overly optimistic if researchers fail to prevent bias, and this is often the case. Therefore, the gold standard is independent cohort validation, which implies using the model on a new set of samples/subjects that did not contribute, in any way, to model generation.

We observed that only 15% of the studies used any type of validation with only 8% of all studies used independent validation.

Effect Sizes

Effect sizes for each of these studies were included as reported (for an explanation of these metrics, see Section Methods). The most reported metric was R^2 in 101 studies with Pearson's R being

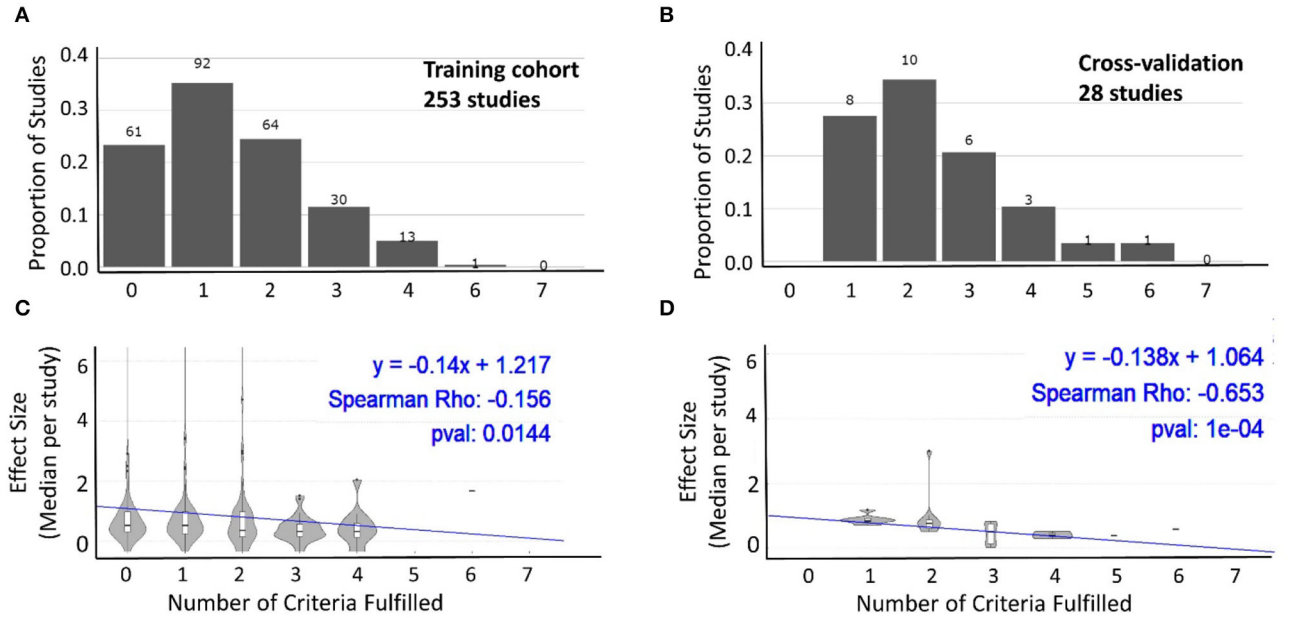

FIGURE 4 | Relationship between technical quality of the study and reported effect size. The proportion of studies fulfilling the sum of the seven technical quality criteria (zero weakest experimental design to seven strongest experimental design) for 253 studies with training cohorts **(A)** and cross-validation cohorts **(B)**. The number of studies in each category is listed above the bars. Effect sizes reported by studies categorized based on the number of technical quality criteria they fulfilled (0–7) for training cohort (**C**; $n = 253$) and cross-validation (**D**; $n = 28$) results. In both cohorts, the reported effect sizes decreased as the number of technical quality criteria fulfilled by these studies increased.

reported in 53 studies, HR in 46 studies, OR in 43 studies, and Spearman's ρ in 29 studies. Values of p were reported alongside these metrics in 202/302 studies.

Overall, we observed a highly selective, rather than comprehensive use of statistical outcomes that reflect effect sizes. This selectivity limits the ability to compare effect sizes between different studies.

Association Between Study Quality and Effect Size

It is estimated that between 51% and 89% of the published literature in biomedical sciences is not reproducible (15, 23, 24) and poor study design, based on small sample sizes (11, 13) and the failure to prevent bias (25–27) is the major contributor to this reproducibility crisis. Indeed, as outlined in the introduction, previous studies highlighted an inverse relationship between the technical quality of study design (10) [including cohort sizes (11, 13)] and reported effect sizes, validating the notion that the technical quality of study design is a major determinant of the generalizability of gained scientific knowledge.

To assess whether we can identify analogous inverse relationships between reported effect sizes and our pre-defined systematic grading of technical quality of the reported study design, we performed two types of analyses. In first analysis, we compiled all studies that reported any effect size separately for the training (**Figure 4A**) and cross-validation cohorts (**Figure 4B**). We then assessed whether there is any relationship between the number of technical quality criteria a study fulfilled vs. the reported effect size. For both the training cohort data (**Figure 4C**) and cross-validation (**Figure 4D**), we observed an inverse relationship between the technical quality of the study and the reported effect size.

Because the above strategy ignored cohort size, which is an important determinant of model generalizability, in the second analysis we construed the two-dimensional assessment of the study design (**Figure 5A**), integrating both grading of reported technical quality with reported sample sizes. Using means ± one SD of all studies, we identified low-quality studies (i.e., at least one SD below the average for both technical quality and sample size) vs. high-quality studies (i.e., at least one SD above the average for both domains). We observed significantly higher reported standardized effect sizes for low quality studies compared with high quality studies (**Figure 5B**). As expected, effect sizes for the remaining studies were centered between the low- and high-quality studies.

Effect Sizes for EDSS-Based Models of MS Progression and MS Severity

To facilitate the interpretation of any future models, we compared the strength of models for different predictors using EDSS-based MS progression (**Table 1**) and MS severity outcomes [MSSS and age-related MSS (ARMSS); **Table 2**]. EDSS-based outcomes are the most broadly used in MS field. We found them to be modeled most, and they are accepted by regulatory agencies for assessing the therapeutic efficacy of MS drugs. For each outcome and predictor pair, we provide the highest reported

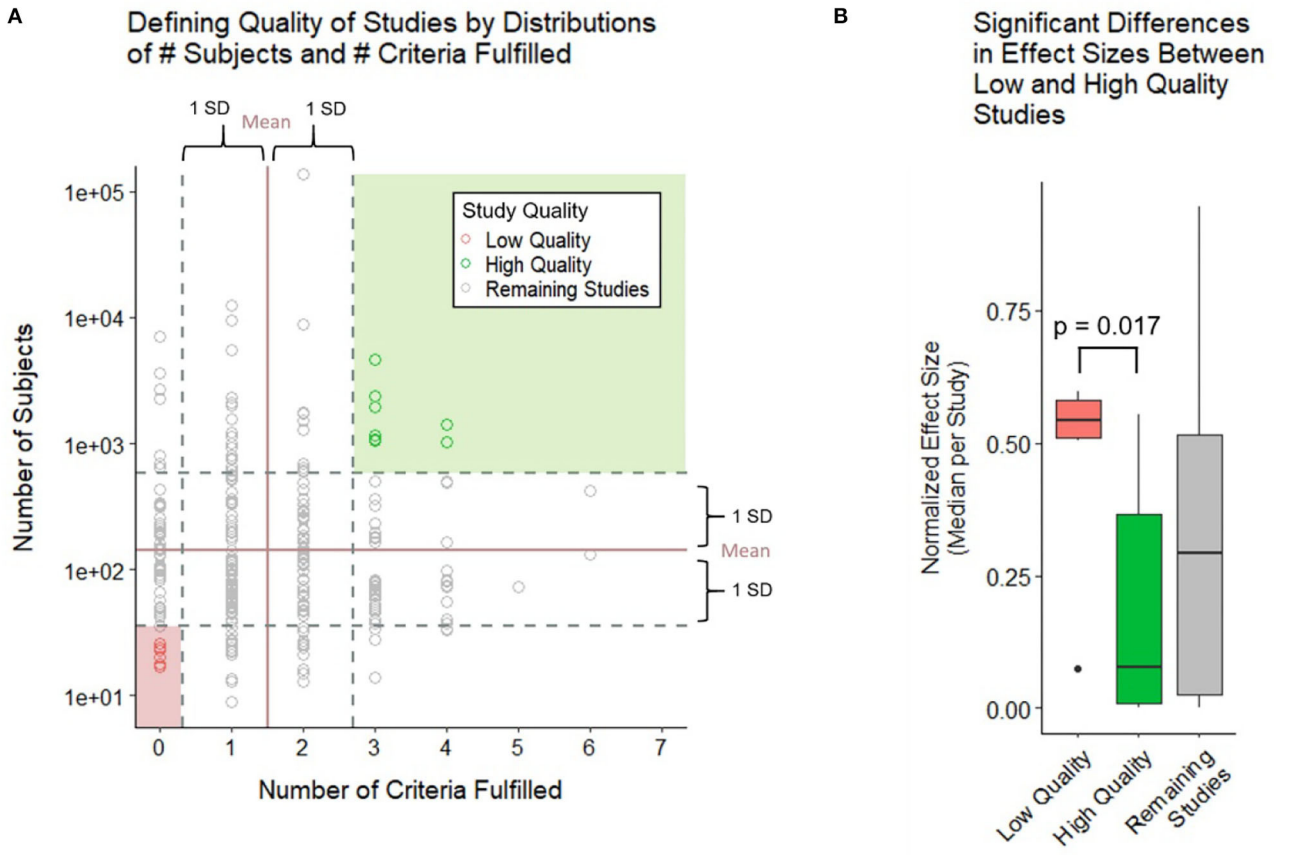

FIGURE 5 | Relationship between technical quality and sample size of the study and reported effect sizes. **(A)** Study quality is defined by the number of subjects and number of criteria fulfilled with high quality studies falling 1 SD above both criteria and low-quality studies falling 1 SD below both criteria. **(B)** Boxplot compares the normalized effect sizes between low- and high-quality studies using a two-sample Wilcoxon (Mann–Whitney) test. Low-quality studies were found to have higher effect sizes at a significant *p*-value of 0.017.

effect size and the effect size reported by the study of highest technical quality. Whenever available, we also reported effect sizes for cross-validation and independent validation studies.

For modeling MS progression using the ordinal EDSS (**Table 1**), we found comparable highest reported effect sizes between studies that used clinical (i.e., $R^2 = 0.67$) and MRI ($R^2 = 0.64$) predictors. The decrease in effect size for best-in class studies was larger for clinical predictors (i.e., $R^2 = 0.26$) than for MRI predictors ($R^2 = 0.52$). Only MRI predictors reported cross-validation results, which further decreased the effect size to $R^2 = 0.19$. We identified no independent validation cohorts. Blood biomarker predictors achieved a much lower effect size in predicting EDSS: the strongest effect size ($R^2 = 0.19$) was reported by a study that included only 23 subjects and achieved the technical quality score of 1, whereas the highest quality study reported $R^2 = 0.06$. We identified no cross-validation or independent validation studies for blood predictors of EDSS. Finally, we identified no studies reporting genetic or CSF biomarker-based predictors of EDSS.

For predicting MS severity (**Table 2**) measured by MSSS, the strongest reported effect size was $R^2 = 0.45$ for MRI and $R^2 = 0.24$ for clinical predictors. However, these were derived from small training cohorts ($n = 67$ for MRI and $n = 54$ for clinical predictors) and were not validated. We identified several studies using genetic predictors of MS severity; effect sizes for independent validation of MSSS and ARMSS were reported only as correlation coefficients and ranged from Pearson's $R = 0.17$–0.2. We did not identify any blood or CSF biomarker-based models of MS severity.

Shiny-App Exploration Tool

To facilitate independent exploration of the rich data set we collected beyond the Excel worksheet containing all extracted data and deposited as **Supplementary Table S1**, we also developed the Shiny App that allows selective filtering of the data (e.g., to isolate specific predictors, specific outcomes, and specific statistical metrics of effect sizes). It can be found at the following link: https://jliu159.shinyapps.io/MS_Models_LitSearch_Data_Exploration/. This tool was designed to facilitate comparisons of any future models with the reviewed literature. A user manual can be found in the **Supplementary Material**.

TABLE 1 | This set of tables shows the models of expanded disability status scale (EDSS; modeled as ordinal scale) using the following predictor types: clinical, magnetic resonance imaging (MRI), and blood, respectively.

Cohort	Study type	R^2 (PMID, #QC, *N*)	|Spearman ρ| (PMID, #QC, *N*)	|Pearson R| (PMID, #QC, *N*)
Outcome: EDSS	**Predictor: Clinical**			
Training	Strongest effect size	0.67 (31218917, 1, 100)	0.77 (18184917, 0, 161)	0.51 (32615409, 1, 38)
	Highest quality	0.26 (26362898, 2, 362)	0.61 (31218917, 1, 100)	-
Cross-validation	Strongest effect size	-	-	-
	Highest quality	-	-	-
Independent validation	Strongest effect size	-	-	-
	Highest quality	-	-	-
Outcome: EDSS	**Predictor: MRI**			
Training	Strongest effect size	0.64 (33598931, 1, 115)	0.82 (24508617, 1, 9)	0.36 (20373349, 0, 107)
	Highest quality	0.52 (30657011, 3, 366)	0.49 (18556361, 4, 74)	0.26 (26115736, 3, 195)
Cross-validation	Strongest effect size	0.19 (32924846, 2, 250)	-	-
	Highest quality	-	-	-
Independent validation	Strongest effect Size	-	-	-
	Highest quality	-	-	-
Outcome: EDSS	**Predictor: Blood**			
Training	Strongest effect size	0.19 (31801106, 1, 23)	-	0.47 (31801106, 1, 23)
	Highest quality	0.06 (30564615, 3, 117)	-	0.15 (22354743, 2, 68)
Cross-validation	Strongest effect size	-	-	-
	Highest quality	-	-	-
Independent validation	Strongest effect size	-	-	-
	Highest quality	-	-	-

Effect sizes were reported for studies with the strongest effect sizes as well as for studies with the highest quality. The following metrics were explored: R^2, Spearman's ρ, and Pearson's R. PMID, PubMed unique Identifier; #QC, sum of technical quality criteria that the study fulfilled; n, number of subjects in the study.

DISCUSSION

Technological advances make measuring thousands of genes, transcripts, proteins, and metabolites and hundreds of imaging and clinical biomarkers relatively easy and common. Thanks to analogous computational advances, these measurements can be aggregated into models that are expected to elucidate disease mechanisms and provide clinical (e.g., prognostic) value. These are valuable developments; however, to fulfill the expectations of providing reproducible knowledge and clinical value, these technological advances must be paired with the rigor of experimental design.

This review shows great potential to improve modeling of clinical disease characteristics in MS. It is startling that 21% of the published studies failed to implement *any* of the seven attributes of a strong experimental design (9, 11–13, 15) to limit bias and enhance reproducibility. An additional 36% of the studies reviewed implemented only one of the seven technical criteria, making this the median attribute of experimental design quality in MS models. This is clearly suboptimal.

This inferior experimental design is compounded by the frequent use of small sample sizes (i.e., fewer than 100 subjects): in fact, for MRI and blood non-genetic biomarker studies, the median cohort sizes were <100. Considering the complexity of disease mechanisms in polygenic diseases like MS, a modeling cohort of <100 patients with MS cannot comprise the entire spectrum of disease heterogeneity. Moreover, such small studies are highly susceptible to bias (11, 13), especially when <20% used blinding, <25% adjusted for covariates, and <30% addressed missingness or adjusted the threshold of significance for the number of comparisons performed (sometimes more than hundreds).

Evidence from other scientific areas (10, 11, 13, 14), supported by this paper, shows that poor experimental design, intensified by small cohort sizes, overestimates effect sizes. This is inevitable, as statistical power is positively associated with cohort and effect sizes (25). Consequently, the only way for small studies to reach statistical significance is for them to demonstrate unusually high effect sizes. These high effect sizes are almost always inflated as abnormalities in individual transcripts, proteins, or metabolites are only mild or moderate, with severe disturbances being incompatible with life (28).

Another underappreciated aspect of complex modeling algorithms is their incredible overfitting power. Contrary to laymen's understanding, it is surprisingly easy to derive seemingly strong models in training cohorts, especially if one measures a comparably higher number of biomarkers to the number of subjects. Such disproportional richness of

TABLE 2 | This set of tables includes EDSS-based multiple sclerosis (MS) severity outcomes MS Severity Score (MSSS) and age-related MSS (ARMSS), showing the studies that reported the highest effect sizes and those that achieved the highest technical quality, reporting R^2, Spearman's ρ, and Pearson's R.

Cohort	Study type	R^2 (PMID, #QC, *N*)	|Spearman ρ| (PMID, #QC, *N*)	|Pearson *R*| (PMID, #QC, *N*)
Outcome: MSSS	**Predictor: MRI**			
Training	Strongest effect size	0.45 (24122185, 1, 67)	-	-
	Highest quality	-	-	-
Cross-validation	Strongest effect size	-	-	-
	Highest quality	-	-	-
Independent validation	Strongest effect size	-	-	-
	Highest quality	-	-	-
Outcome: MSSS	**Predictor: Blood**			
Training	Strongest effect size	0.24 (20965962, 2, 54)	-	0.19 (22354743, 2, 68)
	Highest quality	-	-	-
Cross-validation	Strongest effect size	-	-	-
	Highest quality	-	-	-
Independent validation	Strongest effect size	-	-	-
	Highest quality	-	-	-
Outcome: MSSS	**Predictor: Genes**			
Training	Strongest effect size	0.16 (20378664, 2, 605)	-	-
	Highest quality	-	-	-
Cross-validation	Strongest effect size	-	-	0.58 (31396954, 6, 205)
	Highest quality	-	-	-
Independent validation	Strongest effect size	-	0.06 (31396954, 6, 94)	0.20 (31396954, 6, 94)
	Highest quality	-	-	-
Outcome: ARMSS	**Predictor: Genes**			
Training	Strongest effect size	-	-	-
	Highest quality	-	-	-
Cross-validation	Strongest effect size	-	-	0.58 (31396954, 6, 205)
	Highest quality	-	-	-
Independent validation	Strongest effect size	-	0.12 (31396954, 6, 94)	0.17 (31396954, 6, 94)
	Highest quality	-	-	-

PMID, PubMed unique Identifier; #QC, sum of technical quality criteria that the study fulfilled; n, number of subjects in the study.

predictors poses a high probability of spurious associations between predictors and the outcome(s), akin to the example we introduced in Section Methods when explaining the ease of making the wrong conclusion if we fail to consider how many "comparisons" were performed during the modeling strategy. Thus, the validation of such models is essential: the probability that the same spurious (i.e., not caused by biology) relationship(s) will occur again in the completely independent set of observations is low. However, validation was included only in 15% of all studies, and most of these (56%) used cross-validation rather than independent validation. Indeed, <8% of all studies validated their model(s) on a completely new set of subjects (i.e., independent validation cohort), which is the gold standard.

Cross-validation (also called rotation estimation or OOB testing) reuses some of the training cohort data by partitioning or resampling the data to train and test models on different iterations. For example, a training cohort may be randomly partitioned (many times) to generate "internal" training and validation splits; this partitioning may be as large as 50:50 split or as small as leaving out only one sample. The model then tests the accuracy of the predictions of these OOB samples. Because cross-validation does not require any new data sets, it should be included in all studies, not just 10% of them. Although cross-validation is certainly better than no validation, it may still overestimate the power/accuracy of the classifier in comparison to true independent validation (29). We have *always* observed decreases in model performance (e.g., predictive accuracy) from training cohort to cross-validation and from cross-validation to independent validation (30–32). These decreases happen regardless of whether we use clinical data (33), functional data (34), MRI data (32, 35), soluble biomarkers (30, 36), or genes (31); and they are often substantial, especially when

comparing cross-validation with true independent validation [e.g., from R^2 0.72 in the training cohort to 0.64 in the 5-fold cross-validation with 10 repetitions to 0.01 in the independent validation (34)]. Please note that the effect sizes for the EDSS-based outcomes summarized in **Tables 1**, **2** also show decreasing effect sizes with increasing quality of experimental design, and from training to cross-validation results. Finally, we emphasize that an exceptionally low *p*-value achieved in the training cohort (even in the cross-validation cohort) does not guarantee the dramatic loss of model accuracy observed in the independent validation cohort (15, 31).

Cross-validation frequently overestimates the accuracy of the model because it often includes a circular argument: somewhere in the modeling process the OOB samples contributed to model construction. For example, we already mentioned that "overfitting" tends to happen when models are generated from a disproportionally large number of predictors in comparison to the number of observations. To avoid this problem, the data analyst may "constrict" the number of predictors for model development, e.g., by correlating predictors with the modeling outcome and selecting only predictors with significant correlations. If this initial step was done in all training cohort observations (which is usually the case), the OOB samples were "compromised"; they contributed to model development and, therefore, will likely overestimate the model effect size in comparison to independent validation.

Furthermore, these early modeling steps (such as quality control, outlier removal, and feature selection), if performed unblinded, may introduce bias and are often omitted from the publication altogether [a problem called "selective reporting" (37–39)]. Consequently, bias may not be identified during the review process. Another source of bias that leads to major misinformation in the scientific literature is publication bias (40): when so-called "positive" studies (i.e., those that achieved arbitrary the value of $p < 0.05$) are published, but "negative" studies, including negative independent validation studies, frequently remain unpublished. This collectively causes unrealistically optimistic view of the reproducibility of the published results.

We initiated this work with the goal of identifying opportunities to advance the modeling of MS outcomes. Based on this work, we endorse the following recommendations:

Enhance the Experimental Design of Future Studies

To minimize bias and maximize reproducibility, no modeling study should fulfill less than four criteria of sound experimental design, and all should include at minimum cross-validation. Studies should also be of sufficient size, including all MS phenotypes, to increase the probability that the results will be generalizable.

Include Most Common Outcomes (E.g., EDSS-Based) as Comparators

Although modeling new and possibly better clinical or functional outcomes (including PROs) are desirable, unless EDSS-based outcomes are included, it is impossible to compare different models and understand their clinical utility.

Prioritize Modeling Continuous (or Ordinal) Over Dichotomized Outcomes

Even though the EDSS is an ordinal scale and EDSS-based severity outcomes (i.e., MSSS and ARMSS) are continuous, 71/138 (51%) studies used the EDSS in a dichotomized manner: e.g., predicting progression (yes/no) within a certain period. Of the 71 studies that used dichotomized EDSS-based outcomes, dichotomization was not uniform across studies. For example, EDSS worsening was defined as a 1-point increase in one study, a 0.5-point increase in another study, and a 0.5- or 1-point increase depending on some EDSS threshold, which varied between EDSS 4 and 6. Without justification for a specific definition of EDSS dichotomization and assurance that this definition was selected before data analyses, non-uniform selection of EDSS-based outcomes may lead to bias, while also preventing comparison between studies. Such call for greater standardization of clinical outcomes has been made previously in the MS field (16). We strongly recommend that even studies that chose to dichotomize the EDSS-based outcome include models that predict the EDSS as an ordinal scale and MSSS/ARMSS as continuous scales. Predicting when and how much progression will occur is a mathematically harder problem than predicting whether a patient is likely to progress. While the dichotomized model may predict that two patients will progress in the next 5 years, the continuous model may predict that one patient will progress 3 EDSS points starting next year and another will progress 0.5 EDSS points by the 5th year. This level of granularity, if validated, provides a greater biological insight into the mechanisms of disease progression and a stronger information gain for clinical management. Because the data (i e., EDSS) are already collected, applying different modeling strategies and reporting their outcomes are not difficult.

Report Broad and Accurate Metrics of Model Accuracy

We observed highly inadequate reporting of model accuracy metrics, at times limited only to the p-value. Values of p do not reliably reflect model accuracy; in fact, one can get a low p-value for a model that has an inverse relationship with a measured outcome. Or, in large cohorts, a clinically insignificant model (explaining <1% of the variance) may have a surprisingly low p-value. For continuous outcomes, correlation coefficients only reflect the strength of the association between measured and predicted outcomes, but not the accuracy of the model: e.g., let us imagine that measured and predicted outcomes are distributed in perfect (positive) line, resulting in correlation coefficients of 1. However, while the measured EDSS has spread of values between 0 and 10, the predicted EDSS may have a different spread of values: e.g., 4–6 or 1–2. In fact, such "mis-calibrated" models are quite common. The R2, reflecting the proportion of the variance explained by the model is preferable to correlation coefficients. However, the best indicator of model accuracy reflects how closely the model predictions match the absolute values of the

measured outcomes (i.e., 1:1 line), such as Lin's concordance coefficient (CCC). Current statistical packages, including freely available options such as R, can calculate all these statistical parameters. Their reporting will provide a better assessment of model accuracy and would facilitate comparison between studies.

Addressing the Clinical Utility of the Models

Not all models have, or must have clinical utility; as indicated above, molecular, genetic, or cellular biomarker predictors might be useful by simply linking specific pathophysiological processes or pathways to MS clinical outcomes. However, even these models should assess and publish metrics of clinical utility, such as receiver operating characteristic (ROC), accuracy, sensitivity/specificity, and positive and negative predictive values, so that clinicians correctly understand their potential clinical value (or lack thereof).

Validation of the Most Promising Observations in the Independent Cohort(s)

The low rate of independent validation (i.e., 8% of the studies) observed in this meta-analysis is, unfortunately, consistent with similar reports of very low independent validation rates (17). Because a "lack of validated predictive tools in MS" has been recognized before (18), funders need to devote more funding to high-quality, definite independent validation studies. Analogously, reviewers and readers should recognize that training cohort data, even cross-validation, has high probability to overestimate the generalizability of the model(s), and reward publications that include independent validation cohorts.

Deposit the Raw Data

Most journals do not limit the amount of **Supplementary Data**. Data sharing is essential to independently validate the algorithms that underlie published models, but also to explore stronger algorithms/models.

CONCLUSIONS

Finally, as evidenced by the summary of current EDSS-based models, we identified a strong need to develop validated models of MS clinical outcomes using cellular or molecular biomarkers. Vast majority of the models reviewed used clinical or MRI predictors. Although they may provide clinical value, they are less likely to yield the mechanistic insight into MS progression or MS severity necessary for the development of effective treatments for progressive MS or treatments that would abrogate the accumulation of disability in patients treated by current disease-modifying agents that successfully limit the formation of new lesions.

While most of these recommendations have no financial or logistical implications (i.e., they can be performed immediately on existing cohorts as they relate to the analytical steps of model development), increasing cohort sizes, and especially the inclusion of independent validation cohorts, requires substantial financial and human resources and cannot be accomplished without funders recognizing the importance of such properly powered studies and prioritizing them for financial support.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JL and EK performed the literature search and extracted all data for this meta-analysis. JL analyzed the data, generated the figures and Shiny App, and contributed to this paper. BB construed the project conceptually, guided and supervised all aspects of this study, and contributed to the writing of this paper. All authors critically reviewed and edited this paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2022.884089/full#supplementary-material

REFERENCES

1. Markovic-Plese S, McFarland HF. Immunopathogenesis of the multiple sclerosis lesion. *Curr Neurol Neurosci Rep.* (2001) 1:257–62. doi: 10.1007/s11910-001-0028-4
2. Harris JO, Frank JO, Patronas N, McFarlin DE, McFarland HF. Serial gadolinium-enhanced magnetic resonance imaging scans in patients with early, relapsing-remitting multiple sclerosis: implication for clinical trials and natural history. *Ann Neurol.* (1991) 29:548–55. doi: 10.1002/ana.410290515
3. Weideman AM, Tapia-Maltos MA, Johnson K, Greenwood M, Bielekova B. Meta-analysis of the age-dependent efficacy of multiple sclerosis treatments. *Front Neurol.* (2017) 8:577. doi: 10.3389/fneur.2017.00577
4. Komori M, Blake A, Greenwood M, Lin YC, Kosa P, Ghazali D, et al. CSF markers reveal intrathecal inflammation in progressive multiple sclerosis. *Ann Neurol.* (2015) 78:3–20. doi: 10.1002/ana.24408
5. Magliozzi R, Howell OW, Reeves C, Roncaroli F, Nicholas R, Serafini B, et al. A Gradient of neuronal loss and meningeal inflammation in multiple sclerosis. *Ann Neurol.* (2010) 68:477–93. doi: 10.1002/ana.22230
6. Campbell GR, Mahad DJ. Mitochondrial changes associated with demyelination: consequences for axonal integrity. *Mitochondrion.* (2012) 12:173–9. doi: 10.1016/j.mito.2011.03.007
7. Lassmann H, van Horssen J, Mahad D. Progressive multiple sclerosis: pathology and pathogenesis. *Nat Rev Neurol.* (2012) 8:647–56. doi: 10.1038/nrneurol.2012.168

8. Manouchehrinia A, Westerlind H, Kingwell E, Zhu F, Carruthers R, Ramanujam R, et al. Age related multiple sclerosis severity score: disability ranked by age. *Mult Scler.* (2017) 23:1938–46. doi: 10.1177/1352458517690618
9. Fanelli D, Costas R, Ioannidis JP. Meta-assessment of bias in science. *Proc Natl Acad Sci U S A.* (2017) 114:3714–9. doi: 10.1073/pnas.1618569114
10. Hackam DG, Redelmeier DA. Translation of research evidence from animals to humans. *JAMA.* (2006) 296:1731–2. doi: 10.1001/jama.296.14.1731
11. Button KS, Ioannidis JP, Mokrysz C, Nosek BA, Flint J, Robinson ES, et al. Power failure: why small sample size undermines the reliability of neuroscience. *Nat Rev Neurosci.* (2013) 14:365–76. doi: 10.1038/nrn3475
12. IntHout J, Ioannidis JP, Borm GF, Goeman JJ. Small studies are more heterogeneous than large ones: a meta-meta-analysis. *J Clin Epidemiol.* (2015). doi: 10.1016/j.jclinepi.2015.03.017
13. Ioannidis JP. Why most discovered true associations are inflated. *Epidemiology.* (2008) 19:640–8. doi: 10.1097/EDE.0b013e31818131e7
14. Ioannidis JP, Greenland S, Hlatky MA, Khoury MJ, Macleod MR, Moher D, et al. Increasing value and reducing waste in research design, conduct, and analysis. *Lancet.* (2014) 383:166–75. doi: 10.1016/S0140-6736(13)62227-8
15. Ioannidis JPA. Why replication has more scientific value than original discovery. *Behav Brain Sci.* (2018) 41:e137. doi: 10.1017/S0140525X18000729
16. Riley RD, Ensor J, Snell KI, Debray TP, Altman DG, Moons KG, et al. External validation of clinical prediction models using big datasets from e-health records or IPD meta-analysis: opportunities and challenges. *BMJ.* (2016) 353:i3140. doi: 10.1136/bmj.i3140
17. Xu Y, Goodacre R. On splitting training and validation set: a comparative study of cross-validation, bootstrap and systematic sampling for estimating the generalization performance of supervised learning. *J Anal Test.* (2018) 2:249–62. doi: 10.1007/s41664-018-0068-2
18. Li JS, Hamann A, Beaubien E. Outlier detection methods to improve the quality of citizen science data. *Int J Biometeorol.* (2020) 64:1825–33. doi: 10.1007/s00484-020-01968-z
19. Vanderaa C, Gatto L. Replication of single-cell proteomics data reveals important computational challenges. *Expert Rev Proteomics.* (2021) 18:835–43. doi: 10.1080/14789450.2021.1988571
20. Potvin O, Mouiha A, Dieumegarde L, Duchesne S. Alzheimer's Disease Neuroimaging I. Normative data for subcortical regional volumes over the lifetime of the adult human brain. *Neuroimage.* (2016) 137:9–20. doi: 10.1016/j.neuroimage.2016.05.016
21. Williams JL, Chu HC, Lown MK, Daniel J, Meckl RD, Patel D, et al. Weaknesses in experimental design and reporting decrease the likelihood of reproducibility and generalization of recent cardiovascular research. *Cureus.* (2022) 14:e21086. doi: 10.7759/cureus.21086
22. Jafari M, Ansari-Pour N. Why, when and how to adjust your p-Values? *Cell J.* (2019) 20:604–7. doi: 10.22074/cellj.2019.5992
23. Begley CG, Ellis LM. Drug development: raise standards for preclinical cancer research. *Nature.* (2012) 483:531–3. doi: 10.1038/483531a
24. Freedman LP, Cockburn IM, Simcoe TS. The economics of reproducibility in preclinical research. *PLoS Biol.* (2015) 13:e1002165. doi: 10.1371/journal.pbio.1002165
25. Ioannidis JP. Why most published research findings are false. *PLoS Med.* (2005) 2:e124. doi: 10.1371/journal.pmed.0020124
26. Young NS, Ioannidis JP, Al-Ubaydli O. Why current publication practices may distort science. *PLoS Med.* (2008) 5:e201. doi: 10.1371/journal.pmed.0050201
27. Ioannidis JP. An epidemic of false claims. Competition and conflicts of interest distort too many medical findings. *Sci Am.* (2011) 304:16. doi: 10.1038/scientificamerican0611-16
28. Bielekova B, Vodovotz Y, An G, Hallenbeck J. How implementation of systems biology into clinical trials accelerates understanding of diseases. *Front Neurol.* (2014) 5:102. doi: 10.3389/fneur.2014.00102
29. Ramspek CL, Jager KJ, Dekker FW, Zoccali C, van Diepen M. External validation of prognostic models: what, why, how, when and where? *Clin Kidney J.* (2021) 14:49–58. doi: 10.1093/ckj/sfaa188
30. Barbour C, Kosa P, Komori M, Tanigawa M, Masvekar R, Wu T, et al. Molecular-based diagnosis of multiple sclerosis and its progressive stage. *Ann Neurol.* (2017) 82:795–812. doi: 10.1002/ana.25083
31. Jackson KC, Sun K, Barbour C, Hernandez D, Kosa P, Tanigawa M, et al. Genetic model of MS severity predicts future accumulation of disability. *Ann Hum Genet.* (2019). doi: 10.1111/ahg.12342
32. Pham L, Harris T, Varosanec M, Morgan V, Kosa P, Bielekova B. Smartphone-based symbol-digit modalities test reliably captures brain damage in multiple sclerosis. *NPJ Digit Med.* (2021) 4:36. doi: 10.1038/s41746-021-00401-y
33. Weideman AM, Barbour C, Tapia-Maltos MA, Tran T, Jackson K, Kosa P, et al. New multiple sclerosis disease severity scale predicts future accumulation of disability. *Front Neurol.* (2017) 8:598. doi: 10.3389/fneur.2017.00598
34. Messan KS, Pham L, Harris T, Kim Y, Morgan V, Kosa P, et al. Intra-individual reproducibility as essential determinant of clinical utility of smartphone-based neurological disability tests. *medRxiv*. (2021):2021. doi: 10.1101/2021.06.01.21258169
35. Kosa P, Ghazali D, Tanigawa M, Barbour C, Cortese I, Kelley W, et al. Development of a sensitive outcome for economical drug screening for progressive multiple sclerosis treatment. *Front Neurol.* (2016) 7:131. doi: 10.3389/fneur.2016.00131
36. Barbour C, Kosa P, Varosanec M, Greenwood M, Bielekova B. Molecular models of multiple sclerosis severity identify heterogeneity of pathogenic mechanisms. medRxiv. (2020):2020. doi: 10.1101/2020.05.18.20105932
37. Landis SC, Amara SG, Asadullah K, Austin CP, Blumenstein R, Bradley EW, et al. A call for transparent reporting to optimize the predictive value of preclinical research. *Nature.* (2012) 490:187–91. doi: 10.1038/nature11556
38. Kong XZ, Group ELW, Francks C. Reproducibility in the absence of selective reporting: An illustration from large-scale brain asymmetry research. *Hum Brain Mapp.* (2020).
39. Friese M, Frankenbach J. p-Hacking and publication bias interact to distort meta-analytic effect size estimates. *Psychol Methods.* (2020) 25:456–71. doi: 10.1037/met0000246
40. Stanley TD, Doucouliagos H, Ioannidis JP. Finding the power to reduce publication bias. *Stat Med.* (2017) 36:1580–98. doi: 10.1002/sim.7228

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ORIGINAL RESEARCH
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Peptidylarginine Deiminase 2 Autoantibodies Are Linked to Less Severe Disease in Multiple Sclerosis and Post-treatment Lyme Disease

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Background: Peptidylarginine deiminase 2 (PAD2) mediates the post-translational conversion of arginine residues in proteins to citrullines and is highly expressed in the central nervous system (CNS). Dysregulated PAD2 activity has been implicated in the pathogenesis of several neurologic diseases, including multiple sclerosis (MS). In this study, we sought to define the cellular and regional expression of the gene encoding for PAD2 (i.e. *PADI2*) in the human CNS using publicly available datasets and evaluate whether anti-PAD2 antibodies were present in patients with various neurologic diseases.

Methods: A total of 491 study participants were included in this study: 91 people with MS, 32 people with neuromyelitis optica (NMO), 281 people with post-treatment Lyme disease (PTLD), and 87 healthy controls. To measure *PADI2* expression in the CNS from healthy individuals, publicly available tissue and single cell RNA sequencing data was analyzed. Anti-PAD2 antibodies were measured in the serum of study participants using anti-PAD2 ELISA. Clinical and demographic variables were compared according to anti-PAD2 antibody positivity for the MS and PTLD groups and correlations between anti-PAD2 levels and disease severity were examined.

Results: *PADI2* expression was highest in oligodendrocytes (mean ± SD; 6.4 ± 2.2), followed closely by astrocytes (5.5 ± 2.6), microglia/macrophages (4.5 ± 3.5), and oligodendrocyte precursor cells (3.2 ± 3.3). There was an increased proportion of anti-PAD2 positivity in the MS (19.8%; $p = 0.007$) and PTLD groups (13.9%; $p = 0.057$) relative to the healthy controls (5.7%), and these antibodies were not detected in NMO patients. There was a modest inverse correlation between anti-PAD2 levels and disease severity in people with MS ($\tau = -0.145$, $p = 0.02$), with levels being the highest in those with relapsing-remitting disease. Similarly, there was a modest inverse correlation between anti-PAD2 levels and neurocognitive score ($\tau = -0.10$, $p = 0.027$) in people with PTLD, with difficulty focusing, memory changes, fatigue, and difficulty finding words contributing most strongly to the effect.

Conclusion: *PADI2* expression was observed in diverse regions and cells of the CNS, and anti-PAD2 autoantibodies were associated with less severe symptoms in subsets of patients with MS and PTLD. These data suggest that anti-PAD2 antibodies may attenuate inflammation in diseases of different etiologies, which are united by high *PADI2* expression in the target tissue.

Keywords: PAD2, citrullination, autoantibodies, central nervous system, multiple sclerosis, Lyme disease

INTRODUCTION

Growing evidence suggests a role for the peptidylarginine deiminase 2 (PAD2) enzyme in the pathogenesis of neurodegenerative, neuroinflammatory, and autoimmune diseases (1, 2). PAD2 belongs to a family of five calcium-dependent enzymes that convert arginine residues in proteins to the non-classical amino acid citrulline, in a process known as citrullination (3). PAD2 is normally expressed in a variety of tissues in the body, with the brain being among the highest PAD2-expressing tissues (4). PAD2 plays important physiological roles in several cellular processes (5), and is known to be expressed by oligodendrocytes where it regulates gene transcription and citrullination of myelin basic protein (MBP) (6, 7).

PAD2 dysregulation and increased expression have been observed in the central nervous system (CNS) of people with various neuroinflammatory and neurodegenerative diseases, including multiple sclerosis (MS) (1, 8). MS is a demyelinating disease of the CNS with both neurodegenerative and autoimmune components (9), and is classified into three general subtypes based on the pattern of disease flare and progression: relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), and primary progressive MS (PPMS). Increased PAD2 and citrullinated protein expression are observed in normal appearing white matter in the CNS of people with MS, thought to be driven by hypomethylation of the PAD2 promoter leading to increased PAD2 expression in these regions (10, 11). Increased citrullination of MBP by PAD2 leads to conformational changes in the myelin sheath, increased protease accessibility, and destabilization, which can disrupt nerve impulses and is hypothesized to reveal new MBP epitopes for immune recognition (8, 12).

PAD2 has also been implicated in playing a pathogenic role in the systemic autoimmune disease rheumatoid arthritis (RA). Many parallels exist between the role of PAD2 in MS and RA, including elevated expression of PAD2 in the target tissue and the striking efficacy of PAD inhibition in reducing disease severity in mouse models of both diseases (10, 13–15). PAD2 is found at high levels in the joint tissue and extracellularly in the synovial fluid from patients with RA (2, 16). Citrullination of a group of proteins by PAD2 is implicated in their targeting by anti-citrullinated antibodies, hallmark serological findings in RA (17). Citrullinated proteins have also been shown to stimulate pro-inflammatory cytokine secretion by macrophages, *via* ligation of toll like receptor 4 (TLR4) (18). Interestingly, we recently found autoantibodies to PAD2 in a subset of people with RA with less severe and progressive joint disease (19), suggesting that anti-PAD2 antibodies may attenuate the pathogenic role of PAD2 in RA.

In this study, we sought to define the cellular and regional expression of the gene encoding for PAD2 (i.e. *PADI2*) in the CNS and evaluate whether anti-PAD2 antibodies were present in patients with MS and other neurologic diseases with known or suspected autoimmune etiology. Neuromyelitis optica (NMO) is a demyelinating disease of the CNS characterized by inflammation of the optic nerve and spinal cord (20). Autoantibodies that target aquaporin-4 are used as biomarkers to facilitate diagnosis in NMO (20), but not all patients are positive, suggesting that other antigens may also be targeted. Post-treatment Lyme disease (PTLD) is a heterogeneous condition of unknown etiology that occurs in a subset of people who are treated for Lyme disease but do not return to baseline health and can have persistent systemic, musculoskeletal, and neurocognitive symptoms (21). The discovery of autoantibodies with reactivity to CNS tissue and observed microglial activation in PTLD suggests that an autoimmune response to CNS antigens may occur in some individuals (22, 23). Although PAD2 has not been previously studied in NMO or PTLD, both are diseases with neurologic and autoimmune components (20, 22), in which PAD2 dysregulation may affect disease pathologies. Understanding the CNS expression and immunologic targeting of PAD2 in neurological diseases has important mechanistic and clinical implications, as defining mechanisms that downregulate PAD2 expression or activity may hold promise for the treatment of these disorders.

MATERIALS AND METHODS

Study Population

Sera from a total of 491 study participants were included in this study. Of these, 91 were people with MS, 32 were people with NMO, 281 were people with PTLD, and 87 were healthy controls. Data and sera from people with MS came from the Johns Hopkins Multiple Sclerosis Center, with recruitment and eligibility criteria described extensively elsewhere (24). Data and sera from people with NMO came from the Johns Hopkins NMO Clinic. Individuals were included in this study if they had a diagnosis of NMO as defined by the 2006 Wingerchuk criteria (25), and all but one individual was positive for aquaporin-4

Abbreviations: PTLD, post-treatment Lyme disease; PLQS, Post-Lyme Questionnaire of Symptoms; SLICE, Studies of Lyme disease Immunology and Clinical Events

TABLE 1 | Demographics and anti-PAD2 positivity.

	HC ($n = 87$)*	MS ($n = 91$)*	NMO ($n = 32$)	PTLD ($n = 281$)*
Age (years), mean ± SD	39.9 ± 13.5	48.8 ± 12.5	49.5 ± 11.3	48.10 ± 15.74
Male, *n* (%)	33 (38.4%)	25 (28.4%)	8 (25.0%)	158 (56.2%)
White, *n* (%)	60 (69.8%)	65 (73.9%)	19 (59.4%)	257* (92.1%)
Anti-PAD2+, *n* (%)	5 (5.7%)	18 (19.8%)	0 (0%)	39 (13.9%)

MS, multiple sclerosis; NMO, neuromyelitis optica; HC, healthy controls; SD, standard deviation; Anti-PAD2+, anti-PAD2 antibody positive individuals.
**Demographic data was available for 88 people with MS and 86 healthy controls, and race data was available for 279 people with PTLD.*

antibodies. Data and sera from people with PTLD and a subset of the healthy controls ($n = 22$) came from the Studies of Lyme Disease Immunology and Clinical Events (SLICE) at the Johns Hopkins Lyme Disease Research Center, as previously described, with the exception that people with PTLD included in the current study were not required to have 6 months' illness duration (26). Data and sera from the remaining healthy controls ($n = 65$) came from an ongoing observational study of healthy donors at the Johns Hopkins Division of Rheumatology. Healthy volunteers who are not pregnant and who do not have a history of cancer, autoimmune disease, or active tuberculosis/HIV/hepatitis infections were eligible for the study. None of the healthy controls had a known history of Lyme disease. All participants signed written informed consent approved by the Johns Hopkins Institutional Review Board.

Demographic variables measured from all study participants include age, sex, and race. Clinical variables measured from people with MS include duration of illness and disease severity measured by the MS severity score (MSSS), as previously described (27, 28). People with MS were grouped according to their MS type, RRMS ($n = 41$), SPMS ($n = 31$), or PPMS ($n = 16$). Three people with MS did not have a documented MS classification, so were exclude from subtype analysis. Anti-aquaporin 4 antibody status was available for individuals with NMO, but no additional clinical information was available. Clinical variables measured from people with PTLD at the time of the study visit included duration of prior antibiotic treatment, duration of illness from onset of initial Lyme disease, two-tier Lyme disease serologic status (29), neurologic Lyme disease status, defined by medical records confirming Bell's Palsy, neuropathy, meningitis, or encephalitis with positive two-tier serology, and symptom severity. Symptom severity associated with PTLD, including the severity of neurologic symptoms, was measured using PLQS as previously described (26). The researchers were blinded to these clinical variables when conducting the experiments to minimize bias.

In silico Analyses of *PADI2* Expression

Single cell sequencing data was retrieved from Gene Expression Omnibus (GEO) database accession no. GSE67835 (30), which contains sequence data from 466 cells (oligodendrocytes ($n = 38$), astrocytes ($n = 62$), microglia/macrophages ($n = 16$), oligodendrocytes precursor cells ($n = 18$), hybrid cells ($n = 46$), microglia/macrophage ($n = 16$), neurons ($n = 131$), and endothelial cells ($n = 20$). Cells were isolated from human cortical tissue from eight adults and individual cells were classified into the categories above as described (31). Processing and visualization of the data was carried out in the R statistical language as previously described (32, 33). We retrieved selected brain region expression data for *PADI2* from two public data sets. The first was data from five donors with 26 brain regions included in the 2010 Allen Institute for Brain Science, Allen Human Brain Atlas (34). Microarray data from three *PADI2* specific probes were normalized across all brains as previously described (https://help.brain-map.org/display/humanbrain/Documentation). The original search can be reproduced at http://human.brain-map.org/microarray/search/show?exact_match=false&search_term=PADI2&search_type=gene&page_num=0. The second dataset was from The Genotype-Tissue Expression (GTEx) Project. The Genotype-Tissue Expression (GTEx) Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health, and by National Cancer Institute (NCI), National Human Genome Research Institute (NHDRI), National Heart, Lung, and Blood Institute (NHLBI), National Institute on Drug Abuse (NIDA), National Institute of Mental Health (NIMH), and National Institute of Neurological Disorders and Stroke (NINDS). The data used for the analyses described in this manuscript were obtained from the GTEx Portal (https://gtexportal.org/home/) on 09/10/2020 using Ensembl Gene ID ENSG00000117115.12. The 17,382 samples included in this dataset are from 54 tissue regions from 948 donors. Details for the exact number of samples for each brain region can be found here: https://gtexportal.org/home/tissueSummaryPage. Data were normalized as previously described (https://gtexportal.org/home/documentationPage#staticTextAnalysisMethods).

Anti-PAD2 ELISA

Anti-PAD2 antibodies were measured in the serum of study participants using an in-house generated anti-PAD2 enzyme-linked immunosorbent assay (ELISA), as previously described (19). Briefly, recombinant human PAD2 protein containing N-terminal 6 × histidine and T7 tags was bacterially expressed and purified. The histidine tag was removed by thrombin digestion and 100 ng PAD2 protein was coated into each well of a high-binding polystyrene 96-well EIA plate (Costar) overnight. A dilution of 1:250 patient or healthy control sera was used for the primary antibody and a 1:7,500 dilution of goat anti-human IgG HRP was used for the secondary antibody. An 8-point standard curve was present on each plate comprised of dilutions of a known anti-PAD2 positive serum and was used to calculate anti-PAD2 Arbitrary Units (AU) for each sample. Blank wells coated in PBS alone were used to determine the background binding of each serum and these values were subtracted from those obtained from PAD2-coated wells. The cutoff for positivity was set at 4.5 AU as previously defined (6).

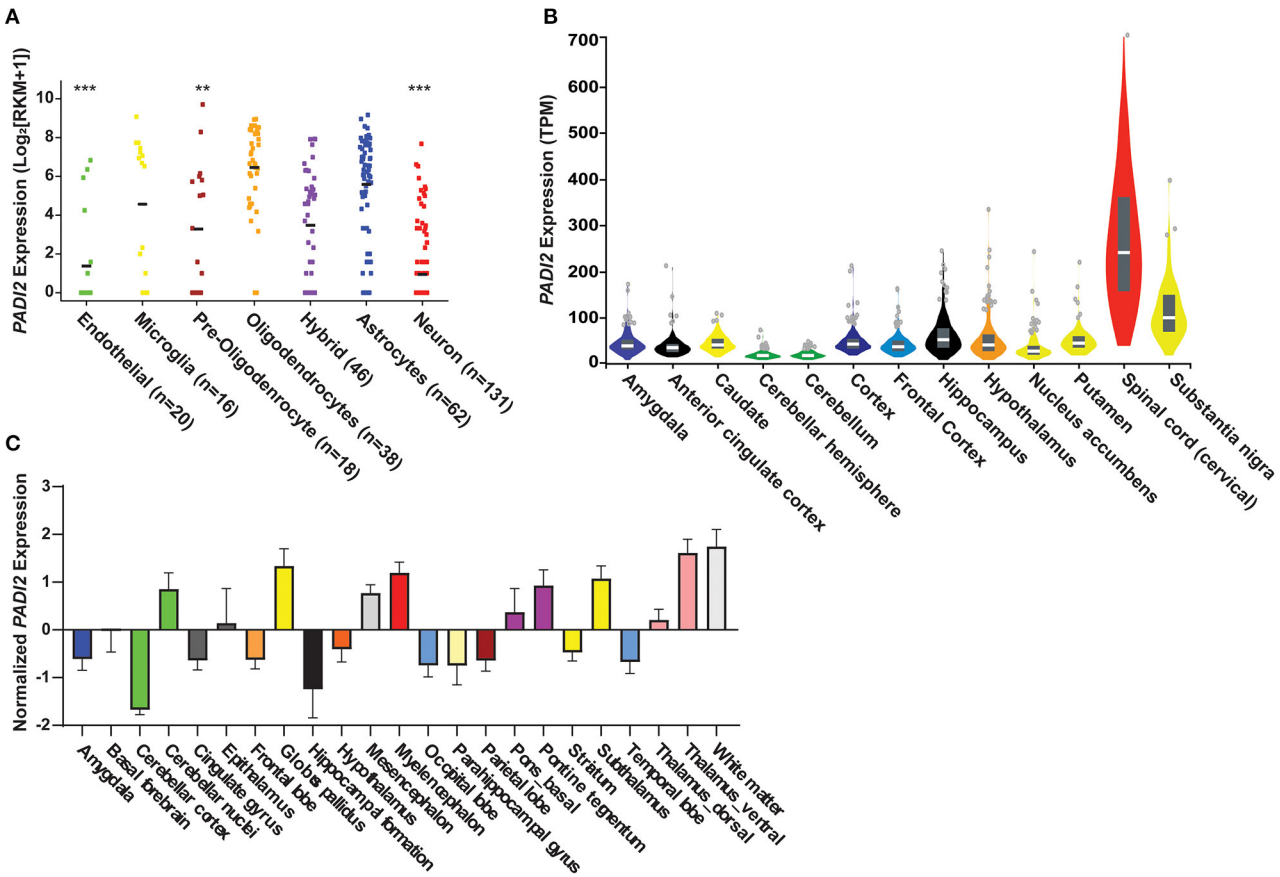

FIGURE 1 | PAD2 transcripts are detectable in glia in the human central nervous system. **(A)** *in silico* analyses of single cell RNA-sequencing data from the GEO human cortex show *PADI2* expression in reads per kilobase of transcript per million (RPKM; *y*-axis) in various cell types (*x*-axis) of the CNS. The number of cells analyzed in each group is provided after the region in paratheses. The mean RPKM of oligodendrocytes was compared to that to the mean of each other cell type in the dataset by ANOVA with Kruskal–Wallis test to corrected for multiple comparisons. ****p-value <0.0001; **p-value <0.01. **(B)** *PADI2* expression from bulk RNA sequencing of different CNS regions was downloaded from the GTEx database and gene expression in transcript per million (TPM) is plotted (*y*-axis) for different brain regions and the spinal cord (*x*-axis). Image is modified from (https://gtexportal.org/home/gene/PADI2). **(C)** Transcript expression of *PADI2* in different regions of the brain measured by microarray was downloaded from The Allen Institute. Normalized *PADI2* gene expression (*y*-axis) is shown for multiple brain regions (*x*-axis).

Statistical Analyses

GraphPad Prism version 8.3.0 and SAS statistical software (version 9.4, SAS Institute, Cary, NC) were used for all statistical analyses and graphs. A p-value ≤ 0.05 was considered statistically significant throughout. Brown–Forsythe ANOVA with a Dunnett's T3 correction for multiple hypotheses analyses were performed to compare *PADI2* expression in oligodendrocytes to other cell types. Fisher's exact tests were used to determine if the proportions of anti-PAD2 antibody positive people differed between groups, and median anti-PAD2 antibody levels between each group were compared using analysis of variance (ANOVA, Kruskal–Wallis), corrected for multiple comparisons. Clinical and demographic variables were compared according to anti-PAD2 antibody positivity for the MS and PTLD groups using Student's t-tests or Mann–Whitney tests for normally and non-normally distributed variables, Chi-squared tests or Fisher's exact tests for categorical variables, as appropriate. The median anti-PAD2 antibody level in each MS subtype was compared using a one-way ANOVA (Kruskal–Wallis) corrected for multiple comparisons. Correlations between anti-PAD2 antibody levels and MS disease severity or PTLD symptom severity were examined using the Kendall's Tau correlation coefficient.

RESULTS

PAD2 Is Expressed Broadly Throughout the CNS

We quantified the expression levels of *PADI2* within specific cells and regions of the CNS by *in silico* analysis of publicly available transcriptomic data (30, 31, 34). This analysis revealed that *PADI2* transcripts are detectable in multiple cell types in the CNS (**Figure 4A**). *PADI2* expression was highest in oligodendrocytes [mean reads per kilobase of transcript per million (RPKM) ± standard deviation; 6.4 ± 2.2], as expected. We compared the expression of *PADI2* in all other cell types to oligodendrocytes

TABLE 2 | Characteristics of people with MS according to anti-PAD2 antibody status.

	Anti-PAD2+ ($n = 17$)	Anti-PAD2– ($n = 71$)	*p*-Value
Age (years), mean ± SD	50.8 ± 14.6	48.3 ± 12.0	0.47
Male, *n* (%)	4 (23.5%)	21 (29.6%)	0.78
White, *n* (%)	14 (82.4%)	51 (71.8%)	0.54
Duration of illness* (years), mean ± SD	16.6 ± 14.9	11.7 ± 9.1	0.10
Treatment*			
Avonex, *n* (%)	2 (11.8%)	3 (4.2%)	0.25
Betaseron, *n* (%)	0 (0%)	1 (1.4%)	1.0
Rebif, *n* (%)	3 (17.6%)	7 (9.9%)	0.40
Copaxone, *n* (%)	6 (35.3%)	20 (28.2%)	0.57
Gilenya, *n* (%)	0 (0%)	1 (1.4%)	1.0
Lemtrada, *n* (%)	0 (0%)	1 (1.4%)	1.0
Tecfidera, *n* (%)	3 (17.6%)	6 (8.5%)	0.37
Rituximab, *n* (%)	0 (0%)	3 (4.2%)	1.0
Tysabri, *n* (%)	2 (11.8%)	12 (16.9%)	1.0

Anti-PAD2+, anti-PAD2 antibody positive; Anti-PAD2–, anti-PAD2 antibody negative.
**Duration of illness data was available for n = 87 and treatment data was available for n = 70 people.*

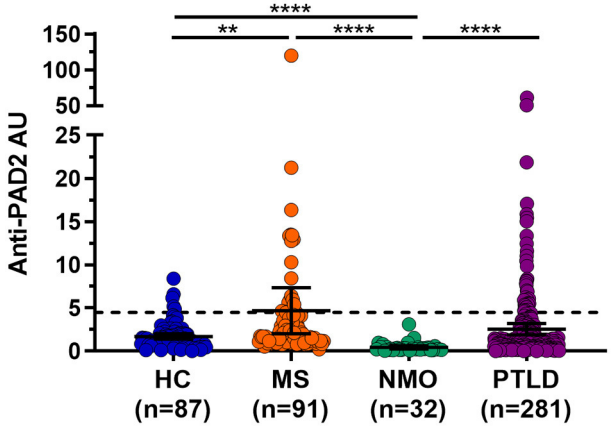

FIGURE 2 | Anti-PAD2 antibody levels in all patient groups. Anti-PAD2 Arbitrary Units (AU) for healthy controls (HC; $n = 87$) or people with multiple sclerosis (MS; $n = 91$), neuromyelitis optica (NMO; $n = 32$), and post-treatment Lyme disease syndrome (PTLD; $n = 281$) as measured by ELISA are shown. The dotted line represents the cutoff value for positivity at 4.5 AU. The median and 95% confidence interval of each group are shown. ****Mann–Whitney *p*-value <0.0001 and ** < 0.01.

by Brown–Forsythe ANOVA with a Dunnett's T3 correction for multiple hypotheses. Astrocytes expressed *PADI2* at similarly high levels as oligodendrocytes (5.5 ± 2.6 RPKM, $p = 0.38$), followed by microglia/macrophages (4.5 ± 3.5 RPKM, $p = 0.29$), which demonstrated bimodal expression of *PADI2*, with one subpopulation of cells expressing high levels of *PADI2* and other low levels. Oligodendrocytes precursor cells (3.2 ± 3.3 RPKM, $p = 0.006$), neurons (0.9 ± 1.8 RPKM, $p < 0.0001$) and endothelial cells (1.3 ± 2.4 RPKM, $p < 0.0001$) were the lowest expressors of *PADI2*. Analysis of the GTEx database indicated that *PADI2* was expressed in multiple regions of the CNS with the highest expression observed in the spinal cord and substantia nigra (**Figure 4B**). Analysis of gene expression data from the Allen Human Brain Atlas (34) showed similar results, with *PADI2* transcripts detectable in multiple regions of the human brain, including the white matter, basal ganglia, midbrain, thalamus, and pons (**Figure 4C).** Together, the data reveal widespread expression of *PADI2* within multiple CNS regions and cell types.

Anti-PAD2 Antibodies Are Found in People With MS and PTLD

Given the widespread expression of *PADI2* throughout the brain and spinal cord, and the finding that PAD2 is a known autoantigen in RA (19), we reasoned that PAD2 may become a target of the immune responses in individuals with CNS symptoms in whom an autoreactive process has been implicated. To address this hypothesis, we performed an ELISA to determine the prevalence of anti-PAD2 antibodies in the sera of individuals with diseases known or suspected to have an autoimmune process affecting the CNS: MS ($n = 91$), NMO ($n = 32$), and PTLD ($n = 281$). Sera from a group of healthy donors was included as a control population ($n = 87$). Demographic variables for each group including age, sex, and race are shown in **Table 1**. The average age of the healthy controls was lower than in the disease groups, but age was similar among the MS, NMO, and PTLD groups. In addition, the PTLD group contained more individuals who identified as male and white. Analysis of anti-PAD2 antibodies revealed an increased proportion of anti-PAD2 positivity in the MS (19.8%, 95% CI: 12.9%−29.1%; $p = 0.007$) and PTLD groups (13.9%, 95% CI: 10.3%−18.4%; $p = 0.057$) relative to the healthy controls (5.7%, 95% CI: 2.5%−12.8%; **Table 1**), with significantly higher median antibody levels in people with MS compared to healthy controls [median (interquartile range) of 1.96 (1.17–4.14) vs. 1.34 (0.78–1.98) anti-PAD2 AU; $p_{adj} = <0.001$; **Figure 2**]. Patients with NMO had significantly lower levels of anti-PAD2 antibodies [0.21 (0.15–0.46) anti-PAD2 AU] than patients with MS, PTLD, or healthy controls ($p_{adj} < 0.0001$ for all; **Figure 2**). Together, these data revealed that anti-PAD2 antibodies are present in a subset of people with MS and PTLD.

Anti-PAD2 Antibodies Are Associated With Less Severe MS Symptoms and Relapsing-Remitting Disease

The finding of anti-PAD2 antibodies in a subset of people with MS coupled to the previously reported association of anti-PAD2 antibodies with less severe disease in RA, led us to explore whether anti-PAD2 antibodies were associated with disease severity in MS (**Table 2**). There were no significant differences in demographic variables, disease duration, or current treatment between anti-PAD2 positive and negative people with MS. However, there was a modest and inverse correlation

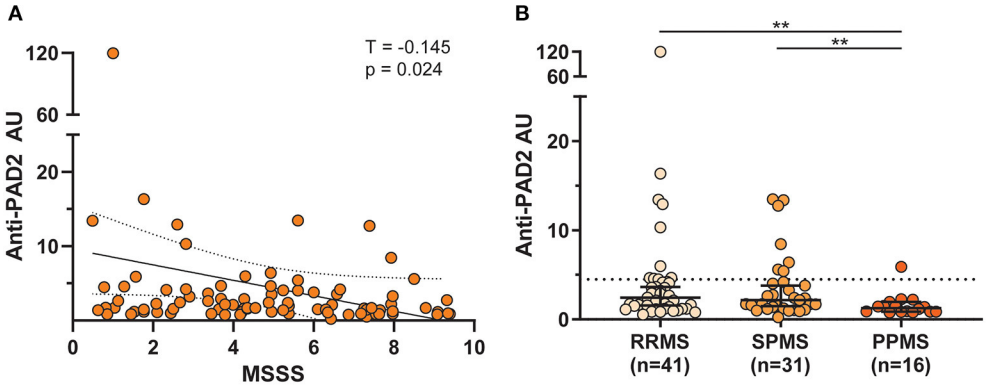

FIGURE 3 | Anti-PAD2 antibody levels by MS subtype and disease severity. **(A)** Scatterplot showing the multiple sclerosis severity score (MSSS) plotted against anti-PAD2 arbitrary units (AU) of all comers with MS with available clinical data ($n = 88$). A univariate analysis was performed between MSSS and anti-PAD2 AU and the Kendall's Tau correlation coefficient (T), p-value, trendline (solid black line), and 95% confidence intervals (dotted black lines) are shown. **(B)** Anti-PAD2 Arbitrary Units (AU) in people with RRMS ($n = 41$), SPMS ($n = 31$), and PPMS ($n = 16$) were plotted and compared using a Kruskal–Wallis test adjusted for multiple comparisons. The median and 95% confidence interval are shown. $^{**}p < 0.01$.

between anti-PAD2 antibody levels and disease severity, as measured by MSSS in a univariate analysis ($\tau = -0.145$, $p = 0.02$) of all patients with MS, but this trend was not maintained in a multivariable model adjusting for age, sex, and treatment (**Figure 3A**). In addition, when assessed by MS subtype, anti-PAD2 antibody levels were significantly higher in people with RRMS and SPMS, compared to those with PPMS (**Figure 3B**). While 22.0% of people with RRMS (9/41) and 22.6% of people with SPMS (7/31) were anti-PAD2 positive, these antibodies were only detected in one individual with PPMS (1/16; 6.3%).

Anti-PAD2 Antibodies Are Associated With Less Severe PTLD Symptoms

Given the clinical heterogeneity in PTLD and the finding that anti-PAD2 antibodies associated with less severe disease in MS, we sought to define whether these autoantibodies associated with neurologic symptom severity in PTLD. When grouped by anti-PAD2 antibody status, anti-PAD2 positive people with PTLD were significantly older, but did not differ by race, sex, two-tier serologic positivity for Lyme disease, duration of illness, duration of antibiotic treatment since the onset of their Lyme disease, or diagnosis of neurologic Lyme disease during the acute infection (**Table 3**). In a univariate analysis, there was a modest and inverse correlation between anti-PAD2 antibody levels and neurocognitive score ($p = 0.027$), with difficulty focusing or concentrating, memory changes, fatigue, and difficulty finding words contributing most strongly to the effect, but this trend was not maintained in a multivariable model adjusting for age and duration of antibiotic treatment (**Figure 4A**). Analysis of other systemic symptoms assessed by the PLQS revealed that difficulty sleeping and changes in the frequency or urgency of urination also negatively correlated with anti-PAD2 antibodies in PTLD (**Figure 4B**). Given that age positively correlated with anti-PAD2 antibodies, we performed a case-control study with 30 individuals with PTLD who had the lowest scores on the PLQS (<5) and 30 individuals with the highest neurological scores (≥17) matched for age, sex, and disease duration. Consistent with the previous observations, anti-PAD2 antibody levels were significantly higher in patients with a lower burden of neurocognitive symptoms ($p = 0.036$).

TABLE 3 | Characteristics of people with PTLD according to anti-PAD2 antibody status.

	Anti-PAD2+ ($n = 39$)	Anti-PAD2– ($n = 242$)	p-Value
Age (years), mean ± SD	52.82 ± 15.44 (18.00, 79.00)	47.34 ± 15.68 (18.00, 82.00)	0.043
Male *n* (%)	20 (51.3%)	138 (57.0%)	0.619
White*, *n* (%)	34 (87.2%)	223 (92.9%)	0.208
Positive two-tier Lyme disease serology*, *n* (%)	18 (46.2%)	101(42.3%)	0.779
Duration of illness (years), mean ± SD	3.53 ± 5.23 (0.13, 27.68)	3.13 ± 3.98 (0.06, 28.59)	0.579
Duration of antibiotic treatment (years), mean ± SD	0.36 ± 0.53 (0.04, 2.46)	0.26 ± 0.41 (0.01, 3.12)	0.174
Neurologic Lyme, *n* (%)	1 (2.6%)	20 (8.3%)	0.328

*Anti-PAD2+, anti-PAD2 antibody positive; Anti-PAD–, anti-PAD2 antibody negative; SD, standard deviation. *Race data was available for n = 240 anti-PAD2- people and positive two-tier Lyme disease serology data was available for n = 239 anti-PAD2- people.*

DISCUSSION

Our study sought to define the expression of *PADI2* in the CNS at the cellular and regional level using publicly available datasets and to determine whether people with CNS pathologies generate autoantibodies to the PAD2 protein. We observed widespread

FIGURE 4 | Anti-PAD2 antibody levels in PTLD inversely correlate with symptom severity. **(A)** A bar graph with the Kendall Tau's correlation coefficients for symptoms measured by the PLQS that are included in the neurocognitive score are shown. **(B)** Kendall Tau's correlation coefficients for symptoms measured by the PLQS that are not included in the neurocognitive score are shown. For **(A)** and **(B)**, the dark purple bars indicate symptoms with a significant $p \leq 0.05$, the light purple bars indicate those with $p \leq 0.1$, and the gray bars indicate symptoms with $p > 0.1$. **(C)** Anti-PAD2 antibody levels were compared in demographically matched people with PTLD who had the lowest (score <5; $n = 17$; dark purple squares) or highest (score ≥17; $n = 17$, light purple circles) burden of neurocognitive symptoms, as measured by the PLQS. Median anti-PAD2 antibody arbitrary units (AU) were compared by group using the Mann–Whitney *U*-test. *$p < 0.05$.

expression of *PADI2* in several cell types and regions within the CNS and a higher prevalence of anti-PAD2 antibodies in people with MS and PTLD compared to either healthy controls or people with NMO. We found an enrichment of anti-PAD2 antibodies in people with relapsing subtypes of MS (RRMS and SPMS), and a modest inverse correlation of anti-PAD2 antibody levels with disease severity. Surprisingly, we also found anti-PAD2 antibodies in a subset of PTLD patients and again observed a modest association with less severe disease. It is interesting to note the lack of anti-PAD2 antibodies in people with NMO, suggesting that the pathologic process is distinct and does not result in autoimmunity to PAD2. Indeed, although NMO is a demyelinating disease of the CNS that shares several clinical features with MS, autoantibodies to aquaporin-4 are diagnostic of the disease and have been identified as a key pathogenic mediator of CNS damage (20), mechanistically setting it apart from MS. Our findings in MS and PTLD parallel our previous observation that anti-PAD2 antibodies are associated with milder symptoms in people with RA, and suggest that anti-PAD2 antibodies may play a role in attenuating inflammation across a spectrum of disorders.

The presence of anti-PAD2 antibodies in subsets of people with RRMS and SPMS and association with a less severe disease in MS suggests that these antibodies may define a mechanistically distinct group of patients in which PAD2 plays a pathogenic role. An important criterion in the diagnosis of MS is the presence of oligoclonal bands (OCBs), indicative of immunoglobins of the IgG subclass, in the cerebrospinal fluid (CSF) (35). OCBs are present in 95% of people with MS and are regarded as important indicators for the diagnosis of MS. However, the antigens targeted by these antibodies, which carry the potential to provide significant insight into MS etiology, remain largely unknown (36). Considering the known role of PAD2 in MS pathogenesis and our discovery that PAD2 is a target antigen in a subset of people with MS, it will be important to define whether PAD2 is targeted by autoantibodies present in the CSF of patients. In addition, longitudinal studies in larger MS cohorts are needed to interrogate whether anti-PAD2 antibodies associate with less severe or progressive disease at the individual level.

The finding that anti-PAD2 antibodies are present in a subset of people with PTLD suggests an underlying immunological component in PTLD pathology in these individuals. Although PTLD is an idiosyncratic disease of unknown etiology, one long-standing hypothesis is that the bacterium that causes Lyme disease, *Borrelia burgdorferi*, may trigger an autoimmune response resulting in persistent symptoms even after successful antibiotic treatment (37). Interestingly, a recent positron emission tomography (PET) imaging study, using a radiotracer specific for activated microglia and reactive astrocytes, demonstrated high levels of signal across multiple brain regions in people with PTLD compared to healthy controls (22). This finding suggested diffuse immune activation in the brains of people with PTLD that may contribute to the development of neurocognitive symptoms (26). Our finding of high PAD2 expression in both microglia and astrocytes as well as in multiple regions of the CNS parallels this observation and suggests that further study of the role of PAD2 in PTLD is warranted. Longitudinal studies are needed to examine whether anti-PAD2 antibodies are present early in Lyme disease infection, are able to predict the development of PTLD in people with acute Lyme, and are associated with changes in the clinical progression of PTLD.

The widespread expression of *PADI2* in the CNS may provide an explanation for why anti-PAD2 antibodies correlate with less severe symptoms in both MS and PTLD. Our working

model is that PAD2 dysregulation in cells expressing high levels of PAD2 may result in higher PAD2 activity that contributes to immune activation in the CNS *via* two primary mechanisms: (1) activation of microglia and astrocytes, and (2) destabilization of the myelin sheath. Macrophages, microglia, and astrocytes have all be shown to express TLR4, and citrullinated proteins have been shown to activate pro-inflammatory cytokine production by macrophages *via* ligation of TLR4 (18). In addition, hyperactivation of PAD2 in oligodendrocytes may promote demyelination *via* citrullination of MBP leading to destabilization of the myelin sheath, increased degradation by proteases, and revelation of neoepitopes for targeting by autoreactive cells (12, 38). Together, these changes may drive CNS inflammation, including the development of autoreactivity to CNS antigens and development of neurocognitive symptoms. Our data suggest that a subset of people with CNS disease develop anti-PAD2 antibodies, which may attenuate PAD2-dependent inflammation and lead to a reduction of symptoms. It will be important to address critical aspects of this model in future studies.

CONCLUSION

We have found circulating anti-PAD2 antibodies in subsets of people with MS and PTLD, which associate with milder neurologic symptoms. Combined with our published data in RA, our current findings reveal that anti-PAD2 antibodies, present in a subset of individuals, associate with less severe symptoms in diseases united by high *PADI2* expression in the target tissue (i.e. the synovium in RA and the CNS in MS and PTLD). While it remains unknown whether PAD2 expression correlates with PAD2 activity at these sites, the implications of our results are that anti-PAD2 antibodies may hold potential as a novel prognostic biomarker to define less severe subsets and inform future development of mechanism-guided therapies that target PAD2 in these diseases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Studies were approved by the Johns Hopkins Institutional Review Board and all participants signed written informed consent approved by the Johns Hopkins Institutional Review Board (IRB00066509, IRB00098663, IRB00035457, NA_00071455, and NA_00011170).

AUTHOR CONTRIBUTIONS

YK performed PAD2 ELISAs, analyzed resulting data, and prepared associated figures and drafted the manuscript. AR and TY analyzed and interpreted PTLD clinical data. TJ analyzed and interpreted cellular and tissue transcriptomics data and prepared corresponding figures. HW prepared PAD2 antigen and optimized PAD2 ELISAs. CC curated and analyzed transcriptomics data. PB analyzed and interpreted MS clinical data. ML provided NMO sera and clinical data. PC provided MS sera and clinical data. JA provided PTLD sera and clinical data. MJS contributed to curation of the SLICE biobank and hypothesis generation. ED led overall study design, data interpretation, and manuscript preparation. All authors contributed to writing the manuscript and approved the final version for publication.

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REFERENCES

1. Witalison EE, Thompson PR, Hofseth LJ. Protein arginine deiminases and associated citrullination: physiological functions and diseases associated with dysregulation. *Curr Drug Targets.* (2015) 16:700–10. doi: 10.2174/1389450116666150202160954
2. Gyorgy B, Toth E, Tarcsa E, Falus A, Buzas EI. Citrullination: a posttranslational modification in health and disease. *Int J Biochem Cell Biol.* (2006) 38:1662–77. doi: 10.1016/j.biocel.2006.03.008
3. Tu R, Grover HM, Kotra LP. Peptidyl arginine deiminases and neurodegenerative diseases. *Curr Med Chem.* (2016) 23:104–14. doi: 10.2174/0929867323666151118120710
4. Lee CY, Wang D, Wilhelm M, Zolg DP, Schmidt T, Schnatbaum K, et al. Mining the human tissue proteome for protein citrullination. *Mol Cell Proteomics.* (2018) 17:1378–91. doi: 10.1074/mcp.RA118.000696
5. Vossenaar ER, Zendman AJ, van Venrooij WJ, Pruijn GJ. Pad, a Growing family of citrullinating enzymes: genes, features and involvement in disease. *Bioessays.* (2003) 25:1106–18. doi: 10.1002/bies.10357
6. Falcao AM, Meijer M, Scaglione A, Rinwa P, Agirre E, Liang J, et al. Pad2-mediated citrullination contributes to efficient oligodendrocyte differentiation and myelination. *Cell Rep.* (2019) 27:1090–102.e10. doi: 10.1016/j.celrep.2019.03.108
7. Schirmer L, Velmeshev D, Holmqvist S, Kaufmann M, Werneburg S, Jung D, et al. Neuronal vulnerability and multilineage diversity in multiple sclerosis. *Nature.* (2019) 573:75–82. doi: 10.1038/s41586-019-1404-z

8. Musse AA, Boggs JM, Harauz G. Deimination of membrane-bound myelin basic protein in multiple sclerosis exposes an immunodominant epitope. *Proc Natl Acad Sci USA*. (2006) 103:4422–7. doi: 10.1073/pnas.0509158103
9. Lassmann H, Bruck W, Lucchinetti CF. The immunopathology of multiple sclerosis: an overview. *Brain Pathol.* (2007) 17:210–8. doi: 10.1111/j.1750-3639.2007.00064.x
10. Calabrese R, Zampieri M, Mechelli R, Annibali V, Guastafierro T, Ciccarone F, et al. Methylation-dependent Pad2 upregulation in multiple sclerosis peripheral blood. *Mult Scler.* (2012) 18:299–304. doi: 10.1177/1352458511421055
11. Mastronardi FG, Noor A, Wood DD, Paton T, Moscarello MA. Peptidyl argininedeiminase 2 Cpg Island in multiple sclerosis white matter is hypomethylated. *J Neurosci Res.* (2007) 85:2006–16. doi: 10.1002/jnr.21329
12. Yang L, Tan D, Piao H. Myelin basic protein citrullination in multiple sclerosis: a potential therapeutic target for the pathology. *Neurochem Res.* (2016) 41:1845–56. doi: 10.1007/s11064-016-1920-2
13. Kawalkowska J, Quirke AM, Ghari F, Davis S, Subramanian V, Thompson PR, et al. Abrogation of collagen-induced arthritis by a peptidyl arginine deiminase inhibitor is associated with modulation of t cell-mediated immune responses. *Sci Rep.* (2016) 6:26430. doi: 10.1038/srep26430
14. Tejeda EJC, Bello AM, Wasilewski E, Koebel A, Dunn S, Kotra LP. Noncovalent protein arginine deiminase (Pad) inhibitors are efficacious in animal models of multiple sclerosis. *J Med Chem.* (2017) 60:8876–87. doi: 10.1021/acs.jmedchem.7b01102
15. Willis VC, Gizinski AM, Banda NK, Causey CP, Knuckley B, Cordova KN, et al. N-Alpha-benzoyl-N5-(2-Chloro-1-Iminoethyl)-L-ornithine amide, a protein arginine deiminase inhibitor, reduces the severity of murine collagen-induced arthritis. *J Immunol.* (2011) 186:4396–404. doi: 10.4049/jimmunol.1001620
16. Damgaard D, Senolt L, Nielsen CH. Increased Levels of Peptidylarginine deiminase 2 in synovial fluid from anti-Ccp-positive rheumatoid arthritis patients: association with disease activity and inflammatory markers. *Rheumatology.* (2016) 55:918–27. doi: 10.1093/rheumatology/kev440
17. Vossenaar ER, Radstake TR, van der Heijden A, van Mansum MA, Dieteren C, de Rooij DJ, et al. Expression and activity of citrullinating peptidylarginine deiminase enzymes in monocytes and macrophages. *Ann Rheum Dis.* (2004) 63:373–81. doi: 10.1136/ard.2003.012211
18. Sokolove J, Zhao X, Chandra PE, Robinson WH. Immune complexes containing citrullinated fibrinogen costimulate macrophages *via* toll-like receptor 4 and fcgamma receptor. *Arthritis Rheum.* (2011) 63:53–62. doi: 10.1002/art.30081
19. Darrah E, Giles JT, Davis RL, Naik P, Wang H, Konig MF, et al. Autoantibodies to peptidylarginine deiminase 2 are associated with less severe disease in rheumatoid arthritis. *Front Immunol.* (2018) 9:2696. doi: 10.3389/fimmu.2018.02696
20. Wingerchuk DM, Weinshenker BG. Neuromyelitis optica (Devic's syndrome). *Handb Clin Neurol.* (2014) 122:581–99. doi: 10.1016/B978-0-444-52001-2.00025-X
21. Aucott JN, Rebman AW, Crowder LA, Kortte KB. Post-treatment Lyme disease syndrome symptomatology and the impact on life functioning: is there something here? *Qual Life Res.* (2013) 22:75–84. doi: 10.1007/s11136-012-0126-6
22. Coughlin JM, Yang T, Rebman AW, Bechtold KT, Du Y, Mathews WB, et al. Imaging glial activation in patients with post-treatment lyme disease symptoms: a pilot study using [(11)C]Dpa-713 pet. *J Neuroinflammation.* (2018) 15:346. doi: 10.1186/s12974-018-1381-4
23. Chandra A, Wormser GP, Klempner MS, Trevino RP, Crow MK, Latov N, et al. Anti-neural antibody reactivity in patients with a history of lyme borreliosis and persistent symptoms. *Brain Behav Immun.* (2010) 24:1018–24. doi: 10.1016/j.bbi.2010.03.002
24. Bhargava P, Steele SU, Waubant E, Revirajan NR, Marcus J, Dembele M, et al. Multiple sclerosis patients have a diminished serologic response to vitamin D supplementation compared to healthy controls. *Mult Scler.* (2016) 22:753–60. doi: 10.1177/1352458515600248
25. Wingerchuk DM, Lennon VA, Pittock SJ, Lucchinetti CF, Weinshenker BG. Revised diagnostic criteria for neuromyelitis optica. *Neurology.* (2006) 66:1485–9. doi: 10.1212/01.wnl.0000216139.44259.74
26. Rebman AW, Bechtold KT, Yang T, Mihm EA, Soloski MJ, Novak CB, et al. The clinical, symptom, and quality-of-life characterization of a well-defined group of patients with posttreatment Lyme disease syndrome. *Front Med.* (2017) 4:224. doi: 10.3389/fmed.2017.00224
27. Bhargava P, Fitzgerald KC, Calabresi PA, Mowry EM. Metabolic alterations in multiple sclerosis and the impact of vitamin D supplementation. *JCI Insight.* (2017) 2:e95302. doi: 10.1172/jci.insight.95302
28. Roxburgh RH, Seaman SR, Masterman T, Hensiek AE, Sawcer SJ, Vukusic S, et al. Multiple sclerosis severity score: using disability and disease duration to rate disease severity. *Neurology.* (2005) 64:1144–51. doi: 10.1212/01.WNL.0000156155.19270.F8
29. From the Centers for Disease Control and Prevention. Recommendations for test performance and interpretation from the second national conference on serologic diagnosis of Lyme disease. *JAMA.* (1995) 274:937. doi: 10.1001/jama.1995.03530120023018
30. Barrett T, Wilhite SE, Ledoux P, Evangelista C, Kim IF, Tomashevsky M, et al. Ncbi geo: archive for functional genomics data sets–update. *Nucleic Acids Res.* (2013) 41(Database issue):D991–5. doi: 10.1093/nar/gks1193
31. Darmanis S, Sloan SA, Zhang Y, Enge M, Caneda C, Shuer LM, et al. A survey of human brain transcriptome diversity at the single cell level. *Proc Natl Acad Sci USA.* (2015) 112:7285–90. doi: 10.1073/pnas.1507125112
32. Team RC. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing (2019).
33. Jaffe AE, Straub RE, Shin JH, Tao R, Gao Y, Collado-Torres L, et al. Developmental and genetic regulation of the human cortex transcriptome illuminate schizophrenia pathogenesis. *Nat Neurosci.* (2018) 21:1117–25. doi: 10.1038/s41593-018-0197-y
34. Hawrylycz MJ, Lein ES, Guillozet-Bongaarts AL, Shen EH, Ng L, Miller JA, et al. An anatomically comprehensive atlas of the adult human brain transcriptome. *Nature.* (2012) 489:391–9. doi: 10.1038/nature11405
35. Freedman MS, Thompson EJ, Deisenhammer F, Giovannoni G, Grimsley G, Keir G, et al. Recommended standard of cerebrospinal fluid analysis in the diagnosis of multiple sclerosis: a consensus statement. *Arch Neurol.* (2005) 62:865–70. doi: 10.1001/archneur.62.6.865
36. Correale J. de los Milagros Bassani Molinas M. Oligoclonal bands and antibody responses in multiple sclerosis. *J Neurol.* (2002) 249:375–89. doi: 10.1007/s004150200026
37. Bolz DD, Weis JJ. Molecular mimicry to Borrelia Burgdorferi: pathway to autoimmunity? *Autoimmunity.* (2004) 37:387–92. doi: 10.1080/08916930410001713098
38. Musse AA Li Z, Ackerley CA, Bienzle D, Lei H, Poma R, et al. Peptidylarginine deiminase 2 (Pad2) overexpression in transgenic mice leads to myelin loss in the central nervous system. *Dis Model Mech.* (2008) 1:229–40. doi: 10.1242/dmm.000729

Conflict of Interest: ED has a licensed agreement and provisional patent for the use of anti-PAD2 antibodies to identify patient subsets in rheumatoid arthritis and other diseases and has received research funds from Pfizer, Bristol Myers Squib, Celgene outside of this current work. ML received consulting fees from Alexion, Viela BioGenentech/Roche/Chugai, Quest Diagnostics, Mitsubishi Pharma and UCB Pharmaceuticals outside of this current work.

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STUDY PROTOCOL
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OzEAN Study to Collect Real-World Evidence of Persistent Use, Effectiveness, and Safety of Ozanimod Over 5 Years in Patients With Relapsing-Remitting Multiple Sclerosis in Germany

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Background: Ozanimod, a sphingosine 1-phosphate receptor 1 and 5 modulator, was approved as a disease-modifying therapy for active relapsing-remitting multiple sclerosis (RRMS) in 2020 and for active ulcerative colitis in 2021. Long-term, real-world studies in a nonselective population are needed. OzEAN is an ongoing study to assess the real-world persistent use, effectiveness, and safety of ozanimod and its impact on quality of life (QoL) in patients with RRMS over a 5-year period.

Methods: This prospective, noninterventional, postmarketing authorization study will enroll ∼1,300 patients (≥18 years of age) with active RRMS. The decision to initiate ozanimod must have been made before and independent from study participation. Enrollment began in March 2021. Recruitment is ongoing and will last for 36 months across 140 sites in Germany. Treatment-naive patients or those having prior experience with a disease-modifying therapy receive oral ozanimod 0.92 mg/day after an initial dose escalation, per the summary of product characteristics recommendations, for up to 60 months. Persistence with ozanimod treatment (primary endpoint) is assessed at month 60. Secondary endpoints include additional physician-reported outcomes [persistence at earlier time points, annualized relapse rate, Expanded Disability Status Scale score, cognition (Symbol Digit Modalities Test), and incidence of adverse events], and patient-reported outcomes assessing patient satisfaction, adherence, and treatment modalities (Treatment Satisfaction Questionnaire for Medication, v1.4), disability (United Kingdom Neurological Disability Rating Scale), QoL (MSQOL-54 questionnaire), fatigue (Fatigue Scale for Motor and Cognitive Functions), and health economics [Work Productivity and Activity Impairment Questionnaire for Multiple Sclerosis (German v2.1); Multiple Sclerosis Health Resource Survey, v3.0]. A Multiple

Sclerosis Documentation System with an internet-based e-health portal allows patients to view files and complete questionnaires. A safety follow-up will occur 3–8 months after the last ozanimod dose for patients who discontinue treatment early. Long-term results are anticipated after study completion in 2029. Yearly interim analyses are planned after enrollment has reached 25%.

Conclusion: This is the first long-term, real-world study of ozanimod in patients with RRMS and, to our knowledge, the first noninterventional study utilizing a patient portal. These data will add to the safety/efficacy profile of ozanimod demonstrated in phase 3 trials.

Clinical Trial Registration: Clinicaltrials.gov, identifier: NCT05335031.

Keywords: relapsing-remitting multiple sclerosis, observational study, real-world evidence, patient-reported outcomes, protocol, trial-in-progress, medication adherence, medication persistence

INTRODUCTION

Ozanimod is a sphingosine 1-phosphate receptor 1 and 5 modulator that blocks lymphocyte egress from lymphoid tissue, reducing the number of circulating lymphocytes in peripheral blood (1). Ozanimod was first approved in the United States in 2020 for the treatment of adults with relapsing forms of multiple sclerosis (RMS) and subsequently approved in multiple countries for adults with active relapsing-remitting multiple sclerosis (RRMS) defined by clinical or imaging results; in 2021, it was approved for the treatment of moderately to severely active ulcerative colitis in the United States and European Union (2, 3).

The ozanimod clinical development program in RMS (**Figure 1**) included a phase 1 pharmacokinetic/pharmacodynamic trial (12 weeks), a phase 2 placebo-controlled trial (24 weeks) with a dose-blinded extension (24 months) (4, 5), and two phase 3 active-controlled trials, RADIANCE (24 months) (6) and SUNBEAM (minimum 12 months) (7). In both phase 3 trials, ozanimod 0.92 mg/day was associated with lower adjusted annualized relapse rate (ARR), fewer gadolinium-enhancing lesions and new or enlarging T2 lesions on brain magnetic resonance imaging (MRI), and reduced brain volume loss compared with intramuscular interferon β-1a 30 μg weekly (6, 7). The most frequent adverse events (AEs) associated with ozanimod treatment were upper respiratory infection, hepatic transaminase elevation, orthostatic hypotension, urinary tract infection, back pain, and hypertension. A pooled analysis of safety results from all trials, including an ongoing open-label extension study (DAYBREAK), were consistent with those of the phase 3 trials and demonstrated no new safety concerns (8). Patients in the parent trials were adults (18–55 years of age) with multiple sclerosis (MS) [diagnosed per 2010 McDonald criteria (9)] with a relapsing clinical course, brain MRI lesions consistent with MS, an Expanded Disability Status Scale (EDSS) (10) score of 0–6.0 (phase 1) or 0–5.0 (phase 2 and 3), and a history of relapses within the past 1–2 years (phase 2 and 3) (4, 6, 7).

Given the recent approvals of ozanimod, long-term studies in a non-selective, real-world population are not yet available, but are needed to evaluate ozanimod in a broader population of patients. Such studies could also provide information on persistence and adherence with ozanimod treatment, disease characteristics and treatment history of patients who are prescribed ozanimod, and patient-reported outcomes and pharmacoeconomic data, outcomes that were not evaluated in the clinical development program.

OzEAN is an ongoing prospective, non-interventional, postmarketing authorization observational cohort study to assess real-world persistent use, effectiveness, and safety of ozanimod, and the impact of treatment on quality of life (QoL), in patients with RRMS in Germany over a 5-year period. These real-world data, including patient-reported QoL outcomes, are of interest to German authorities and support their Health Technology Assessment (11).

METHODS AND ANALYSIS

Study Setting and Treatment

Eligible patients will be enrolled in study sites across Germany. The decision to initiate ozanimod treatment must have been made by the physician before and independent from enrollment into the study. Patients receive oral ozanimod 0.92 mg/day for up to 60 months following a 7-day dose escalation regimen according to the summary of product characteristics (SmPC) (3). During this observation period, patients are evaluated at 16 data collection visits: baseline (visit 1), month 1 (visit 2), quarterly from month 3 to month 24 (visits 3–10), and at 6-month intervals from month 24 to month 60 (visits 11–16; **Figure 2**). Patients who permanently discontinue ozanimod treatment before month 60 are withdrawn from the observational study. A safety follow-up is performed when a new MS therapy is initiated (3–8 months after receiving the final dose of ozanimod) or 8 months (at the latest) after receiving the final dose of ozanimod if no new MS therapy is initiated.

The individual study duration (observation period) per patient includes up to 60 months for the noninterventional treatment documentation period, and approximately 3–8 months for the safety follow-up period in case of premature discontinuation of ozanimod. The overall study duration is 104

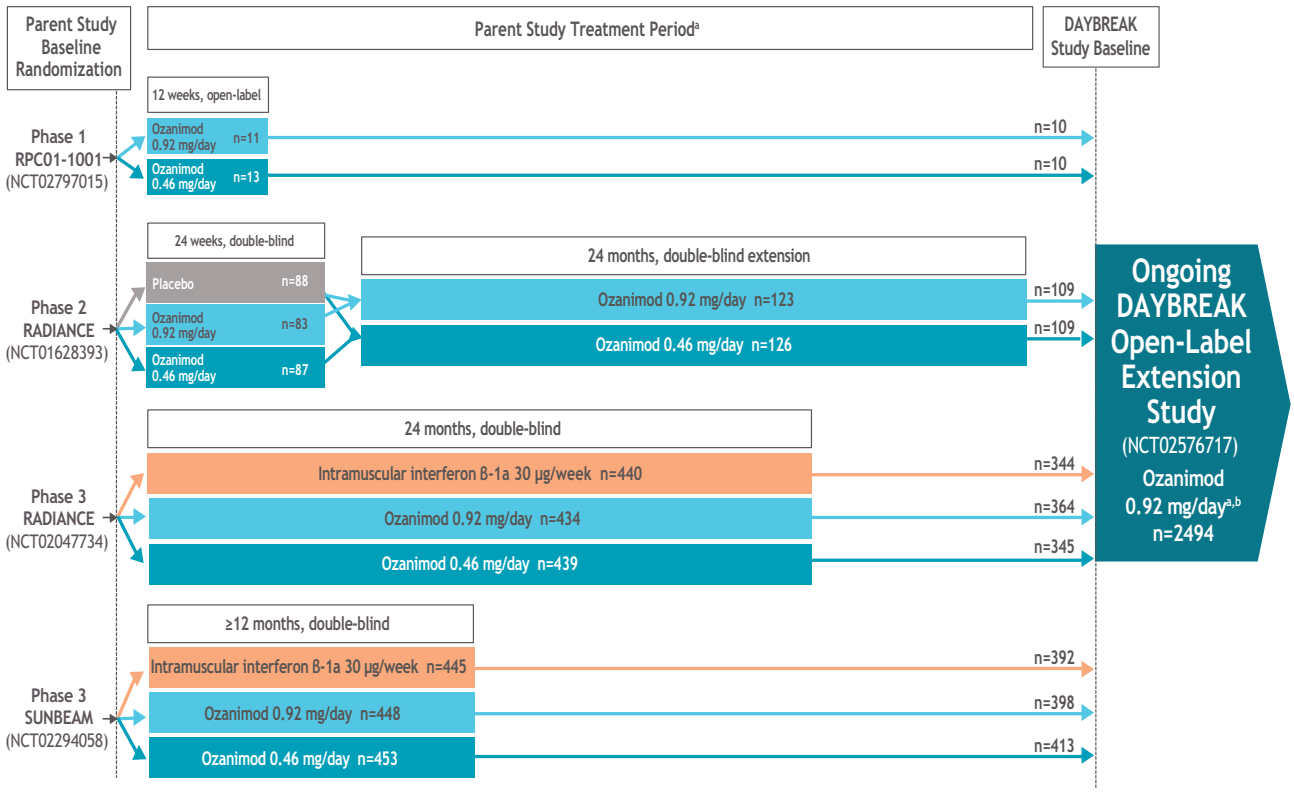

FIGURE 1 | Ozanimod clinical development in RMS program. [a]In all trials, upon initiation of ozanimod, patients received 0.23 mg on days 1–4, 0.46 mg on days 5–7, and then their assigned dose of 0.46 or 0.92 mg on day 8 and thereafter. All patients entering the phase 2 dose-blinded extension period underwent dose escalation, even if treated with ozanimod in the parent trial, to maintain the blind. [b]In DAYBREAK, dose escalation was performed for all patients entering from one of the active-controlled phase 3 trials, irrespective of prior treatment assignment (to maintain the blinding in the parent trials); dose escalation was not performed for those entering from the phase 1 or 2 trials, unless the last dose of ozanimod was >14 days before entering DAYBREAK.

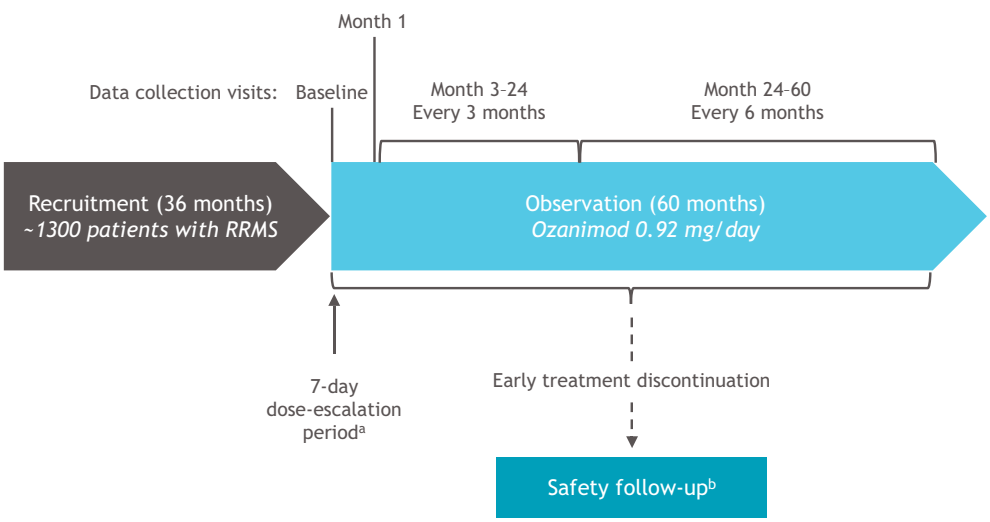

FIGURE 2 | OzEAN study design. MS, multiple sclerosis; RRMS, relapsing-remitting multiple sclerosis. [a]Days 1–4: ozanimod 0.23 mg; days 5–7: ozanimod 0.46 mg; days 8 and thereafter: ozanimod 0.92 mg. [b]Patients who discontinue ozanimod prior to end of study (month 60) will be withdrawn from the OzEAN study and a safety follow-up will be performed 3–8 months after the last dose of ozanimod when a new therapy for MS is started or after 8 months (at the latest) if no subsequent MS therapy is initiated.

TABLE 1 | Eligibility criteria.

Inclusion Criteria	Exclusion Criteria
Males or females aged ≥ 18 years who provided written informed consent	Contraindications specified in the current version of the SmPC
Confirmed diagnosis of RRMS according to ICD-10 and eligible for treatment with ozanimod according to physician's judgement and based on the recommendation of the current SmPC	Known hypersensitivity to the active substance(s) or to any of the excipients of ozanimod as specified in the SmPC
The decision to initiate treatment with ozanimod must have been made by the treating physician before enrollment and independently of this study; retrospective documentation of ozanimod therapy and enrollment of patients that are already on ozanimod therapy is not allowed	Participation in any other clinical studies

ICD-10, International Statistical Classification of Diseases and Related Health Problems, 10th version; RRMS, relapsing-remitting multiple sclerosis; SmPC, Summary of Product Characteristics.

months, including a 36-month recruitment period, 60-month observational period, and up to 8-month safety follow-up period. The entire study period is planned to last from the enrollment of the first patient (March 2021) until March 2029.

Eligibility Criteria

Eligible patients are adults (≥18 years of age) diagnosed with RRMS who are either treatment-naive or have prior experience with a disease-modifying therapy and elected to switch to ozanimod. Patients with contraindications specified in the current version of the SmPC (3), or with known hypersensitivity to ozanimod or any of its excipients, are excluded. Patients may not be participating in any other clinical studies.

Inclusion and exclusion criteria are shown in **Table 1**.

Endpoints and Assessments

At baseline, the following data are collected: patient demography, vital parameters, physical status, prior and concomitant diseases and medications, MS diagnosis, MS history (including prior relapses), prior MS treatment, reason for initiating/switching to ozanimod, and treatment modalities with ozanimod. In addition, physician-reported outcomes [EDSS (10) and Symbol Digit Modalities Test (SDMT) (12, 13)] and patient-reported outcomes (PROs; Treatment Satisfaction Questionnaire for Medication, version 1.4 (TSQM v1.4) (14, 15), United Kingdom Neurological Disability Rating Scale (UKNDS) (16, 17), Multiple Sclerosis Quality of Life Instrument-54 items (MSQOL-54) (18), Fatigue Scale for Motor and Cognitive Functions (FSMC) (19), Work Productivity and Activity Impairment Questionnaire for Multiple Sclerosis, version 2.1 (WPAI-MS v2.1) (20), and Multiple Sclerosis Health Resource Utilization Survey, version 3.0 (MS-HRS v3.0) (21) are completed. Following the baseline visit, these physician-reported outcomes and PROs, together with physician-reported clinical relapses (the occurrence of new symptoms or the worsening of old symptoms) are completed at 3-, 6-, or 12-month intervals during the 60-month observation period (**Table 2**). Alternate forms of the SDMT will be used to avoid practice effects. The following characteristics of ozanimod treatment are documented continuously: initial dose escalation; maintenance dose; temporary interruptions of treatment, including date and reason for interruption, and re-initiation of therapy following treatment interruption; and all medication taken concomitantly with ozanimod and all changes in concomitant medication during the study, including reason for administration. Primary and secondary endpoints are summarized in **Table 3**.

The primary endpoint is persistence with therapy (as reported by physicians), defined as the proportion of patients who remain on continuous treatment with ozanimod (with gaps of ≤ 90 days allowed), from baseline to month 60. Persistence from baseline to months 12, 24, 36, and 48 is a secondary endpoint. Adherence to therapy, defined as the percentage of ozanimod doses taken as prescribed (as reported by patients) (22), at months 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 42, 48, 54, and 60, and at the safety follow-up, is a secondary endpoint.

Additional secondary endpoints include the following physician-reported outcomes: clinical relapse, expressed as ARR at months 12, 24, 36, 48, and 60, and at the safety follow-up (total number of relapses experienced by all patients in this study divided by the total number of days in the study for the patients, and the ratio multiplied by 365); disability, assessed as change in EDSS score from baseline to months 12, 24, 36, 48, and 60, and at the safety follow-up; and cognitive processing speed, measured using the SDMT and quantified as change from baseline in SDMT score, proportion of patients with increase (improvement) or decrease (worsening) in SDMT raw score of ≥ 4 points or 10% from baseline, and proportion of patients with change in SDMT raw score that does not meet criteria for improvement or worsening (stable) at months 6, 12, 18, 24, 30, 36, 42, 48, 54, and 60, and at the safety follow-up.

Secondary endpoints reported by patients (i.e., PROs) include measures of treatment satisfaction, effectiveness, QoL, fatigue, and health economics. Treatment satisfaction is assessed using the TSQM v1.4; secondary endpoints are changes in TSQM v1.4 domains (effectiveness, side effects, convenience, and global satisfaction) and the relationship between each domain and clinical outcomes at months 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 42, 48, 54, and 60, and at the safety follow-up. Disability is assessed using the UKNDS; secondary endpoints are the change from baseline in UKNDS sum score and the proportion of patients with clinically meaningful improvement or worsening of at least 1 grade in each UKNDS subscale at months 6, 12, 18, 24, 30, 36, 42, 48, 54, and 60, and at the safety follow-up. QoL is assessed using the MSQOL-54; secondary endpoints are change from baseline in the physical composite summary (PCS) and mental health composite summary (MCS) scores, and the proportion of patients with a clinically meaningful change [increase (improvement) or decrease (worsening) of ≥5

TABLE 2 | Schedule of enrollment, interventions, and assessments.

Assessment/Data Collected	Observation				End of observation[a]	Safety follow-up[b]
	Enrollment	Month 1	Month 3–24	Month 24–60		
	Baseline		Every 3 months	Every 6 months	End of study	Last treatment + 3–8 months
Baseline						
Informed consent	X					
Inclusion/exclusion criteria	X					
Demography	X					
General medical history	X					
MS history and pretreatment[c]	X					
Concomitant diseases	X					
All previous malignant diseases	X					
All other previous diseases within 5 years prior to study enrollment	X					
Physical status[d]	X					
Treatment						
Treatment modalities[e]	X	X	X	X		X
Concomitant medication	X	X	X	X		X
Subsequent MS treatment					X	X
Physician-reported assessment						
Persistence with therapy			Assessed continuously throughout study			
Clinical relapse (ARR)		X	X[f]	X[f]		X
EDSS	X	X	X[f]	X[f]		X
SDMT	X	X[g]	X[g]	X		X
MRI[h]	X[h]	X[h]	X[h]	X[h]		X[h]
Patient-reported assessment						
Adherence to therapy[i]	X[i]	X[i]	X[i]	X[i]	X[i]	X[i]
TSQM v1.4	X	X	X[j]	X		X
UKNDS	X	X	X[g]	X		X
MSQOL-54	X	X	X[g]	X		X
FSMC	X	X	X[g]	X		X
WPAI-MS v2.1	X	X	X[g]	X		X
MS-HRS v3.0	X	X	X[g]	X		X
Safety assessment						
AEs/SAEs			Assessed continuously throughout study			
Laboratory panel[h]	X[h]	X[h]	X[h]	X[h]		X[h]

AE, adverse event; ARR, annualized relapse rate; EDSS, Expanded Disability Status Scale; FSMC, Fatigue Scale for Motor and Cognitive Functions; MRI, magnetic resonance imaging; MS, multiple sclerosis; MSFC, Multiple Sclerosis Functional Composite; MS-HRS v3.0, Multiple Sclerosis Health Resource Survey, version 3.0; MSQOL-54, Multiple Sclerosis Quality of Life-54; SAE, serious adverse event; SDMT, Symbol Digit Modalities Test; TSQM v1.4, Treatment Satisfaction Questionnaire for Medication, version 1.4; UKNDS, United Kingdom Neurological Disability Rating Scale; WPAI-MS v2.1, Work Productivity and Activity Impairment Questionnaire for Multiple Sclerosis, version 2.1.

[a] *Documentation performed directly after a patient reached the regular end of study (at month 60) or permanently discontinued ozanimod treatment before month 60; if routine data were collected that do not meet any of the documentation time points offered, these data may be entered with the month 60 documentation.*

[b] *Follow-up documentation of potential AEs and subsequent therapy, performed approximately 3–8 months after stopping treatment with ozanimod and when a new MS therapy is initiated, or after 8 months (at the latest) if there is no subsequent MS therapy initiated.*

[c] *Includes MS diagnosis according to International Classification of Diseases, Tenth Revision; first manifestation of MS; course of MS disease and number of relapses within the year before enrollment; and type and duration of prior disease-modifying therapies for MS.*

[d] *Includes vital parameters, physical status.*

[e] *Treatment modalities referring to ozanimod, including (planned) date of first administration of ozanimod, reason for switch to ozanimod, interruptions, re-initiation of therapy following treatment interruption, and reason for discontinuation in case of switch to another MS treatment.*

[f] *At yearly intervals (months 12, 24, 36, 48, and 60) only.*

[g] *At 6-month intervals (months 6, 12, 18, and 24) only.*

[h] *Only if available based on the local clinical routine assessments performed at the study center: MRI (number of lesions) and relevant laboratory measurements, especially those for monitoring ozanimod therapy (e.g., liver parameters, such as alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase, and total bilirubin; differential blood count; or lymphocyte count).*

[i] *Patient-reported qualitative assessment of how often the patient missed doses and how regularly he or she took the medication, by entering the date of starting with a new ozanimod package and selection of the package size. This information is collected electronically via the patient portal application or, alternatively, using paper-based questionnaires provided at the local study center.*

[j] *At 3-month intervals (month 3, 6, 9, 12, 15, 18, 21, and 24).*

TABLE 3 | Primary and secondary endpoints in the OzEAN study.

Domain	Reported by	Assessment	Outcome measure	Time point
Primary endpoint				
Treatment satisfaction	Physician	Persistence with therapy	• Proportion of patients who remain on continuous treatment with ozanimod • Evaluated at month 60	Collected continuously
Secondary endpoints				
Treatment satisfaction	Physician	Persistence with therapy	• Proportion of patients who remain on continuous treatment with ozanimod • Evaluated at months 12, 24, 36, and 48	Collected continuously
	Patient	Adherence to therapy	• Percentage of dose taken as prescribed • Evaluated at months 12, 24, 36, 48, and 60	Months 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 42, 48, 54, and 60, and SFU
	Patient	TSQM v1.4	• Changes in TSQM v1.4 domains • Relationship between each TSQM v1.4 domain and clinical outcomes [a]	Baseline and months 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 42, 48, 54, and 60, and SFU
Effectiveness	Physician	Clinical relapse	• Annualized relapse rate	Months 12, 24, 36, 48, and 60, and SFU
	Physician	EDSS	• Change from baseline in EDSS	Baseline and months 12, 24, 36, 48, and 60, and SFU
	Patient	UKNDS	• Change from baseline in UKNDS sum score • Proportion of patients with a clinically meaningful improvement/worsening of 1 grade in each subscale	Baseline and months 6, 12, 18, 24, 30, 36, 42, 48, 54, and 60, and SFU
Cognitive processing speed	Physician	SDMT	• Change from baseline in SDMT • Proportion of patients with: ○ Increase in raw score of ≥ 4 points or 10% from baseline (improved) ○ Decline in raw score of ≥ 4 points or 10% from baseline (worsened) ○ Raw score change from baseline who do not meet improved or worsened definition (stable)	Baseline and months 6, 12, 18, 24, 30, 36, 42, 48, 54, and 60, and SFU
QoL	Patient	MSQOL-54	• Change from baseline in PCS and MCS • Proportion of patients with: ○ Increase of ≥ 5 points in PCS and/or MCS (improved) ○ Decline of ≥ 5 points in PCS and/or MCS (worsened)	Baseline and months 6, 12, 18, 24, 30, 36, 42, 48, 54, and 60, and SFU
Fatigue	Patient	FSMC	• Change from baseline in FSMC sum score and physical and cognitive subdomains • Proportion of patients with: ○ Decline of ≥ 10 points in sum score (improved) ○ Increase of ≥ 10 points in sum score (worsened) ○ Decline of ≥ 6 points or ≥ 5 points in cognitive and/or physical domain, respectively (improved) ○ Increase of ≥ 6 points or ≥ 5 points in cognitive and/or physical domain, respectively (worsened)	Baseline and months 6, 12, 18, 24, 30, 36, 42, 48, 54, and 60, and SFU
Health economics	Patient	WPAI-MS v2.1	• Change from baseline in WPAI-MS v2.1 domains	Baseline and months 6, 12, 18, 24, 30, 36, 42, 48, 54, and 60, and SFU
	Patient	MS-HRS v3.0	• Resource use/direct and indirect costs	Baseline and months 6, 12, 18, 24, 30, 36, 42, 48, 54, and 60, and SFU
Safety	Physician	Incidence rate for AEs[b]	• Number of new cases per population at risk over the follow-up period (per person-time) • Number of patients with event	Collected continuously

AE, adverse event; EDSS, Expanded Disability Status Scale; FSMC, Fatigue Scale for Motor and Cognitive Functions; MCS, mental health composite summary; MS-HRS v3.0, Multiple Sclerosis Health Resource Survey, version 3.0; MSQOL-54, Multiple Sclerosis Quality of Life-54; PCS, physical composite summary; SDMT, Symbol Digit Modalities Test; TSQM v1.4, Treatment Satisfaction Questionnaire for Medication, version 1.4; UKNDS, United Kingdom Neurological Disability Rating Scale; QoL, quality of life; SFU, safety follow-up; WPAI-MS v2.1, Work Productivity and Activity Impairment Questionnaire for Multiple Sclerosis, version 2.1.

[a] *If effect size Cohen's d (used to indicate the standardized difference between 2 means) > 0.3.*

[b] *Based on the first occurrence of event during the follow-up period.*

points] (23) in PCS or MCS score, at months 6, 12, 18, 24, 30, 36, 42, 48, 54, and 60, and at the safety follow-up. Fatigue is assessed using the FSMC; secondary endpoints include change from baseline in FSMC sum score, physical subscale, and cognitive subscale; proportion of patients with decrease (improvement) or increase (worsening) of ≥10 points in the sum score; proportion of patients with decrease (improvement) or increase (worsening) of ≥6 points in the cognitive domain; and proportion of patients with decrease (improvement) or increase (worsening) of ≥5 points in the physical domain, at months 6, 12, 18, 24, 30, 36, 42, 48, 54, and 60, and at the safety follow-up. Measures of health economics include the WPAI-MS v2.1 and the MS-HRS v3.0; secondary endpoints are the change from baseline in WPAI-MS v2.1 domains at months 6, 12, 18, 24, 30, 36, 42, 48, 54, and 60, and at the safety follow-up, and resource use and direct and indirect costs assessed using the MS-HRS v3.0 at baseline and months 6, 12, 18, 24, 30, 36, 42, 48, 54, and 60, and at the safety follow-up.

Incidence of AEs [number of new cases per population at risk over the follow-up period (per person-time) and number of patients with event] is also a secondary endpoint. AEs are defined as any untoward medical occurrence, which does not necessarily have a causal relationship with treatment.

Sample Size and Recruitment

Planned recruitment is approximately 1,300 patients. Recruitment is ongoing and will occur over 36 months. Enrollment began in March 2021.

To increase representativeness of selected sites, a large number of participating sites (up to 140) of different types (office- and hospital-based sites specialized in neurology), which are located geographically across Germany, are planned. To discourage physicians from selecting specific patients for inclusion in the study, they are instructed and trained to ask all eligible patients consecutively for their participation.

It is assumed that the persistence at year 5 will be 55% overall, including 50% of patients who switched to ozanimod treatment from another MS treatment and 55%−60% of treatment naive patients. A sample size of 1,331 patients is required to cover the estimated persistence rate of 0.55 with a 2-sided 95% confidence interval (CI) of 0.03 in each direction using the large sample normal approximation and after considering a dropout rate of 15%. This interval width is considered appropriate for correct description of the actual persistence rate on a descriptive level.

Data Collection, Management, and Analysis

Data Collection Methods

As part of routine care, the study physician or qualified study staff members at the study site enter data on treatment, MS relapses, EDSS, SDMT, and AEs since the patient's last visit, according to the timeline outlined in **Table 2**, using an electronic case report form (eCRF; **Figure 3**). Laboratory panels and MRIs are performed only as available based on routine clinical assessments at each study center.

PROs (including adherence, TSQM v1.4, UKNDS, MSQOL-54, FSMC, WPAI-MS v2.1, and MS-HRS v3.0), preferably scored directly by the patient, are collected via the Multiple Sclerosis Documentation System 3D [MSDS^{3D} (24, 25)] with a study-specific, internet-based, patient e-health portal (**Figure 3**) according to the timeline in **Table 2**. The patient portal is accessible via computer or tablet and allows patients to download files and complete questionnaires (**Figure 4**). Alternatively, upon patient request, PROs are provided as paper-based versions to be completed and scored at the study site. All PRO questionnaires are made available in the German language. A detailed description of the instruments used in these assessments is available in **Table 4**.

AEs elicited as part of PRO data collection are subject to AE reporting. Investigators must review responses provided by patients to PRO instrument questions that assess safety to determine if AE reporting is warranted. The physician will enter AE data into the eCRF.

In the safety follow-up visit for patients withdrawn from the observational study before month 60, safety-related data (AEs) and data on any subsequent MS treatment and concomitant medication are collected in addition to PROs.

Data Management

Documentation of study data by physicians and authorized site staff is done exclusively online using the eCRF. The data are transmitted via secure connections and stored on secure servers of MedicalSyn GmbH, the eCRF, data management, and patient portal provider. Data entries in the patient portal are checked for plausibility and accuracy using validation programs, which generate automated queries. Open queries are displayed in the current status overview to be resolved. Data management screens new or updated free text entries in the patient portal for hidden AEs. The system follows open queries on a regular basis and communicates queries to the site. The site responds to open queries online.

Statistical Methods

According to the non-interventional design of the study, the statistical analyses are descriptive and exploratory. No statistical hypotheses are formulated. There will be no inferential testing, and no *P* value will be provided. Summary statistics for continuous variables will include number of observations available and number of missing values, minimum, maximum, median, mean, and standard deviation. Summary statistics for discrete variables will be presented with counts and percentages and number of missing variables.

The analysis set for the primary analysis will comprise all patients who received at least 1 dose of ozanimod during the study and for whom at least 1 postbaseline documentation is available. A subgroup analysis will be performed examining pretreatment with disease-modifying therapies (i.e., patients who are treatment-naive compared with patients who switched from another disease-modifying therapy to ozanimod).

If a patient is lost to follow-up, efforts will be undertaken to collect the data from the previous visit. Data management procedures will be implemented to limit the amount of non-reported data. Analysis methods for handling missing data

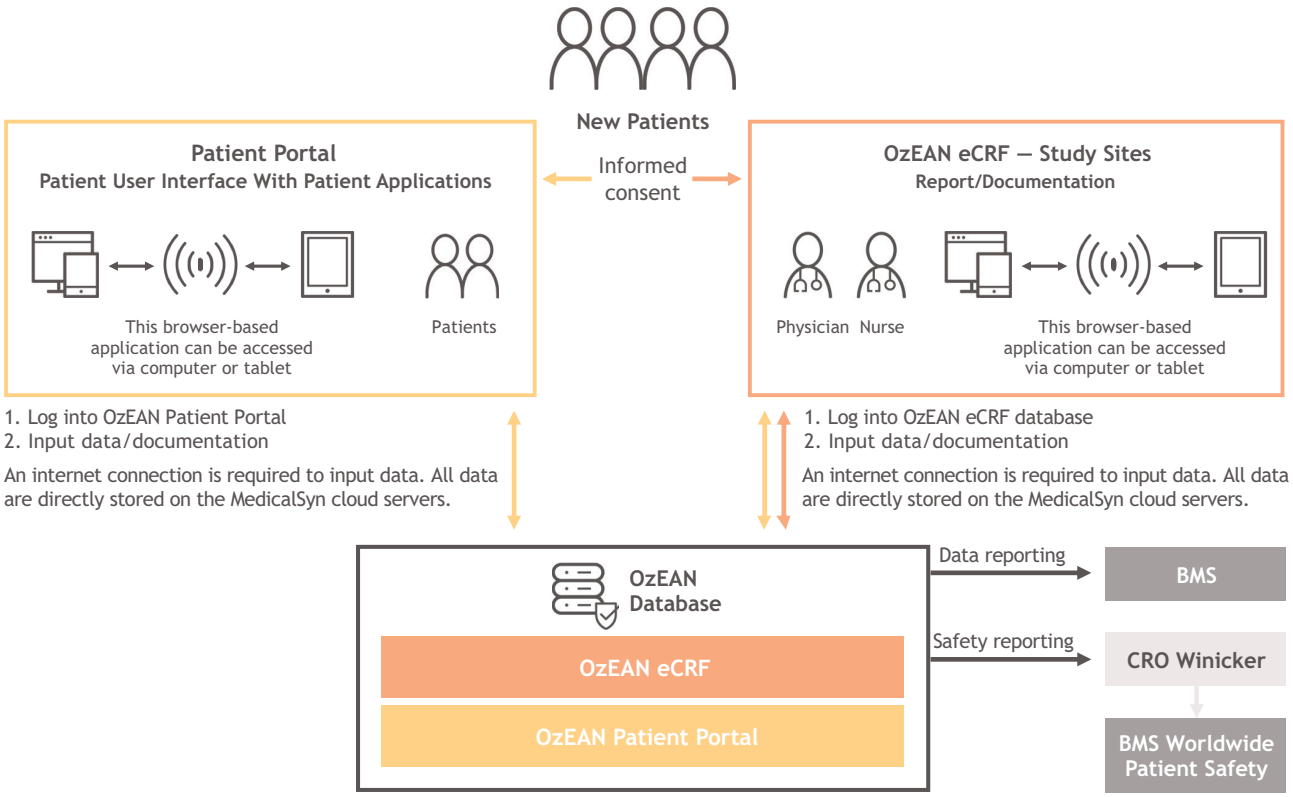

FIGURE 3 | Architecture and data flow within OzEAN eCRF and OzEAN patient portal. Physician-reported assessments are performed at the study site, and data are entered by physicians into the OzEAN eCRF. Patient-reported assessments are completed by patients using the OzEAN patient portal, and data are fed into the OzEAN eCRF. The patient portal interface additionally provides a calendar, medication plan, documents module, and access to surveys (PROs). Data from the OzEAN eCRF are transmitted to Winicker Norimed GmbH (the contract research organization) for storage on secure servers. Data on adverse events are additionally transmitted to the sponsor or its designee as part of standard safety reporting. CRO, contract research organization; eCRF, electronic case report form; PRO, patient-reported outcome.

(e.g., last observation carried forward, imputation, or sensitivity analyses) will be applied.

Treatment (e.g., dosage, duration of treatment, dose modifications, treatment interruptions, and concomitant medications) will be analyzed descriptively.

The analysis of the primary endpoint of persistence rate over 60 months in routine clinical practice will be calculated descriptively as percentage value at 60 months (i.e., the proportion of patients who are on continuous treatment with ozanimod at this time point), including corresponding 95% CIs calculated by the Clopper-Pearson method. A patient will be classified as non-persistent if a medication gap > 90 days occurs before the end of the 60-month documentation period. A sensitivity analysis will be performed evaluating patients who are lost to follow-up as "non-persistent." The persistence rate will be evaluated by Kaplan-Meier methods over the entire study period (patients who are lost to follow-up will be right-censored).

The analysis of persistence rate at months 12, 24, 36, and 48 (secondary endpoint) will be calculated descriptively and summarized as the proportion of patients on continuous treatment at the respective time point, with corresponding 95% CIs.

Medication adherence (secondary endpoint) will be evaluated categorically in terms of the percentage of doses taken as prescribed.

Secondary endpoints of clinical effectiveness (ARR, EDSS, and SDMT) and all PRO measures (changes from baseline, including proportion of patients who achieved a clinically meaningful change from baseline) will be analyzed descriptively. For TSQM v1.4, it is assumed that a clinically meaningful relationship between TSQM v1.4 domains and clinical outcomes is given if the effect size (Cohen's d) is > 0.3 (15).

All AE data will be listed and summarized. AEs will be classified using the Medical Dictionary for Regulatory Activities (MedDRA) classification system. The incidences based on the patient population enrolled as well as incidence density rates (number of events/sum of person-time in years) of all AEs, serious AEs (SAEs), adverse drug reactions (ADRs), and serious ADRs will be summarized by system organ class, preferred term, and relationship to study treatment. AEs leading to discontinuation from treatment will also be summarized and listed separately.

SAS software version 9.2 or higher will be used for analyses.

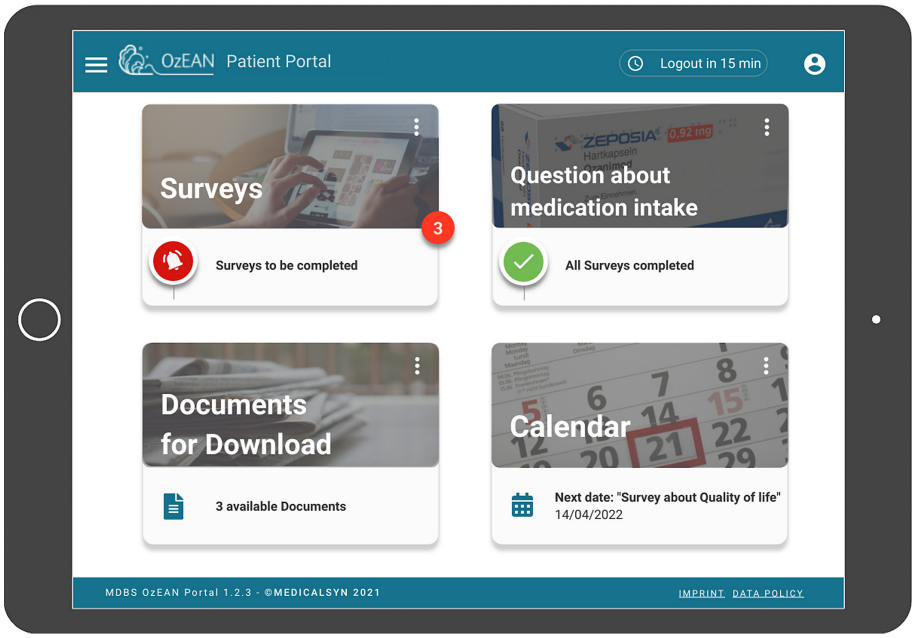

FIGURE 4 | OzEAN patient portal user interface. On the start page of the patient user interface, the patient can select from the following options: surveys (PROs), questions about medication intake (medication plan), documents for download, and calendar. The medication plan displays information on ozanimod treatment. The documents module provides downloadable files (e.g., patient portal user manual and ozanimod SmPC). The calendar displays the patient's start date and participation within the OzEAN study and is equipped with a reminder to take ozanimod. The patient is also able to review and visualize personal longitudinal data graphically. PRO, patient-reported outcome; SmPC, Summary of Product Characteristics.

Monitoring

An external steering committee is planned for data review, data evaluation, and publication in agreement with the leading principal investigator.

An interim analysis will be conducted after enrollment of 25% of the planned number of patients and will describe the baseline data. Thereafter, yearly interim analyses are planned.

AEs are collected during study site visits and via the patient portal, as described previously. All AEs occurring after the first dose of ozanimod until the end of the study (including the safety follow-up), whether related or not to ozanimod treatment, will be recorded and reported to the sponsor or its designee. AEs and SAEs are reported within 24 h. If it is discovered that a patient or female partner of a male patient is pregnant, this is reported to the sponsor within 24 h. SAEs associated with the pregnancy are reported within 24 h. Follow-up information on pregnancy outcomes is forwarded to the sponsor, even if the outcome becomes known after the end of the study.

Representatives of the sponsor and/or its delegates are permitted to visit all study site locations to assess the data quality and study integrity. On site, they review study files and, if allowed by local laws and regulations, patient medical charts to compare them with source documents, discuss the conduct of the study with the investigator, and verify that the facilities remain acceptable. In addition, the study may be evaluated by the sponsor's internal auditors and government inspectors, who are permitted access to CRFs, source documents, other study files, and study facilities.

DISCUSSION

OzEAN is an ongoing study to evaluate ozanimod treatment for RRMS in real-world clinical practice in Germany, with a large sample size, sophisticated methodology, and complex design involving endpoints related to multiple MS symptoms and functional consequences. The findings from this real-world evidence study will provide an important complement to the efficacy and safety results from highly structured randomized controlled trials (26), including phase 3 clinical trials (6, 7) and the ongoing open-label extension study of phase 1–3 trials (DAYBREAK) (27).

The eligibility criteria of OzEAN are less selective than those of the phase 2 and 3 clinical trials, allowing for inclusion of adults (≥18 years) of any age with RRMS who are eligible for ozanimod treatment and elect to initiate ozanimod treatment prior to and independent from study enrollment. There are no requirements in terms of relapse history, EDSS score, MRI parameters, or prior MS treatment. This is beneficial in 2 respects. First, it will be important to establish the efficacy and safety of ozanimod in a heterogeneous and extended population outside of the highly regulated confines of a clinical trial, in which patients are selected based on predetermined criteria that may not be met by a majority of patients in clinical care (28). Second, this will allow for characterization of patients prescribed ozanimod in real-world clinical practice. A question of interest is whether ozanimod is prescribed to patients newly diagnosed with MS, consistent with a shift toward earlier use of high-efficacy disease-modifying

TABLE 4 | Description of Measures in OzEAN.

Physician-Reported Assessment	
Expanded Disability Status Scale (EDSS) (10)	The EDSS is a standardized, widely accepted method to evaluate disability in people with MS. Severity of disability in multiple functional systems (pyramidal, cerebellar, brain stem, sensory, bowel and bladder, visual or optic, cerebral or mental, and other) observed during a standard neurological examination is scored on a numerical scale ranging from 0 (normal) to 10 (death due to MS).
Symbol Digit Modalities Test (SDMT) (12, 13)	The SDMT is a reliable measure of change in cognitive processing speed over time. Patients are given a key showing numbers [0–9] paired with symbols. They are also presented with rows of the same symbols (in random order) and are asked to provide the matching numbers, based on the key. The score is based on the number of correct responses within 90 s, with higher scores indicating better performance. The SDMT has been validated in patients with MS and is typically administered orally in this population. Changes in SDMT raw score of ≥ 4 points or 10% are considered clinically meaningful.
Patient-Reported Assessment	
Treatment Satisfaction Questionnaire for Medication, version 1.4 (TSQM v1.4) (14, 15)	The TSQM v1.4 is a general measure of treatment satisfaction with medication in chronic diseases that has been tested extensively in people with RRMS. It comprises 14 items covering 4 domains: effectiveness, side effects, convenience, and global satisfaction. On individual items, patients rate their satisfaction with a medication, presence/absence and bothersomeness of side effects, extent to which side effects interfere with functioning and impact treatment satisfaction, ease of use and convenience, and confidence in the treatment. Ratings reflect experience with the medication over the previous 2–3 weeks, or since the patient's last use of the medication. Scores range from 0 to 100, with higher scores representing greater satisfaction.
United Kingdom Neurological Disability Rating Scale (UKNDS) (16, 17)	The UKNDS is a simple, user-friendly clinical disability scale that is valid and reliable for the assessment of patients with MS. It is derived from Guy's Neurology Disability Scale and consists of 11 domains: cognition, mood, vision, speech, swallowing, upper limb function, lower limb function, bladder function, bowel function, fatigue, and pain. Each subscale is scored on a 6-point Likert scale ranging from 0 (normal) to 5 (total loss of function/maximum impairment), producing an overall sum score ranging from 0 (best) to 55 (worst). Improvement or worsening of at least 1 grade in each subscale are considered clinically meaningful.
Multiple Sclerosis Quality of Life-54 (MSQOL-54) (18)	The MSQOL-54 is a multidimensional health-related QoL measure that combines both generic and MS-specific items into a single instrument. The generic component is the Short Form-36 Health Survey (SF-36) (42), to which 18 items were added to identify MS-specific issues. There are 54 items distributed into 12 subscales (physical function, role limitations-physical, role limitations-emotional, pain, emotional well-being, energy, health perceptions, social function, cognitive function, health distress, overall quality of life, and sexual function), along with 2 summary scores (physical composite summary [PCS] and mental health composite summary [MCS]). A change in score equivalent to 0.5 SD has been found to have almost universal relevance as a minimum clinically important difference for health-related QoL. Using the 0.5 SD threshold for the SF-36, a change of ≥ 5 points in PCS and MCS has been proposed to be clinically meaningful (23).
Fatigue Scale for Motor and Cognitive Functions (FSMC) (19)	The FSMC includes a cognitive scale and a physical (motor) scale, each consisting of 10 items. Items are scored using a 5-point scale ranging from 1 to 5, yielding a total score range of 20 (no fatigue at all) to 100 (severest grade of fatigue). Based on cut-off values for severity categories, a change from baseline of ≥ 10 in FSMC sum score, ≥ 6 in cognitive subscore, and ≥ 5 in physical subscore should denote a clinically meaningful change.
Work Productivity and Activity Impairment Questionnaire for Multiple Sclerosis (WPAI-MS v2.1) (20)	The WPAI-MS German v2.1 consists of 6 items across 4 domains: absenteeism (work time missed), presenteeism (impairment at work/reduced on-the-job effectiveness), work productivity loss (overall work impairment/absenteeism + presenteeism), and activity impairment. Each domain is measured on a scale of 0% to 100% impairment. The recall period is 7 days. A lower score on the WPAI-MS v2.1 subscales indicates less impairment (i.e., an improvement).
Multiple Sclerosis Health Resource Survey (MS-HRS v3.0) (21)	The MS-HRS v3.0 is a validated, 24-item questionnaire that enables a holistic and longitudinal examination of resource use and costs (direct medical, direct non-medical, and indirect) in patients with MS. It documents social resource use, independent of source of reimbursement, and economic impact on work, family, and leisure. The instrument allows for allocation of a monetary value to a specific disease state, to an event (e.g., a relapse), or to a specific therapy.

MS, multiple sclerosis; QoL, quality of life; RRMS, relapsing-remitting multiple sclerosis; SD, standard deviation.

therapies (29), or reserved for patients previously treated with lower-efficacy agents (i.e., an escalation approach) (30).

The OzEAN study incorporates assessments of persistence and adherence to treatment. Persistence (remaining on continuous treatment) and adherence (taking treatment as prescribed) are important to treatment outcomes but tend to be poor in people with MS (31). Generally, persistence and adherence are better with oral disease-modifying therapies compared with injectables, but there are differences even among oral medications that can impact treatment success (31, 32). Assessment of persistence and adherence to ozanimod treatment is an important element of the OzEAN study and complements the TSQM v1.4 as another index of treatment satisfaction.

The efficacy outcome measures of the OzEAN study complement those of the phase 2 and 3 clinical trials (**Table 5**), which focused on traditional, physician-reported assessments of relapses (ARR) and disability progression (EDSS), MRI parameters (gadolinium-enhancing lesions, T2 lesions, and brain atrophy), and functional measures [the Multiple Sclerosis Functional Composite (MSFC)(33)), which comprises the Timed 25-Foot Walk (lower limb function and walking speed), Nine-Hole Peg Test (upper limb function and dexterity), and either the SDMT or the Paced Auditory Serial Addition Test (cognition) (4, 6, 7, 34, 35). QoL was assessed in the phase 3 studies using the MSQOL-54, but no other PROs were employed (6, 7). The value of assessing symptoms and consequences of MS that are viewed

TABLE 5 | Outcome measures in OzEAN compared with phase 2 and phase 3 studies of ozanimod.

	Phase 2 (4)	Phase 3 SUNBEAM (7)	Phase 3 RADIANCE (6)	OzEAN
Clinical				
ARR	X	X	X	X
Disability progression (EDSS)		X	X	X
MSFC		X	X	
T25FW		X	X	
9HPT		X	X	
SDMT		X		X
PASAT			X	
Persistence with therapy				X
MRI				
Gadolinium-enhancing lesions	X	X	X	X[a]
New or enlarging T2 lesions	X	X	X	X[a]
Brain atrophy		X	X	
Patient-reported outcomes				
MSQOL-54		X	X	X
TSQM v1.4				X
UKNDS				X
FSMC				X
WPAI-MS v2.1				X
MS-HRS v3.0				X
Adherence to therapy				X
Safety and tolerability				
AEs	X	X	X	X
Laboratory values	X	X	X	X[a]

9HPT, Nine-Hole Peg Test; AE, adverse event; ARR, annualized relapse rate; EDSS, Expanded Disability Status Scale; FSMC, Fatigue Scale for Motor and Cognitive Functions; MRI, magnetic resonance imaging; MSFC, Multiple Sclerosis Functional Composite; MS-HRS v3.0, Multiple Sclerosis Health Resource Utilization Survey, version 3.0; MSQOL-54, Multiple Sclerosis Quality of Life-54; PASAT, Paced Auditory Serial Addition Test; SDMT, Symbol Digit Modalities Test; T25FW, Timed 25-Foot Walk; TSQM v1.4, Treatment Satisfaction Questionnaire for Medication, version 1.4; UKNDS, United Kingdom Neurological Disability Rating Scale; WPAI-MS v2.1, Work Productivity and Activity Impairment Questionnaire for Multiple Sclerosis, version 2.1.

[a]*Only if available based on the local clinical routine assessments performed at the respective study site.*

as important by patients, as well as the patient's perspective on treatment outcome and success, is increasingly recognized (26). The OzEAN study employs a number of PROs designed to evaluate not only QoL (MSQOL-54) and cognition (SDMT), but also treatment satisfaction (TSQM v1.4), patient-assessed disability (UKNDS), fatigue (FSMC), functioning at work and in other contexts (WPAI-MS v2.1), and costs associated with MS (MS-HRS v3.0). These PROs are valid, reliable, and responsive to change, with established thresholds for clinically meaningful change (**Table 4**).

To our knowledge, OzEAN is the first non-interventional study in MS to offer a patient portal. While use of the MSDS^{3D} data collection and management system has been incorporated into other real-world studies in MS (36–40), the expansion of this tool to include patient connection via the portal is intended to facilitate study participation, optimally inform patients, and support study patients' compliance. The aim of the patient portal is to obtain a high-resolution picture of the course of the disease with the highest possible data quality, independent from visits, through at-home documentation of digital PRO questionnaires (41). The patient portal complements and optimizes medical care in daily practice, as it enables the patient to play an active role in the study and to increase the e-health interaction between patient and study center.

This study has a number of further strengths. It represents the first collection of real-world data from patients with RRMS initiating treatment with ozanimod according to the SmPC. The results will broaden the understanding of ozanimod's safety and efficacy outside of controlled clinical conditions and in a patient population chosen based only on criteria outlined in the SmPC, and it will provide needed information to physicians and other health care providers on ozanimod treatment in routine clinical care. A high level of external validity can be expected, as the study sites and patient sample are selected to be representative of treatment and care in Germany.

There are also limitations. As OzEAN is an observational study, there is no randomization of patients, no blinding, and no control group. Outcome measures are limited to clinical assessments and PROs, with no MRI endpoints. Patient recall bias is a possible limitation, particularly with the long (6-month) intervals between assessments in the latter portion of the study. There is potential for selection bias with regard to participating sites and patients, as well as attrition bias, but measures are taken to minimize these issues, including participation of a

large number of randomly selected sites of different types and locations across Germany, a large sample size, consecutive enrollment of eligible patients, and prespecified methods for handling missing data from patients lost to follow-up. Finally, the generalizability of the findings may be limited, as all of the study sites are located in Germany, a technologically advanced and predominantly racially/ethnically homogeneous country with a universal healthcare system.

CONCLUSION

In summary, this is the first long-term, real-world study of ozanimod in patients with RRMS. These data will add to the safety and efficacy profile of ozanimod previously demonstrated in the phase 3 trials (6, 7) and the ongoing open-label extension study of phase 1–3 trials (DAYBREAK) (27) in patients with relapsing MS, and they will provide new information on endpoints not previously evaluated in ozanimod clinical trials. To our knowledge, this is the first non-interventional study utilizing a patient portal, which is expected to facilitate study participation and compliance, provide valuable information on PROs, and draw a high-resolution picture of the course of disease independent of study visits in a convenient way (41). Final long-term results are anticipated after study completion in March 2029; yearly interim analyses are planned after enrollment has reached 25%. The OzEAN study aims to assess the utility of ozanimod in clinical practice.

ETHICS AND DISSEMINATION

The required approvals from Ethics Committees, Independent Review Committees, Regulatory Authorities, and/or other local governance bodies were obtained before study initiation. The observational plan, patient questionnaires, and informed consent forms were reviewed and approved by the Independent Ethics Committee. The study was disclosed to the higher federal authority, to the German Association of Statutory Health Insurance Physicians, to the Central Federal Association of the Health Insurance Fund, and to the Association of Private Health Insurance Funds as required by §67 (6) German drug law. In accordance with local regulations, all patients provide written consent before enrollment. Investigators ensure that patients or their legally acceptable representatives are clearly and fully informed about the purpose of the study, potential risks, and the patient's rights and responsibilities when participating in this study.

AUTHOR CONTRIBUTIONS

Study design: TZ, BK, A-MP, ML, and RL. Study investigator: MB, TZ, and MM. Enrolled patients: TZ, SR, MM, and MB. Collection and assembly of data: CRO MedicalSyn. Data interpretation, manuscript preparation, manuscript review and revisions, and final approval of manuscript: All authors.

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REFERENCES

1. Scott FL, Clemons B, Brooks J, Brahmachary E, Powell R, Dedman H, et al. Ozanimod (RPC1063) is a potent sphingosine-1-phosphate receptor-1 (S1P1) and receptor-5 (S1P5) agonist with autoimmune disease-modifying activity. *Br J Pharmacol.* (2016) 173:1778–92. doi: 10.1111/bph.13476
2. Zeposia. *Package Insert*. Princeton, NJ: Bristol Myers Squibb (2021).
3. Zeposia. *Summary of Product Characteristics*. Utrecht: Celgene Distribution BV (2021).
4. Cohen JA, Arnold DL, Comi G, Bar-Or A, Gujrathi S, Hartung JP, et al. Safety and efficacy of the selective sphingosine 1-phosphate receptor modulator ozanimod in relapsing multiple sclerosis (RADIANCE): a randomised, placebo-controlled, phase 2 trial. *Lancet Neurol.* (2016) 15:373–81. doi: 10.1016/S1474-4422(16)00018-1
5. Cohen JA, Comi G, Arnold DL, Bar-Or A, Selmaj KW, Steinman L, et al. Efficacy and safety of ozanimod in multiple sclerosis: dose-blinded extension of a randomized phase II study. *Mult Scler.* (2019) 25:1255–62. doi: 10.1177/1352458518789884
6. Cohen JA, Comi G, Selmaj KW, Bar-Or A, Arnold DL, Steinman L, et al. Safety and efficacy of ozanimod versus interferon beta-1a in relapsing multiple sclerosis (RADIANCE): a multicentre, randomised, 24-month, phase 3 trial. *Lancet Neurol.* (2019) 18:1021–33. doi: 10.1016/S1474-4422(19)30238-8
7. Comi G, Kappos L, Selmaj KW, Bar-Or A, Arnold DL, Steinman L, et al. Safety and efficacy of ozanimod versus interferon beta-1a in relapsing multiple sclerosis (SUNBEAM): a multicentre, randomised, minimum 12-month, phase 3 trial. *Lancet Neurol.* (2019) 18:1009–20. doi: 10.1016/S1474-4422(19)30239-X
8. Selmaj KW, Cohen JA, Comi G, Bar-Or A, Arnold DL, Steinman L, et al. Ozanimod in relapsing multiple sclerosis: pooled safety results from the clinical development program. *Mult Scler Relat Disord.* (2021) 51:102844. doi: 10.1016/j.msard.2021.102844
9. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol.* (2011) 69:292–302. doi: 10.1002/ana.22366
10. Kurtzke JF. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology.* (1983) 33:1444–52. doi: 10.1212/WNL.33.11.1444
11. D'Amico E, Haase R, Ziemssen T. Review: Patient-reported outcomes in multiple sclerosis care. *Mult Scler Relat Disord.* (2019) 33:61–6. doi: 10.1016/j.msard.2019.05.019
12. Smith A. *Symbol Digit Modalities Test: Manual*. Los Angeles, CA: Western Psychological Services (1982).
13. Benedict RH, DeLuca J, Phillips G, LaRocca N, Hudson LD, Rudick R. Validity of the symbol digit modalities test as a cognition performance outcome measure for multiple sclerosis. *Mult Scler.* (2017) 23:721–33. doi: 10.1177/1352458517690821
14. Atkinson MJ, Sinha A, Hass SL, Colman SS, Kumar RN, Brod M, et al. Validation of a general measure of treatment satisfaction, the

Treatment Satisfaction Questionnaire for Medication (TSQM), using a national panel study of chronic disease. *Health Qual Life Outcomes.* (2004) 2:12. doi: 10.1186/1477-7525-2-12
15. Vermersch P, Hobart J, Dive-Pouletty C, Bozzi S, Hass S, Coyle PK. Measuring treatment satisfaction in MS: is the treatment satisfaction questionnaire for Medication fit for purpose? *Mult Scler.* (2017) 23:604–13. doi: 10.1177/1352458516657441
16. Sharrack B, Hughes RA. The Guy's Neurological Disability Scale (GNDS): a new disability measure for multiple sclerosis. *Mult Scler.* (1999) 5:223–33. doi: 10.1177/135245859900500406
17. Rossier P, Wade DT. The Guy's Neurological Disability Scale in patients with multiple sclerosis: a clinical evaluation of its reliability and validity. *Clin Rehabil.* (2002) 16:75–95. doi: 10.1191/0269215502cr447oa
18. Vickrey BG, Hays RD, Harooni R, Myers LW, Ellison GW. A health-related quality of life measure for multiple sclerosis. *Qual Life Res.* (1995) 4:187–206. doi: 10.1007/BF02260859
19. Penner IK, Raselli C, Stöcklin M, Opwis K, Kappos L, Calabrese P. The Fatigue Scale for Motor and Cognitive Functions (FSMC): validation of a new instrument to assess multiple sclerosis-related fatigue. *Mult Scler.* (2009) 15:1509–17. doi: 10.1177/1352458509348519
20. Reilly MC, Zbrozek AS, Dukes EM. The validity and reproducibility of a work productivity and activity impairment instrument. *Pharmacoeconomics.* (1993) 4:353–65. doi: 10.2165/00019053-199304050-00006
21. Ness NH, Haase R, Kern R, Schriefer D, Ettle B, Cornelissen C, et al. The Multiple Sclerosis Health Resource Utilization Survey (MS-HRS): development and validation study. *J Med Internet Res.* (2020) 22:e17921. doi: 10.2196/17921
22. Cramer JA, Roy A, Burrell A, Fairchild CJ, Fuldeore MJ, Ollendorf DA, et al. Medication compliance and persistence: terminology and definitions. *Value Health.* (2008) 11:44–7. doi: 10.1111/j.1524-4733.2007.00213.x
23. Jongen PJ. Health-related quality of life in patients with multiple sclerosis: impact of disease-modifying drugs. *CNS Drugs.* (2017) 31:585–602. doi: 10.1007/s40263-017-0444-x
24. Haase R, Wunderlich M, Dillenseger A, Kern R, Akgün K, Ziemssen T. Improving multiple sclerosis management and collecting safety information in the real world: the MSDS3D software approach. *Expert Opin Drug Saf.* (2018) 17:369–78. doi: 10.1080/14740338.2018.1437144
25. Ziemssen T, Kern R, Voigt I, Haase R. Data Collection in Multiple Sclerosis: The MSDS Approach. *Front Neurol.* (2020) 11:445. doi: 10.3389/fneur.2020.00445
26. Ziemssen T, Hillert J, Butzkueven H. The importance of collecting structured clinical information on multiple sclerosis. *BMC Med.* (2016) 14:81. doi: 10.1186/s12916-016-0627-1
27. Cree BAC, Selmaj KW, Steinman L, Comi G, Bar-Or A, Arnold DL, et al. Long-term safety and efficacy of ozanimod in relapsing multiple sclerosis: up to 5 years follow-up in the DAYBREAK open-label extension study. *Mult Scler J.* (2022).
28. Jalusic KO, Ellenberger D, Rommer P, Stahmann A, Zettl U, Berger K. Effect of applying inclusion and exclusion criteria of phase III clinical trials to multiple sclerosis patients in routine clinical care. *Mult Scler.* (2021) 27:1852–63. doi: 10.1177/1352458520985118
29. Hartung HP, Meuth SG, Thompson AJ. Paradigm shifts: early initiation of high-efficacy disease-modifying treatment in multiple sclerosis. *Mult Scler.* (2021) 27:1473–6. doi: 10.1177/13524585211033190
30. Inojosa H, Proschmann U, Akgün K, Ziemssen T. The need for a strategic therapeutic approach: multiple sclerosis in check. *Ther Adv Chronic Dis. In Press.* (2021) 13:20406223211063032. doi: 10.1177/2040622321106 3032
31. Johnson KM, Zhou H, Lin F, Ko JJ, Herrera V. Real-world adherence and persistence to oral disease-modifying therapies in multiple sclerosis patients over 1 year. *J Manag Care Spec Pharm.* (2017) 23:844–52. doi: 10.18553/jmcp.2017.23.8.844
32. Haase R, Kullmann JS, Ziemssen T. Therapy satisfaction and adherence in patients with relapsing-remitting multiple sclerosis: the THEPA-MS survey. *Ther Adv Neurol Disord.* (2016) 9:250–63. doi: 10.1177/1756285616634247
33. Fischer JS, Jak AJ, Kniker JE, Rudick RA, Cutter G. *Multiple Sclerosis Functional Composite (MSFC) Administration and Scoring Manual.* (2001). National Multiple Sclerosis Society. Available online at: https://www.nationalmssociety.org/NationalMSSociety/media/MSNationalFiles/Brochures/10-2-3-31-MSFC_Manual_and_Forms.pdf (accessed December 22, 2020).
34. Drake AS, Weinstock-Guttman B, Morrow SA, Hojnacki D, Munschauer FE, Benedict RH. Psychometrics and normative data for the multiple sclerosis functional composite: replacing the PASAT with the symbol digit modalities test. *Mult Scler.* (2010) 16:228–37. doi: 10.1177/1352458509354552
35. DeLuca J, Schippling S, Montalban X, Kappos L, Cree BAC, Comi G, et al. Effect of ozanimod on Symbol Digit Modalities Test performance in relapsing MS. *Mult Scler Relat Disord.* (2021) 48:102673. doi: 10.1016/j.msard.2020.102673
36. Ziemssen T, Engelmann U, Jahn S, Leptich A, Kern R, Hassoun L, et al. Rationale, design, and methods of a non-interventional study to establish safety, effectiveness, quality of life, cognition, health-related and work capacity data on Alemtuzumab in multiple sclerosis patients in Germany (TREAT-MS). *BMC Neurol.* (2016) 16:109. doi: 10.1186/s12883-016-0629-9
37. Ziemssen T, Gass A, Wuerfel J, Bayas A, Tackenberg B, Limmroth V, et al. Design of TRUST, a non-interventional, multicenter, 3-year prospective study investigating an integrated patient management approach in patients with relapsing-remitting multiple sclerosis treated with natalizumab. *BMC Neurol.* (2016) 16:98. doi: 10.1186/s12883-016-0625-0
38. Ziemssen T, Kern R, Cornelissen C. Study design of PANGAEA 2.0 a non-interventional study on RRMS patients to be switched to fingolimod. *BMC Neurol.* (2016) 16:129. doi: 10.1186/s12883-016-0648-6
39. Dirks P, Zingler V, Leemhuis J, Berthold H, Hieke-Schulz S, Wormser D, et al. Design of a non-interventional post-marketing study to assess the long-term safety and effectiveness of ocrelizumab in German real world multiple sclerosis cohorts - the CONFIDENCE study protocol. *BMC Neurol.* (2020) 20:95. doi: 10.1186/s12883-020-01667-7
40. Ziemssen T, Hoffmann O, Klotz L, Schreiber H, Weber MS, Rauser B. Gaining first insights on secondary progressive multiple sclerosis patients treated with siponimod in clinical routine: protocol of the noninterventional study AMASIA. *JMIR Res Protoc.* (2020) 9:e19598. doi: 10.2196/19598
41. Voigt I, Benedict M, Susky M, Scheplitz T, Frankowitz S, Kern R, et al. A digital patient portal for patients with multiple sclerosis. *Front Neurol.* (2020) 11:400. doi: 10.3389/fneur.2020.00400
42. Ware JJr, Kosinski M, Keller SD. A 12-Item Short-Form Health Survey: construction of scales and preliminary tests of reliability and validity. *Med Care.* (1996) 34:220–33. doi: 10.1097/00005650-199603000-00003

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Peripheral Hemolysis in Relation to Iron Rim Presence and Brain Volume in Multiple Sclerosis

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Background: Iron rim lesions (IRLs) represent chronic lesion activity and are associated with a more severe disease course in multiple sclerosis (MS). How the iron rims around the lesions arise in patients with MS (pwMS), and whether peripheral hemolysis may be a source of iron in rim associated macrophages, is unclear.

Objective: To determine a potential correlation between peripheral hemolysis parameters and IRL presence in pwMS.

Methods: This retrospective study included pwMS, who underwent a 3T brain MRI between 2015 and 2020 and had a blood sample drawn at ± 2 weeks. Patients with vertigo served as a control group.

Results: We analyzed 75 pwMS (mean age 37.0 years [SD 9.0], 53.3% female) and 43 controls (mean age 38.3 years [SD 9.8], 51.2% female). Median number of IRLs was 1 (IQR 4), 28 (37.3%) pwMS had no IRLs. IRL patients showed significantly higher Expanded Disability Status Scale (EDSS) compared to non-IRL patients (median EDSS 2.3 [IQR 2.9] vs. 1.3 [IQR 2.9], $p = 0.017$). Number of IRLs correlated significantly with disease duration ($r_S = 0.239$, $p = 0.039$), EDSS ($r_S = 0.387$, $p < 0.001$) and Multiple Sclerosis Severity Scale (MSSS) ($r_S = 0.289$, $p = 0.014$). There was no significant difference in hemolysis parameters between non-IRL, IRL patients (regardless of gender and/or disease type) and controls, nor between hemolysis parameters and the number of IRLs. Total brain volume was associated with fibrinogen ($\beta = -0.34$, 95% CI −1.32 to −0.145, $p = 0.016$), and absolute cortical and total gray matter volumes were associated with hemoglobin ($\beta = 0.34$, 95% CI 3.39–24.68, $p = 0.011$; $\beta = 0.33$, 95% CI 3.29–28.95, $p = 0.015$; respectively).

Conclusion: Our data do not suggest an association between hemolysis parameters and IRL presence despite a significant association between these parameters and markers for neurodegeneration.

Keywords: iron rim, hemolysis, multiple sclerosis, disease progression, brain volume

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INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), which leads to focal demyelination in the gray and white matter (WM) (1, 2). In the early relapsing stage of the disease, acute inflammation and blood-brain barrier (BBB) disruption is reflected by gadolinium (Gd) enhancing lesions in MRI. Later, chronic active inflammation behind the BBB can be detected by MRI *via* susceptibility-weighted imaging (SWI), R2* or QSM as a rim of iron-laden microglia and macrophages around the FLAIR-hyperintense lesion (3–6). These so-called iron rim lesions (IRLs) or paramagnetic rim lesions are a subset of chronic active lesions (3, 6–9), and occur in ∼60% of people with MS (pwMS) irrespective of MS course peaking in the late relapsing-remitting MS and early secondary progressive MS stage (10). Approximately 30% of all WM lesions and about 40% of chronic active MS lesions have an iron rim (11). Their presence has been associated with a more severe disease course (12, 13). They display more pronounced black holes compared to non-IRLs, reflecting severe axonal loss and the absence of remyelination (4, 5), and they are associated with elevated serum neurofilament levels (sNfL) (14, 15) and brain atrophy rates (13, 14, 16). Recent work has shown that IRLs expand over time (3, 4, 17), while the iron rims themselves gradually diminish over an extended time period of about 7 years (17). Altogether, IRLs are now considered a potential biomarker for progression and chronic MS course with high neurodestructive potential (5, 18).

Generally, iron is known to accumulate in the human brain with age (19). On the one hand, it is important for normal cellular functions, biochemical reactions such as DNA, RNA, and proteins synthesis, and is involved in myelin synthesis (20, 21). On the other hand, iron is also cytotoxic due to free oxygen or nitrogen radical formation and is known to amplify demyelination, neurodegeneration, and oxidative damage in MS (8). Most cerebral iron is found in the substantia nigra and basal ganglia (22–24). An intact BBB protects the brain from fluctuations in systemic iron levels so that impaired iron homeostasis in the periphery has only minor effects on brain iron metabolism (25). Thus, concentrations of iron and iron-modulating proteins in the serum and cerebrospinal fluid (CSF) differ substantially (26). Since iron metabolism in terms of uptake, transport, and storage is not fully clarified (27), a better understanding of dysregulatory pathways of iron homeostasis will help elucidate the causes of iron accumulation and iron-mediated tissue damage in the brain. Recent advances in the field of iron-dependent lipid peroxidation leading to a cell death called "ferroptosis" have already provided valuable insights that are particularly relevant to the lipid-rich environment of the CNS (28).

Nevertheless, to date, no significant factor influencing the development of IRLs in the CNS has been identified, and it is still unclear why some pwMS have iron rims and others have not. We raise the question, whether peripheral hemolysis in pwMS under conditions of a chronically impaired BBB with potentially higher iron influx into the lesion may favor the formation of iron rims, which in turn are formed by phagocytes which protect brain tissue from free pro-oxidant Fe^{2+}.

Iron is present in serum as both heme and non-heme iron. The former is used for metabolic processes, is highly concentrated in erythrocytes, and is liberated upon hemolysis. Liberated hemoglobin and its iron are bound by the serum protein haptoglobin to avoid its deleterious pro-oxidative effects (29). Main sources for iron-accumulation in the CNS are erythrocytes leaking through the BBB and their decay within the brain and spinal cord or the destruction of iron containing oligodendrocytes and myelin (4, 30). Already, early studies describe a macrocytosis and higher osmotic fragility of erythrocytes in pwMS than in healthy controls, and particularly more pronounced in patients with a relapse (31, 32). Also, a recent experimental study on iron overload found an increased fragility and macrocytosis of erythrocytes, low-grade hemolysis and a significant liberation of hemoglobin from erythrocytes (33). In addition, extracellular methemoglobin (metHb) was observed to cause oxidative damage to myelin components in the CNS after extravasation of blood from plaque veins into plaque tissue (34). This could eventually mean that iron release from myelin together with blood-derived iron amplifies neurodegeneration. Lewin et al. reported that elevated serum free hemoglobin (Hb) correlated with brain atrophy rate in people with secondary progressive MS (35). This has been attributed to chronic, low-grade intravascular hemolysis, which in turn is thought to lead to oxidative damage of oligodendrocytes by serum Hb after its passage through the impaired BBB. Nevertheless, intravascular hemolysis was not reflected by a lower total blood Hb level (35). Altogether, these data indicate that hemolysis may play a role in MS as a cofactor enhancing neurodegeneration. It is already known that elevated iron levels in postmortem brain SWI-MR images and iron deposition seen in histopathology correlate positively in pwMS (30, 36), but it is unknown whether peripheral serum parameters of hemolysis are associated with SWI-detected IRLs.

METHODS

Patients

In this cross-sectional, retrospective study, 75 patients from the Vienna MS database (VMSD) (37) and 43 sex- and age-matched patients with peripheral vertigo as control group were included. All included control patients with peripheral vertigo were clearly clarified as not being centrally affected by our experts of our special outpatient clinic for vertigo based on clinical history, neurological status and imaging. Clinically definite MS was determined according to the established diagnostic criteria (38, 39). All pwMS met the following inclusion criteria: ≥18 years, availability of T1, FLAIR and SWI-based MRI scan at 3T, and a blood sample drawn at ±2 weeks from MRI. None of the patients had a diagnosed hemolytic disease. Data on expanded Disability Status Scale (EDSS) and Multiple Sclerosis Severity Scale (MSSS) according to Roxburgh et al. were obtained at the time of MRI (40), and clinical activity (relapses) was analyzed in a time period ±6 months from MRI. A severe relapse was defined as a relapse that required either treatment with steroids or hospitalization. Disease-modifying treatment (DMT) status

was classified as following: (1) "no DMT" defined as patients receiving no DMT; (2) "moderately effective DMT" (M-DMT) defined as patients receiving either interferon-beta, glatiramer acetate, dimethyl fumarate, or teriflunomide; or (3) "highly effective DMT" (H-DMT) defined as patients receiving either natalizumab, fingolimod, alemtuzumab, cladribine, ocrelizumab or rituximab. MRI acquisition, including sequences such as FLAIR, T1 and SWI-based MRI for assessing lesions and brain volume (see below) and their analysis were performed at the Medical University of Vienna.

Imaging Acquisition

All 3T MRI brain scans were performed on a Siemens Magnetom 3T MRI system, using a 64-channel radio frequency (RF) coil between January 1, 2015, and December 31, 2020. Isovoxel (1 mm^3) 3DFLAIR (TR = 6,000 ms, TE = 288 ms, TI = 2,100 ms), T1 weighted images (TE = 2,16 ms, TR = 1,670 ms and flip angle = 15) with a gadolinium-based contrast administration and SWI sequences (TE = 40 ms, TR = 49,ms, image matrix = 224 × 256, slices = 80, slice thickness = 2 mm) were acquired consecutively.

Evaluation of Lesions and Brain Volume

All supratentorial lesions of the periventricular, juxtacortical and deep white matter in the frontal, parietal and occipital lobes (41), and in the upper parts of the temporal lobes as well as infratentorial lesions of the cerebellum were analyzed in consensus by two independent raters (ADB, NK) highly experienced in MS imaging. IRLs were defined as FLAIR-hyperintense lesions that were partially or completely surrounded by a pronounced and distinct SWI-hypointense rim. The presence of the central plaque vein did not affect the iron rim evaluation. After both raters had made their decision, the unclear lesions were discussed together on the monitor and an agreement was reached. The inter-rater agreement before matching was 98.7%.

Volume of T1 lesions and total brain volume were automatically assessed using the MorphoBox prototype imaging software normalized for age from Siemens Healthineers (42). IRLs were considered valid if a hypointense SWI signal entirely or partially surrounded a hyperintense white matter lesion in FLAIR images. Patients were grouped for the presence of IRLs (no IRLs vs. ≥1 IRLs).

Hemolysis Parameters

Hemolysis parameters included red blood cell (RBC) count, reticulocytes, Hb, hematocrit (Ht), potassium, iron, total bilirubin, free Hb, hemolysis index, lactate dehydrogenase, fibrinogen and aspartate transaminase levels. Blood samples were drawn and analyzed at the Department of Laboratory Medicine, Medical University of Vienna. The quality of blood samples was maintained with the usage of standardized protocols for their collection and storage.

Ethics

The study was approved by the Ethics Committee of the Medical University of Vienna (EC 1599/2021).

Statistics

Statistical analysis was performed using SPSS 26.0 (SPSS Inc, Chicago, IL, USA). Categorical variables were expressed in frequencies and percentages, continuous variables as mean and standard deviation (SD) or median and interquartile range (IQR) as appropriate. Continuous variables were tested for normal distribution by the Kolmogorov–Smirnov test. Univariate comparisons were done by chi-square test, independent *t*-test, Mann-Whitney *U*-test or Kruskal-Wallis test as appropriate.

Hemolysis parameters, clinical (EDSS, MSSS, disease course and duration) and paraclinical parameters [total brain volume, total gray matter (GM) and cortical volume, WM volume, total lesion volume], and the number of IRLs were first univariately analyzed by Spearman correlation analyses. Then, we calculated a linear step-wise regression model with the number of IRLs as the dependent variable and hemolytic parameters as independent variables adjusted for sex, age, DMT and disease duration. The same model was used with MRI parameters (total brain volume, total GM and cortical volume, WM volume, total lesion volume) as dependent variables and hemolytic parameters as independent variables adjusted for sex, age, and disease duration. To test the level of agreement of hemolysis parameters analyzed at different time points, the latter were compared as median values using Friedman's related-samples two-way analysis of variance by ranks with a clinical (relapse)- and radiological (Gd-enhancement)-based subanalysis. Intra-individual variance of hemolysis parameters was calculated using Bland-Altman method.

A value of $p < 0.05$ was considered statistically significant. All multiple analyses were corrected using Bonferroni method.

RESULTS

Seventy-five pwMS (53.3% female, 76.0% relapsing-remitting MS) were included with a mean age of 37.0 (SD 9.0) years and a median disease duration of 6 years (IQR 2–12), a median EDSS of 2.0 (IQR 1–3.5) and a median MSSS of 3.05 (IQR 0.99–5.85). Detailed demographics and characteristics are given in **Table 1**. IRL patients showed significantly higher scores in EDSS ($p = 0.017$) and MSSS ($p = 0.036$) compared to non-IRLs. Patients with IRLs were more commonly prescribed H-DMT (27; 57.4%) compared to patients without IRLs (11; 39.3%) ($p = 0.024$). Thirty-one (41.3%) pwMS experienced a relapse during the observation period (median time to MRI 7 weeks [IQR 4–19]), with 29 (38.7%) pwMS experiencing a severe relapse. No other relevant differences in demographics and clinical characteristics between non-IRL and IRL were found. In the control group, 43 patients (51.2% female) with a mean age of 38.3 years (SD 9.8) were included.

Number of IRLs

Among all patients, 281 IRLs were identified (**Figure 1**). Median number of IRLs in pwMS was 1 (IQR 0–4), 28 (37.3%) patients had no IRLs. Among those, 10 patients (nine females, mean age 36.9 years [SD 8.9], median disease duration of 3.5 years [IQR 1.0–8.3]) experienced a severe relapse. Two of those 10 patients showed Gd-enhancing lesions in the observed MRI. Only one

TABLE 1 | Characteristics of pwMS.

	pwMS ($n = 75$)	Non-IRL patients ($n = 28$)	IRL patients ($n = 47$)	p-value
Demographic and clinical data				
Female[a]	40 (53.3)	18 (64.3)	22 (46.8)	0.142
Age (years)[b]	37.0 (9.0)	35.5 (8.3)	38.0 (9.4)	0.255
Disease duration (years)[c]	6 (2–12)	4 (2.3–8.8)	8 (2–13)	0.180
Clinical activity[a]	31 (41.3%)	10 (35.7%)	21 (44.7%)	0.446
Severe relapse	29 (38.7%)	10 (35.7%)	19 (40.4%)	
EDSS[c]	2.0 (1–3.5)	1.3 (0–2.9)	2.3 (1.1–4)	0.017
MSSS[c]	3.05 (0.99–5.85)	2.39 (0.53–3.23)	3.91 (1.72–5.87)	0.036
RRMS[a]	57 (76.0)	24 (85.7)	33 (70.2)	0.128
DMT[a]				
No DMT	11 (14.7)	2 (7.1)	9 (19.1)	0.024
M-DMT	26 (34.7)	15 (53.6)	11 (23.4)	
IFN	3 (4.0)	3 (10.7)	0 (0.0)	
Glatiramer acetate	7 (9.3)	4 (14.3)	3 (6.4)	
Dimethyl fumarate	14 (18.7)	8 (28.6)	6 (12.8)	
Teriflunomide	2 (2.7)	0 (0.0)	2 (4.3)	
H-DMT	38 (50.7)	11 (39.3)	27 (57.4)	
Fingolimod	13 (17.3)	2 (7.1)	11 (23.4)	
Natalizumab	4 (5.3)	2 (7.1)	2 (4.3)	
Alemtuzumab	6 (8.0)	2 (7.1)	4 (8.5)	
Rituximab	12 (16.0)	5 (17.9)	7 (14.9)	
Cladribine	3 (4.0)	0 (0.0)	3 (6.4)	
MRI data				
No. of IRLs[c]	1 (0–4)	NA	3 (1–9)	NA
No. of total lesions[c]	22 (11–45)	16 (8–49.8)	24 (11–45)	0.266
No. of IRLs/No. of total lesions[c]	0.07 (0.00–0.18)	NA	0.15 (0.08–0.32)	NA
Gd-enhancement[a,†]	15 (20.0)	3 (10.7)	12 (25.5)	0.101
Absolute brain volume (ml)[c]	1,090.9 (1,000.2–1,183.9)	1,096.3 (1,015.9–1,211.9)	1,077.4 (994.9–1,179.6)	0.385
Absolute GM cortical volume (ml)[c]	517.3 (473.0–560.6)	521.3 (473.0–567.9)	514.7 (471.3–560.6)	0.776
GM total volume (ml)[c]	667.7 (603.6–709.9)	671.5 (604.9–723.1)	666.5 (603.6–709.9)	0.648
Absolute WM volume (ml)[c]	422.3 (384.3–479.5)	427.5 (397.3–461.7)	417.6 (373.2–489.3)	0.373
Total lesion volume (ml)[c]	1.1 (0.4–3.6) [0.1–50.1][d]	0.8 (0.2–3.6) [0.1–38.1][d]	1.2 (0.5–3.8) [0.1–50.1][d]	0.612
Absolute ventricular CSF volume (ml)[c]	346.5 (303.4–383.6)	344.3 (303.9–377.4)	348.65 (299.3–387.3)	0.798

CSF, cerebrospinal fluid; DMT, disease-modifying treatment; EDSS, Expanded Disability Status Scale; MSSS, Multiple Sclerosis Severity Scale; GM, gray matter; IRL, iron rim lesion; NA, not applicable; pwMS, patients with multiple sclerosis; RRMS, relapsing-remitting multiple sclerosis; WM, white matter.

[a] *Number and percentage.*

[b] *Mean and standard deviation.*

[c] *Median and interquartile range.*

[d] *Range.*

[†] *In two pwMS, no contrast was administered.*

patient had a SWI-sequence in the follow-up MRI. This patient did not show IRL formation within 1.5 years. Therefore, no predictors for conversion to IRLs could be identified. Number of IRLs correlated significantly with disease duration ($r_s = 0.239$, $p = 0.039$), EDSS ($r_s = 0.387$, $p < 0.001$) and MSSS ($r_s = 0.289$, $p = 0.014$) but not with patients' age ($r_s = 0.151$; $p = 0.194$).

Hemolysis Parameters

We analyzed the median values of hemolysis parameters in pwMS according to the presence of IRLs (no IRLs vs. ≥1 IRLs) (**Figure 2**; **Supplementary Table 1**) with gender (male with/without IRLs vs. female with/without IRLs) and disease course (relapsing vs. progressive MS) -related subanalysis, and compared them to those of controls; however, no differences were found. Besides, no correlation between the number of IRLs and hemolysis parameters was seen (**Supplementary Table 1**).

Furthermore,no significant correlation between hemolysis and clinical parameters was found. However, absolute brain volume was associated with fibrinogen ($\beta = -0.34$; 95% CI −1.32, −0.145; $p = 0.016$), and absolute cortical and total GM volumes were associated with Hb ($\beta = 0.34$; 95% CI 3.39, 24.68;

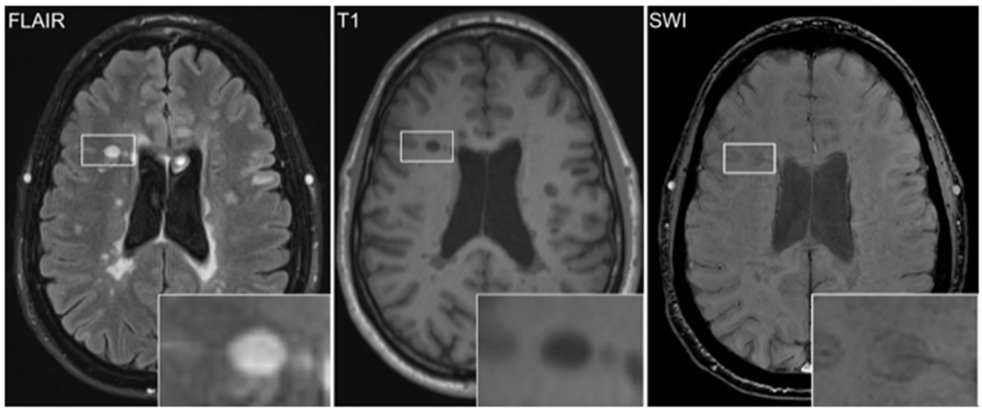

FIGURE 1 | Iron rim lesions (IRLs) can be visualized by MRI *via* susceptibility-weighted imaging (SWI) as a hypointense rim of iron-laden microglia and macrophages surrounding the FLAIR-hyperintense lesion. Increased T1 hypointensity of the IRL indicates severe tissue destruction.

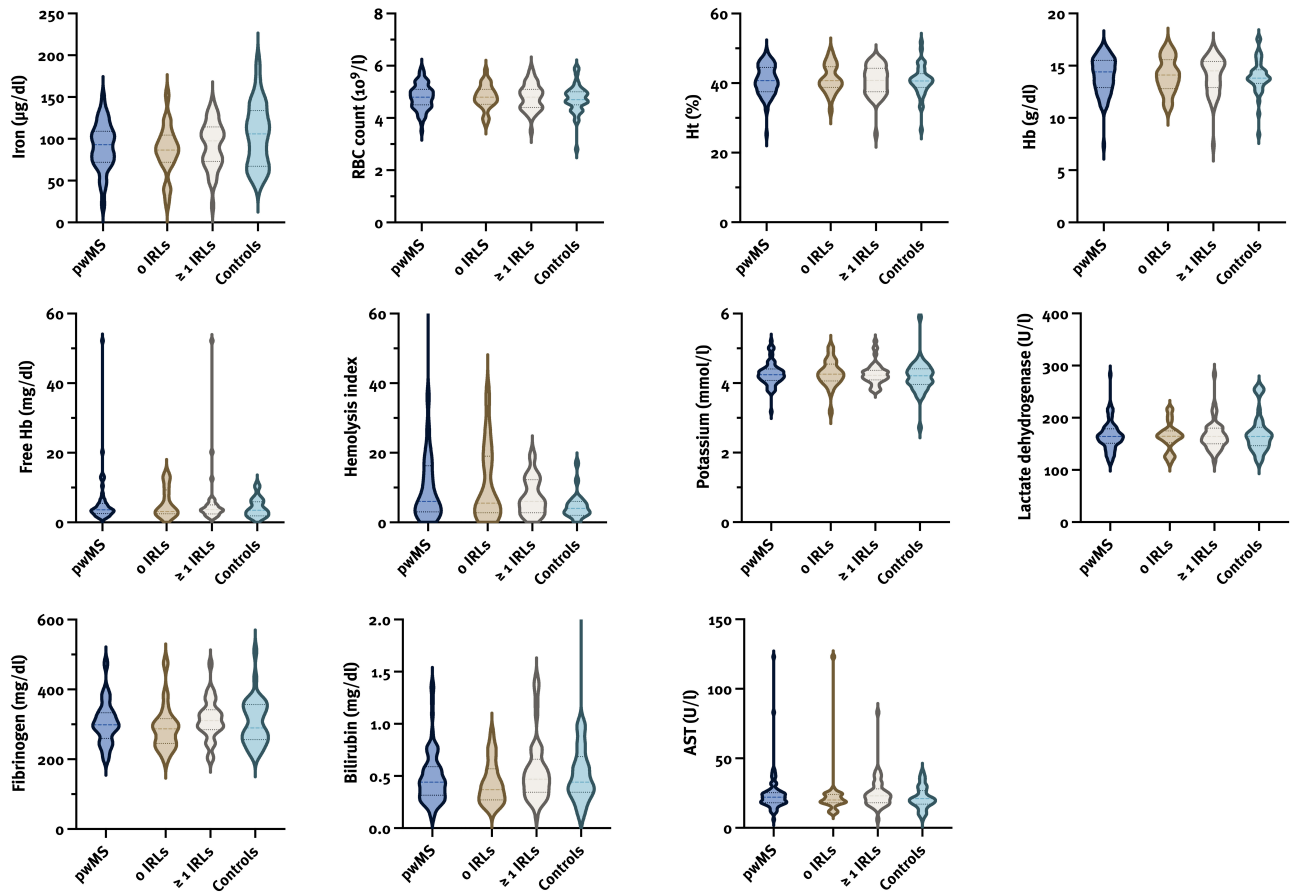

FIGURE 2 | Violin plots for hemolysis parameters in pwMS according to the presence of IRLs and controls. AST, aspartate transaminase; Hb, hemoglobin; Ht, hematocrit; IRL, iron rim lesion; pwMS, patients with multiple sclerosis; RBC, red blood cell.

$p = 0.011$ and $\beta = 0.33$; 95% CI 3.29, 28.95; $p = 0.015$; respectively).

We also analyzed the variability of hemolysis parameters in the period ±6 months from MRI on both a population and individual level (**Supplementary Table 2**; **Figure 3**). As 29/31 (93.5%) of pwMS experienced a severe relapse, no subanalysis based on the relapse severity was performed. The hemolysis parameters remained stable during the observation period regardless of

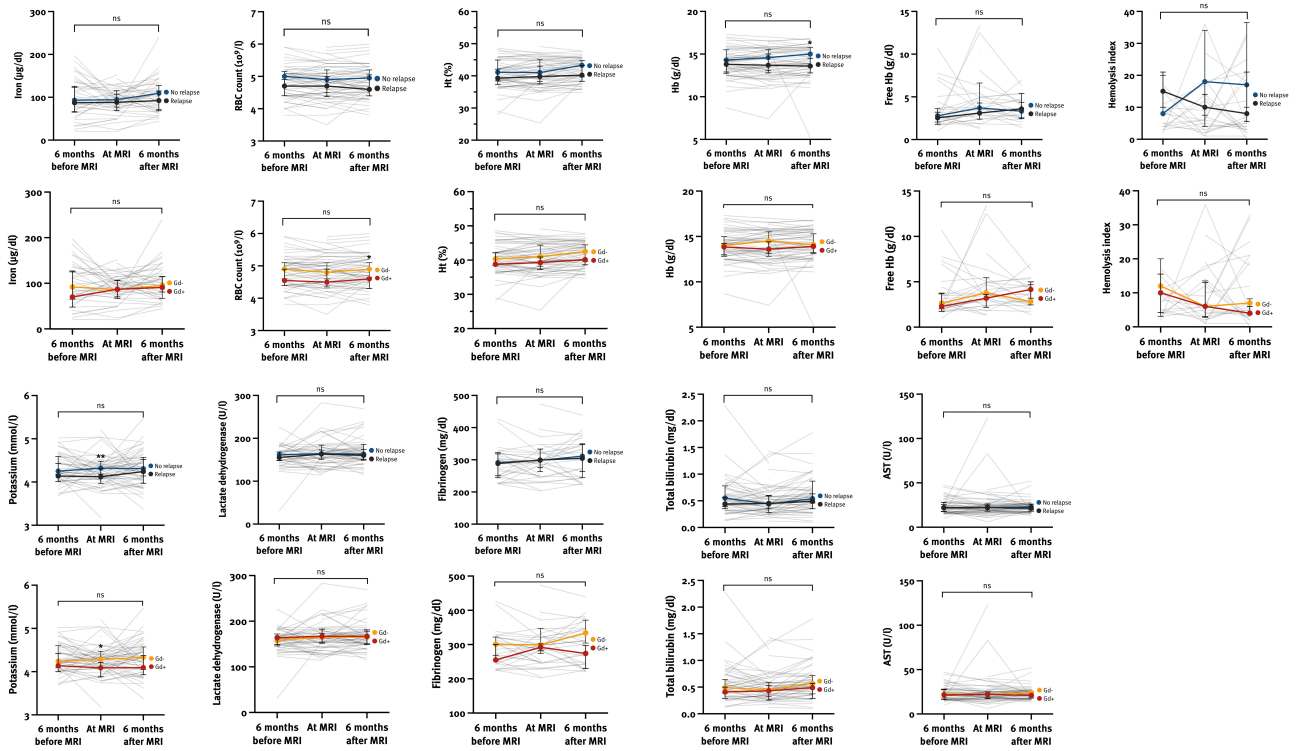

FIGURE 3 | The variability of hemolysis parameters at different time points according to the clinical and radiological activity within the observation period. AST, aspartate transaminase; Gd, gadolinium-enhancing lesion; Ht, hematocrit; Hb, hemoglobin; ns, not significant; RBC, red blood cells. *$p < 0.05$, **$p < 0.01$ calculated by Mann-Whitney *U*-test.

clinical and radiological activity. However, pwMS with a relapse had a lower median Hb 6 months after MRI (13.6 [IQR 12.7–14.6] vs. 14.9 [IQR 13.7–15.8], $p = 0.012$) and a lower median potassium level at MRI (4.12 [IQR 3.97–4.33] vs. 4.35 [IQR 4.18–4.47], $p = 0.006$), and pwMS with at least one Gd-enhancing lesion had a lower median RBC level 6 months after MRI (4.6 [IQR 4.3–4.9] vs. 4.9 [IQR 4.6–5.2], $p = 0.030$) and a lower median potassium level at MRI (4.09 [IQR 3.87–4.27] vs. 4.28 [IQR 4.13–4.42], $p = 0.029$).

DISCUSSION

IRLs are currently being evaluated as imaging biomarkers of chronic activity and their potential future role in therapy monitoring, particularly in progressive MS. Since pwMS with IRLs compared to those without IRLs have a more severe disease course and transit earlier to a progressive stage, it is clinically relevant to find out associated, and perhaps even facilitating, factors for the presence of IRLs. Erythrocyte instability and low grade hemolysis seem to lead to increased free Hb in patients with systemic inflammation and immune activation (32), which has been also associated with the rate of brain atrophy in pwMS (35). Free Hb might enter the brain during active relapses or in the course of the low-grade increase of BBB permeability in progressive MS (43). After its degradation, free iron could contribute to neurodegeneration by amplifying oxidative injury and propagating proinflammatory activation of macrophages and microglia (8, 9). In our study we, thus, analyzed whether there is a direct association between hemolysis and the presence of IRLs in the MS brain.

Our study results confirm that a higher number of IRLs is associated with both longer disease duration and higher disability measured by EDSS, and this was independent of age. Higher EDSS in IRL patients was not associated with a significantly higher relapse activity, at least within 6 months from MRI, compared to non-IRL patients. The distribution of DMT also reflected a more severe clinical course in IRL patients as 57.4% of patients with IRLs received H-DMT, whereas 60.7% of patients without IRLs received no DMT or M-DMT. DMT was not changed within the time period of ±6 months from MRI. In addition, there was a trend for lower brain volume and higher lesion volumes in IRL patients compared with non-IRL patients consistent with recent literature (13, 15, 16)These data further support the view that iron rim lesions are markers for the progression of brain damage, but not for disease activity. Furthermore, IRLs could also serve as a marker for an earlier decision for H-DMT.

Apart from that, our retrospective cross-sectional study of 75 pwMS is the first to assess whether patients with IRLs have elevated levels of peripheral hemolysis parameters compared to patients without IRLs. Tested blood parameters for hemolysis were not significantly related in our cohort to the presence

of IRLs nor with disease course or clinical (relapse) and MRI activity (Gd-enhancement). Despite the absence of a relation between hemolysis parameters and iron rims, MRI parameters (brain volume, GM volume) were associated with fibrinogen and Hb, being in line with a recently published study confirming an association between free Hb and brain atrophy in secondary progressive MS (35). However, several confounding factors may explain this possible association, including an older age of patients with progressive MS, thus being characterized by other comorbidities (e.g., diabetes, atherosclerosis, etc.). It is currently not known whether iron enters the macrophages predominantly *via* erythrocytes or free Hb and/or other forms of iron. Since the results show that hemolysis and IRLs are not significantly associated, the accumulation of iron-containing macrophages forming the iron rim around slowly expanding lesions cannot be explained by a continuous leakage of erythrocytes, Hb or iron through a weakly impaired BBB in the chronic phase of the disease. The fact that macrophages in the iron rim are not progressively loaded with iron but slowly and gradually loose signal intensity fits with the known long-term stability of iron rims observed by MRI (4, 44).

Our observation that there was no significant association between hemolysis and clinical (relapse) and MRI activity (Gd-enhancement) should be taken with caution, as in our study the median time between relapse and MRI was 7 weeks. This may have underestimated the number of patients with Gd^+ lesions, as an open BBB is expected only for 4–6 weeks after relapse onset. However, a possible association between hemolysis parameters and disease activity should be analyzed in a young patient cohort with higher activity, a short disease course and a relapse-related MRI. Since it can be assumed that iron accumulation in the brain depends on the extent of the BBB opening, Gd-enhancing active lesions in patients with early MS might show a significant association with peripheral hemolysis in contrast to pwMS with long disease duration with a low burning chronic inflammation behind an only weakly impaired BBB.

The binding and transport system of iron itself in the CNS is complex and plays a crucial role in iron accumulation in the brain. Thus, it was recently indicated that iron accumulation in the CNS is simply the end stage of many different processes, reflected by altered expressions of different molecules involved in iron influx, efflux and storage, as well as iron sensors (28). Furthermore, not only the failure of iron transport but also inadequate antioxidant defense mechanisms of oligodendroglia and neurons compared with astrocytes influence the extent of iron-induced tissue damage (45–47). In addition, phagocytic cell instability due to long-term iron storage may further lead to cell dystrophy and death, resulting iron release and the propagation of oxidative damage. All of these points underscore the importance of investigating dysfunctional mechanisms of iron transport in a disease like MS. This knowledge may reveal new therapeutic targets to stop the vicious cycle of iron-induced CNS tissue damage. A first hint in this direction may be the observation of decreasing iron content of rim lesions per year in patients treated with dimethyl fumarate compared to patients treated with glatiramer acetate (48). In addition, dimethyl fumarate but not glatiramer acetate reduced inflammatory activity and associated iron levels in human microglia (49). Yet, further studies are necessary to evaluate therapeutic effects on IRLs and the long-term consequences for brain tissue.

The strengths of our study are the detailed characterization of the study cohort provided by the high-quality data from the Vienna MS database and the high-quality standard of MRI scans. The number of IRLs was counted manually by two experienced raters, providing low level of data variability. However, there are some limitations to this study. First, the retrospective and cross-sectional design as well as the relatively small sample size carry inherent potential of bias. Secondly, we did not have a quantitative threshold for partial IRL selection. However, since the inter-rater agreement was 98.7%, we can assume that partial IRLs were reliably detected. Further, ferritin, transferrin and total iron binding capacity, important biomarkers of iron status, could not be analyzed as these parameters were not performed within clinical routine. However, a recently published study found no correlation between different activation stages of MS with ferritin, transferrin, transferrin receptor and soluble transferrin receptor, as well as hepcidin, an important regulator of systemic iron homeostasis (25). Besides, the blood samples were drawn in the median time of 2 weeks before and after the MRI, which might present a less precise picture of the actual state in blood parameters during MRI acquisition. Nevertheless, iron rims are a stable feature with only slow changes over time. Moreover, we also analyzed the variability of hemolysis parameters in the 6-month period before and after MRI, which remained stable at both population and individual levels regardless of disease activity. It should also be noted that not only iron accumulation, but also myelin loss and perilesional white matter are reported to play a role in MR frequency or QSM image contrast (44). Since these considerations are particularly important for the quantitative interpretation of MR frequency or QSM data, and in our study the presence of iron rims was only assessed qualitatively (present or not), we do not see this as an interference with our results.

In conclusion, we did not find a significant association between peripheral hemolysis and IRL presence, which predominantly occur as a subtype of chronic active lesions in the progressive phase of MS behind an already almost completely closed BBB. However, hemolysis is confirmed to play a role in relation to the brain volume, which is the predominant feature in progressive MS. Further studies are needed to clarify the role of hemolysis in young and early diagnosed active pwMS with Gd-enhancing lesions indicating a wide-open BBB.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Medical University of Vienna (EC 1599/2021). Written informed consent for

participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

NK: acquisition of data, data management, statistical analysis and interpretation of data, and drafting of manuscript. GB, GK, TZ, BK, TB, FL, PR, HL, and SH: acquisition of data, interpretation of data, and critical revision of manuscript for intellectual content. AD-B: study concept and design, acquisition of data, interpretation of data, study supervision, and critical revision of manuscript for intellectual content. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2022.928582/full#supplementary-material

REFERENCES

1. Charcot JM. *Le*cons sur les maladies des vieillards et les maladies chroniques*. Paris: A. Delahaye (1868). 257 p.
2. Frischer JM, Bramow S, Dal-Bianco A, Lucchinetti CF, Rauschka H, Schmidbauer M, et al. The relation between inflammation and neurodegeneration in multiple sclerosis brains. *Brain*. (2009) 132(Pt 5):1175–89. doi: 10.1093/brain/awp070
3. Dal-Bianco A, Grabner G, Kronnerwetter C, Weber M, Hoftberger R, Berger T, et al. Slow expansion of multiple sclerosis iron rim lesions: pathology and 7 T magnetic resonance imaging. *Acta Neuropathol.* (2017) 133:25–42. doi: 10.1007/s00401-016-1636-z
4. Dal-Bianco A, Grabner G, Kronnerwetter C, Weber M, Kornek B, Kasprian G, et al. Long-term evolution of multiple sclerosis iron rim lesions in 7 T MRI. *Brain.* (2021). 144:833–47. doi: 10.1093/brain/awaa436
5. Absinta M, Sati P, Schindler M, Leibovitch EC, Ohayon J, Wu T, et al. Persistent 7-tesla phase rim predicts poor outcome in new multiple sclerosis patient lesions. *J Clin Invest.* (2016) 126:2597–609. doi: 10.1172/JCI86198
6. Bagnato F, Hametner S, Yao B, van Gelderen P, Merkle H, Cantor FK, et al. Tracking iron in multiple sclerosis: a combined imaging and histopathological study at 7 Tesla. *Brain*. (2011) 134(Pt12):3602–15. doi: 10.1093/brain/awr278
7. Kaunzner UW, Kang Y, Zhang S, Morris E, Yao Y, Pandya S, et al. Quantitative susceptibility mapping identifies inflammation in a subset of chronic multiple sclerosis lesions. *Brain.* (2019) 142:133–45. doi: 10.1093/brain/awy296
8. Mehta V, Pei W, Yang G, Li S, Swamy E, Boster A, et al. Iron is a sensitive biomarker for inflammation in multiple sclerosis lesions. *PLoS ONE.* (2013) 8:e57573. doi: 10.1371/journal.pone.0057573
9. Hametner S, Wimmer I, Haider L, Pfeifenbring S, Bruck W, Lassmann H. Iron and neurodegeneration in the multiple sclerosis brain. *Ann Neurol.* (2013) 74:848–61. doi: 10.1002/ana.23974
10. Absinta M, Dal-Bianco A. Slowly expanding lesions are a marker of progressive MS - Yes. *Mult Scler.* (2021) 27:1679–81. doi: 10.1177/13524585211013748
11. Ng Kee Kwong KC, Mollison D, Meijboom R, York EN, Kampaite A, Thrippleton MJ, et al. The prevalence of paramagnetic rim lesions in multiple sclerosis: a systematic review and meta-analysis. *PLoS ONE.* (2021) 16:e0256845. doi: 10.1371/journal.pone.0256845
12. Blindenbacher N, Brunner E, Asseyer S, Scheel M, Siebert N, Rasche L, et al. Evaluation of the 'ring sign' and the 'core sign' as a magnetic resonance imaging marker of disease activity and progression in clinically isolated syndrome and early multiple sclerosis. *Mult Scler J Exp Transl Clin.* (2020) 6:2055217320915480. doi: 10.1177/2055217320915480
13. Absinta M, Sati P, Masuzzo F, Nair G, Sethi V, Kolb H, et al. Association of chronic active multiple sclerosis lesions with disability *in vivo*. *JAMA Neurol.* (2019) 76:1474–83. doi: 10.1001/jamaneurol.2019.2399
14. Dal-Bianco A, Schranzer R, Grabner G, Lanzinger M, Kolbrink S, Pusswald G, et al. Iron rims in MS patients as neurodegenerative marker? - A 7 Tesla Magnetic Resonance Study. *Front Neurol.* (2021). doi: 10.3389/fneur.2021.632749
15. Maggi P, Kuhle J, Schadelin S, van der Meer F, Weigel M, Galbusera R, et al. Chronic white matter inflammation and serum neurofilament levels in multiple sclerosis. *Neurology.* (2021) 97:e543–53. doi: 10.1212/WNL.0000000000012326
16. Weber CE, Kramer J, Wittayer M, Gregori J, Randoll S, Weiler F, et al. Association of iron rim lesions with brain and cervical cord volume in relapsing multiple sclerosis. *Eur Radiol.* (2022) 32:2012–22. doi: 10.1007/s00330-021-08233-w
17. Absinta M, Maric D, Gharagozloo M, Garton T, Smith MD, Jin J, et al. A lymphocyte-microglia-astrocyte axis in chronic active multiple sclerosis. *Nature.* (2021) 597:709–14. doi: 10.1038/s41586-021-03892-7
18. Yao B, Hametner S, van Gelderen P, Merkle H, Chen C, Lassmann H, et al. 7 Tesla magnetic resonance imaging to detect cortical pathology in multiple sclerosis. *PLoS ONE.* (2014) 9:e108863. doi: 10.1371/journal.pone.0108863
19. Hallgren B, Sourander P. The effect of age on the non-haemin iron in the human brain. *J Neurochem.* (1958) 3:41–51. doi: 10.1111/j.1471-4159.1958.tb12607.x
20. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of mammalian iron metabolism. *Cell.* (2010) 142:24–38. doi: 10.1016/j.cell.2010.06.028
21. Wang J, Pantopoulos K. Regulation of cellular iron metabolism. *Biochem J.* (2011) 434:365–81. doi: 10.1042/BJ20101825
22. Griffiths PD, Crossman AR. Distribution of iron in the basal ganglia and neocortex in postmortem tissue in Parkinson's disease and Alzheimer's disease. *Dementia.* (1993) 4:61–5. doi: 10.1159/000107298
23. Haacke EM, Cheng NY, House MJ, Liu Q, Neelavalli J, Ogg RJ, et al. Imaging iron stores in the brain using magnetic resonance imaging. *Magn Reson Imaging.* (2005) 23:1–25. doi: 10.1016/j.mri.2004.10.001
24. Drayer B, Burger P, Darwin R, Riederer S, Herfkens R, Johnson GA, et al. MRI of brain iron. *Am J Roentgenol.* (1986) 147:103–10. doi: 10.2214/ajr.147.1.103
25. Bsteh G, Haschka D, Tymoszuk P, Berek K, Petzer V, Hegen H, et al. Serum hepcidin levels in multiple sclerosis. *Mult Scler J Exp Transl Clin.* (2019) 5:2055217319885984. doi: 10.1177/2055217319885984
26. Moos T. Brain iron homeostasis. *Dan Med Bull.* (2002) 49:279–301.
27. Singh N, Haldar S, Tripathi AK, Horback K, Wong J, Sharma D, et al. Brain iron homeostasis: from molecular mechanisms to clinical significance and therapeutic opportunities. *Antioxid Redox Signal.* (2014) 20:1324–63. doi: 10.1089/ars.2012.4931
28. David S, Jhelum P, Ryan F, Jeong SY, Kroner A. Dysregulation of iron homeostasis in the central nervous system and the role of ferroptosis in neurodegenerative disorders. *Antioxid Redox Signal.* (2022). doi: 10.1089/ars.2021.0218. [Epub ahead of print].
29. Schaer DJ, Vinchi F, Ingoglia G, Tolosano E, Buehler PW. Haptoglobin, hemopexin, and related defense pathways-basic science, clinical perspectives, and drug development. *Front Physiol.* (2014) 5:415. doi: 10.3389/fphys.2014.00415
30. Hametner S, Dal Bianco A, Trattnig S, Lassmann H. Iron related changes in MS lesions and their validity to characterize MS lesion types and dynamics with Ultra-high field magnetic resonance imaging. *Brain Pathol.* (2018) 28:743–9. doi: 10.1111/bpa.12643
31. Plum CM. Studies in multiple sclerosis. *I Acta Psychiatr Neurol Scand Suppl.* (1959) 128:1–94.
32. Caspary EA, Sewell F, Field EJ. Red blood cell fragility in multiple sclerosis. *Br Med J.* (1967) 2:610–1. doi: 10.1136/bmj.2.5552.610

33. Wimmer I, Scharler C, Kadowaki T, Hillebrand S, Scheiber-Mojdehkar B, Ueda S, et al. Iron accumulation in the choroid plexus, ependymal cells and CNS parenchyma in a rat strain with low-grade haemolysis of fragile macrocytic red blood cells. *Brain Pathol.* (2021) 31:333–45. doi: 10.1111/bpa.12920
34. Bamm VV, Henein MEL, Sproul SLJ, Lanthier DK, Harauz G. Potential role of ferric hemoglobin in MS pathogenesis: Effects of oxidative stress and extracellular methemoglobin or its degradation products on myelin components. *Free Radic Biol Med.* (2017) 112:494–503. doi: 10.1016/j.freeradbiomed.2017.08.022
35. Lewin A, Hamilton S, Witkover A, Langford P, Nicholas R, Chataway J, et al. Free serum haemoglobin is associated with brain atrophy in secondary progressive multiple sclerosis. *Wellcome Open Res.* (2016) 1:10. doi: 10.12688/wellcomeopenres.9967.2
36. Walsh AJ, Lebel RM, Eissa A, Blevins G, Catz I, Lu JQ, et al. Multiple sclerosis: validation of MR imaging for quantification and detection of iron. *Radiology.* (2013) 267:531–42. doi: 10.1148/radiol.12120863
37. Bsteh G, Hegen H, Riedl K, Altmann P, Auer M, Berek K, et al. Quantifying the risk of disease reactivation after interferon and glatiramer acetate discontinuation in multiple sclerosis: The VIAADISC score. *Eur J Neurol.* (2021) 28:1609–16. doi: 10.1111/ene.14705
38. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol.* (2011) 69:292–302. doi: 10.1002/ana.22366
39. Thompson AJ, Banwell BL, Barkhof F, Carroll WM, Coetzee T, Comi G, et al. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *Lancet Neurol.* (2018) 17:162–73. doi: 10.1016/S1474-4422(17)30470-2
40. Roxburgh RH, Seaman SR, Masterman T, Hensiek AE, Sawcer SJ, Vukusic S, et al. Multiple sclerosis severity score: using disability and disease duration to rate disease severity. *Neurology.* (2005) 64:1144–51. doi: 10.1212/01.WNL.0000156155.19270.F8
41. Filippi M, Preziosa P, Banwell BL, Barkhof F, Ciccarelli O, De Stefano N, et al. Assessment of lesions on magnetic resonance imaging in multiple sclerosis: practical guidelines. *Brain.* (2019) 142:1858–75. doi: 10.1093/brain/awz144
42. Schmitter D, Roche A, Marechal B, Ribes D, Abdulkadir A, Bach-Cuadra M, et al. An evaluation of volume-based morphometry for prediction of mild cognitive impairment and Alzheimer's disease. *Neuroimage Clin.* (2015) 7:7–17. doi: 10.1016/j.nicl.2014.11.001
43. Kwon EE, Prineas JW. Blood-brain barrier abnormalities in longstanding multiple sclerosis lesions. An immunohistochemical study. *J Neuropathol Exp Neurol.* (1994) 53:625–36. doi: 10.1097/00005072-199411000-00010
44. Wiggermann V, Hametner S, Hernandez-Torres E, Kames C, Endmayr V, Kasprian G, et al. Susceptibility-sensitive MRI of multiple sclerosis lesions and the impact of normal-appearing white matter changes. *NMR Biomed.* (2017) 30:e3727. doi: 10.1002/nbm.3727
45. Fischer MT, Wimmer I, Hoftberger R, Gerlach S, Haider L, Zrzavy T, et al. Disease-specific molecular events in cortical multiple sclerosis lesions. *Brain.* (2013) 136(Pt 6):1799–815. doi: 10.1093/brain/awt110
46. Juurlink BH, Thorburne SK, Hertz L. Peroxide-scavenging deficit underlies oligodendrocyte susceptibility to oxidative stress. *Glia.* (1998) 22:371–8. doi: 10.1002/(SICI)1098-1136(199804)22:4<371::AID-GLIA6>3.0.CO;2-6
47. Thorburne SK, Juurlink BH. Low glutathione and high iron govern the susceptibility of oligodendroglial precursors to oxidative stress. *J Neurochem.* (1996) 67:1014–22. doi: 10.1046/j.1471-4159.1996.67031014.x
48. Zinger N, Ponath G, Sweeney E, Nguyen TD, Lo CH, Diaz I, et al. Dimethyl fumarate reduces inflammation in chronic active multiple sclerosis lesions. *Neurol Neuroimmunol Neuroinflamm.* (2022) 9:1138. doi: 10.1212/NXI.0000000000001138
49. De Kleijn KMA, Martens GJM. Molecular effects of FDA-approved multiple sclerosis drugs on glial cells and neurons of the central nervous system. *Int J Mol Sci.* (2020) 21:4229 doi: 10.3390/ijms21124229

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Bridging Therapies With Injectable Immunomodulatory Drugs in the Management of Multiple Sclerosis: A Delphi Survey of an Italian Expert Panel of Neurologists

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Background: In multiple sclerosis (MS), bridging therapies are usually administered when switching from one therapy to another. Such treatments generally consist of injectable immunomodulatory drugs (interferon or glatiramer acetate), whose efficacy, safety, and tolerability data are consolidated for use even in fragile patients. We performed a nationwide survey to gather expert opinions regarding the most appropriate use of bridging therapies in MS.

Methods: An independent steering committee of Italian neurologists with expertise in MS treatment identified critical issues in the use of bridging therapies and formulated a questionnaire. This questionnaire was used to conduct a Delphi web survey, involving a panel of Italian neurologists with experience in MS treatment. Their anonymous opinions were collected in three sequential rounds. Consensus was defined as an interquartile range (IQR) ≤ 2.

Results: Responses were obtained from 38 experts (100%) in all three rounds. Injectable immunomodulatory drugs were considered first-line therapy in patients with mild-to-moderate disease activity and in women planning to become pregnant. In addition, the experts were confident about prescribing these drugs in patients at risk of cancer recurrence, while the panel agreed to discontinue any treatments in patients with uncontrolled cardiovascular or metabolic disorders. Moreover, bridging therapy with injectable immunomodulatory drugs was considered appropriate in order to protect the patient from disease reactivation when a prolonged washout was needed and also while waiting for the completion of the immunization schedule.

Conclusion: The results of this nationwide survey confirm that, among Italian neurologists, there was wide agreement on the use of bridging therapies with injectable

immunomodulatory drugs in several conditions in order to minimize the risk of disease reactivation when a prolonged washout was required or when the immunization schedule still needed to be completed in patients planning to become pregnant and in patients at risk of cancer recurrence.

Keywords: multiple sclerosis, bridging therapy, Delphi survey, MS management, injectable immunomodulatory drugs

INTRODUCTION

The term "bridging therapy" is used in medicine to indicate a transitional period to another stage of therapy or health. This concept is well-known and widely applicable in the field of transplantation (1, 2) and anticoagulant treatment (e.g., heparin bridge) (3). Therapeutic plasma exchange and intravenous immunoglobulins are examples of rapid but short-acting immunomodulatory treatments used as a bridge while waiting for slower-acting immunosuppressive therapies to become effective in other autoimmune neurologic diseases, such as myasthenia gravis (particularly when glucocorticoid use has to be avoided or minimized).

In multiple sclerosis (MS), bridging therapies may be administered when switching from one therapy to another. Such treatments generally consist of injectable immunomodulatory drugs (interferon or glatiramer acetate), whose efficacy, safety, and tolerability data are consolidated for use even in fragile patients. In the past, monthly pulses of intravenous steroids were suggested as an option to prevent reactivation of MS in subjects switching from natalizumab to alemtuzumab or in patients discontinuing fingolimod (4). Moreover, if the chosen disease-modifying treatment (DMT) could not be administered immediately, due, for example, to persistent leukopenia, a bridging therapy with corticosteroids, interferons, or glatiramer acetate was considered a valid option to fill this treatment gap.

However, while the concept of bridging therapy in MS is relatively new and still not adequately defined in terms of duration, it still might play an important role in MS decision-making strategies. In 2019, interferon labeling was updated to indicate that it could be safely used during pregnancy and breastfeeding, suggesting its potential role as a bridging treatment in female patients with MS with mild disease activity who plan on becoming pregnant in the short term (5–7).

The aim of this survey was to obtain expert opinions on the use of bridging therapies with injectables in MS from 38 Italian neurologists highly qualified in treating MS.

MATERIALS AND METHODS

An independent steering committee of seven Italian neurologists with expertise in the treatment of MS identified critical issues concerning bridging therapies and generated a 16-item questionnaire.

This questionnaire was used to conduct a Delphi web survey with an expert panel consisting of 38 neurologists from 25 Italian MS centers.

The Delphi technique is considered an effective way to gain and measure group agreement in healthcare consensus development methods (8). It is an anonymous structured approach that uses repeated administration (rounds) of the same questionnaire given to a panel of experts (8, 9). Anonymity can reduce the effects of status, personality, and group pressure that can arise in meetings and can help resolve several difficulties typically due to group decision dynamics. Questionnaire items are provided by a small group of experts, called the board, and submitted to the entire panel. During the following rounds, the administrator who manages the process, called the facilitator, provides participants with a statistical summary of the responses from all respondents from the previous round and invites the experts to provide reasons if there is no consensus of opinion (9).

Three consensus rounds were executed over nearly 5 months (from December 2019 to April 2020). All responses were aggregated to maintain respondent anonymity. Review and approval of this study by an ethics committee were not necessary since the collected data consisted of neurologist opinions. In each round, the participants were invited to respond by scaling each statement based on the degree of agreement (ranging from 1 = no agreement to 7 = maximum agreement).

The interquartile range (IQR) was used as a measure of the deviation of the individual expert's opinion from the opinion of the whole panel (median value). The IQR is the difference between the 3rd and 1st quartile in which the middle 50% of evaluations were located.

Consensus was defined as an IQR $\leq$2 and agreement with the statement when the 1st quartile was $\geq$4. For all 16 questions, the following statistical parameters were calculated: median, 1st and 3rd quartile, and IQR. Stata 16.1 was used for all analyses and graphs.

RESULTS

Responses were obtained from 38 experts (100%) in all three rounds. Between the second and third rounds, 39% and 23% of the respondents changed their responses, respectively. All statements are shown in **Table 1**.

High positive consensus was obtained for12 statements, while two statements reached a negative consensus (Items 9 and 12). In one case, the panel disagreed with the statement but did not reach a consensus (Item 11), and, in another case, there was indecision regarding the statement (Item 15; **Figures 1**, **2**, **Supplementary Figure S1**).

TABLE 1 | A Delphi questionnaire.

1. The onset of drug action plays a key role in choosing bridging treatment.
2. At diagnosis, I administer injectable immunomodulatory drugs in patients with mild-to-moderate disease activity and in women who wish to become pregnant in the short term.
3. Clinical evidence regarding the safety profile of interferon beta and glatiramer acetate during pregnancy is strong.
4. Clinical evidence regarding the safety profile of interferon beta during breastfeeding is strong.
5. I prescribe an approved immunomodulatory therapy during pregnancy.
6. I prescribe an approved immunomodulatory therapy during breastfeeding.
7. Clinical evidence regarding the safety profile of injectable immunomodulatory drugs on cancer risk is strong.
8. In patients with MS with a history of previous cancer, I prescribe an injectable immunomodulatory therapy.
9. In patients with MS with uncontrolled cardiovascular and metabolic diseases, I discontinue any treatment.
10. I perform an extended infection risk assessment at the time of diagnosis.
11. I perform an extended infection risk assessment only when switching to second-line therapies.
12. I performan extended infection risk assessment only when patients are therapy free.
13. During the infection risk assessment, it is important to prescribe injectable immunomodulatory drugs to protect the patient from disease reactivation.
14. While waiting for the immunization schedule to be completed, bridging therapy with injectable immunomodulatory drugs is appropriate.
15. I use injectable immunomodulatory drugs in patients with a not-yet-well-defined prognosis due to pending clinical or instrumental data or a short temporal window from the disease onset.
16. When switching MS treatments, I minimize the risks associated with a prolonged washout by administering bridging therapies.

The respondents stated that the time necessary for the onset of drug activity played a critical role in choosing a bridging therapy. At the time of diagnosis, injectable immunomodulatory drugs were confirmed to be the first choice in patients with mild-to-moderate disease activity and in women who were planning to become pregnant in the short term. Neurologists agreed that scientific evidence supporting the safety of interferon and glatiramer acetate administration during pregnancy was robust, although the label of glatiramer acetate suggested avoiding its use unless the benefits outweighed the risks. The neurologists also agreed that scientific evidence regarding interferon use during breastfeeding was robust. In clinical practice, they prescribed immunomodulatory treatments approved for pregnancy and breastfeeding in patients who were pregnant or breastfeeding. Moreover, all experts were confident about prescribing injectable immunomodulatory drugs in patients at risk of cancer recurrence.

The respondents stated that they discontinued any immunomodulatory treatment in patients with uncontrolled cardiovascular or metabolic disease.

There was agreement on the statement that an extensive infection risk assessment should be performed at the time of diagnosis. However, a consensus was not reached when they were asked if they actually performed this extensive assessment before switching to second-line therapies (Item 11). It was agreed that an extended infection risk assessment should be performed only in immunosuppressive drug-free patients to avoid the risk of latent infection reactivation and interference with laboratory tests.

During the evaluation of infection risk, the experts highlighted the critical issue of protecting patients from disease reactivation by administering injectable immunomodulatory drugs as a bridging therapy. This behavior was considered appropriate also while waiting for the immunization schedule to be completed.

Item 15 resulted in indecision among neurologic health professionals regarding the use of injectable immunomodulatory drugs in patients with a not-yet-well-defined prognosis due to pending clinical findings and/or instrumental assessment or a short temporal window from the disease onset.

Regarding switching from one DMT to another, the neurologists were in favor of using a bridging therapy in order to minimize the risk of disease reactivation when prolonged washout was required in individual patients. When the various items were discussed, it was clearly intended that bridging therapy duration would outlast the 8–12 weeks required for injectables to be effective (10, 11).

DISCUSSION

The objective of this Delphi analysis was to obtain consensus on the choice and most appropriate use of bridging therapy in MS. In summary, 14 statements achieved a consensus in the survey. There was positive consensus on 12 statements and negative consensus on two statements.

A rapid onset of action was confirmed to be a critical issue driving the choice of bridging treatment, and this approach may play a key role during the current pandemic period. Interferon beta does not increase the risk linked to SARS-CoV-2, and, indeed, some studies have highlighted the protective effect of this drug as indicated as a potential antiviral treatment of coronavirus-related diseases (COVID-19, MERS, and SARS) (12–17). According to literature data, Italian neurologists participating in this survey consider interferon and glatiramer acetate as first-line treatment in patients with mild-to-moderate disease activity at early stages (18). Although there are no evidence-based guidelines on decision-making in family planning, these first-line treatments are considered appropriate strategies in women with MS who desire to become pregnant in the short term (19).

Until a few years ago, clinical treatment guidelines recommended that injectables, such as interferon be discontinued at pregnancy occurrence (20, 21). However, interferons are now considered safe in pregnancy and have obtained approval for use during pregnancy in Europe (5–7). Moreover, all injectables are no longer contraindicated during breastfeeding according to the recent label updates (2019 for interferons and 2022 for glatiramer acetate). This modified prescription label now allows interferons to be recommended from conception, during the whole gestational period, and while breastfeeding (22). Therefore, a switch to interferon may be considered for female patients with MS on oral first-line DMTs that need to be discontinued due to pregnancy planning (i.e., dimethylfumarate, teriflunomide).

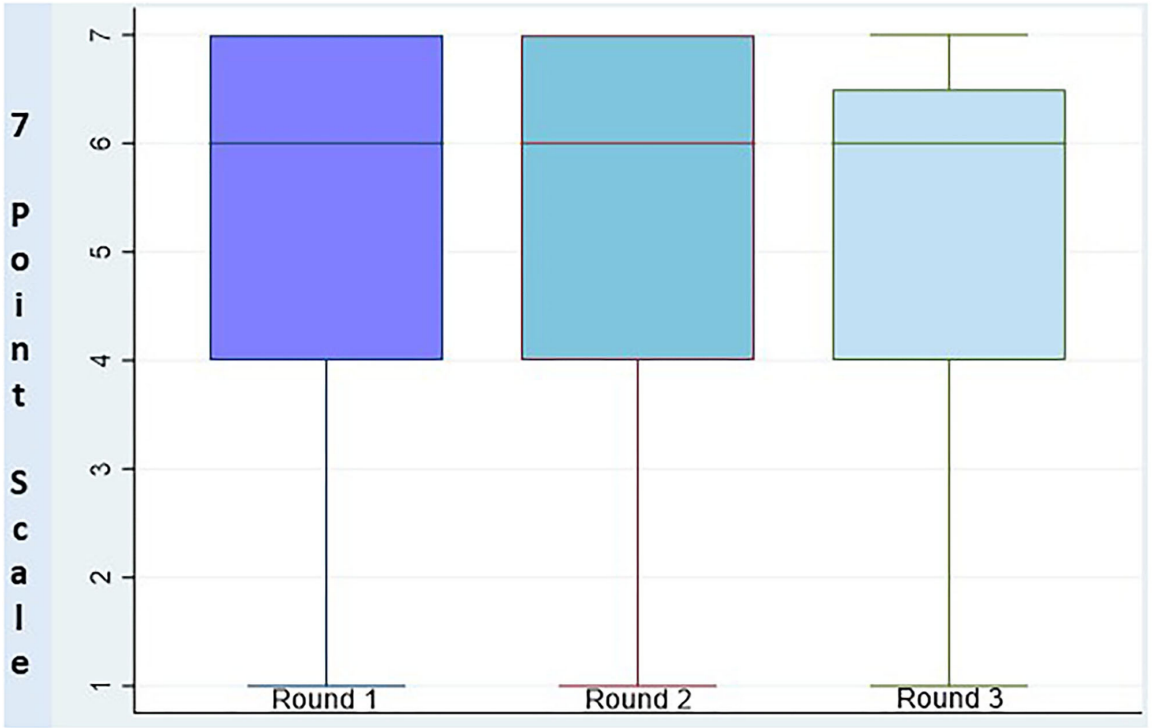

FIGURE 1 | Distribution of responses between rounds.

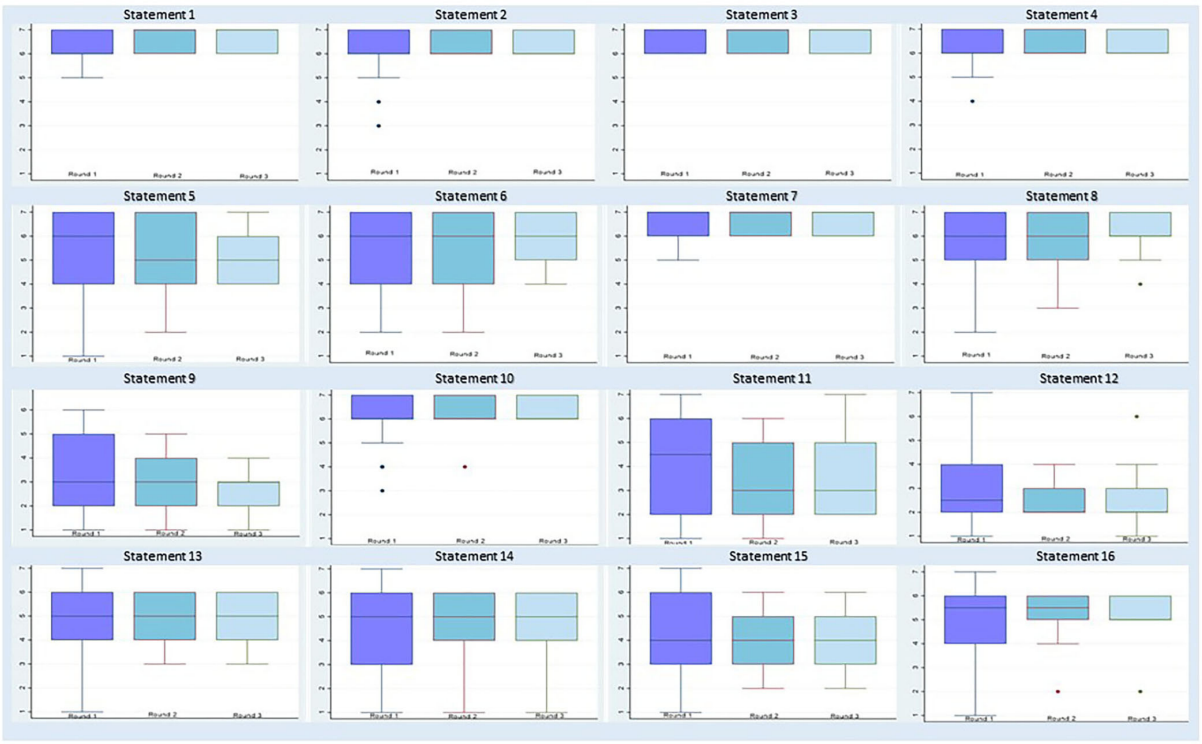

FIGURE 2 | Distribution of responses for each item per round.

In regard to currently available DMTs, several of which have immunosuppressive effects, screening patients with MS for potential malignancy risk has become crucial, especially in older patients in whom comorbidity risk is higher. Since interferon and glatiramer acetate are considered to have a favorable and well-documented safety profile and were not associated with cancer in clinical trials (23), they tend to be preferred in patients with MS with comorbidities and, in particular, in people at risk of cancer or cancer recurrence. Some disorders, including uncontrolled cardiovascular and metabolic diseases, remain a critical issue and neurologists are less confident in prescribing even injectable DMTs in these conditions due to the perceived overall benefit-risk ratio.

According to prescription label recommendations, screening for chronic infections (e.g., hepatitis B and C, tuberculosis) is required before initiating specific DMTs. Patients who test positive for latent infections must be treated before starting these drugs. In the last few years, however, an extended infection risk assessment has been widely recommended regardless of the DMT product label. To avoid possible false-negative results due to the interference of immunosuppressive drugs, this assessment should be performed in therapy-free patients. Moreover, an extensive infection risk assessment performed at the time of diagnosis in naïve patients may avoid delays in switching to a second-line treatment during the disease course and may help to identify potential subclinical comorbidities. This beneficial approach, however, is not always applied in clinical practice. In light of these considerations, prescribing a bridging therapy with injectable immunomodulatory drugs (with a slightly prevalent use of high-dosage subcutaneous interferon beta) may protect patients from disease reactivation during the evaluation of infection risk or while waiting to complete the immunization schedule, thus minimizing the risks associated with a prolonged washout. Although not detailed, it is worth noting that, for all clinical conditions considered in the Delphi panel, the time interval intended to be covered by bridging therapy outlasted the known interval required for the injectables to be active as DMTs (i.e., longer than 2–3 months).

A limitation of this study is related to the Delphi technique itself; in particular, the opinions reported are those of a select group of experts from a few Italian centers, and their approach may not be representative of Italian neurologists and clinical practice in other countries. Another limitation is related to the type of bridging drugs investigated. We specifically considered bridging with injectables and not bridging when switching from some second-line therapies to prevent rebound or bridging with natalizumab in patients on second-line DMTs in case of pregnancy desire. Thus, expert consensus is still needed regarding the unaddressed bridging of second-line DMTs. More importantly, the present study only evaluated the potential role of injectables used as bridging therapy in specific clinical conditions according to MS neurologists, but it did not address their effectiveness as bridging therapy. Nonetheless, the present Delphi study paves the way toward future clinical studies specifically designed to assess the effectiveness of injectables as bridging therapy for the various clinical conditions identified by the MS expert panel. To our knowledge, this is the first survey based on a panel of experts (neurologists) that has tried to obtain consensus on the use of bridging therapy with injectables in MS management.

CONCLUSIONS

The results of this nationwide survey confirm that Italian neurologists agree on the use of bridging therapy with injectable immunomodulatory drugs in several conditions in order to minimize the risk of disease reactivation when a prolonged washout is required or the immunization schedule still needs to be completed in patients who plan on becoming pregnant and in patients at risk of cancer recurrence.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

VP performed the statistical analysis. GM, MM, and AC wrote the first draft of the manuscript. DC, MS, CG, and EF wrote sections of the manuscript. All authors contributed to conception and design of the study, contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2022.898741/full#supplementary-material

Supplementary Figure S1 | Data for each item per round.

REFERENCES

1. Prasad MA, Kulik LM. The role of bridge therapy prior to orthotopic liver transplantation. *J Natl ComprCancNetw*. (2014) 12:1183–90. quiz 1191. doi: 10.6004/jnccn.2014.0113
2. Shigemura N, Gilbert S, Bhama JK, Crespo MM, Zaldonis D, Pilewski JM, et al. Lung transplantation after lung volume reduction surgery. *Transplantation*. (2013) 96:421–5. doi: 10.1097/TP.0b013e31829853ac
3. Baser O, Supina D, Sengupta N, Wang L. Anticoagulation bridging therapy patterns in patients undergoing total hip or total knee replacement in a US health plan: real-world observations and implications. *Am Health Drug Benefits*. (2011) 4:240−8.
4. Berger B, Baumgartner A, Rauer S, Mader I, Luetzen N, Farenkopf U, et al. Severe disease reactivation in four patients with relapsing-remitting multiple sclerosis after fingolimod cessation. *J Neuroimmunol*. (2015)282:118–22. doi: 10.1016/j.jneuroim.2015.03.022
5. European Medicines Agency. *Human Medicine European Public Assessment Report (EPAR): Rebif*. Available online at: https://www.ema.europa.eu/documents/product-information/rebif-epar-product-information_en.pdf (accessed September 21, 2021).
6. European Medicines Agency. *Human Medicine European Public Assessment Report (EPAR): Plegridy*. Available online at: https://www.ema.europa.eu/en/documents/product-information/plegridy-epar-product-information_en.pdf (accessed September 21, 2021).
7. European Medicines Agency. *Human Medicine European Public Assessment Report (EPAR): Avonex*. Available online at: https://www.ema.europa.eu/documents/product-information/avonex-epar-product-information_en.pdf (accessed September 21, 2021).
8. Murphy MK, Black NA, Lamping DL, McKee CM, Sanderson CF, Askham J, et al. Consensus development methods, and their use in clinical guideline development. *Health Technol Assess*. (1998) 2:i–iv.1–88. doi: 10.3310/hta2030
9. Hasson F, Keeney S, McKenna H. Research guidelines for the Delphi survey technique. *J Adv Nurs*. (2000) 32:1008–15. doi: 10.1046/j.1365-2648.2000.t01-1-01567.x
10. De Stefano N, Curtin F, Stubinski B, Blevins G, Drulovic J, Issard D, et al. Rapid benefits of a new formulation of subcutaneous interferon beta-1a in relapsing-remitting multiple sclerosis. *MultScler*. (2010) 16:888–92. doi: 10.1177/1352458510362442
11. Comi G, Filippi M, Wolinsky JS. European/Canadian multicenter, double-blind, randomized, placebo-controlled study of the effects of glatiramer acetate on magnetic resonance imaging–measured disease activity and burden in patients with relapsing multiple sclerosis. European/Canadian Glatiramer Acetate Study Group. *Ann Neurol*. (2001) 49:290–7. doi: 10.1002/ana.64
12. Sallard E, Lescure FX, Yazdanpanah Y, Mentre F, Peiffer-Smadja N. Type 1 interferons as a potential treatment against COVID-19. *Antiviral Res*. (2020) 178:104791. doi: 10.1016/j.antiviral.2020.104791
13. Sheahan TP, Sims AC, Leist SR, Schäfer A, Won J, Brown AJ, et al. Comparative therapeutic efficacy of remdesivir and combination lopinavir, ritonavir, and interferon beta against MERS-CoV. *Nat Commun*. (2020) 11:222. doi: 10.1038/s41467-019-13940-6
14. Mantlo E, Bukreyeva N, Maruyama J, Paessler S, Huang C. Antiviral activities of type I interferons to SARS-CoV-2 infection. *Antiviral Res*. (2020)179:104811. doi: 10.1016/j.antiviral.2020.104811
15. Hung IF, Lung KC, Tso EY, Liu R, Chung TW, Chu MY, et al. Triple combination of interferon beta-1b, lopinavir-ritonavir, and ribavirin in the treatment of patients admitted to hospital with COVID-19: an open-label, randomised, phase 2 trial. *Lancet*. (2020) 395:1695–704. doi: 10.1016/S0140-6736(20)31042-4
16. Dastan F, Nadji SA, Saffaei A, Marjani M, Moniri A, Jamaati H, et al. Subcutaneous administration of interferon beta-1a for COVID-19: a non-controlled prospective trial. *Int Immunopharmacol*. (2020) 85:106688. doi: 10.1016/j.intimp.2020.106688
17. Severa M, Farina C, Salvetti M, Coccia EM. Three decades of interferon-β in multiple sclerosis: can we repurpose this information for the management of SARS-CoV2 infection? *Front Immunol*. (2020) 11:1459. doi: 10.3389/fimmu.2020.01459
18. Melendez-Torres GJ, Armoiry X, Court R, Patterson J, Kan A, Auguste P, et al. Comparative effectiveness of beta-interferons and glatiramer acetate for relapsing-remitting multiple sclerosis: systematic review and network meta-analysis of trials including recommended dosages. *BMC Neurol*. (2018) 18:162. doi: 10.1186/s12883-018-1162-9
19. Coyle PK, Oh J, Magyari M, Oreja-Guevara C, Houtchens M. Management strategies for female patients of reproductive potential with multiple sclerosis: an evidence-based review. *MultSclerRelatDisord*. (2019) 32:54–63. doi: 10.1016/j.msard.2019.04.003
20. Sandberg-Wollheim M, Frank D, Goodwin TM, Giesser B, Lopez-Bresnahan M, Stam-Moraga M, et al. Pregnancy outcomes during treatment with interferon beta-1a in patients with multiple sclerosis. *Neurology*. (2005) 65:802–6. doi: 10.1212/01.wnl.0000168905.97207.d0
21. Boskovic R, Wide R, Wolpin J, Bauer DJ, Koren G. The reproductive effects of beta interferon therapy in pregnancy: a longitudinal cohort. *Neurology*. (2005)65:807–11. doi: 10.1212/01.wnl.0000180575.77021.c4
22. Hellwig K, Geissbuehler Y, Sabidó M, Popescu C, Adamo A, Klinger J, et al. Pregnancy outcomes in interferon-beta-exposed patients with multiple sclerosis: results from the European Interferon-beta Pregnancy Registry. *JNeurol*. (2020) 267:1715–23. doi: 10.1007/s00415-020-09762-y
23. Melamed E, Lee MW. Multiple sclerosis and cancer: the ying-yang effect of disease modifying therapies. *Front Immunol*. (2020) 10:2954. doi: 10.3389/fimmu.2019.02954

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Consensus on early detection of disease progression in patients with multiple sclerosis

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Background: Early identification of the transition from relapsing-remitting multiple sclerosis (RRMS) to secondary progressive MS (SPMS) can be challenging for clinicians, as diagnostic criteria for SPMS are primarily based on physical disability and a holistic interpretation.

Objective: To establish a consensus on patient monitoring to identify promptly disease progression and the most useful clinical and paraclinical variables for early identification of disease progression in MS.

Methods: A RAND/UCLA Appropriateness Method was used to establish the level of agreement among a panel of 15 medical experts in MS. Eighty-three items were circulated to the experts for confidential rating of the grade of agreement and recommendation. Consensus was defined when ≥66% agreement or disagreement was achieved.

Results: Consensus was reached in 72 out of 83 items (86.7%). The items addressed frequency of follow-up visits, definition of progression, identification of clinical, cognitive, and radiological assessments as variables of suspected or confirmed SPMS diagnosis, the need for more accurate assessment tools, and the use of promising molecular and imaging biomarkers to predict disease progression and/or diagnose SPMS.

Conclusion: Consensus achieved on these topics could guide neurologists to identify earlier disease progression and to plan targeted clinical and therapeutic interventions during the earliest stages of SPMS.

KEYWORDS

multiple sclerosis, early detection, secondary progressive multiple sclerosis, consensus, disease progression

Introduction

MS is a chronic, inflammatory, immune-mediated disease of the CNS characterized by demyelination and axonal degeneration (1). Most patients (∼85%) initiate with a relapsing–remitting course (RRMS) which can evolve to a secondary progressive form characterized by irreversible disability accumulation independent of relapses (SPMS) (2). Time from disease onset until conversion to SPMS varies widely among studies (3–5). A median time of 32.4 years has been recently reported (3), which is considerably higher than that observed a decade ago (21.4 years) (4), most likely due to the use of more efficacious emerging RRMS treatments.

Identifying the transition from RRMS to SPMS remains a challenge for physicians, as both phenotypes overlap as a continuum, and combined signs of early progression may present differently among patients. Diagnosis is often guided by a confirmed increase in physical disability independently of relapses, decline in cognitive functions, and the onset of persistent symptoms reported by patients. SPMS is thus frequently diagnosed retrospectively, with an estimated average 2–3-year delay between detection of the first signs of suspected progression and confirmed diagnosis of SPMS (6, 7). Several promising cerebrospinal fluid and blood plasma biomarkers have shown great potential as early markers of neurodegeneration and progression independent of relapses and are being integrated as part of the long-term patient monitoring in some specialized MS units (8).

An unequivocal definition of SPMS based on the Expanded Disability Status Scale (EDSS) and previous relapses has been proposed by Lorscheider et al. as a potential tool for timely SPMS diagnosis (7). Despite its accuracy for identifying the onset of progression (87%), the definition relies on the EDSS as a single diagnostic tool, an approach that is not free from limitations (7). Besides the EDSS, other disability-related measures, such as the Timed 25-Feet Walk Test (T25FWT) or 9-Hole Peg Test (9-HPT) significantly predicted conversion to SPMS (9, 10).

The growing knowledge of the underlying pathogenic processes involved in MS progression has led to the development of new drugs targeting SPMS patients (11). To maximize the potential therapeutic impact of such drugs, there is an imperative need to identify and treat SPMS patients in a timely manner. In response to this unmet need, an effort to develop a consensus document by a panel of 15 Spanish MS experts was undertaken.

The main purpose of this consensus is to identify early disease progression to help clinicians in detecting early signs of progression and make the most appropriate and timely therapeutic decisions in their practice. We present here the main topics of agreement on the most relevant aspects for early detection of progression identified by the panel of experts.

Materials and methods

Overview of the method of consensus

The RAND/UCLA Appropriateness Method (RAM) was used (12). The RAM is based on the Delphi method and integrates the review of scientific evidence with the opinion of experts regarding the appropriateness of a medical decision and/or intervention. The RAM has previously been applied to formalize the grade of agreement among experts on the management and diagnosis of MS patients (13, 14), and in other diseases (15, 16).

Expert panel composition

The experts were selected based on their publication record and long-term experience in specialized MS units. The panel was defined to represent the breadth of knowledge, experience, and opinions of national MS experts, covering all national territories.

The working group was divided into two subgroups: a steering committee and a rating group. The former was constituted by 3 experts who were involved in drafting the initial proposal of statements. The latter was formed by 15 experts, including the 3 members of the steering committee, and rated the pre-defined statements (henceforth the experts). The RAND/UCLA method was conducted with an experienced facilitator.

First stage: Statements definition

The steering committee drafted a list of guidance statements including the identification of clinical features [functional and

EDSS assessments [37 statements], cognitive assessments (16), additional assessments (9)], radiological characteristics (7), and biomarkers (7). An on-site meeting of the steering committee was held (25th April, 2019) to share the individual proposals and prepare the first draft of the questionnaire. After the meeting, the proposed statements were reviewed individually by the three members, resulting in the validation of an initial questionnaire with 72 guidance statements.

Second stage: Statements rating

The rating group gave feedback on each statement in a two-round process. In the first round, each statement was submitted to the rating group, who privately rated their grade of agreement on a 4-point Likert scale and the grade of recommendation using a 5-point Likert scale (Figure 1). Each member sent the ratings to a facilitator, who integrated the responses that were given in the on-site meeting (16th May, 2019). During this meeting, the rating group discussed their rating, re-rate scores, modify the original list and include new statements; a new version of the questionnaire with 83 statements was created.

In the second round, the revised version of the questionnaire was sent to the experts again, who privately re-rated all the statements and send them to the facilitator. The expert panel was convened for a last on-site meeting, where the results of the ratings for each statement from all the members were shared, the wording of the statements was refined, and the final document with the guidance statements that reached consensus was approved.

A descriptive analysis was conducted. The median value of each statement was calculated based on the numerical value of the 4 or 5 possible ratings in the 4 or 5-point Likert scale, respectively. Based on the median value, statements with a higher proportion of agreement ("Totally agree" and "Agree") were grouped vs. those with low agreement ("Totally disagree" and "In disagreement"). Consensus in favor was established when the sum of "Totally agree" and "Agree" was ≥66.6% of experts' responses. Consensus against was established when the sum of "Totally disagree" and "In disagreement" was ≥66.6% of experts' responses. A lack of consensus was considered when none of the above assumptions were met.

Likewise, statements with a higher proportion of recommendation ("Recommended" and "Essential") were grouped vs. those with low recommendation ("Not recommended" and "Depending on availability"). The response "According to clinical criteria/optional" was established as neutral. Consensus in favor was established when the sum of "Recommended" and "Essential" was ≥66.6% of experts' responses, and consensus against when the sum of "Not recommended" and "Depending on availability" was ≥66.6% of experts' responses. A lack of consensus was considered when none of the above assumptions were met. Percentages have been rounded off to whole figures.

Results

Consensus (grade of agreement) was reached in 72 out of 83 statements (86.7%). Tables 1–3 present the variables in which an isolated change: (i) allows to suspect progression (Table 1), (ii) does not allow diagnosis of progression (Table 2), and (iii)

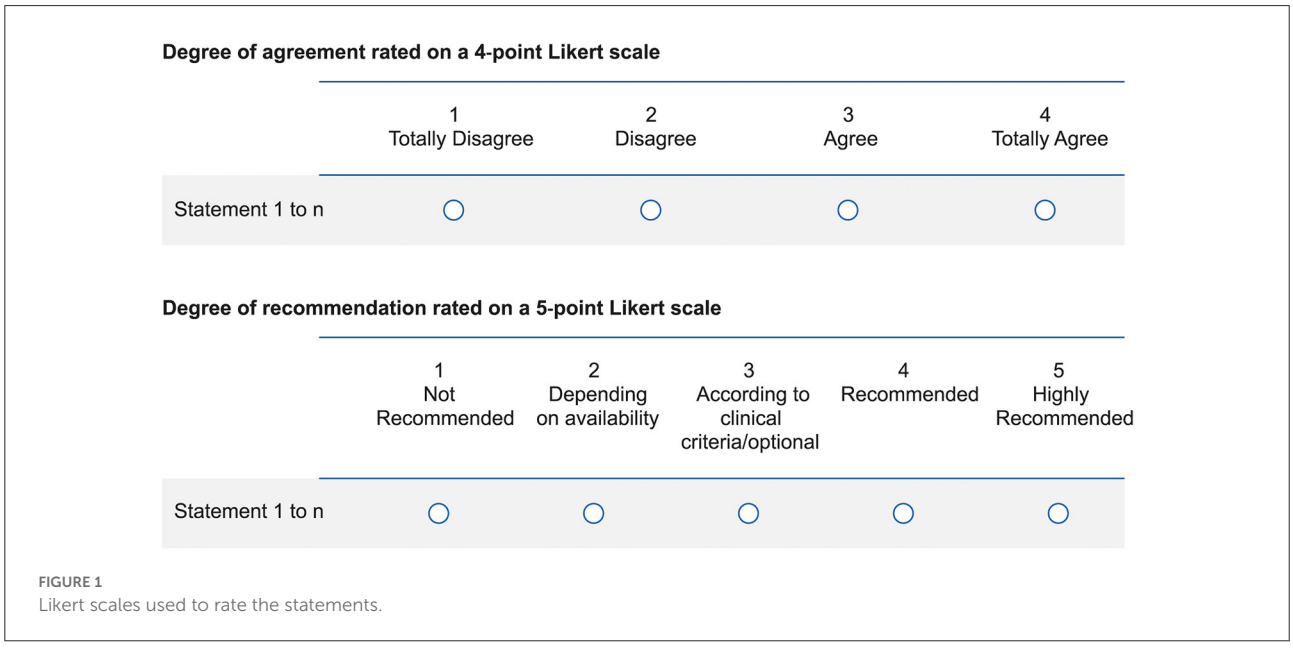

FIGURE 1
Likert scales used to rate the statements.

TABLE 1 Variables whose isolated change allows to suspect diagnosis of progression.

Statement	Consensus in favor			
	Grade of agreement		Grade of recommendation	
	(%)[a]	Median	(%)[b]	Median
A confirmed worsening of 2 points in any functional system (except the visual system)	80	Agree	87	Recommended
A confirmed worsening of 2 points in any functional system (except the visual system),				
with a disease duration <10 years	93	Agree	NC	Recommended
with a disease duration between 10 and 20 years	87	Agree	73	Recommended
with a disease duration > 20 years	73	Agree	80	Recommended
if the patient is <35 years old	87	Agree	73	Recommended
if the patient is between 35 and 45 years old	87	Agree	80	Recommended
if the patient is > 45 years old	87	Agree	80	Recommended
A confirmed 20% time increase in:				
the 25FTW	93	Agree	80	Recommended
the 9HPT	87	Agree	67	Recommended
the 25FTW and the 9HPT	100	Agree	87	Recommended
the 2MWT	87	Agree	80	Recommended
A confirmed 20% reduction in the SDMT	93	Agree	67	Recommended
A confirmed 20% worsening in at least two subtests of the BRB-N or BICAMS battery	87	Agree	80	Recommended
An isolated worsening of cognitive function	87	Agree	67	Recommended
A change in the degree of brain atrophy that is maintained and/or confirmed over time	80	Agree	71	Recommended
A change in the degree of spinal cord atrophy that is maintained and/or confirmed over time	100	Agree	87	Recommended
The presence of diffuse hyperintensity in the brain white matter or confluence of lesions	80	Agree	NC	Recommended
The presence of meningeal ectopic lymphoid follicles	67	Agree	NC	Recommended

[a]Sum of the percentages of responses obtained for "Totally agree" and "Agree." If no consensus was reached (i.e., <66%) NC is shown. [b]Sum of the percentages of responses obtained for "Recommended" and "Essential." If no consensus was reached (i.e., <66%) NC is shown.

suggests that additional assessments to diagnose progression is required (Table 3). All the statements that reached consensus are shown in Supplementary Tables S1–S7. Also, the statements that did not reach consensus are shown in Supplementary Table S8. The description in this section focuses on summarizing the percentage of experts who agreed with the statements.

Identification of progression by clinical features

Functional and EDSS assessments

Experts agreed on monitoring patients who are clinically and radiologically stable when treated with immunomodulator (93%) or immunosuppressant (73%) drugs every 6 months. In those patients with clinical and radiological instability related to the disease-modifying treatment (DMT) or with suspected disease progression, it was recommended to increase monitoring frequency to every 3 months (80%). A consensus was also reached on determining the frequency of these patients' follow-up on a case-by-case basis (>80%).

The EDSS score was considered the best variable to define progression by 93% of the experts and all agreed that based on Lorscheider et al. (7) progression could be defined as an increase in EDSS, by 1 or 0.5 points if the baseline EDSS was $\leq$ 5.5 or $\geq$ 6, respectively, considering a minimal EDSS of 4, a minimal pyramidal function of 2 and a confirmation of progression over at least 3 months. However, and regardless of the variable used for the assessment, experts agreed that the minimum time to establish the diagnosis of confirmed disability progression not associated with relapses is 6 months (87%). They also considered that a confirmed worsening of 2 points in any isolated functional system (except the visual system), even without changes in the EDSS, suggests progression (80%), regardless of disease duration [<10 years [93%], between 10–20 years [87%], > 20 years [73%]] and patient age [<35 years, between 35 and 45 years, > 45 years [87%]]. A confirmed minimum 20% increase in the performance of tests evaluating function (25FTW, 9HPT, or 2-min walk test) considered individually was rated sufficient to suspect progression (>87%) but not to confirm it (>93%). Similarly, experts agreed that if a patient experiences repeated falls, even if the EDSS or other scales remain unchanged, progression

TABLE 2 Variables whose isolated change does not allow diagnosis of progression.

Statement	Consensus in favor			
	Grade of agreement		Grade of recommendation	
	(%)[a]	Median	(%)[b]	Median
A confirmed worsening by 2 points in any functional system (except the visual system)	67	Disagree	NC	According to clinical criteria/optional
A confirmed worsening by 2 points in any functional system (except the visual system) with a disease duration <10 years	67	Disagree	NC	According to clinical criteria/optional
A confirmed 20% time increase in:				
the 25FTW	93	Disagree	93	Not recommended
the 9HPT	100	Disagree	NC	Not recommended
the 2MWT	93	Disagree	66	Not recommended
Experiencing repeated falls	93	Disagree	NC	Not recommended
A confirmed 20% reduction in the SDMT	93	Agree	67	Recommended
A confirmed 20% worsening in at least two subtests of the BRB-N or BICAMS battery	87	Agree	80	Recommended

[a]Sum of the percentages of responses obtained for "Totally disagree" and "Disagree." If no consensus was reached (i.e., <66%) NC is shown. [b]Sum of the percentages of responses obtained for "Not recommended" and "Depending on availability." If no consensus was reached (i.e., <66%) NC is shown.

TABLE 3 Variables whose isolated change indicates that more accurate progression diagnostic tools should be used.

Statement	Consensus in favor			
	Grade of agreement		Grade of recommendation	
	(%)[a]	Median	(%)[b]	Median
A confirmed reduction from 500 to 300 meters in a patient capable of wandering 500 meters or more without help or rest	100	Totally agree	93	Essential
Transition from walking independently to needing any kind of support or help to walk	100	Totally agree	100	Essential
Changes in the QoL questionnaires	80	Agree	73	Recommended
A worsening of spasticity	87	Agree	73	Recommended
A change in the degree of brain atrophy	93	Totally agree	93	Recommended
A change in the degree of spinal atrophy	93	Totally agree	93	Recommended

[a]Sum of the percentages of responses obtained for "Totally agree" and "Agree." If no consensus was reached (i.e., <66%) NC is shown. [b]Sum of the percentages of responses obtained for "Recommended" and "Essential." If no consensus was reached (i.e., <66%) NC is shown.

of disability should be suspected (100%) but not confirmed (93%). Nonetheless, when some of these variables are considered together, and a confirmed 20% increase in the 25FTW and 9HPT is accompanied by an increase in the EDSS (based on the definition described above), a diagnosis of progression can be confirmed (87%).

Cognitive assessments

Experts agreed (80%) on performing at least one annual cognitive assessment that includes the largest number of domains, such as the brief repeatable battery of neuropsychological tests (BRB-N, 93%). If applying the BRB-N is not possible, a shorter neuropsychological battery such as the brief international cognitive assessment for MS (BICAMS, 93%), or the symbol digit modalities tests (SDMT) is recommended (100%). Disease progression can be suspected by a confirmed minimum worsening of 20% in two subtests of the BRB-N or BICAMS batteries (87%), or in the SDMT (93%), but diagnosis based only on results of these tests is not recommended.

Other assessments

Experts agreed to evaluate, at least once per year, QoL (80%), depression (73%), fatigue (73%), and spasticity (74%), the latter in case of alterations in the pyramidal functional system. A full consensus was achieved on asking patients proactively and in a structured manner if they have perceived changes in their symptoms that may lead to suspect progression. Seventy-four percent of the experts agreed that changes in fatigue and depression scales rarely confirm the diagnosis of progression.

Identification of progression by radiological characteristics

A high grade of agreement was reached on suspecting disease progression based on a change in the increase of brain atrophy or spinal cord atrophy. Moreover, experts considered that detecting a change in brain or spinal cord atrophy should indicate that more accurate clinical diagnostic tools of disease progression should be used.

Identification of progression by biomarkers

Presence of ectopic meningeal lymphoid follicles, serum light-chain neurofilaments (sNfL) levels, and optical coherence tomography (OCT) measurements were rated as valid biomarkers supporting detection or suspicion of progression (73, 87, and 67%, respectively). All experts agreed that data collected from wearables and digital devices will become relevant for early identification of disease progression in the future.

Discussion

Due to the absence of standard criteria for transition identification from RRMS to a secondary progressive course, the diagnosis of SPMS is retrospective and based entirely on clinical judgment. As the reluctance to diagnose SPMS decreases with the arrival of new treatments specific for SPMS patients, consensus statements on SPMS diagnosis will be a key resource for clinicians on the complex decision-making process during this transition from RRMS to SPMS. Here, a formal consensus method was used to make feasible recommendations for a timely and more accurate identification of disease progression. The expert panel reached consensus on most of the statements and with low variation between the grade of agreement and the grade of recommendation, reflecting the robustness of statement identification.

Statements concerned relevant dimensions such as clinical, radiological and biomarkers. Experts agreed on monitoring patients every 6 months when they are clinically and radiologically stable, and to increase the frequency to every 3 months when patients are unstable or with suspected progression. These follow-ups imply a higher frequency compared to the minimum annual monitoring previously suggested (2). Nevertheless, adaptation of monitoring on a case-by-case basis was also acknowledged, indicating that the frequency should be dictated by the patient's characteristics (17).

In terms of defining SPMS, full consensus was reached on adopting the definition developed by Lorscheider et al. (7) for EDSS $\geq$ 4, which has proved to enable the diagnosis of SPMS more than 3 years earlier than the diagnosis date assigned by the physician. In Lorscheider et al., (7) reducing the time needed to confirm progression from 6 to 3 months only led to a marginal increase in sensitivity (from 88 to 89%), while decreasing specificity (92 to 86%). Based on their daily clinical practice and healthcare experience, the consensus group agreed that a higher specificity should prevail and thus 6 months was defined as the time needed to establish progression.

Using this definition, a study conducted in 15,717 patients from the MSBase registry showed that older age and longer disease duration, among other factors, were independently associated with an increased risk of SPMS (3). In line with these findings, we agreed that older age or longer disease duration together with a worsening of 2 points in any functional system—excluding the visual system—leads to suspect progression but does not allow to confirm diagnosis (18).

Indeed, no single functional assessment was considered sufficient to diagnose progression. Experts agreed that diagnosis can be confirmed when there is a minimum 20% increase in the 25FTW and the 9HPT, along with an increase in EDSS based on the definition given by Lorscheider et al. (7). This consensus concurs with previous research demonstrating that composite measures of disability progression such as the EDSS-Plus (EDSS, 9HPT and T25FW) refine the identification of disability progression in clinically definite SPMS patients (10). However, no evidence has been generated yet on the superiority of the EDSS-Plus vs. the EDSS alone to measure disability worsening in the RRMS course. The utility of using these measures in the early identification of progression proposed here should be confirmed by future research. The use of composite endpoints is essential in the clinical setting but it also needs to be considered in the design of clinical trials (19). The T25FW and 9HPT are especially suitable to assess disease progression as they do not have practice effects, which allows to assume that changes in scores are due to the patient's status rather than measurement variability (20). Regardless of the variable used, the minimum time to establish the diagnosis of confirmed progression of disability not associated with relapses was agreed to be 6 months.

The evaluation of cognitive functions, such as information processing speed (IPS) by the SDMT, together with the EDSS, probably detects more progression events as they measure different aspects of disability (21). IPS is the main cognitive domain affected by progression in MS (22) and SDMT is one of the most valid and efficient tools to detect its impairment (23). The assumption of an additive value by combining these measurements has been further supported by the absence of a strong correlation between the SDMT and EDSS (20) and by worsening on the SDMT independently from worsening on the EDSS (23). In line with this, we believe that, in addition to functional assessment, cognitive domains should be assessed at least annually in RRMS patients by a neuropsychologist or other trained healthcare professional. The assessment should include

as many domains as possible, using batteries such as the BRB-N or BICAMS, and if these batteries cannot be applied due to constraints in time and/or resources, full consensus was reached on applying at least the SDMT. However, all experts agreed on conducting a comprehensive neuropsychological study by a neuropsychologist when progression of cognitive decline is suspected. These statements concur with the recommendations by the National MS Society, which indicate using the SDMT to evaluate progression of cognitive impairment, and performing a more comprehensive assessment when significant cognitive decline is detected (24).

Fatigue, QoL, depression, and spasticity were recommended by experts to be assessed at least annually, even if changes in these measurements do not allow to diagnose progression *per se*. Detection of changes in patient-reported outcomes (PROs) may be useful to predict patients at a higher risk to progress in the near future (25). Asking patients' perception of the progression of their own disability was considered of key importance by all experts. Information from the patients' perspective and their awareness of change could contribute to the early detection of progression onset, and a systematic review of changes in patients' narrative may reveal non-obvious early signs of progression.

At present, brain and spinal cord volume measures have a limited role in MS diagnostic criteria (26) or disease course classification (2). Despite increasing studies showing promising results for the use of MRI markers to detect conversion to SPMS (27, 28), translating group-based results to the individual level is not straightforward (29). Individual cut-off values for brain and spinal cord volume changes discriminating RRMS from SPMS are not yet clearly defined, which hampers their practical application in the clinical setting. However, because global brain volume and cervical cord area are associated with and predict disability, their measurement in clinical practice have been recommended (30). Accordingly, we emphasized the relevance but also the limitations of radiological assessments by considering that detecting changes in brain or spinal cord atrophy and the presence of diffuse hyperintensity or meningeal ectopic lymphoid follicles allow suspicion—but not diagnosis—of disease progression.

Experts also agreed that evidence of potential biomarkers such as sNfL levels, meningeal ectopic lymphoid follicles, and OCT measurements is promising (31–33), and that these biomarkers, together will digital devices, will prove useful in detecting disease progression in the near future.

One limitation of the present consensus statements could be that only experts from the Spanish clinical practice participated in the study. However, consensus statements on the identification of progression by clinical and radiological features and by biomarkers are expected to be a useful resource for neurologist worldwide, who still face the challenge of identifying conversion to SPMS with limited guide and no standard criteria.

Conclusion

These consensus statements could help clinicians on the early identification of SPMS, in a context where no standard diagnostic criteria are available. Early identification of progression in MS is fundamental since it facilitates a better therapeutic management of the disease. Although by the consensus has been agreed that diagnosis of SPMS should be confirmed based only on clinical assessments, input from cognitive, PROs, imaging assessments, and systematic review of patients' perceptions of their functional status should also be considered for suspecting progression. As research in MS management continues to evolve and potential biomarkers might be validated in the near future, periodic updates of this document should be performed.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

JM-L, BC, and CO-G: conceptualization, investigation, methodology, project administration, resources, supervision, validation, visualization, and writing-review and editing. AR-A, SE, GI, CD, JR, MH, CC, JP-G, JA, DU, LC-F, and AG-M: investigation, resources, and validation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2022.931014/full#supplementary-material

Supplementary information file – Includes all the supporting files for this submission.

References

1. Reich DS, Lucchinetti CF, Calabresi PA. Multiple sclerosis. *N Engl J Med.* (2018) 378:169–80. doi: 10.1056/NEJMra1401483

2. Lublin FD, Reingold SC, Cohen JA, Cutter GR, Sorensen PS, Thompson AJ, et al. Defining the clinical course of multiple sclerosis: the 2013 revisions. *Neurology.* (2014) 83:278–86. doi: 10.1212/WNL.0000000000000560

3. Fambiatos A, Jokubaitis V, Horakova D, Kubala Havrdova E, Trojano M, Prat A, et al. Risk of secondary progressive multiple sclerosis: a longitudinal study. *Mult Scler.* (2020) 26:79–90. doi: 10.1177/1352458519868990

4. Koch M, Kingwell E, Rieckmann P, Tremlett H. The natural history of secondary progressive multiple sclerosis. *J Neurol Neurosurg Psychiatry.* (2010) 81:1039–43. doi: 10.1136/jnnp.2010.208173

5. Cree BA, Gourraud PA, Oksenberg JR, Bevan C, Crabtree-Hartman E, Gelfand JM, et al. Long-term evolution of multiple sclerosis disability in the treatment era. *Ann Neurol.* (2016) 80:499–510. doi: 10.1002/ana.24747

6. Katz Sand I, Krieger S, Farrell C, Miller AE. Diagnostic uncertainty during the transition to secondary progressive multiple sclerosis. *Mult Scler.* (2014) 20:1654–7. doi: 10.1177/1352458514521517

7. Lorscheider J, Buzzard K, Jokubaitis V, Spelman T, Havrdova E, Horakova D, et al. Defining secondary progressive multiple sclerosis. *Brain.* (2016) 139:2395–405. doi: 10.1093/brain/aww173

8. Khalil M, Teunissen CE, Otto M, Piehl F, Sormani MP, Gattringer T, et al. Neurofilaments as biomarkers in neurological disorders. *Nat Rev Neurol.* (2018) 14:577–89. doi: 10.1038/s41582-018-0058-z

9. Tomic D, Kappos L, Meier DP, Häring D, Meinert R, Giovannoni G, et al. Predictors of conversion to secondary progressive multiple sclerosis in patients with relapsing–remitting multiple sclerosis (P2.393). *Neurology.* (2018) 90:P2.393.

10. Cadavid D, Cohen JA, Freedman MS, Goldman MD, Hartung HP, Havrdova E, et al. The edss-plus, an improved endpoint for disability

progression in secondary progressive multiple sclerosis. *Mult Scler.* (2017) 23:94–105. doi: 10.1177/1352458516638941

11. Faissner S, Plemel JR, Gold R, Yong VW. Progressive multiple sclerosis: from pathophysiology to therapeutic strategies. *Nat Rev Drug Discov.* (2019) 18:905–22. doi: 10.1038/s41573-019-0035-2

12. Fitch KB, Aguilar SJ, Burnand MD, LaCalle B, Lazaro JR, van het Loo P, et al. *The Rand/Ucla Appropriateness Method User's Manual*. Santa Monica, CA: RAND Corporation (2001).

13. Stoevelaar H, Barkhof F, Berger T, Centonze D, Papeix C, Tourbah A, et al. *Application of the Rand/Ucla Method to Explore the Appropriateness of Current and Emerging Treatments for Relapsing-Remitting Multiple Sclerosis*. Boston, MA: ACTRIMS-ECTRIMS (2014).

14. Cristiano E, Rojas JI, Abad P, Adoni T, Barahona J, Becker J, et al. Consensus recommendations for the diagnosis and treatment of primary progressive multiple sclerosis in Latin America. *J Neurol Sci.* (2018) 393:4–13. doi: 10.1016/j.jns.2018.07.024

15. Sanders DB, Wolfe GI, Benatar M, Evoli A, Gilhus NE, Illa I, et al. International consensus guidance for management of myasthenia gravis. Executive summary. *Neurology.* (2016) 87:419–25. doi: 10.1212/WNL.0000000000002790

16. Weizman AV, Nguyen GC, Seow CH, Targownik L, Murthy SK, Boland K, et al. Appropriateness of biologics in the management of crohn's disease using Rand/Ucla appropriateness methodology. *Inflamm Bowel Dis.* (2018) 25:328–35. doi: 10.1093/ibd/izy333

17. Gafson A, Craner MJ, Matthews PM. Personalised medicine for multiple sclerosis care. *Mult Scler.* (2017) 23:362–9. doi: 10.1177/1352458516672017

18. Roxburgh RHSR, Seaman SR, Masterman T, Hensiek AE, Sawcer SJ, Vukusic S, et al. Multiple Sclerosis Severity Score. Using disability and disease duration to rate disease severity. *Neurology.* (2005) 64:1144–51. doi: 10.1212/01.WNL.0000156155.19270.F8

19. Chataway J. *How to Design Rcts for Spms*. Stockholm: ECTRIMS (2019).

20. Goldman MD, LaRocca NG, Rudick RA, Hudson LD, Chin PS, Francis GS, et al. Evaluation of multiple sclerosis disability outcome measures using pooled clinical trial data. *Neurology.* (2019) 93:e1921–31. doi: 10.1212/WNL.0000000000008519

21. Kappos L, Vermersch P, Cree B, Benedict R, Giovannoni G, Bar-Or A, et al. A novel functional composite endpoint to characterize disease progression in patients with secondary progressive multiple sclerosis (S12.006). *Neurology.* (2019) 92:S12.006.

22. Costa SL, Genova HM, DeLuca J, Chiaravalloti ND. Information processing speed in multiple sclerosis: past, present, and future. *Mult Scler.* (2017) 23:772–89. doi: 10.1177/1352458516645869

23. Strober L, DeLuca J, Benedict RH, Jacobs A, Cohen JA, Chiaravalloti N, et al. Symbol digit modalities test: a valid clinical trial endpoint for measuring cognition in multiple sclerosis. *Mult Scler.* (2019) 25:1781–90. doi: 10.1177/1352458518808204

24. Kalb R, Beier M, Benedict RH, Charvet L, Costello K, Feinstein A, et al. Recommendations for cognitive screening and management in multiple sclerosis care. *Mult Scler.* (2018) 24:1665–80. doi: 10.1177/1352458518803785

25. Conway D, Thompson N, Meng X, Johnson K, Fox R. *Patient Reported Outcomes and Performance Measures at Secondary Progressive Multiple Sclerosis Diagnosis.* Stockholm: ECTRIMS (2019). doi: 10.1177/1352458520936214

26. Thompson AJ, Banwell BL, Barkhof F, Carroll WM, Coetzee T, Comi G, et al. Diagnosis of multiple sclerosis: 2017 revisions of the mcdonald criteria. *Lancet Neurol.* (2018) 17:162–73. doi: 10.1016/S1474-4422(17)30470-2

27. Genovese AV, Hagemeier J, Bergsland N, Jakimovski D, Dwyer MG, Ramasamy DP, et al. Atrophied brain T2 lesion volume at Mri Is associated with disability progression and conversion to secondary progressive multiple sclerosis. *Radiology.* (2019) 293:424–33. doi: 10.1148/radiol.2019190306

28. Zivadinov R, Bergsland N, Dwyer MG. Atrophied brain lesion volume, a magnetic resonance imaging biomarker for monitoring neurodegenerative changes in multiple sclerosis. *Quant Imaging Med Surg.* (2018) 8:979–83. doi: 10.21037/qims.2018.11.01

29. Rocca MA, Battaglini M, Benedict RHB, De Stefano N, Geurts JJG, Henry RG, et al. Brain Mri atrophy quantification in ms: from methods to clinical application. *Neurology.* (2017) 88:403–13. doi: 10.1212/WNL.0000000000003542

30. Sastre-Garriga J, Pareto D, Battaglini M, Rocca MA, Ciccarelli O, Enzinger C, et al. Magnims consensus recommendations on the use of brain and spinal cord atrophy measures in clinical practice. *Nat Rev Neurol.* (2020) 16:171–82. doi: 10.1038/s41582-020-0314-x

31. Canto E, Barro C, Zhao C, Caillier SJ, Michalak Z, Bove R, et al. Association between serum neurofilament light chain levels and long-term disease course among patients with multiple sclerosis followed up for 12 years. *JAMA Neurol.* (2019) 76:1359–66. doi: 10.1001/jamaneurol.2019.2137

32. Costello F, Burton JM. Retinal imaging with optical coherence tomography: a biomarker in multiple sclerosis? *Eye Brain.* (2018) 10:47–63. doi: 10.2147/EB.S139417

33. Howell OW, Reeves CA, Nicholas R, Carassiti D, Radotra B, Gentleman SM, et al. Meningeal inflammation is widespread and linked to cortical pathology in multiple sclerosis. *Brain.* (2011) 134:2755–71. doi: 10.1093/brain/awr182

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The association between blood MxA mRNA and long-term disease activity in early multiple sclerosis

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Background: Myxovirus resistance protein A (MxA) is a protein that is upregulated by interferon-beta. Homeostatic MxA mRNA levels are potentially correlated with inflammatory disease activity in multiple sclerosis (MS) and could have an important role in MS pathology.

Aim: To investigate the association between myxovirus resistance protein A (MxA) mRNA levels in blood and disease activity and progression in MS over a long-term follow-up period.

Methods: Baseline blood MxA mRNA levels were determined in a prospective cohort of 116 untreated patients with a clinically isolated syndrome (CIS) or early relapsing remitting MS (RRMS), and related to long-term relapses, radiological disease activity, clinical scores [Expanded Disability Status Scale (EDSS), timed-25-foot walk (T25FW), 9-hole-peg test (9HPT)], MS type, and disease modifying therapy (DMT) use.

Results: Low MxA mRNA levels were associated with the occurrence of ≥9 T2-lesions on MRI imaging and the occurrence of relapses during long-term follow-up (median 11 years, IQR 5.91–13.69 years). MxA mRNA levels were not associated with EDSS, T25FW, 9HPT, and MS subtype.

Conclusion: Baseline MxA mRNA levels are associated with long-term development of T2-lesions on MRI-scans in our cohort. This confirms the relevance of the endogenous interferon-beta system in the occurrence of MS disease activity.

KEYWORDS

demyelinating disease, multiple sclerosis, MRI, myxovirus resistance protein A (MxA), mRNA

Introduction

The disease course of multiple sclerosis (MS) can vary greatly between patients. Inflammatory disease activity is an important determinant of the disease course in patients with relapsing forms of MS. It can be monitored by the occurrence of relapses and the presence of new or enlarging T2-lesions or contrast-enhancing lesions (CELs) on MRI (1).

Myxovirus resistance protein A (MxA) is one of the proteins specifically upregulated by interferon-beta. MxA mRNA levels are used in clinical practice to determine bioactivity of interferon-beta and its consequent effectiveness to suppress or prevent inflammatory disease activity in MS patients. Low MxA mRNA levels after interferon-beta injection indicate lack of treatment efficacy, caused by formation of neutralizing antibodies (NAbs) against interferon-beta (2–4). In addition to its function as a biomarker for bioactivity related to interferon-beta treatment, the association between spontaneous MxA mRNA level and inflammatory disease activity in MS has been investigated (5, 6). One hypothesis is that in patients with substantial inflammatory disease activity endogenous interferons are less effective, in which case it would be expected that low MxA mRNA levels are associated with an active disease course associated with increased inflammation (5, 6). In 2010 Van der Voort et al. investigated the association between homeostatic MxA mRNA levels and inflammatory disease activity in MS. Low blood MxA mRNA levels were associated with a higher number of CELs on baseline MRI, higher frequency of relapses and a shorter time to first relapse (7). To provide more insight into the role of the endogenous interferon-beta system in the severity of inflammatory disease activity in MS, it is of interest to investigate if this association between homeostatic MxA mRNA levels and clinical and radiological disease activity in MS is still present in the long-term. Therefore, we reevaluated this well-documented, prospective cohort to determine whether the association of homeostatic low MxA mRNA levels with inflammatory disease activity and disability is still present during long-term follow-up.

Methods

Study design and data collection

The patient cohort that was studied has been described previously (7). Patients were originally selected from a prospective cohort of patients that presented with a CIS or were diagnosed with RRMS in the 6 months before inclusion. Only patients that were not treated with disease modifying therapy (DMT) at the moment of blood collection were included. Expanded Disability Status Scale (EDSS), timed-25 foot walk test (T25FW) and 9-hole peg test (9HPT) were performed at baseline and during follow-up by experienced raters. In addition, data on MS type and DMT use over the course of the disease were collected. The average time interval between baseline and follow-up visits was 18.2 months (SD 8.1 months).

At baseline, peripheral blood was collected in PAXgene tubes, which were kept at room temperature for at least 2 h after blood collection and then frozen at −80°C. Automated RNA isolation was performed in the VU Medical Center Amsterdam on the BioRobot MDX (Qiagen) according to the instructions of the manufacturer (PaxGene Blood RNA MDx kit). MxA mRNA expression was assessed by one-step real-time quantitative RT-PCR with Taqman probes and normalized to housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression level to correct for experimental variations (7, 8). To monitor for active infections, total leukocyte counts and differentiation were also determined at the moment of blood collection. MxA mRNA expression was then measured as described previously (8), and divided into two categories based on a median split.

Brain MRI including FLAIR/T2 sequences and a 2DT1-post contrast series were obtained at baseline. Baseline scans were performed on 1.0 or 1.5 T scanners as previously described (7). Follow-up scans were performed for clinical purposes according to a standardized MRI protocol that always included FLAIR and dual-echo T2 sequences. 2DT1 post contrast sequences were obtained when necessary according to clinical guidelines. The number of new or enlarging T2-lesions during any of the follow-up moments, and the presence of contrast-enhancing lesions (CELs) in case of gadolinium administration was evaluated by neuroradiologists with extensive experience with MS and related diseases. For the analyses, the total number of new T2-lesions during the follow-up period was used and divided into two categories (<9 vs. ≥ 9 new T2-lesions). These categories were chosen because the distinction between <9 vs. ≥ 9 new T2-lesions has in the past been part of the MRI-criteria for MS diagnosis, and was for that reason well-documented in our cohort (9, 10).

Definitions

Relapses were defined as the onset of new or recurrent symptoms that last more than 24 h, that are accompanied by new objective abnormalities on a neurological examination and not explained by other non-MS causes. Radiological disease activity was defined as the presence of new T2-lesions and/or gadolinium enhanced lesions on follow-up brain MRI.

For the longitudinal analysis on clinical progression, a clinically significant change was defined as: a 20% increase on the T25FW (11), a 20% increase on the 9HPT (12), and a significant increase in EDSS was scored as a $\geq$1.5-point increase if baseline EDSS was 0, a $\geq$1-point increase if baseline EDSS was 1.0–5.0, and a $\geq$0.5-point increase if baseline score was $\geq$5.5 (13). In addition, "EDSS-plus" progression was assessed,

TABLE 1 Baseline characteristics.

Baseline characteristics	n = 116
Age at onset, y, mean (SD)	32.9 (9.1)
Age at baseline, y, mean (SD)	34.3 (9.3)
Sex, n (%) female	74 (63.8)
EDSS, median (IQR)	2.0 (1.5–3.0)
T25FW, median (IQR)	3.8 (3.4–4.2)
9HPT, median (IQR)	
Dominant hand	17.3 (15.3–19.1)
Non-dominant hand	18.3 (17.0–19.9)
MS subtype, *n* (%)	
Clinically isolated syndrome	49 (42.2)
Relapsing-remitting	67 (57.8)
MxA mRNA/GAPDH*, median (IQR)	0.08 (0.02–0.12)

SD, standard deviation; IQR, interquartile range; EDSS, Expanded Disability Status Scale; T25FW, timed 25-foot walk test; 9HPT, 9-hole peg test; CIS, clinically isolated syndrome; RRMS, relapsing-remitting MS; MxA, Myxovirus resistance protein A; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
*MxA mRNA expression was normalized to the expression level of housekeeping gene GAPDH.

TABLE 2 Follow-up characteristics.

Follow-up characteristics	n = 116
Follow-up duration	
Months, median (IQR)	132.77 (71.0–164.4)
Years, median (IQR)	11.06 (5.9–13.7)
EDSS, median (IQR)	3.0 (2.0–4.0)
T25FW, median (IQR)	4.2 (3.7–5.1)
9HPT, median (IQR)	
Dominant hand	18.9 (16.8–21.6)
Non-dominant hand	20.4 (18.0–23.3)
MS subtype, n (%)	
CIS	12 (10.3)
RRMS	93 (80.2)
SPMS	8 (6.9)
PPMS	3 (2.6)
Relapse during follow up, n (%)	
Yes	80 (69.0)
No	36 (31.0)
Radiological disease activity during follow-up, n (%)	
Yes	105 (90.5)
No	11 (9.5)

SD, standard deviation; IQR, interquartile range; EDSS, Expanded Disability Status Scale; T25FW, timed 25-foot walk test; 9HPT, 9-hole peg test; CIS, clinically isolated syndrome; RRMS, relapsing-remitting MS; SPMS, secondary progressive MS; PPMS, primary progressive MS.

defined as a significant worsening of EDSS, 9HPT and/or TWT during follow-up.

Statistical analysis

Cross-sectional analyses were conducted using Mann Whitney for categorical variables and Kruskal Wallis for continuous variables. Longitudinal analyses were conducted with linear regression for continuous outcome variables and logistic regression for dichotomous and categorical outcome variables. All analyses were corrected for sex, age at baseline and, if relevant, follow-up duration, use of DMT, and the number of T2-lesions on baseline MRI. Statistical analyses were performed using IBM SPSS Statistics version 26.0.

Results

Baseline

In total, 116 patients were included in the analyses between November 2002 and March 2007. As previously described (7), 74 (63.8%) were female. At baseline, 67 patients (57.8%) were diagnosed with RRMS and 49 (42.2%) with a CIS, according to the standard diagnostic criteria at that time (14, 15). Median MxA mRNA level was 0.075 (IQR 0.015–0.123). Median follow-up duration was 11.05 years (IQR 5.91–13.69 years). Baseline characteristics are depicted in Table 1.

All patients were treatment naive at the moment of blood collection. At the moment of blood sampling, 23 patients were experiencing a clinical relapse, whereas in other patients, blood sampling was done during remission. Previous analysis showed that MxA mRNA levels were lower in patients with a relapse at the time of blood sampling [median 0.036 (IQR 0.009–0.075)], compared to those in remission [median 0.084 (IQR 0.021–0.145)] ($p = 0.002$) (7). To exclude possible bias caused by the timing of blood collection, all analyses were done for the complete patient population (116 patients) as well as for the subgroup of patients in which blood collection was done at remission (93 patients). None of the patients reported any viral infections at the moment of blood sampling, and leukocyte counts and differentiation were normal (7).

Follow-up

At the end of the follow-up period, 93 patients (80.2%) had a diagnosis of RRMS, 12 (10.3%) of CIS, 8 (6.9%) of secondary progressive MS (SPMS) and 3 (2.6%) of primary progressive MS (PPMS). Thirty-two patients (27.6%) converted from CIS to RRMS during follow-up. No significant association was found between baseline MxA mRNA level and MS type at follow-up, or between baseline MxA mRNA and conversion from CIS to RRMS during follow-up. Follow-up characteristics are depicted in Table 2.

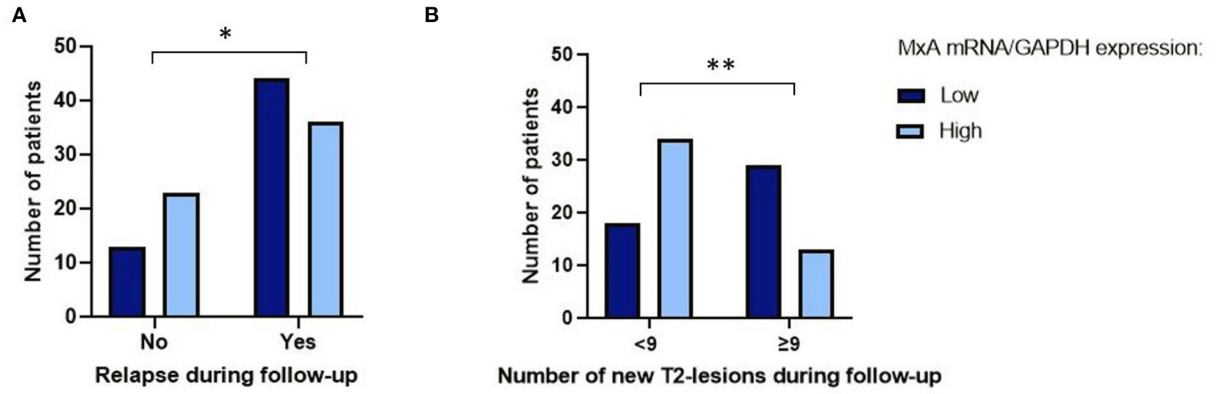

FIGURE 1
Baseline MxA mRNA level vs. occurrence of relapses and new T2-lesions on MRI during follow-up. **(A)** Number of patients with low vs. high MxA mRNA/GAPDH expression that experience at least one relapse during follow-up. *Results of logistic regression analysis: B = −0.81, Exp(B) = 0.45, $p = 0.070$, 95% CI 0.19–1.07. **(B)** Number of patients with low vs. high MxA mRNA/GAPDH expression that have <9 vs. ≥9 new T2-lesions on MRI during follow-up. **Results of logistic regression analysis: B = −1.86, Exp(B) = 0.16, $p = 0.012$, 95% CI 0.04–0.66. All patients included. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Relapses

Eighty patients experienced at least one relapse during follow-up. Median number of relapses during follow-up was 1 (IQR 0–3). A baseline MxA mRNA level of <0.075 was associated with the occurrence of at least one relapse during follow-up, although not statistically significant in the complete patient group [B = −0.81, Exp(B) = 0.45, $p = 0.070$, 95% CI 0.19–1.07] (see also Figure 1). When repeated in only the patients that were in remission during blood collection, this effect was statistically significant [B = −1.18, Exp(B) = 0.31, $p = 0.025$, 95%CI 0.11–0.86]. In addition, low baseline MxA mRNA level (<0.075) was associated with a higher number of relapses during follow-up [complete patient group: U = 1295, Z = −2.18, $p = 0.029$, remission group only: U = 831, Z = −1.83, $p = 0.068$]. No significant association was found between baseline MxA mRNA level and time to first relapse.

Clinical scores

Median EDSS at follow-up was 3.0 (IQR 2.0–4.0). During follow-up, 47.7% of patients experienced a significant increase in EDSS. No significant association was found between baseline MxA mRNA level and the occurrence of a significant increase in EDSS during follow-up [B = −0.16, Exp(B) = 0.85, $p = 0.678$, 95% CI 0.40–1.82]. In addition, no significant association was found between baseline MxA mRNA level and the occurrence of a significant increase in T25FW [B = 0.50, Exp(B) = 1.65, $p = 0.241$, 95 CI 0.72–3.79] or 9HPT score [dominant hand: B = 0.01, Exp(B) = 1.0, $p = 0.998$, 95% CI 0.05–20.15, non-dominant hand: B = 0.12, Exp(B) = 1.13, $p = 0.929$, 95% CI 0.09–14.94] during follow-up. Also, no significant association was found between baseline MxA mRNA level and "EDSS-plus" worsening during follow-up [B = 0.02, Exp (B) = 1.02, $p = 0.965$, 95% CI 0.47–2.19]. All analyses were repeated in the remission group only, which also showed no significant association between these clinical scores and baseline MxA mRNA level.

DMT use

Sixty-three patients (54.3%) started using DMT at any moment during follow-up. Fifty-two patients (44.8%) did not; of one patient this was unknown. At the end of the follow-up period, 42 patients (33.6%) used first-line DMT (any of the interferons, glatiramer acetate, teriflunomide, dimethylfumarate) and 16 patients (13.8%) used second-line DMT (natalizumab, fingolimod, ocrelizumab). Baseline MxA mRNA level was not associated with the start of DMT during follow-up, with the type of DMT used at follow-up, or with the use of first line or second line DMT during follow-up.

MRI parameters

Median number of MRI-scans done during 11 years of follow-up was 6 per patient (IQR 4–10). Ninety percent of patients experienced MRI-activity during follow-up, meaning any new T2-lesions or CELs on any MRI scan available during follow-up. A baseline MxA mRNA level of <0.075 was associated with a cumulative number of ≥9 new T2-lesions during follow-up, compared to <9 new T2-lesions during follow-up. This association was present when including all patients in the analysis [B = −1.86, Exp(B) = 0.16, $p = 0.012$, 95% CI 0.04–0.66] (see also Figure 1). In the remission group, the same effect was seen, although not statistically significant [B = −1.51, Exp(B) = 0.22, $p = 0.079$, 95% CI 0.04–1.19]. No

significant association was found between baseline MxA mRNA level and time to first MRI activity.

Discussion

In our prospective cohort of CIS and early RRMS patients we found low spontaneous MxA mRNA levels in the first months after diagnosis to be associated with the occurrence of a larger number of new T2 lesions during a median follow-up period of 11 years. Low baseline MxA mRNA levels were also associated with the occurrence, and a higher number of, relapses during follow-up. No significant association was found between spontaneous MxA mRNA level and clinical scores (EDSS, T25FW, 9HPT) and MS type at follow-up.

MxA mRNA and MxA protein are present at stable low levels in blood under normal circumstances, and are rapidly upregulated by interferon type I in a dose-dependent manner. They are therefore known as reliable markers for interferon type I responsiveness (16). Endogenous interferon type I pathways play various important roles in the human immune system. For example, a dysregulation of type I interferon pathways is found in various chronic inflammatory autoimmune diseases, such as Sjögren's syndrome and systemic lupus erythematosus (SLE) (17–19).

In MS, an increased activation of type I interferon pathways has been associated with an upregulation of immunoregulatory cytokines and reduced T-cell responses, which might be associated with a dampening of inflammatory disease activity (20–22). Therefore, it is conceivable that in patients with a more active inflammatory disease course, characterized by the occurrence of (more) relapses and new T2-lesions and/or enhancing lesions on MRI, endogenous interferon-beta pathways are insufficiently capable to upregulate and protect against inflammatory activity, as reflected by low blood levels of MxA mRNA in these patients.

Interesting in this regard is the growing evidence of the role of viral infections in the pathogenesis of MS. One of the main functions of type I interferon system is its antiviral function, and people with a defective interferon type I system are likely more susceptible to viral infections or a more severe course of infectious disease (23, 24). Regarding MxA, it is known that MxA proteins, amongst many other proteins, also possess antiviral properties: in case of certain viral infections, such as influenza and measles, MxA mRNA and MxA protein are rapidly upregulated by endogenous interferon type I and play a role in the inhibition of multiplication of these viruses (16, 25). An impairment in interferon type I pathways may lead to an impaired upregulation of MxA mRNA, resulting in lower MxA mRNA and protein levels.

Multiple studies have shown a potential triggering effect of viral infections in the development of MS, especially Epstein-Bar virus (EBV) infections, of which increasing evidence is found for a major causal role in MS pathogenesis (26, 27). The altered interferon type I system that is found in MS could possibly be related to this phenomenon, considering the importance of the type I interferon system in viral immunity, such as immunity against EBV infections (20–22, 28, 29). This alteration of the interferon type I system might be reflected by a change in MxA mRNA level.

In 2010, we described the association between spontaneous MxA mRNA levels and disease activity with a median follow-up period of 44 months (8). There was a lower relapse rate and a longer time to new relapses in patients with high spontaneous MxA mRNA levels. Even though there was no significant association between MxA mRNA level and annualized number of new T2-lesions or the occurrence of CELs during follow-up, the proportion of patients with no or low number of T2-lesions after a follow-up period of 1 year was higher in patients with a high spontaneous MxA mRNA level at baseline. The current study extends these findings by confirming the association between MxA mRNA levels and clinical and radiological disease activity over a follow-up period of 11 years.

It must be noted that this is an observational study in a real-world setting. The decisions on follow-up and treatment were made based on the clinical treatment protocols at that moment. At the time of treatment initiation, interferon and glatiramer acetate were the only available treatment options in CIS or early MS. During the period of follow-up, higher efficacy compounds were admitted to the market (e.g., natalizumab, fingolimod, ocrelizumab), resulting in a change in the treatment landscape of MS. Additionally, not all MRI-scans were made with gadolinium contrast administered during follow-up.

Despite these limitations, it would be of interest to validate the results found in this study in a larger patient group. Our study suggests that an impairment in the type I interferon system could play an important role in inflammatory MS pathology, as reflected by low MxA mRNA levels, which are associated with long-term inflammatory disease activity in our study. In addition to providing more insight into the mechanisms of inflammatory disease activity in MS, homeostatic MxA mRNA level could also be of interest as an easy-to-use prognostic biomarker for long-term inflammatory disease activity in MS. Currently, the best prognostic factors for long-term disease activity are MRI measurements. Number of active lesions on brain MRI and spinal cord lesions on MRI are known to be associated with disability in MS. In addition to using MRI, the development of easy-to-use biomarkers that predict inflammatory disease activity would greatly benefit clinical decision making regarding treatment of MS patients and improve personalized patient care. For example, if the occurrence of long-term inflammatory disease activity can be predicted easily and precisely, treatment decisions can be adjusted based on this knowledge, such as the decision when to start with DMT, and which DMT should be started.

In conclusion, our long-term clinical and radiological follow-up data suggest an important mechanistic effect of the endogenous type-1 interferon system reflected in MxA mRNA in the expression of inflammatory pathology of MS. If confirmed in other populations, MxA mRNA could also be an interesting candidate as prognostic biomarker for long-term inflammatory disease activity in MS.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Medical Ethical Committee of the Amsterdam UMC, location VUmc. The patients/participants provided their written informed consent to participate in this study.

Author contributions

EC, ES, and JK contributed to conception and design of the study, analysis and interpretation of data, and drafted the manuscript. LP, ZV, BU, FB, CO, BU, and CT contributed to the interpretation of data and revision of the manuscript for intellectual content. All authors contributed to the article and approved the submitted version.

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Conflict of interest

LP received funding for courses from Novartis and Biogen. FB acts as a consultant to Biogen-Idec, Janssen Alzheimer Immunotherapy, Bayer-Schering, Merck-Serono, Roche, Novartis, Genzyme, and Sanofi-aventis. BU reports personal fees from Genzyme, Biogen Idec, Teva Pharmaceutical Industries, Merck Serono, and Roche. CT has served on advisory boards for Roche, has received non-financial support in the form of research consumables from ADx NeuroSciences and Euroimmun, and has performed contract research or received grants from Probiodrug, Biogen, Esai, Toyama, Janssen Prevention Center, Boehringer, Axon Neuroscience, EIP Pharma, PeopleBio, and Roche. JK has accepted speaker and consulting fees from Merck, Biogen, Roche, Teva, Genzyme, and Novartis.

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References

1. Lublin FD, Reingold SC, Cohen JA, Cutter GR, Sorensen PS, Thompson AJ, et al. Defining the clinical course of multiple sclerosis: the 2013 revisions. *Neurology.* (2014) 83:278–86. doi: 10.1212/WNL.0000000000000560

2. Deisenhammer F, Reindl M, Harvey J, Gasse T, Dilitz E, Berger T. Bioavailability of interferon beta 1b in MS patients with and without neutralizing antibodies. *Neurology.* (1999) 52:1239–43. doi: 10.1212/WNL.52.6.1239

3. Vallittu AM, Halminen M, Peltoniemi J, Ilonen J, Julkunen I, Salmi A, et al. Neutralizing antibodies reduce MxA protein induction in interferon-beta-1a-treated MS patients. *Neurology.* (2002) 58:1786–90. doi: 10.1212/WNL.58.12.1786

4. Malucchi S, Gilli F, Caldano M, Marnetto F, Valentino P, Granieri L, et al. Predictive markers for response to interferon therapy in patients with multiple sclerosis. *Neurology.* (2008) 70:1119–27. doi: 10.1212/01.wnl.0000304040.29080.7b

5. Tao Y, Zhang X, Chopra M, Kim MJ, Buch KR, Kong D, et al. The role of endogenous IFN-β in the regulation of Th17 responses in patients with relapsing-remitting multiple sclerosis. *J Immunol.* (2014) 192:5610–7. doi: 10.4049/jimmunol.1302580

6. Al-Masri AN, Heidenreich F, Walter GF. Interferon-induced Mx proteins in brain tissue of multiple sclerosis patients. *Eur J Neurol.* (2009) 16:721–6. doi: 10.1111/j.1468-1331.2009.02573.x

7. van der Voort LF, Vennegoor A, Visser A, Knol DL, Uitdehaag BM, Barkhof F, et al. Spontaneous MxA mRNA level predicts relapses in patients with recently diagnosed MS. *Neurology.* (2010) 14:1228–33. doi: 10.1212/WNL.0b013e3181f6c556

8. van der Voort LF, Kok A, Visser A, Oudejans CB, Caldano M, Gilli F, et al. Interferon-beta bioactivity measurement in multiple sclerosis: feasibility for routine clinical practice. *Mult Scler.* (2009) 15:212–8. doi: 10.1177/1352458508096877

9. Barkhof F, Filippi M, Miller DH, Scheltens P, Campi A, Polman CH, et al. Comparison of MRI criteria at first presentation to predict conversion to clinically definite multiple sclerosis. *Brain.* (1997) 120 (Pt 11):2059–69. doi: 10.1093/brain/120.11.2059

10. McDonald WI, Compston A, Edan G, Goodkin D, Hartung HP, Lublin FD, et al. Recommended diagnostic criteria for multiple sclerosis: guidelines from the

International Panel on the diagnosis of multiple sclerosis. *Ann Neurol.* (2001) 50:121–7. doi: 10.1002/ana.1032

11. Motl RW, Cohen JA, Benedict R, Phillips G, LaRocca N, Hudson LD, et al. Validity of the timed 25-foot walk as an ambulatory performance outcome measure for multiple sclerosis. *Mult Scler.* (2017) 23:704–10. doi: 10.1177/1352458517690823

12. Feys P, Lamers I, Francis G, Benedict R, Phillips G, LaRocca N, et al. The Nine-Hole Peg Test as a manual dexterity performance measure for multiple sclerosis. *Mult Scler.* (2017) 23:711–20. doi: 10.1177/1352458517690824

13. Uitdehaag BMJ. Disability outcome measures in phase III clinical trials in multiple sclerosis. *CNS Drugs.* (2018) 32:543–58. doi: 10.1007/s40263-018-0530-8

14. Poser CM, Paty DW, Scheinberg L, McDonald WI, Davis FA, Ebers GC, et al. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann Neurol.* (1983) 13:227–31.

15. Polman CH, Reingold SC, Edan G, Filippi M, Hartung HP, Kappos L, et al. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". *Ann Neurol.* (2005) 58:840–6. doi: 10.1002/ana.20703

16. Ronni T, Melén K, Malygin A, Julkunen I. Control of IFN-inducible MxA gene expression in human cells. *J Immunol.* (1993) 150:1715–26.

17. Wildenberg ME, van Helden-Meeuwsen CG, van de Merwe JP, Drexhage HA, Versnel MA. Systemic increase in type I interferon activity in Sjögren's syndrome: a putative role for plasmacytoid dendritic cells. *Eur J Immunol.* (2008) 38:2024–33. doi: 10.1002/eji.200738008

18. Lee PY, Kumagai Y, Li Y, Takeuchi O, Yoshida H, Weinstein J, et al. TLR7-dependent and FcgammaR-independent production of type I interferon in experimental mouse lupus. *J Exp Med.* (2008) 205:2995–3006. doi: 10.1084/jem.20080462

19. Fava A, Petri M. Systemic lupus erythematosus: diagnosis and clinical management. *J Autoimmun.* (2019) 96:1–13. doi: 10.1016/j.jaut.2018.11.001

20. Börnsen L, Romme Christensen J, Ratzer R, Hedegaard C, Søndergaard HB, Krakauer M, et al. Endogenous interferon-β-inducible gene expression and interferon-β-treatment are associated with reduced T cell responses to myelin basic protein in multiple sclerosis. *PLoS ONE.* (2015) 10:e0118830. doi: 10.1371/journal.pone.0118830

21. Hesse D, Krakauer M, Lund H, Søndergaard HB, Limborg SJW, Soelberg Sørensen P, et al. Disease protection and interleukin-10 induction by endogenous interferon-β in multiple sclerosis? *Eur J Neurol.* (2011) 18:266–72. doi: 10.1111/j.1468-1331.2010.03116.x

22. Deczkowska A, Baruch K, Schwartz M. Type I/II interferon balance in the regulation of brain physiology and pathology. *Trends Immunol.* (2016) 37:181–92. doi: 10.1016/j.it.2016.01.006

23. Samuel CE. Antiviral actions of interferons. *Clin Microbiol Rev.* (2001) 14:778–809, table of contents. doi: 10.1128/CMR.14.4.778-809.2001

24. Ivashkiv LB, Donlin LT. Regulation of type I interferon responses. *Nat Rev Immunol.* (2014) 14:36–49. doi: 10.1038/nri3581

25. Schnorr JJ, Schneider-Schaulies S, Simon-Jödicke A, Pavlovic J, Horisberger MA, ter Meulen V. MxA-dependent inhibition of measles virus glycoprotein synthesis in a stably transfected human monocytic cell line. *J Virol.* (1993) 67:4760–8.

26. Tarlinton RE, Martynova E, Rizvanov AA, Khaiboullina S, Verma S. Role of viruses in the pathogenesis of multiple sclerosis. *Viruses.* (2020) 12:643. doi: 10.3390/v12060643

27. Bjornevik K, Cortese M, Healy BC, Kuhle J, Mina MJ, Leng Y, et al. Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. *Science.* (2022) 375:296–301. doi: 10.1126/science.abj8222

28. Rafiee N, Ravanshad M, Asadi B, Kianfar R, Maleki A. Investigation of IL-2 and IFN-γ to EBV peptides in stimulated whole blood among multiple sclerosis patients and healthy individuals. *Intervirology.* (2021) 64:203–8. doi: 10.1159/000517002

29. Liu X, Sadaoka T, Krogmann T, Cohen JI. Epstein-Barr Virus (EBV) tegument protein BGLF2 suppresses type I interferon signaling to promote EBV reactivation. *J Virol.* (2020) 94:e00258–20. doi: 10.1128/JVI.00258-20

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Impact of histone modifier-induced protection against autoimmune encephalomyelitis on multiple sclerosis treatment

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Multiple sclerosis is a progressive demyelinating central nervous system disorder with unknown etiology. The condition has heterogeneous presentations, including relapsing-remitting multiple sclerosis and secondary and primary progressive multiple sclerosis. The genetic and epigenetic mechanisms underlying these various forms of multiple sclerosis remain elusive. Many disease-modifying therapies approved for multiple sclerosis are broad-spectrum immunomodulatory drugs that reduce relapses but do not halt the disease progression or neuroaxonal damage. Some are also associated with many severe side effects, including fatalities. Improvements in disease-modifying treatments especially for primary progressive multiple sclerosis remain an unmet need. Several experimental animal models are available to decipher the mechanisms involved in multiple sclerosis. These models help us decipher the advantages and limitations of novel disease-modifying therapies for multiple sclerosis.

KEYWORDS

central nervous system, epigenetics, experimental autoimmune encephalomyelitis, histone deacetylases, multiple sclerosis, myelin oligodendrocyte glycoprotein, T helper cells, tolerance

Introduction

Clinical manifestations of multiple sclerosis

More than 2.8 million people live with multiple sclerosis (MS) worldwide, and the prevalence has been increasing (1). The mean age of diagnosis of MS is 32 years, with twice the number of female patients compared with male patients afflicted with this disease. However, the basis of sexual dimorphism in MS manifestation remains elusive, as in other autoimmune diseases. MS is a prototypical organ-specific autoimmune disease of the central nervous system (CNS), affecting the brain and spinal cord (2–4). Most (85%) patients with MS manifest relapsing-remitting MS (RRMS), characterized by alternate periods of relapses and remissions for decades after an initial episode of neurological dysfunction, clinically isolated syndrome. Relapses accompany

CNS inflammation and demyelination detectable as white matter lesions by magnetic resonance imaging. Accumulating disabilities during relapses in most (80%) patients with MS leads to secondary progressive MS (SPMS), characterized by decreased brain volume and increased axonal loss without associated inflammatory lesions. A minor fraction (10%) of patients with MS continue to decline progressively from the beginning of diagnosis without relapses. Variations of MS include progressive-relapsing and pediatric disease and severe Marburg variant. The hallmark of MS is sharply demarcated demyelinating plaque with axons relatively preserved, whereas in neuromyelitis optica (MNO), both axons and myelin are involved, resulting in necrotic cavitation. Severe involvement of optic nerves and the spinal cord is a characteristic of the opticospinal MS (OSMS) subtype, which is more prevalent in African Americans (5, 6). Compared with Whites, African Americans had an older age at onset, experienced greater disability, progressed faster, had increased risk for SPMS, experienced transverse myelitis more often, and were likely to have motor symptoms and the OSMS subtype. The classic multifocal MS is rare in Japanese, who manifest OSMS with features similar to those of the relapsing form of NMO in Western populations, and was proposed to be the same as the NMO disorder, rather than a form of MS (7). However, in Brazilian patients, OSMS is recognized as a milder MS phenotype distinct from NMO (8). While antibody-dependent aquaporin four loss occurred in some patients with NMO, antibody-independent astrocytopathy was found in several demyelinating conditions, including Baló's disease, NMO, and MS (9). In addition to these complexities, MS is also rare among Samis, Turkmen, Uzbeks, Kazakhs, Kyrgyzis, native Siberians, North and South Amerindians, Chinese, Japanese, African blacks, and New Zealand Maoris, in contrast to a high propensity of Sardinians, Parsis, and Palestinians to develop MS (10). The different susceptibilities of distinct racial and ethnic groups are essential determinants of the uneven geographic distribution of MS.

The clinical manifestations of MS include temporary vision loss, sensory and motor problems, fatigue, impaired bowel and sexual functions, cognitive deficits, and paralysis (2–4). Distinct forms of MS appear to correlate with the spatiotemporal dissemination of lesional sites within the CNS (2–4, 11). The hallmarks of MS pathology include the breakdown of the blood–brain barrier, accumulation of immune cell infiltrates, oligodendrocyte loss, demyelination, astrogliosis, axonal degeneration, and disruption of neuronal signaling (Figure 1). Substantial T-cell infiltration occurs in patients with acute and relapsing disease but is spared during later stages of MS, despite an unabated neuronal disability. Intrinsic neuronal deficits such as those associated with Alzheimer's disease are thought to play a role, especially during the advanced stage of MS (11).

Genetics of MS

Although the etiology of MS remains elusive, genes within the human leukocyte antigenic (HLA) loci, such as *HLA-A*02:01*, *HLA-DRB1*15:01*, *HLA-DRB5*, *HLA-C*, and *TNF*, have been firmly associated with MS susceptibility (12). In African Americans, classic/multifocal MS is associated with *DRB1*15* alleles, whereas OSMS is not (5). Not only the *DRB*1501* allele but also the extended *DRB1*1501-DQB1*0602* haplotype is commonly found in northern Europeans with MS (5). The *HLA-DPB1*0501* haplotype is not uniquely associated with the OSMS subtype, which is relatively more common in Japan (13). Interleukin-2 (IL-2) and its receptor IL-2R play a crucial role in MS and are also crucial for T-cell tolerance (14). In addition, the soluble form of the IL-2R (sIL-2R) plays a role in MS. IL-7 and IL-7Rα form a non-redundant ligand–receptor system and plays a critical role in T-cell activation. Peripheral blood mononuclear cells of patients with MS display deletion of exon 4 of the IL-7 transcript and splice variants lacking exons 5, 6, and 7 (15). A closer analysis of the impact of these genetic variations is necessary for a better understanding of MS pathogenesis.

The pivotal role of T helper cells in MS

Cerebrospinal fluid (CSF)-infiltrating $CD4^+$ T cells of patients with MS proliferated and secreted interferon- γ (IFN-γ), a characteristic of the Th1 subset, but not IL-17 when challenged with the myelin oligodendrocyte glycoprotein 35–55 (MOG_{35-55}) peptide *in vitro* (16), a proposed candidate CNS determinant in MS (17). However, others reported the abundance of IL-17-expressing Th17 cells in the peripheral blood, CSF, and brain lesions of patients with MS, which increased during relapses (18). Increasing evidence also indicates a role of central memory Th17.1 (Th1/17) cells, which share the hallmarks of Th1 and Th17 cells, respectively, in IFN- γ and IL-17 production, in MS (18). In addition to Th17 cells, follicular helper T cells that promote the germinal center formation, B-cell differentiation, and antibody production are also implicated in several autoimmune diseases, including MS (19). The intrathecal inflammatory environment in patients with RRMS promotes the recruitment of peripheral follicular helper T cells to the CNS without increasing their ability to migrate (20). Since the follicular helper T cells failed to transfer demyelinating disease in mice (21), it is unlikely that they have pathological consequences in patients with MS. The role of follicular T helper cells in MS remains to be proven. Although MHC class I-restricted $CD8^+$ cells were found in the brain lesions of patients with MS, they were also present in patients with infections and other brain diseases, providing inconclusive evidence for their involvement in MS (22).

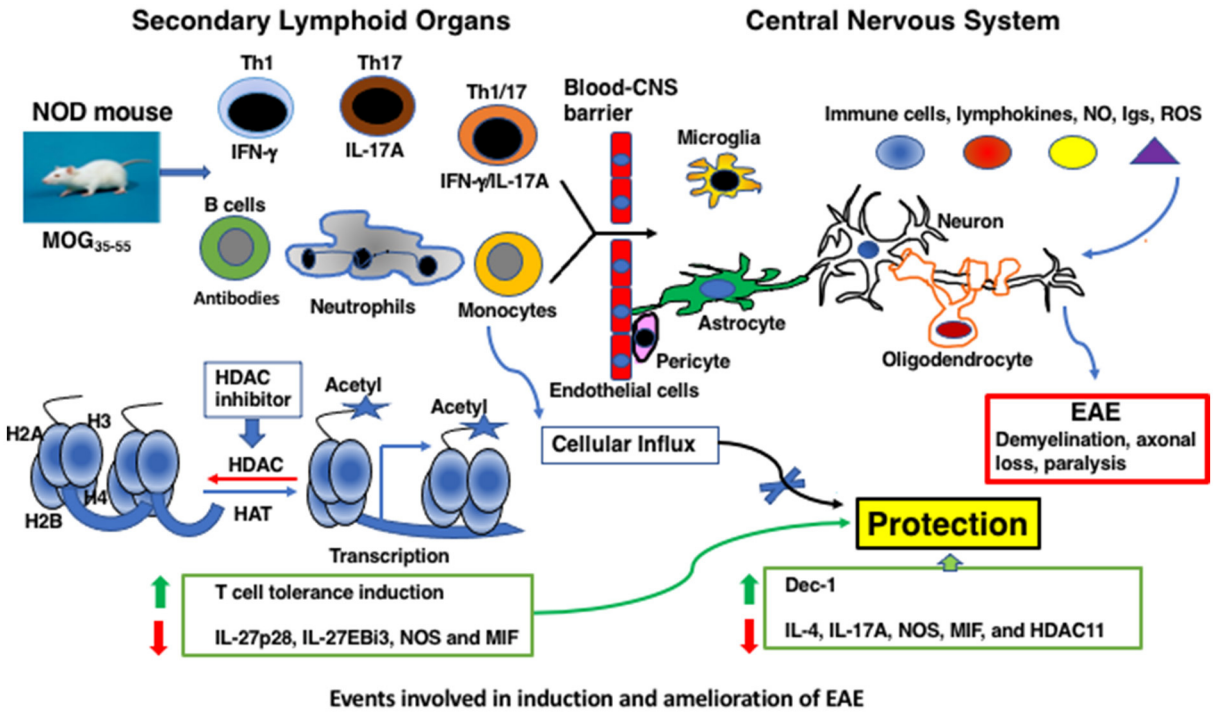

FIGURE 1
Events involved in induction and amelioration of EAE.

Epstein–Barr virus (EBV) infection and MS

Infection with EBV is associated with monoclonal or oligoclonal B-cell expansion in many autoimmune diseases, including Hashimoto's thyroiditis, Grave's disease, Sjögren's syndrome, rheumatoid arthritis, systemic lupus erythematosus syndrome, and MS (23). Whereas infectious mononucleosis increases the risk of MS, the vast majority (90–95%) of the world population infected with EBV at some point in life does not develop MS (24). Although elevated EBV nuclear antigen 1 IgG titers are associated with gadolinium-enhancing brain lesions, the lack of correlation between acute viral reactivation in the peripheral blood and MS lesions suggests a limited role for EBV infection in driving the disease activity (25). Despite the increased level of EBV viral load in patients with RRMS compared with controls, there was no statistically significant difference in EBV and human herpes virus-6 (HHV-6) copy numbers between the patients and controls (26). In addition, the frequency of NK and $CD8^{+}$ T cells increased during relapse, which was not associated with EBV and HHV6 plasma viral loads. Although EBV infection has been hypothesized to contribute to MS development in the context of other predisposing conditions, such as the HLA genotype, vitamin D deficiency, smoking, and altered T-cell responses (23), evidence for this hypothesis remains to be garnered.

Disease-modifying therapies for MS

Several disease-modifying therapies (DMTs) were approved for MS treatment by the Food and Drug Administration (FDA) [reviewed in (27, 28)]. These include self-injectables such as the anti-virals IFN-β-1a and b, first-line treatment, and peginterferon beta-1a provided moderate protection against RRMS (29, 30). However, the production of antibodies against IFN-β and the lack of the effect on Th17 cells, considered encephalitogenic, (31) remained a major concern. Glatiramer acetate designed based on four amino acids from myelin basic protein (MBP) was designed to induce clinical disease in animals but was well tolerated with low/moderate efficacy on RRMS (32). Several orally administered drugs, including teriflunomide, provided moderate effects on RRMS (33, 34). Dimethyl fumarate and diroximel fumarate (35, 36), and fingolimod/FTY720 (37), the first approved oral drug for MS, had moderate beneficial effects on RRMS but with several side effects, including progressive multifocal leukoencephalopathy (PML). Modulators of sphingosine-1-phosphate receptor 1 (S1PR1) and S1PR5, and siponimod decreased oligodendrocyte and axonal loss (38). Ozanimod and SIPR1 and 5 agonist reduced plasma neurofilament light-chain concentrations (39), and the selective S1PR1 modulator ponesimod (40) and cladribine, a deoxyadenosine analog (41), provided moderate benefits to patients with MS. Notably, many intravenous infusion

strategies were implemented for MS treatment. Mitoxantrone, a general immunosuppressant, was the first-line treatment with high efficacy for MS (42). The first humanized monoclonal antibody (mAb) used for MS treatment, natalizumab (43), is directed against anti-α4β1-integrins and blocks the entry of immune cells into the CNS. Although it is highly effective, it causes PML in John Cunningham virus-seropositive patients. The first humanized mAb, anti-CD52 antibody (campath-1/alemtuzumab), originally used for treating graft *vs*. host disease proved to be highly efficacious for MS treatment but associated with significant side effects (44, 45). Several B-cell-depleting anti-CD20 mAbs, such as rituximab, ocrelizumab, ofatumumab, and ublituximab, were highly efficacious for MS treatment but with PML occurrence in some cases (46–50). Most of these drugs were designed to regulate adaptive immune cells prominent during the early, but not the late, stage of MS (3, 27, 28). Some of these therapies reduce relapses but do not prevent the progression of MS and the accumulation of disabilities. The first-line treatments for MS, such as glatiramer acetate (32), dimethyl fumarate (35), and natalizumab (anti-IFN-β-1b) (43), affect T cells variously. Whereas IFN-β-1a/b reduced relapses without affecting Th17 cells (31), glatiramer acetate (32) and dimethyl fumarate suppress Th1 while upregulating Th17 cells (27). Alemtuzumab decreases central memory T cells (27). Fingolimod targets the SIPR and blocks T-cell transmigration into the CNS. This treatment results in cardiac complications, varicella–zoster, and herpes simplex virus reactivation, and exacerbation of MS (27, 28, 37). Natalizumab, a humanized monoclonal antibody, selectively targets the α4 subunit of the cell adhesion molecule, very late antigen 4, and prevents leukocyte adhesion and diapedesis at the blood–brain barrier, leading to PML in John Cunningham-virus seropositive patients (2, 3, 27, 28, 43). Systemic administration of anti-CD20 monoclonal B-cell-depleting antibody rituximab in patients with PPMS reduced gadolinium-enhancing lesions and relapses for 48 weeks (46). However, long-term therapy with ocrelizumab, a humanized depleting anti-CD20 mAb, provided modest protection against PPMS (47). Earlier and continuous treatment of patients with PPMS with ocrelizumab over 6.5 years provided sustained benefits on measures of disease progression (48). Since CNS B cells residing in meningeal ectopic lymphoid follicles are associated with subpial inflammation in patients with SPMS, inadequate penetration of the anti-CD20 antibody across the blood–brain barrier into the CNS could explain the lack of protection observed in some studies. Rituximab administered intrathecally also failed to provide clinical benefits in the phase 1b clinical trial on progressive MS (49). Other B-cell-depleting antibodies including ofatumumab (50) and ublituximab, a novel glycoengineered anti-CD20 mAb (51) that was administered SC unlike other mAbs, induced modest protection against MS.

Since 2018, several second-generation molecules with reduced gastrointestinal side effects have been approved for the treatment of MS by the FDA (52). Diroximel fumarate, the second-generation version of dimethyl fumarate, is lymphopenic and modifies monocytes. Oral formulations of S1PR modulators such as siponimod, ozanimod, and ponesimod target S1PR1 and SIPR5 have potentially better safety profiles. Ofatumumab, an anti-CD20 antibody administered subcutaneously, and glycoengineered anti-CD20 antibody, ublituximab, and oral compounds such as teriflunomide and cladribine were also approved for MS treatment (52).

Several other DMTs outnumbering those approved for MS treatment failed to meet the primary study endpoint and progress to a subsequent clinical trial because of commercial decisions. These include antibodies against the IL-12/23 p40 subunit (53), anti-CD25 (54, 55), CTLA-4-Ig (56), and anti-IL-17A (57). The mAbs targeting different subsets of B cells, tabalumab inhibited B-cell activation factor (BAFF), and atacicept induced depletion of mature B cells and suppressed antibody formation (58). However, they failed to deplete memory B cells and inhibit relapsing MS. Moreover, GNbAC1, a humanized mAb directed against an endogenous retroviral protein (59), and raltegravir (Isentress), the HIV integrase strand inhibitor (60), did not have an impact on MS disease activity. Interestingly, natalizumab failed to demonstrate a significant protective effect in patients with SPMS (61, 62). In addition, the anti-CD20 antibody, rituximab, shown to have superior protection in RRMS, has been abandoned due to the expiry of the patent (61, 62).

In addition to these non-specific drug therapies, several attempts were made to induce antigen-specific tolerance in encephalitogenic T cells, which would ensure stable and adequate protection against autoimmune diseases without off-target effects [reviewed in Refs. (63, 64)]. These include the administration of synthetic peptides corresponding to the T-cell epitopes mapped within myelin components such as MBP, MOG, proteolipid proteins (PLP), and altered ligand peptides. Moreover, T-cell receptor (TCR) vaccination constituting attenuated autologous antigen-specific T cells and autologous peripheral blood mononuclear cells chemically coupled with myelin peptides were also undertaken. None of these maneuvers induced T-cell tolerance as assessed by the ability of peripheral blood T cells to proliferate and produce IFN-γ in response to a challenge with the corresponding immunizing peptide *in vitro*. Significantly, they also did not improve the clinical outcome in patients with MS. Thus, effective methods of inducing antigen-specific tolerance in encephalitogenic T cells without causing adverse reactions remain an unmet need.

Experimental models of MS

Myelin antigen-induced experimental autoimmune encephalomyelitis

The MS-like disease, experimental autoimmune encephalomyelitis (EAE), has been successfully induced in monkeys, guinea pigs, rats, and mice, following immunization with the whole-brain and spinal cord extracts and peptides derived from myelin proteins, such as MBP, PLP, and MOG [reviewed in Ref. (65)]. The mouse is a popular choice for studying MS variations primarily due to the availability of genetically defined inbred strains and transgenic and gene knockout mice. SJL/J mice immunized with the $PLP_{139-151}$ peptide or peptides derived from MBP exhibited relapsing-remitting EAE (RR-EAE) (66), and this model would allow the development of novel DMTs for RRMS. Immunization with rat MOG induced classic EAE in congenic C3H.SW (H-2^b) mice, while causing atypical EAE characterized by ataxia, proprioception defects, and axial rotary clinical presentation in C3HeB/Fej (H-2^k) mice (66, 67). Atypical EAE was also induced in IFN-γ knockout mice on the BALB/c background immunized with MBP-derived peptides (68). In one study, granulocytes were implicated in atypical EAE (66), while others found the participation of granulocytes in both classic EAE and atypical EAE (68). The brain seems primarily involved in atypical EAE, while the spinal cord is considered the primary target of classic EAE and RR-EAE (66, 68). Since the brain is primarily involved in MS (2–4), atypical EAE models may provide valuable tools for further understanding the mechanisms of brain lesions and their prevention.

MOG is a member of the immunoglobulin superfamily expressed exclusively in the CNS myelin. The MOG_{35-55} region proved to be an immunodominant epitope eliciting T- and B-cell responses and EAE in most strains of mice (65, 69–80). MOG_{35-55} was identified as an autoantigen in patients with MS (17). Immunization of C57BL/6 (H-2^b) mice with the MOG_{35-55} peptide elicited EAE (78–80). However, MOG_{35-55} peptide immunization induced a robust and long-lasting progressive EAE (PEAE) in non-obese diabetic (NOD) (H-2^{g7}) mice (70–77). Interestingly, pronounced remissions were observed in some (70–72), but not in other, studies (73–77), indicating variations in PEAE. Genetic drift and gene deletions could be attributed to the inconsistency in remissions in NOD mice bred in different geographical locations—Oceania, Europe, and the United States. The detection of T cells recognizing MOG_{35-55} peptide in patients with MS (17) provided an impetus to explore EAE specifically induced by this peptide autoantigen, although other myelin peptide antigens also elicited EAE in multiple strains of mice (65, 69) (Table 1). Moreover, NOD mice develop several autoimmune diseases, including type 1 diabetes (81) and other endocrine gland-related autoimmune conditions, such as thyroiditis, sialitis, and Sjögren's syndrome (82–84). Thus, NOD mice offer a unique opportunity to study the mechanisms of self-reactive T-cell-mediated neurodegeneration in an autoimmune environment. Significantly, PEAE induced in NOD mice lasts throughout the life of the mice with increasing disabilities (70–77), unlike the non-autoimmune-prone C57BL/6 mice (Table 1) (78–80). Biozzi ABH mice also develop PEAE when immunized with the whole spinal cord homogenate (85). Immunization of Lewis rats with $gpMBP_{68-84}$ (86) and dark Agouti rats with MOG_{1-125} also induced classic EAE (87). Thus, EAE is a well-studied model system of MS and is amenable to investigating the efficacy of novel treatment options.

Other demyelinating disease models

Infection of mice with the neurotropic picornavirus Theiler's murine encephalomyelitis virus (TMEV) induces a disease similar to PPMS involving the brain, brainstem, and spinal cord (88). The TMEV infects macrophage/microglia, oligodendrocytes, and astrocytes during the chronic phase. Axonal damage in MS and EAE occurs secondary to inflammatory demyelination (outside-in model) (89). By contrast, TMEV infection induces demyelinating lesions that develop from the axon to the myelin (inside-out model) (90). Although TMEV infection cannot occur naturally in rodents or humans (91), it is a valuable model for studying the efficacy of drugs to prevent axonal degeneration independent of immune mechanisms. Feeding of C57BL/6 mice with the copper-chelating agent cuprizone induced demyelination, oligodendrocyte death, and profound activation of astrocytes and microglia (91). Removing cuprizone from the diet led to the regeneration of oligodendrocytes from the pool of oligodendrocyte progenitors and the formation of myelin sheaths, indicating the reversible nature of the disease. Interestingly, lysolecithin injection produced focal areas of demyelination in SJL/J mice, rats, and rabbits due to direct toxic effects on myelin sheath without affecting other cells and axons (91). These models help study the process of de- and remyelination independent of the involvement of immune mechanisms.

EAE models for investigation of MS therapeutics

EAE models have traditionally been used to benchmark the efficacy of various disease-modifying therapies. However, several inconsistencies between mice and humans concerning the outcome of these attempts have been intensely debated (91–93). A few established MS therapies, including glatiramer acetate (copolymer 1), mitoxantrone, and natalizumab, were

TABLE 1 Regulation of EAE by HDAC inhibitors.

Model	Clinical manifestation	Drug	Drug administration	Clinical efficacy	Functional effect	Effects on gene expression	References
C57BL/6	Acute, monophasic EAE	TSA, HDAC class I, IIa, and IV inhibitor-hydroxamate	Prophylactic—SC injection.	Reduced EAE.	Caspase inhibition.	Upregulation of genes encoding anti-oxidants, neuroprotection and neuronal differentiation.	(79)
C57BL/6	Acute, monophasic EAE	Vorinostat (SAHA)-HDAC class I and IIa inhibitor-hydroxamate	Prophylactic—intragastric, daily.	Reduced EAE.	Limits CNS inflammation and demyelination. Suppresses Th1, Th17 cells, and costimulatory molecules.	Not determined.	(80)
C57BL/6	Acute, monophasic EAE	Valproic acid, HDAC class I inhibitor	Prophylactic—day 3 or therapeutic-day 12 onward —IP injection or oral administration.	Reduced EAE.	Suppression of spinal cord inflammation, demyelination, and T cells.	Reduction of caspase-3,−8, and−9 mRNA in T cells.	(81)
NOD	Primary, progressive EAE	TSA, HDAC class I, IIa, and IV inhibitor-hydroxamate	Prophylactic- days 0 to 45 or therapeutic- days 15 to 45-SC injection.	Diminished PEAE.	Reduced expansion and infiltration of granulocytes, Th1, Th1/17, and Th17 cells and their infiltration into the CNS. Diminished spinal cord inflammation, demyelination, and axonal loss. Induction of antigen-specific T cell tolerance.	Transcriptional repression of IL-17A, IL-27 p28, IL-27 Ebi3, iNos, and MIF in the peripheral lymphoid compartment. Reduced transcription of IL-4, IL-17A, iNos, MIF, aryl hydrocarbon receptor, and Hdac11 but increased expression of DEC-1 mRNA in the CNS.	(75–78)
NOD	Primary, progressive EAE	Panobinostat, Givinostat (hydroxamate, pan-lysine inhibitor), and Entinostat	Therapeutic-day 20 onward-oral	No effect on PEAE or mortality.	Reduced T cell proliferation *in vitro*.	Reduced transcription of *Tbet* and *Rorgt* but not *Gata3* or *Foxp3* in lymphoid cells.	(78)
Lewis rat	Acute, monophasic	Valproic acid	Prophylactic and therapeutic-oral.	Reduced EAE	Th1/Th17-Th2 shift, attenuated infiltration of macrophages and lymphocytes in the spinal cord.	Suppressed mRNA levels of IFN-γ, TNF-α, IL-1β, MMP9, iNos, Tbet and increased IL-4 in the spinal cord.	(87)
Dark Agouti rat	Acute, monophasic	Valproic acid	Therapeutic-IP injection of multiple doses every day for many days.	A modest decrease in chronic EAE without affecting the peak response.	Reduced T cell proliferation and decreased Th17 cells.	Increased *Sox8* and *Mog* expression in the brain. Reduced demyelination in the spinal cord.	(88)

Mice were immunized with MOG_{35-55} peptide, Lewis rats with $gpMBP_{68-84}$ peptide, and DA rats with MOG_{1-125} peptide.

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tested in animal models, which turned out to be potent non-specific suppressors and unsuitable for all patients with MS (27). Some DMTs were investigated in EAE models retrospectively after disappointing outcomes in human trials (91, 92). The failures of translational therapies for MS treatment could be due to differences in genetics, the extent of blood–brain barrier disruption, and individual variability in the responsiveness of patients to treatment. Emphasis has also been placed on discovering reliable biomarkers of MS and improving the design of CNS drug delivery (93). Most of the multifocal symptoms of classic MS have not been reproduced in rodent models. This limitation should be kept in mind when discussing the lack of efficacy of the DMTs for MS treatment since this disease is highly heterogenous and sometimes manifest with other comorbidities.

Epigenetic approaches to control EAE

In EAE, adaptive immune T and B cells, the innate immune granulocytes, and the CNS-resident cells such as microglia, astrocytes, and oligodendrocytes collectively contribute to neurodegeneration. Gene expression is a highly regulated process, and aberrant expression of mRNA encoding cytokines and chemokines contributes to pathological manifestations. Although the genome-wide association studies have implicated genes encoding human leukocyte antigens in MS pathogenesis (94), environmental factors such as Epstein–Barr virus infection, smoking, and vitamin D deficiency may influence gene expression *via* epigenetic mechanisms (95). Epigenetics is the heritable changes in gene expression without altering the DNA sequence, which can provide a mechanism by which external factors, including drugs, produce various phenotypic variations with identical genotypes (96). Discordance in the rate of MS among monozygotic twins suggests that susceptible genes alone are not enough to manifest the neuronal disease, implying the participation of epigenetic mechanisms in disease manifestation (97). DNA methylation (98) and microRNAs (99) have been proposed to play a role in MS. However, direct evidence supporting the contention that modulation of these epigenetic mechanisms can result in neuroprotection is lacking.

Histone acetylation is the most well-characterized posttranslational mechanism of histone modifications, facilitating an open chromatin configuration and gene transcription (96) (Figure 1). The balance between acetylation by histone acetyltransferases and their regulation by histone deacetylases (HDACs) dictates the outcome of transcription of many protein-coding genes (96) and, interestingly, a non-coding microRNA (100). Trichostatin A (TSA), a hydroxamate member, was initially developed for cancer treatment (101) and is the most potent broad-spectrum HDAC inhibitor (102). TSA inhibits the transcription of class I, IIa, IIb, and IV HDACs (76). When C57BL/6 mice were immunized with MOG_{35-55} and treated with large doses of TSA s.c throughout the investigation, a modest reduction in the EAE score was noted (78) (Table 1). Similarly, daily oral administration of vorinostat, another hydroxamate that inhibits class I and IIa HDACs (102) throughout the period of investigation, also reduced the acute EAE in C57BL/6 mice (79). Interestingly, the class I HDAC inhibitor and the antiepileptic drug valproic acid when administered prophylactically or therapeutically reduced acute EAE in C57BL/6 mice (80). Notably, s.c administration of a lower dose of TSA prophylactically up to 45 days on alternate days provided irreversible and prolonged protection against PEAE in NOD mice (74). Consistent with these encouraging results of HDAC inhibitors to treat neurodegenerative diseases in mice, oral treatment of Lewis rats (86) or i.p administration of DA rats (87) with valproic acid reduced EAE induced by immunization with $gpMBP_{68-84}$ and MOG_{1-125} peptides, respectively. In contrast to the success of reducing the clinical scores by TSA and valproic acid in C57BL/6 and NOD mice, oral administration of another hydroxamate panobinostat, givinostat, a pan-lysine inhibitor, or entinostat therapeutically from day 20 onward failed to afford protection against PEAE (77). These data indicate that not all HDAC inhibitors can serve as potent DMTs for ongoing neurodegeneration.

Neuroprotection provided by TSA, vorinostat (SAHA), and valproic acid corroborated with reduced CNS inflammation and demyelination in mice (74, 75, 79, 80). Significantly, inhibition of axonal degeneration during PEAE was also prominently mediated by TSA (74). Reduced T-cell proliferation and suppression of Th17 cells were noted in HDAC inhibitor-treated rodents (74, 79, 86, 87). Neuroprotection was also accompanied by decreased $CD4^{+}CD44^{+}$ cells, a characteristic of activated/memory cells (103), and reduced ability of T cells to produce IFN-γ, IL-17A, and GM-CSF in response to a challenge with MOG_{35-55} *in vitro* (74). Histone hyperacetylation rendered T cells unresponsive to the MOG_{35-55} antigen challenge while retaining their ability to respond to polyclonal stimulation (74), akin to anergy (104). By contrast, daily oral administration of HDAC inhibitors such as panobinostat, givinostat, and entinostat from the start of clinical signs (day 20) failed to protect NOD mice from PEAE or fatality, despite reduced T-cell proliferation *in vitro* and diminished transcription of *Tbet* and *Rorγt* (77). However, the antiepileptic drug valproic acid (54) and the anti-cancer drug, TSA (74), administered therapeutically (after the disease onset, Table 1) provided robust neuroprotection and thus may be useful in a clinical setting.

Regulation of the innate immune system in EAE by HDAC inhibitors

In MS, innate immune cells, such as infiltrating macrophages and dendritic cells, and CNS-resident microglia, have been

implicated in the reactivation of T cells during the effector phase of neurodegeneration (2, 3). In NOD mice, PEAE development was associated with the expansion of *mature* (MHC class II^+) $CD11b^+Ly\text{-}6G^+$ neutrophils and, to a lesser extent (MHC class II^+) $CD11b^+Ly\text{-}6C^+$ mature monocytes in the peripheral lymphoid compartment before the onset of the peak clinical disease (75). Participation of neutrophils in monophasic EAE of C57BL/6 mice was indicated by increased neutrophils in the bone marrow, blood, and spleen during the early phase of the disease (105). Studies suggested a role for neutrophils in MS during the initial formation of lesions in the brain, but not during the advanced stages of the disease, probably owing to the short-lived nature of neutrophils (106). Treatment with TSA concurrently afforded neuroprotection and diminished the frequency of neutrophils in secondary lymphoid organs and their influx into the spinal cord (75), indicating a role for these cells in the PEAE model (Figure 1). Thus, in addition to myelin-specific T-cell tolerance induction, selective regulation of the innate immune system appears to be an integral part of the regulation of neurodegeneration by the HDAC inhibitor TSA.

Implications of HDAC inhibitor-induced regulation of EAE to MS treatment

Impact of immune regulation

Immune responses elicited by immunization with the whole spinal cord homogenate or various peptides derived from the CNS-associated MBP, PLP, and MOG have been extensively studied in mice and rats that develop monophasic EAE, PEAE, and atypical EAE (65–80, 86, 87). Various methodologies such as ELISA, Western blot, flow cytometry, and quantitative reverse transcriptase-mediated polymerase chain reaction (RTq-PCR) have provided significant insights into the underlying immune mechanisms of EAE. However, consensus on whether any given immune mediator can serve as a biomarker indicating the stage and severity of the chronic disease remains enigmatic. Most studies focused on immune mediators typically at the peak of the clinical disease after *in vitro* activation with T-cell ligands. A systematic and comprehensive analysis of *basal levels* of 41 genes frequently implicated in neurodegeneration and their regulation by TSA treatment was assessed using RTq-PCR in the CNS and secondary lymphoid organs longitudinally during the prolonged course of PEAE (27 weeks) without overt activation *in vitro* (76). These studies indicated that immunization of NOD mice with MOG_{35-55} increased the expression levels of mRNAs encoding IL-4 and IL-17A in the CNS during the chronic phase, days 21–54. The reduction in the level of IL-17A gene in TSA-treated mice is consistent with the proposed role of IL-17A in EAE (107). Prolonged expression of *Nos2* in the CNS (76) is in line with the association of iNOS-positive macrophages, astrocytes, and granulocytes in demyelinating pathology (108). Increased numbers of neutrophils in the spleen and spinal cord and their downregulation by the histone modifier treatment support this contention (75).

On the other hand, in the peripheral lymphoid tissues, genes encoding the heterodimeric chains of IL-27, IL-27p28, and IL-27EBi3, implicated in EAE (109), were overexpressed in PEAE mice, which were reduced by TSA treatment. Augmentation of the transcriptional repressors by histone acetylation could indirectly cause a reduction in gene expression. Notably, *in vitro* activation of peripheral lymphoid cells from TSA-treated mice exhibited compromised expression of both intracellular and secreted IL-17A and IFN-γ (74). Interestingly, TSA treatment reduced the infiltration of Th1 and Th17 cells from the periphery into the spinal cord (74) (Figure 1). This is similar to the suppressive effect of valproic acid on the influx of T cells into the spinal cord of EAE Lewis rats (86). These data demonstrate that the infiltration of T lymphocytes into the CNS is crucial for neurodegeneration, and their retardation by HDAC inhibitors facilitates neuroprotection.

Although migration inhibitory factor (MIF) has been proposed to be crucial for EAE (110), surprisingly, it was not transcriptionally upregulated in the CNS and lymphoid tissues of NOD mice manifesting PEAE (76). Yet, TSA treatment repressed the constitutive expression of *Mif* in protected mice. Surprisingly, several other genes implicated in EAE, including GM-CSF (111), prominent chemokine CCL2 (112), transcription factors T-bet (113), and RORγt (114), were neither overexpressed in the PEAE mice nor downregulated by TSA treatment (76). However, in EAE rats, valproic acid treatment suppressed the mRNA levels of IFN-γ, TNF-α, IL-1β, MMP9, iNos, and Tbet and increased IL-4 in the spinal cord (86). The transcription factor FoxP3 mRNA was neither upregulated in the PEAE model nor modulated by chromatin modifier treatment (76), similar to the lack of suppression of FoxP3 transcription in another study (77). TSA treatment also did not alter the numbers of $FoxP3^+$ T regulatory cells in NOD mice (74, 76). Although the transcription factor FoxP3 is essential for the generation of T regulatory cells (115), it is contentious whether these cells are involved in the regulation of EAE (116, 117). Studies in mice indicated the upregulation of genes encoding anti-oxidants, neuroprotection, and neuronal differentiation by TSA treatment (78), while the expression of *Sox8* and *Mog* was upregulated in valproic acid-treated rat brains (87). Valproic acid administration also reduced the genes crucial for apoptosis, and caspase-3,−8, and−9 in T cells (78). Collectively, these data indicate that the HDAC inhibitors modulate the transcription of several genes crucially involved in neurodegeneration.

The role of histone deacetylases in EAE and their modulation by TSA

Surprisingly, immunization of NOD mice with MOG_{35-55} upregulated the transcription of Hdac11 in the CNS, but none of the 11 Hdacs in the peripheral lymphoid cells (76). The wide-spectrum HDAC inhibitor, TSA, did not diminish the Hdac11 enzymatic activity *in vitro* (118), indicating the lack of correlation between Hdac expression and Hdac activity. Nevertheless, the data demonstrating the selective upregulation of *Hdac11* in the spinal cord of PEAE mice and its downregulation by TSA treatment have implications to the control of MS by histone modifiers. The use of high-resolution *in situ* hybridization and imaging revealed abundant expression of *Hdac11* in the hippocampus and Purkinje cells of rat brains, suggesting a role in locomotor activity and ataxic syndromes, respectively (119). However, it is unclear whether in PEAE mice, *Hdac11* expression is localized to these cells and downregulated by TSA treatment. Knockout of *Hdac11* reduced the infiltration of monocytes and myeloid DC into the CNS, expression of CCL2, clinical severity, and demyelination (120). Although both TSA treatment and *Hdac11* gene knockout resulted in amelioration of EAE, the protective effect of *Hdac11* deletion observed may be secondary to the absence of *Hdac11* in the CNS and unrelated to the impact on monocytes and CCL2 expression (120). Nevertheless, by extrapolation, repression of *Hdac11* could be beneficial in treating patients with MS with broad-spectrum HDAC inhibitors, such as TSA. Although *Hdacs* other than *Hdac11* was not regulated by the histone modifier either in the peripheral lymphoid tissues or in the CNS (74), HDAC3 mRNA was reportedly increased in the peripheral blood mononuclear cells of patients with RRMS (121). However, another study failed to validate this observation (122), indicating uncertainty of the role of *HDAC3* in MS. Interestingly, TSA treatment prevented the manifestation of type 1 diabetes in NOD mice associated with the transcriptional repression of *Hdac4*, *Hdac8*, and *Hdac9*, but not *Hdac11*, in the spleen (123). However, TSA administration did not influence the transcription of *Hdac* genes expressed in the target organ pancreas. These data suggest that the overexpression of specific Hdac is tissue- and disease-specific, which could be utilized to manipulate hard-to-treat diseases, including MS.

Implications of HDAC inhibition to MS treatment

Targeting multiple HDAC isoforms might be necessary for specific indications and proof-of-concept studies. The involvement of specific HDACs crucial for various forms of MS has not yet been delineated. Studying the expression level of different HDAC genes in particular cell types in the secondary lymphoid organs and the CNS is essential for designing selective HDAC inhibitors for MS treatment. Based on the data obtained, it is possible to create more selective compounds that could prove safer by reducing off-target effects. In addition to the downregulation of many genes, the expression of the transcription factor Dec1 (Bhlhe40) was upregulated in the CNS of TSA-treated mice (76). Thus, HDAC inhibitors such as TSA with broad specificity might provide benefits against complex neurodegenerative diseases by concurrently repressing and increasing the transcription of multiple genes. The wide range of the action of the broad-spectrum HDAC inhibitor is likely to provide protection against complex neurodegenerative diseases like MS. Consistently, therapeutic intervention with HDAC inhibitors has been proposed to enhance synaptic plasticity, learning, and memory in Alzheimer's disease, Huntington's disease, and Parkinson's disease (124). Lysine acetylation of non-histones constitutes a significant portion of the acetylome in mammalian cells and is involved in several cellular functions, including gene transcription (125). However, it is unclear whether HDAC inhibitors can also acetylate non-histones and alter gene transcription in conjunction with gene regulation mediated by acetylation of histone tails. Nevertheless, changes in gene expression due to inhibition of HDACs by small-molecule inhibitors could have substantial impact on regulating disease pathogenesis.

Recent work has unraveled the inheritance of non-DNA sequence-based epigenetic information, epimutations, across several generations in yeast to humans (126). The signals that underpin these epimutations, including DNA methylation, histone modification, and non-coding RNAs, and the underlying mechanisms are beginning to be understood (127). Treatment of the nematode *Auanema freiburgensis* with class I HDAC inhibitors butyrate and valproic acid, and the broad-spectrum HDAC inhibitor TSA increased the acetylation of histones 3 and 4 (128). Notably, they also exerted transgenerational effects on the offspring by producing increased numbers of hermaphrodites, suggesting that histone acetylation represents the histone code. The HDAC inhibitors have successfully ameliorated several diseases, including type 1 diabetes (123, 129–132), EAE (74–76), asthma (133), lupus (134, 135), and colitis (136), in animal models, indicating their usefulness to treat a variety of diseases. Accumulating data indicate that histone modifier-mediated hyperacetylation in lymphoid cells and the target tissues is associated with the amelioration of type 1 diabetes (129) and PEAE (74), and selective regulation of genes. It remains to be seen whether the changes in gene expression observed following treatment with HDAC inhibitors have transgenerational consequences.

Conclusion

This review discusses the effects of HDAC inhibitors on EAE regulation (Table 1) and, by extrapolation, their utility in treating MS. Neuroprotection in mice was accompanied by the repression of mostly non-overlapping sets of genes induced by immunization with myelin antigens and a few constitutively expressed genes in the peripheral lymphoid system and the CNS. Notably, TSA administration contrived the expansion of granulocytes and induced T-cell tolerance in the periphery while reducing the influx of immune cells into the CNS (Figure 1). Lessons learned from the EAE models require validation, which may provide impetus to investigate the efficacy of histone modifiers for treating MS variants efficiently. Since HDAC inhibitors such as valproic acid and hydroxamates are currently used in patients for ailments unrelated to MS and are well tolerated, these small-molecule inhibitors may be used for treating MS.

Author contributions

SJ conceived and executed the project and wrote the first draft of the manuscript. AJ conducted most of the experiments reported in this article and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Walton C, King R, Rechtman L, Kaye W, Leray E, Marrie RA, et al. Rising prevalence of multiple sclerosis worldwide: Insights from the Atlas of MS, third edition. *Mult Scler.* (2020) 26:1816–21. doi: 10.1177/1352458520970841

2. Weissert R. The immune pathogenesis of multiple sclerosis. *J Neuroimmune Pharmacol.* (2013) 8:857–66. doi: 10.1007/s11481-013-9467-3

3. Dendrou CA, Fugger L, Friese MA. Immunopathology of multiple sclerosis. *Nat Rev Immunol.* (2015) 15:545–58. doi: 10.1038/nri3871

4. Kearney H, Miller DH, Ciccarelli O. Spinal cord MRI in multiple sclerosis–diagnostic, prognostic and clinical value. *Nat Rev Neurol.* (2015) 11:327–38. doi: 10.1038/nrneurol.2015.80

5. Cree BA, Reich DE, Khan O, De Jager PL, Nakashima I, Takahashi T, et al. Modification of multiple sclerosis phenotypes by African ancestry at HLA. *Arch Neurol.* (2009) 66:226–33. doi: 10.1001/archneurol.2008.541

6. Kister I, Bacon T, Cutter GR. How multiple sclerosis symptoms vary by age, sex, and race/ethnicity. *Neurol Clin Pract.* (2021) 11:335–41. doi: 10.1212/CPJ.0000000000001105

7. Kira J. Neuromyelitis optica and Asian phenotype of multiple sclerosis. *Ann N Y Acad Sci.* (2008) 1142:58–71. doi: 10.1196/annals.1444.002

8. Papais Alvarenga RM, Araújo ACRAE, Nascimento ACB, Araujo NEC, Meneguette NS, Neri VC, et al. Is Asian type MS an MS phenotype, an NMO spectrum disorder, or a MOG-IgG related disease? *Mult Scler Relat Disord.* (2020) 42:102082. doi: 10.1016/j.msard.2020.102082

9. Kira J. Autoimmunity in neuromyelitis optica and opticospinal multiple sclerosis: astrocytopathy as a common denominator in demyelinating disorders. *J Neurol Sci.* (2011) 311:69–77. doi: 10.1016/j.jns.2011.08.043

10. Rosati G. The prevalence of multiple sclerosis in the world: an update. *Neurol Sci.* (2001) 22:117–39. doi: 10.1007/s100720170011

11. Frischer JM, Bramow S, Dal-Bianco A, Lucchinetti CF, Rauschka H, Schmidbauer M, et al. The relation between inflammation and neurodegeneration in multiple sclerosis brains. *Brain.* (2009) 132:1175–89. doi: 10.1093/brain/awp070

12. Hollenbach JA, Oksenberg JR. The immunogenetics of multiple sclerosis: a comprehensive review. *J Autoimmun.* (2015) 64:13–25. doi: 10.1016/j.jaut.2015.06.010

13. Fukazawa T, Kikuchi S, Miyagishi R, Miyazaki Y, Yabe I, Hamada T, et al. HLA-dPB1*0501 is not uniquely associated with opticospinal multiple sclerosis in Japanese patients. Important role of DPB1*0301. *Mult Scler.* (2006) 12:19–23. doi: 10.1191/135248506ms1252oa

14. Peerlings D, Mimpen M, Damoiseaux J. The IL-2–IL-2 receptor pathway: Key to understanding multiple sclerosis. *J Transl Autoimmun.* (2021) 4:100123. doi: 10.1016/j.jtauto.2021.100123

15. Rane L, Vudattu N, Bourcier K, Graniar E, Hillert J, Seyfert V, et al. Alternative splicing of interleukin-7 (IL-7) and interleukin-7 receptor alpha (IL-7Ralpha) in peripheral blood from patients with multiple sclerosis (MS). *J Neuroimmunol.* (2010) 222:82–6. doi: 10.1016/j.jneuroim.2010.02.014

16. Cruciani C, Puthenparampil M, Tomas-Ojer P, Jelcic I, Docampo MJ, Planas R, et al. T-cell specificity influences disease heterogeneity in multiple sclerosis. *Neurol Neuroimmunol Neuroinflamm.* (2021) 8:e1075. doi: 10.1212/NXI.0000000000001075

17. Bernard CC, Johns TG, Slavin A, Ichikawa M, Ewing C, Liu J, et al. Myelin oligodendrocyte glycoprotein: a novel candidate autoantigen in multiple sclerosis. *J Mol Med (Berl).* (1997) 75:77–88. doi: 10.1007/s001090050092

18. Moser T, Akgün K, Proschmann U, Sellner J, Ziemssen T. The role of TH17 cells in multiple sclerosis: therapeutic implications. *Autoimmun Rev.* (2020) 9:102647. doi: 10.1016/j.autrev.2020.102647

19. Ueno H, Banchereau J, Vinuesa CG. Pathophysiology of T follicular helper cells in humans and mice. *Nat Immunol.* (2015) 16:142–52. doi: 10.1038/ni.3054

20. Holm Hansen R, Talbot J, Højsgaard Chow H, Bredahl Hansen M, Buhelt S, Herich S, et al. Increased intrathecal activity of follicular helper T cells in patients with relapsing-remitting multiple sclerosis. *Neurol Neuroimmunol Neuroinflamm.* (2022) 9:e200009. doi: 10.1212/NXI.0000000000200009

21. Quinn JL, Kumar G, Agasing A, Ko RM, Axtell RC. Role of TFH cells in promoting T helper 17-induced neuroinflammation. *Front Immunol.* (2018) 9:382. doi: 10.3389/fimmu.2018.00382

22. Machado-Santos J, Saji E, Tröscher AR, Paunovic M, Liblau R, Gabriely G, et al. The compartmentalized inflammatory response in the multiple sclerosis brain is composed of tissue-resident CD8+ T lymphocytes and B cells (2018). *Brain.* (2018) 141:2066–82. doi: 10.1093/brain/awy151

23. Pender MP. The essential role of Epstein-Barr virus in the pathogenesis of multiple sclerosis. *Neuroscientist.* (2011) 17:351–67. doi: 10.1177/1073858410381531

24. Thacker EL, Mirzaei F, Ascherio A. Infectious mononucleosis and risk for multiple sclerosis: a meta-analysis. *Ann Neurol.* (2006) 59:499–503. doi: 10.1002/ana.20820

25. Farrell RA, Antony D, Wall GR, Clark DA, Fisniku L, Swanton J, et al. Humoral immune response to EBV in multiple sclerosis is associated with disease activity on MRI. *Neurology.* (2009) 73:32–8. doi: 10.1212/WNL.0b013e3181aa29fe

26. Salehi Z, Beheshti M, Nomanpour B, Khosravani P, Naseri M, Sahraian MA, et al. The association of EBV and HHV-6 viral load with different NK and CD8+ T cell subsets in the acute phase of relapsing-remitting multiple sclerosis. *Cell J.* (2021) 23:626–32.

27. Wingerchuk DM, Carter JL. Multiple sclerosis: current and emerging disease-modifying therapies and treatment strategies. *Mayo Clin Proc.* (2014) 89:225–40. doi: 10.1016/j.mayocp.2013.11.002

28. Hauser, S. L., and Cree, B. A. C. (2020). Treatment of multiple sclerosis: a review. *Am J Med.* 133:1380–90.e2. doi: 10.1016/j.amjmed.2020.05.049

29. The IFNB Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group. Interferon beta-1b in the treatment of multiple sclerosis: final outcome of the randomized controlled trial. *Neurology.* (1995) 45:1277–85. doi: 10.1212/WNL.45.7.1277

30. Calabresi P, Kieseier B, Arnold D, Balcer L, Boyko A, Pelletier J, et al. Clinical efficacy and safety of peginterferon beta-1a in relapsing multiple sclerosis:data from the pivotal phase 3 ADVANCE Study. *Neurology.* (2013) 80 (Meeting Abstracts 1):S31–006.

31. Axtell RC, Raman C, Steinman L. Type I interferons: beneficial in Th1 and detrimental in Th17 autoimmunity. *Clin Rev Allergy Immunol.* (2013) 44:114–20. doi: 10.1007/s12016-011-8296-5

32. Dhib-Jalbut S. Mechanisms of action of interferons and glatiramer acetate in multiple sclerosis. *Neurology.* (2002) 58:S3–9. doi: 10.1212/WNL.58.8_suppl_4.S3

33. Miller A, Kappos L, Comi G, Confavreux C, Freedman M, Olsson T, et al. Teriflunomide efficacy and safety in patients with relapsing multiple sclerosis: results from TOWER, a second, pivotal, phase 3 placebo-controlled study. *Neurology.* (2013) 80 (Meeting Abstracts 1): S01.004.

34. Coyle PK, Khatri B, Edwards KR, Meca-Lallana JE, Cavalier S, Rufi P, et al. Teriflunomide real-world evidence: global differences in the phase 4 Teri-PRO study. *Mult Scler Relat Disord.* (2019) 31:157–64. doi: 10.1016/j.msard.2019.03.022

35. van Oosten BW, Killestein J, Barkhof F, Polman CH, Wattjes MP. PML in a patient treated with dimethyl fumarate from a compounding pharmacy [published correction appears in N Engl J Med 368:1950]. *N Engl J Med.* (2013) (2013) 368:1658–9. doi: 10.1056/NEJMc1215357

36. Wray S, Then Bergh F, Wundes A, Arnold DL, Drulovic J, Jasinska E, et al. Efficacy and safety outcomes with diroximel fumarate after switching from prior therapies or continuing on DRF: results from the phase 3 EVOLVE-MS-1 Study. *Adv Ther.* (2022) 39:1810–31. doi: 10.1007/s12325-022-02068-7

37. Montalban X, Comi G, O'Connor P, Gold S, de Vera A, Eckert B, et al. Oral fingolimod (FTY720) in relapsing multiple sclerosis: impact on health-related quality of life in a phase II study. *Mult Scler.* (2011) 17:1341–50. doi: 10.1177/1352458511411061

38. Kappos L, Li DK, Stüve O, Hartung HP, Freedman MS, Hemmer B, et al. Safety and efficacy of siponimod (BAF312) in patients with relapsing-remitting multiple sclerosis: dose-blinded, randomized extension of the Phase 2 BOLD Study. *JAMA Neurol.* (2016) 73:1089–98. doi: 10.1001/jamaneurol.2016.1451

39. Harris S, Comi G, Cree BA, Arnold DL, Steinman L, Sheffield JK, et al. Plasma neurofilament light chain concentrations as a biomarker of clinical and radiologic outcomes in relapsing multiple sclerosis: post hoc analysis of phase 3 ozanimod trials. *Eur J Neurol.* (2021) 28:3722–30. doi: 10.1111/ene.15009

40. Kappos L, Fox RJ, Burcklen M, Freedman MS, Havrdová EK, Hennessy B, et al. Ponesimod compared with teriflunomide in patients with relapsing multiple sclerosis in the active-comparator phase 3 OPTIMUM Study: a randomized clinical trial. *JAMA Neurol.* (2021) 78:558–67. doi: 10.1001/jamaneurol.2021.0405

41. Giovannoni G, Soelberg Sorensen P, Cook S, Rammohan K, Rieckmann P, Comi G, et al. Safety and efficacy of cladribine tablets in patients with relapsing-remitting multiple sclerosis: results from the randomized extension trial of the CLARITY study. *Mult Scler.* (2018) 24:1594–604. doi: 10.1177/1352458517727603

42. Foo EC, Russell M, Lily O, Ford HL. Mitoxantrone in relapsing-remitting and rapidly progressive multiple sclerosis: ten-year clinical outcomes post-treatment with mitoxantrone. *Mult Scler Relat Disord.* (2020) 44:102330. doi: 10.1016/j.msard.2020.102330

43. Perumal J, Balabanov R, Su R, Chang R, Balcer L, Galetta S, et al. Natalizumab in early relapsing-remitting multiple sclerosis: a 4-year, open-label study. *Adv Ther.* (2021) 38:3724–42. doi: 10.1007/s12325-021-01722-w

44. Cohen JA, Coles AJ, Arnold DL, Confavreux C, Fox EJ, Hartung HP, et al. Alemtuzumab versus interferon beta 1a as first-line treatment for patients with relapsing-remitting multiple sclerosis: a randomised controlled phase 3 trial. *Lancet.* (2012) 380:1819–28. doi: 10.1016/S0140-6736(12)61769-3

45. Baker D, Ali L, Saxena G, Pryce G, Jones M, Schmierer K, et al. The irony of humanization: alemtuzumab, the first, but one of the most immunogenic, humanized monoclonal antibodies. *Front Immunol.* (2020) 11:124. doi: 10.3389/fimmu.2020.00124

46. Hauser SL, Waubant E, Arnold DL, Vollmer T, Antel J, Fox RJ, et al. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N Engl J Med.* (2008) 358:676–88. doi: 10.1056/NEJMoa0706383

47. Montalban X, Hauser SL, Kappos L, Arnold DL, Bar-Or A, Comi G, et al. Ocrelizumab versus placebo in primary progressive multiple sclerosis. *N Engl J Med.* (2017) 376e:209–20. doi: 10.1056/NEJMoa1606468

48. Wolinsky JS, Arnold DL, Brochet B, Hartung HP, Montalban X, Naismith RT, et al. Long-term follow-up from the ORATORIO trial of ocrelizumab for primary progressive multiple sclerosis: a post-hoc analysis from the ongoing open-label extension of the randomised, placebo-controlled, phase 3 trial. *Lancet Neurol.* (2020) 19:998–1009. doi: 10.1016/S1474-4422(20)30342-2

49. Bergman J, Burman J, Gilthorpe JD, Zetterberg H, Jiltsova E, Bergenheim T, et al. Intrathecal treatment trial of rituximab in progressive MS: an open-label phase 1b study. *Neurology.* (2018) 91:e1893–901. doi: 10.1212/WNL.0000000000006500

50. Gärtner J, Hauser SL, Bar-Or A, Montalban X, Cohen JA, Cross AH, et al. Efficacy and safety of ofatumumab in recently diagnosed, treatment-naive patients with multiple sclerosis: results from ASCLEPIOS I and II. *Mult Scler.* (2022) 28:1562–75. doi: 10.1177/13524585221078825

51. Fox E, Lovett-Racke AE, Gormley M, Liu Y, Petracca M, Cocozza S, et al. A phase 2 multicenter study of ublituximab, a novel glycoengineered anti-CD20 monoclonal antibody, in patients with relapsing forms of multiple sclerosis. *Mult Scler.* (2021) 27:420–9. doi: 10.1177/1352458520918375

52. Faissner S, Gold R. Efficacy and safety of multiple sclerosis drugs approved since 2018 and future developments. *CNS Drugs.* (2022) 36:803–17. doi: 10.1007/s40263-022-00939-9

53. Segal BM, Constantinescu CS, Raychaudhuri A, Kim L, Fidelus-Gort R, Kasper LH, et al. Repeated subcutaneous injections of IL12/23 p40 neutralising antibody, ustekinumab, in patients with relapsing-remitting multiple sclerosis: a phase II, double-blind, placebo-controlled, randomised, dose-ranging study. *Lancet Neurol.* (2008) 7:796–804. doi: 10.1016/S1474-4422(08)70173-X

54. Gold R, Giovannoni G, Selmaj K, Havrdova E, Montalban X, Radue EW, et al. Daclizumab high-yield process in relapsing-remitting multiple sclerosis (SELECT): a randomised, double-blind, placebo-controlled trial. *Lancet.* (2013) 381:2167–75. doi: 10.1016/S0140-6736(12)62190-4

55. Kappos L, Cohan S, Arnold DL, Robinson RR, Holman J, Fam S, et al. Safety and efficacy of daclizumab beta in patients with relapsing multiple sclerosis in a 5-year open-label study (EXTEND): final results following early termination. *Ther Adv Neurol Disord.* (2021) 14:1756286420987941. doi: 10.1177/1756286420987941

56. Viglietta V, Bourcier K, Buckle GJ, Healy B, Weiner HL, Hafler DA, et al. CTLA4Ig treatment in patients with multiple sclerosis: an open-label, phase 1 clinical trial. *Neurology.* (2008) 71:917–24. doi: 10.1212/01.wnl.0000325915.00112.61

57. Havrdová E, Belova A, Goloborodko A, Tisserant A, Wright A, Wallstroem E, et al. Activity of secukinumab, an anti-IL-17A antibody, on brain lesions in RRMS: results from a randomized, proof-of-concept study. *J Neurol.* (2016) 263:1287–95. doi: 10.1007/s00415-016-8128-x

58. Baker D, Pryce G, James LK, Schmierer K, Giovannoni G. Failed B cell survival factor trials support the importance of memory B cells in multiple sclerosis. *Eur J Neurol.* (2020) 27:221–8. doi: 10.1111/ene.14105

59. Curtin F, Porchet H, Glanzman R, Schneble HM, Vidal V, Audoli-Inthavong ML, et al. A placebo randomized controlled study to test the efficacy and safety of GNbAC1, a monoclonal antibody for the treatment of multiple sclerosis - Rationale and design. *Mult Scler Relat Disord.* (2016) 9:95–100. doi: 10.1016/j.msard.2016.07.002

60. Gold J, Marta M, Meier UC, Christensen T, Miller D, Altmann D, et al. A phase II baseline versus treatment study to determine the efficacy of raltegravir (Isentress) in preventing progression of relapsing remitting multiple sclerosis as determined by gadolinium-enhanced MRI: the INSPIRE study. *Mult Scler Relat Disord.* (2018) 24:123–8. doi: 10.1016/j.msard.2018.06.002

61. Rolfes L, Pawlitzki M, Pfeuffer S, Huntemann N, Wiendl H, Ruck T, et al. Failed, interrupted, or inconclusive trials on immunomodulatory treatment strategies in multiple sclerosis: update 2015-2020. *BioDrugs.* (2020) 34:587–610. doi: 10.1007/s40259-020-00435-w

62. Krämer J, Wiendl H. What have failed, interrupted, and withdrawn antibody therapies in multiple sclerosis taught us? *Neurotherapeutics.* (2022) 19:785–807. doi: 10.1007/s13311-022-01246-3

63. Lutterotti A, Martin R. Antigen-specific tolerization approaches in multiple sclerosis. *Expert Opin Investig Drugs.* (2014) 23:9–20. doi: 10.1517/13543784.2014.844788

64. Steinman L. The re-emergence of antigen-specific tolerance as a potential therapy for MS. *Mult Scler.* (2015) 21:1223–38. doi: 10.1177/1352458515581441

65. Procaccini C, Rosa D, Pucino V, Formisano V, Matarese LG. Animal models of multiple sclerosis. *Eur J Pharmacol.* (2015) 759:182–91. doi: 10.1016/j.ejphar.2015.03.042

66. Stromnes IM, Cerretti LM, Liggitt D, Harris RA, Goverman JM. Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. *Nat Med.* (2008) 14:337–42. doi: 10.1038/nm1715

67. Pierson ER, Wagner C, Goverman JM. The contribution of neutrophils to CNS autoimmunity. *Clin Immunol.* (2018) 189:23–8. doi: 10.1016/j.clim.2016.06.017

68. Abromson-Leeman S, Bronson R, Luo Y, Berman M, Leeman R, Leeman J, et al. T-cell properties determine disease site, clinical presentation, and cellular pathology of experimental autoimmune encephalomyelitis. *Am J Pathol.* (2004) 165:1519–33. doi: 10.1016/S0002-9440(10)63410-4

69. Shetty A, Gupta SG, Varrin-Doyer M, Weber MS, Prod'Homme T, Molnarfi N, et al. Immunodominant T-cell epitopes of MOG reside in its transmembrane and cytoplasmic domains in EAE. *Neurol Neuroimmunol Neuroinflamm.* (2014) 1:e22. doi: 10.1212/NXI.0000000000000022

70. Slavin A, Ewing C, Liu J, Ichikawa M, Slavin J, Bernard CC, et al. Induction of a multiple sclerosis-like disease in mice with an immunodominant epitope of myelin oligodendrocyte glycoprotein. *Autoimmunity.* (1998) 28:109–20. doi: 10.3109/08916939809003872

71. Hidaka Y, Inaba Y, Matsuda K, Itoh M, Kaneyama T, Nakazawa Y, et al. Cytokine production profiles in chronic relapsing-remitting experimental autoimmune encephalomyelitis: IFN-γ and TNF-α are important participants in the first attack but not in the relapse. *J Neurol Sci.* (2014) 340:117–22. doi: 10.1016/j.jns.2014.02.039

72. Baker D, Nutma E, O'Shea H, Cooke A, Orian JM, Amor S, et al. Autoimmune encephalomyelitis in NOD mice is not initially a progressive multiple sclerosis model. *Ann Clin Transl Neurol.* (2019) 6:1362–72. doi: 10.1002/acn3.792

73. Basso AS, Frenkel D, Quintana FJ, Costa-Pinto FA, Petrovic-Stojkovic S, Puckett L, et al. Reversal of axonal loss and disability in a mouse model of progressive multiple sclerosis. *J Clin Invest.* (2008) 118:1532–43. doi: 10.1172/JCI33464

74. Jayaraman A, Soni A, Prabhakar BS, Holterman M, Jayaraman S. The epigenetic drug trichostatin A ameliorates experimental autoimmune encephalomyelitis *via* T cell tolerance induction and impaired influx of T cells into the spinal cord. *Neurobiol Dis.* (2017) 108:1–12. doi: 10.1016/j.nbd.2017.07.015

75. Jayaraman A, Sharma M, Prabhakar B, Holterman M, Jayaraman S. Amelioration of progressive autoimmune encephalomyelitis by epigenetic regulation involves selective repression of mature neutrophils during the preclinical phase. *Exp Neurol.* (2018) 304:14–20. doi: 10.1016/j.expneurol.2018.02.008

76. Jayaraman A, Avgush K, Kulam R, Soni A, Khan A, Kerdjoudj M, et al. Treatment of autoimmune encephalomyelitis with a histone deacetylase inhibitor. Analyzing the role of immune-response genes. *Free Neuropathol.* (2020) 1:19. doi: 10.17879/freeneuropathology-2020-2819

77. Buonvicino D, Ranieri G, Chiarugi A. Treatment with non-specific HDAC inhibitors administered after disease onset does not delay evolution in a mouse model of progressive multiple sclerosis. *Neuroscience.* (2021) 465:38–45. doi: 10.1016/j.neuroscience.2021.04.002

78. Camelo S, Iglesias AH, Hwang D, Due B, Ryu H, Smith K, et al. Transcriptional therapy with the histone deacetylase inhibitor trichostatin A ameliorates experimental autoimmune encephalomyelitis. *J Neuroimmunol.* (2005) 164:10–21. doi: 10.1016/j.jneuroim.2005.02.022

79. Ge Z, Da Y, Xue Z, Zhang K, Zhuang H, Peng M, et al. Vorinostat, a histone deacetylase inhibitor, suppresses dendritic cell function and ameliorates experimental autoimmune encephalomyelitis. *Exp Neurol.* (2012) 241:56–66. doi: 10.1016/j.expneurol.2012.12.006

80. Lv J, Du C, Wei W, Wu Z, Zhao G, Li Z, et al. The antiepileptic drug valproic acid restores T cell homeostasis and ameliorates pathogenesis of experimental autoimmune encephalomyelitis. *J Biol Chem.* (2012) 287:28656–65. doi: 10.1074/jbc.M112.356584

81. Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K, Tochino Y, et al. Breeding of a non-obese, diabetic strain of mice. *Jikken Dobutsu.* (1980) 29:1–13. doi: 10.1538/expanim1978.29.1_1

82. Damotte D, Colomb E, Cailleau C, Brousse N, Charreire J, Carnaud C, et al. Analysis of susceptibility of NOD mice to spontaneous and experimentally induced thyroiditis. *Eur J Immunol.* (1997) 27:2854–62. doi: 10.1002/eji.1830271117

83. Noorchashm H, Noorchashm N, Kern J, Rostami SY, Barker CF, Naji A, et al. B-cells are required for the initiation of insulitis and sialitis in nonobese diabetic mice. *Diabetes.* (1997) 46:941–6. doi: 10.2337/diab.46.6.941

84. Abughanam G, Maria OM, Tran SD. Studying Sjögren's syndrome in mice: What is the best available model? *J Oral Biol Craniofac Res.* (2021) 11:245–55. doi: 10.1016/j.jobcr.2020.12.001

85. Pryce G, O'Neill JK, Croxford JL, Amor S, Hankey DJ, East E, et al. Autoimmune tolerance eliminates relapses but fails to halt progression in a model of multiple sclerosis. *J Neuroimmunol.* (2005) 165:41–52. doi: 10.1016/j.jneuroim.2005.04.009

86. Zhang Z, Zhang ZY, Wu Y, Schluesener HJ. Valproic acid ameliorates inflammation in experimental autoimmune encephalomyelitis rats. *Neuroscience.* (2012) 221:140–50. doi: 10.1016/j.neuroscience.2012.07.013

87. Castelo-Branco G, Stridh P, Guerreiro-Cacais AO, Adzemovic MZ, Falcão AM, Marta M, et al. Acute treatment with valproic acid and l-thyroxine ameliorates clinical signs of experimental autoimmune encephalomyelitis and prevents brain pathology in DA rats. *Neurobiol Dis.* (2014) 71:220–33. doi: 10.1016/j.nbd.2014.08.019

88. Lipton HL, Twaddle G, Jelachich ML. The predominant virus antigen burden is present in macrophages in Theiler's murine encephalomyelitis virus induced demyelinating disease. *J Virol.* (1995) 69:2525–33. doi: 10.1128/jvi.69.4.2525-2533.1995

89. Tsunoda I, Kuang L-Q, Libbey JE, Fujinami RS. Axonal injury heralds virus-induced demyelination. *Am J Pathol.* (2003) 162:1259–69. doi: 10.1016/S0002-9440(10)63922-3

90. Tsunoda I, Tanaka T, Saijoh Y, Robert SF. Targeting inflammatory demyelinating lesions to sites of Wallerian degeneration. *Am J Pathol.* (2007) 171:1563–75. doi: 10.2353/ajpath.2007.070147

91. Mix E, Meyer-Rienecker H, Hartung HP, Zettl UK. Animal models of multiple sclerosis—potentials and limitations. *Prog Neurobiol.* (2010) 92:386–404. doi: 10.1016/j.pneurobio.2010.06.005

92. Pachner AR. The neuroimmunology of multiple sclerosis: fictions and facts. *Front Neurol.* (2022) 12:796378. doi: 10.3389/fneur.2021.796378

93. Villoslada P, Steinman L. New targets and therapeutics for neuroprotection, remyelination and repair in multiple sclerosis. *Expert Opin Investig Drugs.* (2020) 29:443–59. doi: 10.1080/13543784.2020.1757647

94. International Multiple Sclerosis Genetics Consortium, Wellcome Trust Case Control Consortium 2. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature.* (2011) 476:214–19. doi: 10.1038/nature10251

95. Koch MW, Metz LM, Kovalchuk O. Epigenetic changes in patients with multiple sclerosis. *Nat Rev Neurol.* (2013) 9:35–43. doi: 10.1038/nrneurol.2012.226

96. Allis CD, Jenuwein T. The molecular hallmarks of epigenetic control. *Nat Rev Genet.* (2016) 17:487–500. doi: 10.1038/nrg.2016.59

97. Ebers GC, Bulman DE, Sadovnick AD, Paty DW, Warren S, Hader W, et al. A population-based study of multiple sclerosis in twins. *N Engl J Med.* (1986) 315:1638–42. doi: 10.1056/NEJM198612253152603

98. Iridoy Zulet M, Pulido Fontes L, Ayuso Blanco T, Lacruz Bescos F, Mendioroz Iriarte M. Epigenetic changes in neurology: DNA methylation in multiple sclerosis. *Neurologia.* (2017) 32:463–8. doi: 10.1016/j.nrleng.2015.03.020

99. Mycko MP, Baranzini SE. microRNA and exosome profiling in multiple sclerosis. *Mult Scler.* (2020) 26:599–604. doi: 10.1177/1352458519879303

100. Jayaraman A, Zhao T, Jayaraman S. Histone modifier differentially regulates gene expression and unravels survival role of microRNA-494 in leukemic T cell. *Microrna.* (2021) 10:39–50. doi: 10.2174/2211536610666210412153322

101. Marks PA, Richon VM, Breslow R, Rifkind RA. Histone deacetylase inhibitors as new cancer drugs. *Curr Opin Oncol.* (2001) 13:477–83. doi: 10.1097/00001622-200111000-00010

102. Ruijter D, van Gennip AJ, Caron AH, Kemp HN, van Kuilenburg SAB. Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem J.* (2003) 370:737–49. doi: 10.1042/bj20021321

103. Croft M, Duncan DD, Swain SL. Response of naive antigen-specific CD4+ T cells in vitro: characteristics and antigen-presenting cell requirements. *J Exp Med.* (1992) 176:1431–7. doi: 10.1084/jem.176.5.1431

104. Schwartz RH, Mueller DL, Jenkins MK, Quill H. T-cell clonal anergy. *Cold Spring Harb Symp Quant Biol.* (1989) 54:605–10. doi: 10.1101/SQB.1989.054.01.072

105. Rumble JM, Huber AK, Krishnamoorthy G, Srinivasan A, Giles DA, Zhang X, et al. Neutrophil-related factors as biomarkers in EAE and MS. *J Exp Med.* (2015) 212:23–35. doi: 10.1084/jem.20141015

106. Casserly CS, Nantes JC, Whittaker Hawkins RF, Vallières L. Neutrophil perversion in demyelinating autoimmune diseases: Mechanisms to medicine. *Autoimmun Rev.* (2017) 16:294–307. doi: 10.1016/j.autrev.2017.01.013

107. Nakahama T, Hanieh H, Nguyen NT, Chinen I, Ripley B, Millrine D, et al. Aryl hydrocarbon receptor-mediated induction of the microRNA-132/212 cluster promotes interleukin-17-producing T-helper cell differentiation. *Proc Natl Acad Sci USA.* (2013) 110:11964–9. doi: 10.1073/pnas.1311087110

108. Tran EH, Hardin-Pouzet H, Verge G, Owens T. Astrocytes and microglia express inducible nitric oxide synthase in mice with experimental allergic encephalomyelitis. *J Neuroimmunol.* (1997) 74:121–9. doi: 10.1016/S0165-5728(96)00215-9

109. Goldberg R, Wildbaum G, Zohar Y, Maor G, Karin N. Suppression of ongoing experimental autoimmune encephalomyelitis by neutralizing the function of the p28 subunit of IL-27. *J Immunol.* (2004) 173:6465–71. doi: 10.4049/jimmunol.173.10.6465

110. Powell ND, Papenfuss TL, McClain MA, Gienapp IE, Shawler TM, Satoskar AR, et al. Cutting edge: macrophage migration inhibitory factor is necessary for progression of experimental autoimmune encephalomyelitis. *J Immunol.* (2005) 175:5611–4. doi: 10.4049/jimmunol.175.9.5611

111. McQualter JL, Darwiche R, Ewing C, Onuki M, Kay TW, Hamilton JA, et al. Granulocyte macrophage colony-stimulating factor: a new putative therapeutic target in multiple sclerosis. *J Exp Med.* (2001) 194:873–82. doi: 10.1084/jem.194.7.873

112. Moreno M, Bannerman P, Ma J, Guo F, Miers L, Soulika AM, et al. Conditional ablation of astroglial CCL2 suppresses CNS accumulation of M1 macrophages and preserves axons in mice with MOG peptide EAE. *J Neurosci.* (2014) 34:8175–85. doi: 10.1523/JNEUROSCI.1137-14.2014

113. O'Connor RA, Cambrook H, Huettner K, Anderton SM. T-bet is essential for Th1-mediated, but not Th17-mediated, CNS autoimmune disease. *Eur J Immunol.* (2013) 43:2818–23. doi: 10.1002/eji.201343689

114. Martinez NE, Sato F, Omura S, Kawai E, Takahashi S, Yoh K, et al. RORγt, but not T-bet, overexpression exacerbates an autoimmune model for multiple sclerosis. *J Neuroimmunol.* (2014) 276:142–9. doi: 10.1016/j.jneuroim.2014.09.006

115. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science.* (2003) 299:1057–61. doi: 10.1126/science.1079490

116. Danikowski KM, Jayaraman S, Prabhakar B. Regulatory T cells in multiple sclerosis and myasthenia gravis. *J Neuroinflammation.* (2017) 14:117. doi: 10.1186/s12974-017-0892-8

117. Jayaraman S, Prabhakar B. Immune tolerance in autoimmune central nervous system disorders. In: Mitoma H, Manto M, editor. *Neuroimmune diseases; from cells to the living brain.* Berlin: Springer (2019). p. 143–66. doi: 10.1007/978-3-030-19515-1_5

118. Bradner JE, West N, Grachan ML, Greenberg EF, Haggarty SJ, Warnow T, et al. Chemical phylogenetics of histone deacetylases. *Nat Chem Biol.* (2010) 6:238–43. doi: 10.1038/nchembio.313

119. Broide RS, Redwine JM, Aftahi N, Young W, Bloom FE, Winrow CJ, et al. Distribution of histone deacetylases 1-11 in the rat brain. *J Mol Neurosci.* (2007) 31:47–58. doi: 10.1007/BF02686117

120. Sun L, Telles E, Karl M, Cheng F, Luetteke N, Sotomayor EM, et al. Loss of HDAC11 ameliorates clinical symptoms in a multiple sclerosis mouse model. *Life Sci Alliance.* (2018) 1:e201800039. doi: 10.26508/lsa.201800039

121. Zhang F, Shi Y, Wang L, Sriram S. Role of HDAC3 on p53 expression and apoptosis in T cells of patients with multiple sclerosis. *PLoS ONE.* (2011) 6:e16795. doi: 10.1371/journal.pone.0016795

122. Tegla CA, Azimzadeh P, Andrian-Albescu M, Martin A, Cudrici CD, Trippe R, et al. SIRT1 is decreased during relapses in patients with multiple sclerosis. *Exp Mol Pathol.* (2014) 96:139–48. doi: 10.1016/j.yexmp.2013.12.010

123. Jayaraman A, Arianas M, Jayaraman S. Epigenetic modulation of selected immune response genes and altered functions of T lymphocytes and macrophages collectively contribute to autoimmune diabetes protection. *BBA Adv.* (2021) 1:100031. doi: 10.1016/j.bbadva.2021.100031

124. Konsoula Z, Barile FA. Epigenetic histone acetylation and deacetylation mechanisms in experimental models of neurodegenerative disorders. *J Pharmacol Toxicol Methods.* (2012) 66:215–20. doi: 10.1016/j.vascn.2012.08.001

125. Narita T, Weinert BT, Choudhary C. Functions and mechanisms of non-histone protein acetylation. *Nat Rev Mol Cell Biol.* (2019) 20:156–74. doi: 10.1038/s41580-018-0081-3

126. Fitz-James MH, Cavalli G. Molecular mechanisms of transgenerational epigenetic inheritance. *Nat Rev Genet.* (2022) 23:325–41. doi: 10.1038/s41576-021-00438-5

127. Turner BM. Histone acetylation and an epigenetic code. *Bioessays.* (2000) 22:836–45. doi: 10.1002/1521-1878(200009)22:9<836::AID-BIES9>3.0.CO;2-X

128. Robles P, Turner A, Zuco G, Adams S, Paganopolou P, Winton M, et al. Parental energy-sensing pathways control intergenerational offspring sex determination in the nematode *Auanema freiburgensis. BMC Biol.* (2021) 19:102. doi: 10.1186/s12915-021-01032-1

129. Patel T, Patel V, Singh R, Jayaraman S. Chromatin remodeling resets the immune system to protect against autoimmune diabetes in mice. *Immunol Cell Biol.* (2011) 89:640–9. doi: 10.1038/icb.2010.144

130. Jayaraman S, Patel A, Jayaraman A, Patel V, Holterman M, Prabhakar B, et al. Transcriptome analysis of epigenetically modulated genome indicates signature genes in manifestation of type 1 diabetes and its prevention in NOD mice. *PLoS ONE.* (2013) 8:e55074. doi: 10.1371/journal.pone.0055074

131. Jayaraman S. Type 1 diabetes and epigenetics. In: Zhang R, editor. *The epigenetics of autoimmunity. Translational Epigenetics Series, Volume 5.* Amsterdam: Elsevier Inc. (2018), p. 188–205. doi: 10.1016/B978-0-12-809912-4.00009-X

132. Patel V, Jayaraman A, Jayaraman S. Epigenetic reprogramming ameliorates type 1 diabetes by decreasing the generation of Th1 and Th17 subsets and restoring self-tolerance in CD4+ T cells. *Int Immunopharmacol.* (2022) 103:108490. doi: 10.1016/j.intimp.2021.108490

133. Choi J-H, Oh S-W, Kang M-S, Kwon HJ, Oh G-T, Kim D-Y, et al. Trichostatin A attenuates airway inflammation in mouse asthma model. *Clin Exp Allergy.* (2005) 35:89–96. doi: 10.1111/j.1365-2222.2004.02006.x

134. Mishra N, Reilly CM, Brown DR, Ruiz P, Gilkeson GS. Histone deacetylase inhibitors modulate renal disease in the MRL-lpr/lpr mouse. *J Clin Invest111.* (2003) 539–52. doi: 10.1172/JCI16153

135. Reilly CM, Thomas M, Gogal Jr R, Olgun S, Santo A, Sodhi R, et al. The histone deacetylase inhibitor trichostatin A upregulates regulatory T cells and modulates autoimmunity in NZB/W F1 mice. *J Autoimmun.* (2008) 31:123–30. doi: 10.1016/j.jaut.2008.04.020

136. Glauben R, Batra A, Fedke I, Zeitz M, Lehr HA, Leoni F, et al. Histone hyperacetylation is associated with amelioration of experimental colitis in mice. *J Immunol.* (2006) 176:5015–22. doi: 10.4049/jimmunol.176.8.5015

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Genetic risk variants for multiple sclerosis are linked to differences in alternative pre-mRNA splicing

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Background: Multiple sclerosis (MS) is a chronic immune-mediated disease of the central nervous system to which a genetic predisposition contributes. Over 200 genetic regions have been associated with increased disease risk, but the disease-causing variants and their functional impact at the molecular level are mostly poorly defined. We hypothesized that single-nucleotide polymorphisms (SNPs) have an impact on pre-mRNA splicing in MS.

Methods: Our study focused on 10 bioinformatically prioritized SNP–gene pairs, in which the SNP has a high potential to alter alternative splicing events (ASEs). We tested for differential gene expression and differential alternative splicing in B cells from MS patients and healthy controls. We further examined the impact of the SNP genotypes on ASEs and on splice isoform expression levels. Novel genotype-dependent effects on splicing were verified with splicing reporter minigene assays.

Results: We were able to confirm previously described findings regarding the relation of MS-associated SNPs with the ASEs of the pre-mRNAs from *GSDMB* and *SP140*. We also observed an increased *IL7R* exon 6 skipping when comparing relapsing and progressive MS patients to healthy subjects. Moreover, we found evidence that the MS risk alleles of the SNPs rs3851808 (*EFCAB13*), rs1131123 (*HLA-C*), rs10783847 (TSFM), and rs2014886 (*TSFM*) may contribute to a differential splicing pattern. Of particular interest is the genotype-dependent exon skipping of *TSFM* due to the SNP rs2014886. The minor allele T creates a donor splice site, resulting in the expression of the exon 3 and 4 of a short *TSFM* transcript isoform, whereas in the presence of the MS risk allele C, this donor site is absent, and thus the short transcript isoform is not expressed.

Conclusion: In summary, we found that genetic variants from MS risk loci affect pre-mRNA splicing. Our findings substantiate the role of ASEs with respect to the genetics of MS. Further studies on how disease-causing genetic variants

may modify the interactions between splicing regulatory sequence elements and RNA-binding proteins can help to deepen our understanding of the genetic susceptibility to MS.

KEYWORDS

B cells, genetic disease risk, splicing reporter minigene assay, multiple sclerosis, single-nucleotide polymorphisms, TSFM, alternative splicing

Introduction

Multiple sclerosis (MS) is a chronic immune-mediated and neurodegenerative disease of the central nervous system (CNS) (1, 2). Approximately 2.8 million people worldwide suffer from MS, with women being affected two to three times more often than men and with an average age at diagnosis of 32 years (3, 4). MS is classified into three different clinical courses: relapsing–remitting MS (RRMS) as the most common form (~85% of initial diagnoses), secondary progressive MS (SPMS), and primary progressive MS (PPMS) (~15% of initial diagnoses) (5–7). Clinically, RRMS is characterized by episodes of disease (relapses) followed by a partial recovery of symptoms (remissions). As the neurological deficits worsen with disease progression, approximately 80% of the RRMS cases convert to SPMS within 25 years after the diagnosis (6, 8, 9). PPMS and SPMS are characterized by a continuous worsening of symptoms without significant recovery. The symptoms of MS include, among others, limited mobility, impaired vision, and cognitive deficits (10). The severity of disability is usually determined by the Expanded Disability Status Scale (EDSS) (11).

The immune system plays a key role in the pathophysiology of MS. Immune cells infiltrate the CNS across the blood–brain barrier, leading to demyelination, neuroaxonal damage, the loss of synapses, and reactive gliosis (1, 8, 12). The disruption of neuronal signal transmission then results in clinical symptoms. Approaches to the management of MS include the treatment of acute relapses (13, 14), symptomatic therapies (15), and therapies to prevent relapses and slow the progression of disability (16–18). B cells are central players in the pathogenesis of MS as they mediate cytokine production, antigen presentation, intrathecal antibody synthesis, and the formation of ectopic follicles (19). As new research findings on MS immunopathology further underlined the functional role of B cells, disease-modifying drugs that mediate the depletion of B cells are now commonly used (20–24).

The etiology of MS is still unclear. However, environmental and lifestyle factors, such as smoking, adolescent obesity, and Epstein–Barr virus (EBV) infection, as well as genetic predisposition have been identified as risk factors contributing to the development of MS (25–29). Single-nucleotide polymorphisms (SNPs), the variations of single base pairs at specific genome locations, are the most common type of genetic risk factors (30, 31). Genome-wide association studies (GWASs) have been used to identify associations between SNP alleles and disease. In the most recent GWAS of MS, 233 SNPs have been associated with a significantly increased risk of developing MS [MS-associated lead SNPs (MS SNPs)] (32). However, considering the tendency of proximal SNPs to be inherited together (33), SNPs that are in linkage disequilibrium (LD) with an MS SNP are also associated with MS. Most disease-associated SNPs are considered to have regulatory implications, which means that they are colocalized with quantitative trait loci (QTLs) and thus can affect, e.g., gene expression (eQTL) or alternative splicing (sQTL) (34–38).

Precursor messenger RNA (pre-mRNA) splicing is a physiological process in the cell nucleus by which the introns (intragenic regions) of a pre-mRNA are cut out and the remaining exons (expressed regions) are joined together to form a mature mRNA molecule (39). The cotranscriptional splicing process is coordinated by a complex interplay of *cis*-elements, *trans*-acting factors, and the spliceosome complex, which consists of five small nuclear ribonucleoproteins (snRNPs) (40, 41). The important sequences within the pre-mRNA are 5' and 3' splice sites (donor and acceptor, respectively), the branch point, the polypyrimidine tract, and exonic or intronic motifs to enhance or silence splicing (42–45). The RNA-binding proteins (RBPs) that recognize these sequences are important for the recruitment of the spliceosome complex. The regulation of the splicing process enables the use of different splice sites, which, in turn, leads to alternative splicing and thus to an altered exon usage compared to the canonical splicing. This allows for the generation of various mRNAs from one pre-mRNA, resulting in a broad transcriptome diversity.

There are five basic types of alternative splicing events (ASEs). While during *exon skipping*, an exon is excised and not inserted into the mRNA, during *intron retention*, an intron is not removed and remains in the mRNA molecule. The use of different splice sites can also result in *mutually exclusive exons*, where only one of two possible exons occurs in the mRNA, or in

exons with different lengths due to the use of different *acceptor* or *donor splice sites* (46). In addition to the physiological role of alternative splice sites, genetic variants, such as SNPs, can alter the splicing pattern and thereby contribute to the risk of developing diseases (47). As the majority of ASEs in the human EST database are not conserved in mice (48), investigations on the splicing pattern in the experimental autoimmune encephalomyelitis (EAE) mouse model for MS are limited, and thus studies with MS patients are needed. We previously reviewed studies in which ASEs in association with MS have been investigated and found that alternative splicing in MS has been little studied so far (49). The most prominent example is exon 6 skipping in the transcript for the interleukin-7 receptor (*IL7R*) dependent on SNP rs6897932 (50).

In this study, we investigated ASEs related to SNPs in genetic loci associated with the risk of MS. For this purpose, we used a bioinformatic approach to identify SNPs that potentially alter splicing in MS. We then measured the expression of genes and of individual exons and exon–exon junctions in B cells from MS patients and healthy individuals and analyzed whether the expression is related to MS and/or the SNP. We further used splicing reporter minigene assays to verify alternative pre-mRNA splicing dependent on the genotype of the SNPs. Our study provides new insights into the molecular pathomechanisms of MS by exploring the putative functional role of genetic variants associated with disease susceptibility.

Methods

This study is divided into *in silico*, *ex vivo*, and *in vitro* parts (Figure 1). A detailed description of all methods is provided in the supplement (Supplementary file).

Selection of multiple sclerosis–associated genetic variants that may alter pre-mRNA splicing

Using publicly available microarray data sets and a literature-based screening, we identified differentially spliced candidate genes in MS that are encoded less than 250 kb away

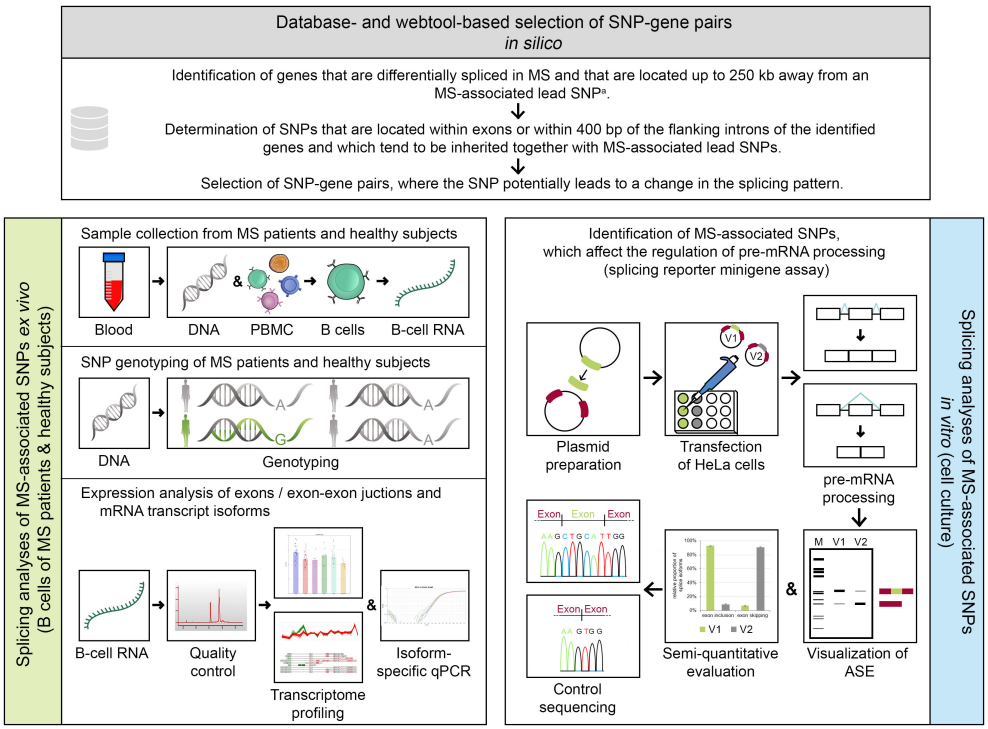

FIGURE 1
Methodical overview of the study. An *in silico* approach (serial workflow) was employed to identify single-nucleotide polymorphism (SNP)–gene pairs, where the SNP has the potential to alter the splicing pattern of a gene. For the selected SNP–gene pairs, *ex vivo* and *in vitro* analyses were conducted. [a] Multiple sclerosis (MS)–associated lead SNP according to the genome-wide association study (GWAS) of MS from 2019 (32). ASE, alternative splicing event; bp, base pairs; GWAS, genome-wide association study; kb, kilobases; MS, multiple sclerosis; PBMC, peripheral blood mononuclear cells; qPCR, quantitative real-time PCR polymerase chain reaction; SNP, single-nucleotide polymorphism; V1/V2, minigene construct variants, which differ in only one base and thus represent the two allelic variants of a SNP (here, V1 shows constitutive splicing and V2 shows alternative splicing).

from an MS SNP (32). We then determined SNPs that are at least in mild LD ($r^2 > 0.1$ and $D' > 0.7$) with the MS SNPs and are located within exons or adjacent intronic regions (up to 400 bp from the exon) of the genes. By using the splice prediction tool Human Splicing Finder (51) and the POSTAR2 database (52), we finally prioritized 10 SNP–gene pairs, in which the SNP has the potential to alter the splicing pattern of the gene (hereafter referred to as splice SNP).

Study cohort

As part of the research projects of the Neuroimmunology research group at Rostock University Medical Center, a total of 121 blood samples were collected and DNA and B-cell RNA were extracted as described previously (53). The subjects were divided into the following three subgroups: healthy controls, PPMS patients, and RRMS patients. MS patients were diagnosed according to the 2017 revisions of the McDonald criteria (54). The diagnosis, treatment, and monitoring of the patients followed routine clinical practice. For further details on the study cohort and the B-cell samples, the reader is referred to our previously published study (53).

Single-nucleotide polymorphism genotyping

The genotyping of the 10 bioinformatically determined splice SNPs was performed with the DNA extracted from the blood samples. For the genotyping, we used custom TaqMan® Array Cards (Applied Biosystems). Data analysis was performed in an automated manner using the TaqMan Genotyper Software (version 1.6, Applied Biosystems). The genotype assignments were manually validated. In case of failed genotyping, the SNP was not considered for further analyses.

Transcriptome analysis

The isolated B-cell RNA samples were used to perform high-density microarray measurements. This was done with Clariom D arrays for human (Thermo Fisher Scientific), which allow to examine the expression of more than 130,000 protein-coding and non-protein-coding genes (transcript clusters, TC probe sets). The arrays are designed using six oligonucleotide probes for each probe selection region (PSR), mostly identical with an exon, and four probes for all presumptive exon–exon junctions (junction probe set, JUC), which enables the analysis of expression differences with respect to single exons or exon–exon junctions. Sample preparation and microarray hybridization were conducted as described in the supplementary methods (Supplementary file). Based on the transcriptome data for all 121 samples, we tested for differential gene expression and splicing pattern differences in MS patients *vs.* healthy controls as well as in the dependence of the splice SNP genotypes. The analysis of the microarray data was accomplished by using the Transcriptome Analysis Console (TAC) software (version 4.0.2, Applied Biosystems).

Verification of splice isoform expression *via* quantitative real-time PCR

After the transcriptome analysis, sufficient material was available for 109 of the 121 B-cell RNA samples to perform transcript isoform expression measurements by quantitative (real-time) PCR (qPCR) assays. Custom TaqMan® Gene Expression Array Cards (Thermo Fisher Scientific) were used for this analysis. For each of the 10 SNP–gene pairs, two qPCR assays were used to distinguish the different transcript isoforms resulting from the specific ASEs under scrutiny (e.g., exon skipping *vs.* exon inclusion). If a transcript isoform could not be detected within 45 PCR cycles, the missing C_T values were imputed with the R package nondetects (55). Primary data analysis was conducted by using the ExpressionSuite software (version 1.3, Thermo Fisher Scientific). The data were normalized and converted to the linear scale (Supplementary file).

Splicing reporter minigene assay

Seven SNP–gene pairs were subjected to splicing reporter minigene assays. The minigene assay is based on the principle of the transient transfection of cells with a vector containing the genomic region of interest cloned between two constitutive exons. Our minigene constructs were generated using the pDESTsplice vector and synthesized genomic sequences cloned into the pDONR221 vector (BioCat). The pDESTsplice vector was kindly gifted by Stefan Stamm (56) (Addgene plasmid #32484; http://n2t.net/addgene:32484; RRID: Addgene_32484). For each SNP–gene pair, our minigene assays always consisted of two minigene constructs that differed in a single base and thus represented the two SNP allele variants. HeLa cells were transiently transfected with the minigene constructs. RNA from the HeLa cells was isolated 24 h after the transfection and used for RT-PCR. The PCR products were visualized by gel electrophoresis and validated by sequencing. The distribution of splice isoforms was evaluated by determining the intensity of the PCR product bands on the gel with the Image Studio Lite software version 5.2 (LI-COR Biosciences).

Statistics

Statistical analyses were performed in R (version 4.0) and the TAC software (version 4.0.2). For descriptive statistics, the (robust) means and standard deviations (SD) per group were either calculated in R or directly obtained from the TAC software. We computed linear models and performed pairwise comparisons with Tukey *post-hoc* tests by using either the limma (57) framework in TAC or the R packages car (58) and stats. For the evaluation of the minigene assay outcomes, we performed two-way analyses of variance (ANOVA) to test whether the relative transcript abundance can be explained by an interaction between the splice SNP allele and the splice isoform. For all analyses, a significance level of $\alpha = 0.01$ was chosen to indicate significant differences in expression and splicing, respectively. This cutoff was chosen to provide a balance between multiple testing and exploratory investigations. The data were visualized with bar plots and beeswarm plots.

Results

Prioritization of splice single-nucleotide polymorphisms in multiple sclerosis–associated genetic loci

We identified a total of 10 SNP–gene pairs in which the splice SNP has the potential to influence pre-mRNA splicing and for which we sought an experimental validation of the determined ASEs in this work (Table 1; Figure S1). For three SNP–gene pairs (genes: *GSDMB*, *IL7R*, and *SP140*), an aberrant alternative splicing in MS has already been described in the literature (49).

TABLE 1 Prioritized SNPs from MS–associated genetic regions that potentially alter the splicing pattern of eight genes.

Gene	MS SNP identifier	Splice SNP identifier	Splice SNP position[b]	Alleles splice SNP[c]	Global allele frequency splice SNP	MS RA	LD (EUR)		Exon (Ensembl transcript ID)	Dist. splice SNP to exon (bp)[b]	Splicing motif[d]	ASE
							r^2	D'				
CLEC16A	rs2286974	rs11074944	chr16:11003696	G/A	G: 91.15%; A: 8.85%	G	0.10	1.00	exon 11 (ENST00000409790)	+ 391 (3')	ISE/ISS	alt. 5' donor site
CLEC16A	rs6498163	rs3214361	chr16:11125905	C/-	C: 60.88%; -: 39.12%	C	0.17	0.83	exon 22 (ENST00000409790)	- 74 (5')	branch point	alt. last exon
EFCAB13	rs11079784	rs3851808	chr17:47347778	C/T	T: 61.41%; C: 38.59%	C	0.55	0.98	exon 9 and 10 (ENST00000331493)	- 30 (5', exon 9)	branch point	exon skipping
GSDMB[a]	rs9909593	rs11078928	chr17:39908216	T/C	T: 62.74%; C: 37.26%	C	0.90	0.98	exon 6 (ENST00000418519)	- 2 (5')	acceptor site	exon skipping
HLA-C	rs9266629	rs1131123	chr6:31271601	G/T	G: 51.57%; T: 48.43%	T	0.13	0.71	exons 2-3 (ENST00000640219)	0 (exon 3)	donor site, ESE/ESS	intron retention
IL7R[a]	rs10063294	rs6897932	chr5:35874473	C/T	C: 76.97%; T: 23.03%	C	0.30	1.00	exon 6 (ENST00000303115)	0	ESE/ESS	exon skipping
NCAPH2	rs140522	rs2782	chr22:50523425	C/T	C: 66.04%; T: 33.96%	T	0.75	0.99	exons 19-20 (ENST00000299821)	0 (exon 20)	ESE/ESS	intron retention, alt. last exon
SP140[a]	rs35540610	rs28445040	chr2:230245867	C/T	C: 85.07%; T: 14.93%	T	0.73	0.99	exon 7 (ENST00000420434)	0	ESE/ESS	exon skipping
TSFM	rs701006	rs2014886	chr12:57783654	C/T	C: 59.61%; T: 40.39%	C	0.62	0.93	exons 3 and 4 (ENST00000417094)	+ 2 (3', exon 3)	donor site	exon skipping
TSFM	rs701006	rs10783847	chr12:57802664	G/A	G: 55.36%; A: 44.64%	G	0.62	0.92	exons 6 and 7 (ENST00000550559)	0 (exon 7)	ESE/ESS	exon skipping, alt. last exon

Ten SNPs (splice SNPs) that are in LD with nine MS SNPs from the latest GWAS (32) were identified. Those splice SNPs are located in exons or in the adjacent intronic sequences of eight genes and are suspected to alter the splicing pattern. According to splice prediction algorithms, databases, and the existing literature, the splice SNPs potentially lead to the alterations of the branch point, an ESE/ESS, an ISE/ISS, an acceptor splice site, or a donor splice site (splicing motif). We identified four different types of ASEs: alt. 5' donor site (n = 1), alt. last exon (n = 3), exon skipping (n = 6), and intron retention (n = 2). The allele distribution according to dbSNP build 151 and the splice SNP allele correlating with the MS risk allele of the MS SNP are indicated (MS RA). [a] For 3 of the 10 SNP–gene pairs, alternative splicing in MS has already been described in the literature (49). [b] Distances and positions according to the GRCh38 reference genome assembly. [c] Allele variant annotation for the + strand of the reference genome. [d] It is usually difficult to distinguish whether a genetic variant weakens a splicing enhancer or augments a splicing silencer. alt., alternative; ASE, alternative splicing event; bp, base pairs; dist., distance; ESE, exonic splicing enhancer; ESS, exonic splicing silencer; EUR, European population; GWAS, genome-wide association study; ISE, intronic splicing enhancer; ISS, intronic splicing silencer; LD, linkage disequilibrium; MS, multiple sclerosis; MS SNP, MS-associated lead single-nucleotide polymorphism; r^2 and D'; the measures of LD between MS SNP and splice SNP; SNP, single-nucleotide polymorphism.

The splice SNPs are located within an exonic region (n = 5) or within 400 bp of the adjacent intronic regions (n = 5), with all but one of the intronic SNPs being located less than 100 bp from the exon. Two of the 10 splice SNPs are in complete LD (*D'* = 1) with the MS SNP (32), implying that one SNP allele is always inherited together with one specific MS SNP allele.

In total, we determined four different types of ASEs for the 10 SNP–gene pairs. In most cases, exon skipping was found (n = 6). Moreover, we identified the ASEs intron retention (n = 2), alternative 5' donor site (n = 1), and alternative last exon (n = 3). Note that in two cases (*TSFM* exon 6 and 7 skipping and *NCAPH2* intron 19 retention), the ASE coincided with the usage of an alternative last exon.

Characteristics of the study cohort groups

A total of 121 blood samples were collected. We obtained 28 samples from healthy controls, 13 samples from PPMS patients, and 80 samples from RRMS patients. The PPMS patients were treated with glucocorticoids. The RRMS samples were taken from patients receiving alemtuzumab (n = 38), natalizumab (n = 29), cladribine (n = 6), fingolimod (n = 3), glatiramer acetate (n = 3), or interferon beta-1b (n = 1).

The sex ratio was relatively balanced in the PPMS group, whereas there was a non-significant preponderance of women in the healthy control group and the RRMS group (Table 2). In terms of age, the healthy controls, with an average age of 28.0 years, were significantly younger than the MS patients (mean age: PPMS: 58.7 years, RRMS: 36.1 years, $p < 0.001$). The mean disease duration was similar for PPMS patients and RRMS patients. RRMS patients had an average of 0.4 relapses in the year prior to the blood collection and a mean EDSS score of 2.7. PPMS patients had a considerably higher degree of disability, with an average EDSS score of 4.9 ($p < 0.001$). There were no major imbalances in the demographic and clinical data between the SNP genotype groups (Supplementary Table S8, Supplementary file).

Differential gene expression and alternative splicing in B cells

The transcriptome data for the 121 B-cell RNA samples were used to test the prioritized genes for differential gene expression and differential alternative splicing. Comparing the gene expression between the study groups, we found a significantly lower *IL7R* mRNA expression in MS patients as compared to healthy controls (Table 3). For two genes, we observed a significant association with the splice SNP genotype. The transcript levels of *EFCAB13* were significantly higher when the MS risk allele C of splice SNP rs3851808 was present. For *GSDMB*, a significantly lower gene expression was observed in the homozygous carriers of the MS risk allele C of splice SNP rs11078928.

Next, we used the transcriptome data set to examine differences in the expression levels of individual exons and exon–exon junctions that distinguish certain alternative pre-mRNA splice variants. For this purpose, the data for PSR and JUC probe sets, which correspond to the ASEs of the 10 prioritized SNP–gene pairs, were compared between the study groups and the splice SNP genotypes. When the MS patients were compared with the healthy controls, an evidence of differential splicing was found for three genes (Table 4). For the probe set interrogating the exon 6 of *IL7R*, we found significantly higher levels in the healthy group, suggesting that in those individuals, the exon is frequently incorporated into the mRNA. Similarly, we measured significantly higher levels for the probe set corresponding to exon 4 of *TSFM* in healthy controls as compared to patients with MS. In addition, we found that the longer *CLEC16A* exon 11, which belongs to the ENST00000409790 transcript variant, was significantly more abundant in the B cells of MS patients (especially PPMS patients) than in those of healthy controls.

For six SNP–pairs, the levels of exons and junctions were significantly associated with the genotype of the respective splice SNP (Figure 2). In B cells from individuals that were homozygous for the MS risk alleles of the splice SNP, we detected lower levels of *GSDMB* exon 6 and higher levels of the *SP140* exon 6 to exon 8 splice junction. The exons 9 and 10 of *EFCAB13* and the intron 2 of *HLA-C* were found more likely to be included in the mRNA when the MS risk allele is present.

TABLE 2 Basic information on the study cohort.

Group	Samples (n)	Female (n)	Male (n)	Age in years, mean ± SD	Disease duration in years, mean ± SD	EDSS score, mean ± SD (MV)	Relapses in previous year, mean ± SD
Healthy subjects	28	17	11	28.0 ± 8.9	—	—	—
PPMS patients	13	5	8	58.7 ± 9.8	9.7 ± 4.6	4.9 ± 1.7	0.0 ± 0.0
RRMS patients	80	57	23	36.1 ± 10.6	8.0 ± 6.9	2.7 ± 1.3 (10)	0.4 ± 0.7

In this study, a total of 121 blood samples were analyzed. Demographic and clinical data were recorded at the time of blood collection. For 10 samples, no information was available on the patients' current degree of disability as rated by the Expanded Disability Status Scale (11). —, not available; EDSS, Expanded Disability Status Scale; MV, missing values; n, number; PPMS, primary progressive multiple sclerosis; RRMS, relapsing–remitting multiple sclerosis; SD, standard deviation.

TABLE 3 Differential gene expression in the B-cell transcriptome data set.

Gene (transcript cluster)	MS patients vs. healthy controls			Splice SNP	Genotypes		
	Group (n)	Mean ± SD	p-value		RA (n)	Mean ± SD	p-value
CLEC16A (TC1600006893.hg.1)	Healthy (n = 28)	9.14 ± 0.46	0.3654	rs11074944	2 RA (n = 110)	9.19 ± 0.47	0.8570
	PPMS (n = 13)	9.38 ± 0.56			1 RA (n = 11)	9.06 ± 0.67	
	RRMS (n = 80)	9.19 ± 0.48			0 RA (n = 0)	—	
				rs3214361		genotyping failed	
EFCAB13 (TC1700012275.hg.1)	Healthy (n = 28)	9.21 ± 0.64	0.7842	rs3851808	2 RA (n = 22)	9.69 ± 0.55	**0.0007**
	PPMS (n = 13)	9.52 ± 0.87			1 RA (n = 53)	9.36 ± 0.61	
	RRMS (n = 80)	9.31 ± 0.55			0 RA (n = 46)	9.09 ± 0.55	
GSDMB (TC1700010590.hg.1)	Healthy (n = 28)	7.99 ± 0.57	0.3921	rs11078928	2 RA (n = 12)	6.63 ± 1.24	**2.6e-06**
	PPMS (n = 13)	8.67 ± 0.96			1 RA (n = 71)	8.23 ± 0.86	
	RRMS (n = 80)	8.03 ± 1.27			0 RA (n = 38)	8.24 ± 1.20	
HLA-C (TC0600014257.hg.1)	Healthy (n = 28)	15.09 ± 0.85	0.6173	rs1131123*	2 RA (n = 33)	15.25 ± 0.86	0.2320
	PPMS (n = 13)	15.15 ± 0.83			1 RA (n = 73)	15.53 ± 0.91	
	RRMS (n = 80)	15.52 ± 0.91			0 RA (n = 15)	15.05 ± 0.75	
IL7R (TC0500007138.hg.1)	Healthy (n = 28)	11.28 ± 1.86	**2.9e-06**	rs6897932	2 RA (n = 70)	9.86 ± 2.14	0.9673
	PPMS (n = 13)	8.19 ± 0.84			1 RA (n = 41)	9.79 ± 2.02	
	RRMS (n = 80)	9.60 ± 1.94			0 RA (n = 10)	9.93 ± 1.08	
NCAPH2 (TC2200007811.hg.1)	Healthy (n = 28)	6.13 ± 0.32	0.8099	rs2782	2 RA (n = 21)	6.16 ± 0.38	0.5645
	PPMS (n = 13)	6.08 ± 0.36			1 RA (n = 65)	6.17 ± 0.40	
	RRMS (n = 80)	6.20 ± 0.45			0 RA (n = 35)	6.16 ± 0.46	
SP140 (TC0200011020.hg.1)	Healthy (n = 28)	14.48 ± 0.41	0.0178	rs28445040	2 RA (n = 6)	14.79 ± 0.43	0.9303
	PPMS (n = 13)	14.83 ± 0.70			1 RA (n = 48)	14.79 ± 0.54	
	RRMS (n = 80)	14.85 ± 0.51			0 RA (n = 67)	14.76 ± 0.54	
TSFM (TC1200012654.hg.1)	Healthy (n = 28)	5.64 ± 0.34	0.1090	rs2014886*	2 RA (n = 59)	5.75 ± 0.33	0.4091
	PPMS (n = 13)	5.62 ± 0.28			1 RA (n = 51)	5.62 ± 0.36	
	RRMS (n = 80)	5.72 ± 0.34			0 RA (n = 11)	5.61 ± 0.32	
				rs10783847	2 RA (n = 60)	5.75 ± 0.33	0.3408
					1 RA (n = 50)	5.61 ± 0.36	
					0 RA (n = 11)	5.61 ± 0.32	

The expression of the eight prioritized genes in B cells from peripheral blood is reported as Tukey biweight means and standard deviations of log2 signal intensities per group (mean ± SD).A total of 121 samples were analyzed. The numbers of samples according to the study group and splice SNP genotype are given in brackets. Significant expression differences ($p < 0.01$) are shown in bold. We observed significantly lower mRNA levels in patients with MS as compared to healthy controls for IL7R. For EFCAB13 and GSDMB, we saw a genotype-dependent gene expression. * For technical reasons, the designated splice SNP was tagged by a proximal SNP (Supplementary file). —, not available; MS, multiple sclerosis; n, number; PPMS, primary progressive MS; RA, risk allele; RRMS, relapsing–remitting MS; SNP, single-nucleotide polymorphism.

Regarding *HLA-C*, we could only evaluate the ASE type intron retention as there are no PSR/JUC probe sets on Clariom D arrays that represent transcripts in which the intron is spliced out. We also found that the two splice SNPs located in the *TSFM* gene are associated with differential alternative splicing. These two SNPs are in the proximity of the same MS SNP, and the respective ASEs presumably account for a short and long transcript isoform of *TSFM* (ENST00000417094 and ENST00000550559). We observed that the levels of the exon 4 of the short transcript were significantly lower in the presence of the MS risk allele C of splice SNP rs2014886, and that the levels of the exon 7 of the long transcript were significantly higher when the MS risk allele G of splice SNP rs10783847 is present. Note that for all six SNP–gene pairs for which the splice SNP genotype was significantly associated with exon- or junction-specific expression levels, the data always correlated with the number of risk alleles carried, i.e., the average expression of the group of heterozygotes was always between that of the two homozygous groups (Figure 2). The full results of the transcriptome data analysis, including those for probe sets that capture the respective opposite events, are provided in Supplementary Tables S9 and S10 (Supplementary file). The transcriptome data are accessible through GEO Series accession number GSE190847.

Validation of differential splice isoform expression

To confirm that the splice SNPs affect ASEs and consequently the expression of different splice isoforms, we performed qPCR

TABLE 4 Differential alternative splicing in the B-cell transcriptome data set.

Gene (PSR/JUC[a])	MS patients *vs.* healthy controls			Splice SNP	Genotypes		
	Group (n)	Mean ± SD	p-value		RA (n)	Mean ± SD	p-value
CLEC16A (PSR1600149031.hg.1, long exon 11)	Healthy (n = 28)	9.65 ± 0.29	**0.0055**	rs11074944	2 RA (n = 110)	9.86 ± 0.49	0.9622
	PPMS (n = 13)	10.03 ± 0.28			1 RA (n = 11)	10.04 ± 0.35	
	RRMS (n = 80)	9.96 ± 0.52			0 RA (n = 0)	—	
CLEC16A (PSR1600149066.hg.1, exon 22)	Healthy (n = 28)	7.62 ± 0.53	0.3295	rs3214361		genotyping failed	
	PPMS (n = 13)	7.85 ± 0.57					
	RRMS (n = 80)	7.79 ± 0.62					
EFCAB13 (JUC1700073491.hg.1, exon 9 to exon 10 junction)	Healthy (n = 28)	4.74 ± 1.25	0.5450	rs3851808	2 RA (n = 22)	6.73 ± 0.97	**5.8e-23**
	PPMS (n = 13)	5.21 ± 1.75			1 RA (n = 53)	5.01 ± 1.38	
	RRMS (n = 80)	3.96 ± 1.68			0 RA (n = 46)	3.13 ± 0.48	
GSDMB (PSR1700183459.hg.1, exon 6)	Healthy (n = 28)	9.91 ± 0.89	0.3003	rs11078928	2 RA (n = 12)	7.94 ± 0.97	**1.2e-09**
	PPMS (n = 13)	10.91 ± 1.11			1 RA (n = 71)	10.13 ± 1.04	
	RRMS (n = 80)	9.97 ± 1.54			0 RA (n = 38)	10.47 ± 1.44	
HLA-C (PSR0600200977.hg.1, exons 2 and 3 with intron 2)	Healthy (n = 28)	15.90 ± 0.79	0.6744	rs1131123*	2 RA (n = 33)	16.09 ± 0.70	**5.8e-06**
	PPMS (n = 13)	15.87 ± 0.53			1 RA (n = 73)	15.85 ± 0.68	
	RRMS (n = 80)	15.84 ± 0.83			0 RA (n = 15)	14.93 ± 0.93	
IL7R (PSR0500148308.hg.1, exon 6)	Healthy (n = 28)	10.50 ± 1.83	**4.7e-05**	rs6897932	2 RA (n = 70)	9.69 ± 1.89	0.2265
	PPMS (n = 13)	8.10 ± 0.65			1 RA (n = 41)	9.04 ± 1.90	
	RRMS (n = 80)	9.22 ± 1.77			0 RA (n = 10)	9.00 ± 0.58	
NCAPH2 (JUC2200052281.hg.1, exon 19 to exon 20 junction)	Healthy (n = 28)	5.44 ± 0.43	0.3229	rs2782	2 RA (n = 21)	5.52 ± 0.66	0.7605
	PPMS (n = 13)	5.69 ± 0.79			1 RA (n = 65)	5.42 ± 0.56	
	RRMS (n = 80)	5.46 ± 0.65			0 RA (n = 35)	5.56 ± 0.73	
SP140 (JUC0200064656.hg.1, exon 6 to exon 8 junction)	Healthy (n = 28)	8.62 ± 1.25	0.4886	rs28445040	2 RA (n = 6)	11.24 ± 0.61	**1.4e-31**
	PPMS (n = 13)	9.25 ± 1.19			1 RA (n = 48)	9.78 ± 0.68	
	RRMS (n = 80)	8.77 ± 1.24			0 RA (n = 67)	7.97 ± 0.70	
TSFM (PSR1200200788.hg.1, exon 4)	Healthy (n = 28)	5.97 ± 0.55	**0.0066**	rs2014886*	2 RA (n = 59)	5.46 ± 0.50	**5.0e-07**
	PPMS (n = 13)	5.82 ± 0.58			1 RA (n = 51)	5.78 ± 0.53	
	RRMS (n = 80)	5.57 ± 0.56			0 RA (n = 11)	6.65 ± 0.54	
TSFM (PSR1200200803.hg.1, exon 7)	Healthy (n = 28)	3.26 ± 0.40	0.8234	rs10783847	2 RA (n = 60)	3.33 ± 0.45	**0.0011**
	PPMS (n = 13)	3.37 ± 0.49			1 RA (n = 50)	3.15 ± 0.38	
	RRMS (n = 80)	3.20 ± 0.43			0 RA (n = 11)	2.85 ± 0.28	

The expression of specific exons and exon–exon junctions in B cells from the peripheral blood was analyzed for the ASEs of the 10 SNP–gene pairs. Tukey biweight means and standard deviations of log2 signal intensities are reported per group (mean ± SD). Data from a total of 121 samples were analyzed, with the number of samples per study group and splice SNP genotype given in brackets. Significant expression differences ($p < 0.01$) are shown in bold. The data indicated genotype-dependent pre-mRNA splicing for six SNP–gene pairs. * For technical reasons, the designated splice SNP was tagged by a proximal SNP (Supplementary file). [a] Summary statistics for all ASE specific PSR JUCs are provided in Supplementary Tables S9 and S10 (Supplementary file). —, not available; ASE, alternative splicing event; JUC, junction probe set; MS, multiple sclerosis; n, number; PPMS, primary progressive MS; PSR, probe selection region; RA, risk allele; RRMS, relapsing–remitting MS; SNP, single-nucleotide polymorphism.

measurements with 109 of the 121 B-cell RNA samples. Based on these data, we compared the expression of mRNA splice isoforms between MS patients and healthy controls and between the splice SNP genotypes (Table 5).

Overall, the qPCR data well reflected the transcriptome data. In line with the transcriptome data, we saw significantly higher levels of *IL7R* transcripts that contain exon 6 in the qPCR data of healthy controls compared to those of MS patients. In addition, in the presence of the MS risk allele, exons 9 and 10 of *EFCAB13* were included more frequently, exon 7 of *SP140* was skipped more frequently and exon 6 of *GSDMB* and exons 3 and 4 of *TSFM* were included at significantly lower rates (Figure 3). In the case of *TSFM* | rs2014886, the short transcript isoform (ENST00000417094) is only rarely expressed in B cells, which explains the high number of missing values. For splice SNP rs10783847 and *TSFM* exons 6 and 7 (ENST00000550559), a non-significant trend toward preferential exon inclusion has been observed for the carriers of the MS risk allele. In contrast to the transcriptome data, no genotype dependence of the ASE in *HLA-C* (intron 2 retention) was seen in the qPCR data. The detailed results of the qPCR analysis are available in Supplementary Tables S11 and S12 (Supplementary file).

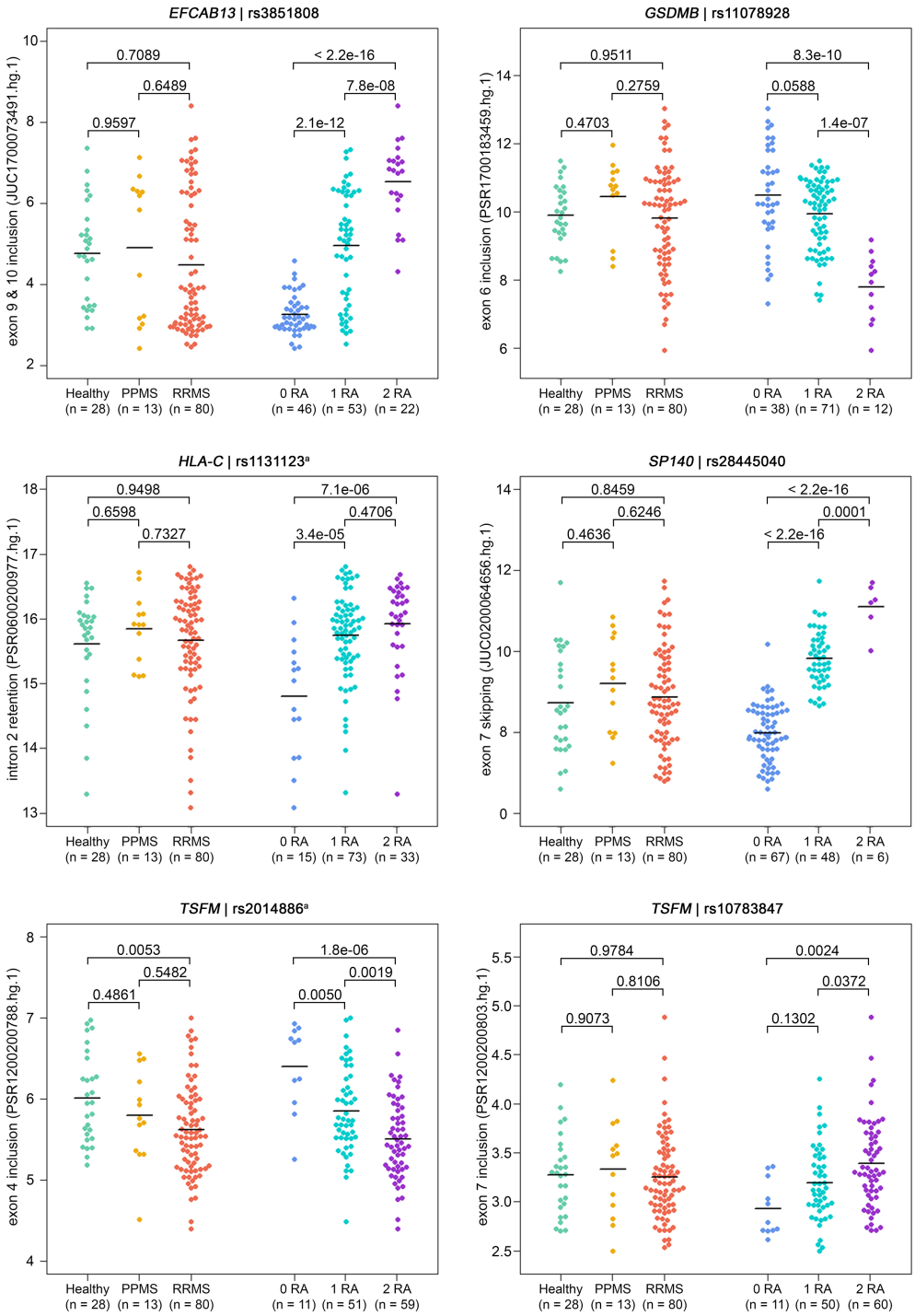

FIGURE 2
Detection of ASEs in transcriptome data from the B cells of MS patients *vs*. healthy controls and in relation to splice SNP genotypes. For all 121 samples, the expression of individual exons and exon–exon junctions was interrogated using PSR and JUCs, respectively. Signal intensities (in log2 scale) and group means (black lines) are depicted for the ASEs of the six SNP–gene pairs for which we found significant associations with the genotype (Table 4). Shown are the comparisons of expression levels between the three study groups (on the left) and between the splice SNP genotypes (on the right). P-values from pairwise Tukey *post-hoc* analyses and the numbers of samples per group are given. The numbering of exons and introns is as specified in Table 1. [a]For technical reasons, the designated splice SNP was tagged by a proximal SNP (Supplementary file). ASE, alternative splicing event; JUC, junction probe set; MS, multiple sclerosis; PPMS, primary progressive MS; PSR, probe selection region; RA, risk allele; RRMS, relapsing–remitting MS; SNP, single-nucleotide polymorphism.

TABLE 5 Differential expression of transcript isoforms in the qPCR data set.

Gene (ASE)	MS patients vs. healthy controls				Splice SNP	Genotypes			
	Group (n)	MV	Mean ± SD	p-value		RA (n)	MV	Mean ± SD	p-value
CLEC16A (long exon 11)	Healthy (n = 25)	0	75.54 ± 20.26	0.7001	rs11074944	2 RA (n = 100)	0	74.24 ± 26.50	0.0206
	PPMS (n = 11)	0	82.60 ± 20.81			1 RA (n = 9)	0	95.93 ± 26.86	
	RRMS (n = 73)	0	75.20 ± 29.92			0 RA (n = 0)	—	—	
CLEC16A (exon 22)	Healthy (n = 25)	0	132.90 ± 52.02	0.0125	rs3214361	genotyping failed			
	PPMS (n = 11)	0	148.62 ± 60.19						
	RRMS (n = 73)	0	108.27 ± 47.59						
EFCAB13 (exon 9 & 10 inclusion)	Healthy (n = 25)	5	19.77 ± 20.13	0.9909	rs3851808	2 RA (n = 18)	0	59.34 ± 42.59	**3.4e-14**
	PPMS (n = 11)	2	19.41 ± 27.52			1 RA (n = 49)	1	21.29 ± 20.96	
	RRMS (n = 73)	11	20.45 ± 32.60			0 RA (n = 42)	17	2.13 ± 3.74	
GSDMB (exon 6 inclusion)	Healthy (n = 25)	0	49.26 ± 50.89	0.8156	rs11078928	2 RA (n = 12)	0	1.19 ± 2.38	**5.4e-06**
	PPMS (n = 11)	0	61.04 ± 51.63			1 RA (n = 64)	0	50.28 ± 37.73	
	RRMS (n = 73)	0	53.02 ± 50.91			0 RA (n = 33)	0	76.99 ± 64.97	
HLA-C (without intron 2)	Healthy (n = 25)	0	5673.15 ± 4759.72	0.5373	rs1131123*	2 RA (n = 29)	0	4851.27 ± 3601.61	0.8859
	PPMS (n = 11)	0	4423.68 ± 3353.48			1 RA (n = 65)	0	4989.49 ± 3830.44	
	RRMS (n = 73)	0	4786.48 ± 3489.30			0 RA (n = 15)	0	4993.27 ± 4210.72	
IL7R (exon 6 inclusion)	Healthy (n = 25)	0	62.06 ± 35.36	**0.0015**	rs6897932	2 RA (n = 64)	0	40.71 ± 38.50	0.8838
	PPMS (n = 11)	0	17.38 ± 12.75			1 RA (n = 37)	0	46.77 ± 38.83	
	RRMS (n = 73)	0	39.26 ± 37.44			0 RA (n = 8)	0	34.17 ± 10.20	
NCAPH2 (without intron 19)	Healthy (n = 25)	0	149.13 ± 42.44	0.0495	rs2782	2 RA (n = 18)	0	147.47 ± 54.08	0.7155
	PPMS (n = 11)	0	153.54 ± 43.05			1 RA (n = 60)	0	124.46 ± 46.71	
	RRMS (n = 73)	0	126.43 ± 49.97			0 RA (n = 31)	0	145.94 ± 46.21	
SP140 (exon 7 skipping)	Healthy (n = 25)	0	88.46 ± 84.25	0.0996	rs28445040	2 RA (n = 5)	0	272.56 ± 129.84	**3.2e-18**
	PPMS (n = 11)	0	153.27 ± 173.82			1 RA (n = 41)	0	156.19 ± 94.40	
	RRMS (n = 73)	0	89.07 ± 78.93			0 RA (n = 63)	0	41.80 ± 28.00	
TSFM (exon 3 & 4 inclusion)	Healthy (n = 25)	19	0.12 ± 0.24	0.7139	rs2014886*	2 RA (n = 53)	47	0.02 ± 0.08	**1.2e-05**
	PPMS (n = 11)	9	0.10 ± 0.20			1 RA (n = 45)	24	0.24 ± 0.37	
	RRMS (n = 73)	48	0.16 ± 0.33			0 RA (n = 11)	5	0.35 ± 0.44	
TSFM (exon 6 & 7 inclusion)	Healthy (n = 25)	6	1.02 ± 0.95	0.8438	rs10783847	2 RA (n = 54)	9	1.45 ± 2.45	0.1827
	PPMS (n = 11)	3	1.32 ± 0.58			1 RA (n = 44)	6	0.98 ± 0.65	
	RRMS (n = 73)	9	1.25 ± 2.12			0 RA (n = 11)	3	0.90 ± 0.76	

Verification of ASE-dependent transcript expression in B cells by isoform-specific assays in a subset of 109 samples. Shown are group means and standard deviations of the qPCR data that were normalized and transformed to linear scale (Mean ± SD). The number of samples in which the corresponding transcript could not be detected and for which C_T values were thus imputed is indicated (MV). The structure of the table is otherwise similar to Table 4, except that for HLA-C the alternative event was considered rather than intron 2 retention due to invalid data for one of the assays used. The full summary statistics for the qPCR data analysis are given in Supplementary Tables S11 and S12 (Supplementary file). Significant expression differences ($p < 0.01$) are shown in bold. For EFCAB13, GSDMB, SP140 and TSFM, we verified the corresponding ASEs as genotype-dependent. * For technical reasons, the designated splice SNP was tagged by a proximal SNP (Supplementary file). —, not available; ASE, alternative splicing event; MS, multiple sclerosis; MV, missing values; n, number; PPMS; primary progressive MS; RA, risk allele; RRMS, relapsing-remitting MS; SNP, single-nucleotide polymorphism.

Multiple sclerosis–associated splice single-nucleotide polymorphism affects splicing pattern of *TSFM*

Since evidence of genotype-dependent splicing was found for 6 of the 10 SNP–gene pairs within the transcriptome and/or qPCR data for our study cohort, we used splicing reporter minigene assays to investigate whether the ASEs are causally related to the splice SNP allele variants. We focused on the seven ASEs that have not yet been previously studied in the samples of MS patients according to our recent systematic review (49), i.e., for *CLEC16A*, the alternative 5' donor site and the alternative last exon, exon skipping for *EFCAB13* and *TSFM*, and intron retention for *HLA-C* and *NCAPH2* (Table 1).

When the MS risk allele of the splice SNP rs2014886 is present, there is a C two nucleotides downstream of *TSFM* exon 3 (variant V1). In this case, we observed *TSFM* exon 3 skipping (Figure 4). On the other hand, when the minigene construct carried the alternative allele T (variant V2), exon 3 was frequently included between the constitutively expressed rat insulin exons (Figures 4B, C). More precisely, the creation of the donor splice site due to the allele T resulted in a significant shift in the expression of the transcript isoforms: from a

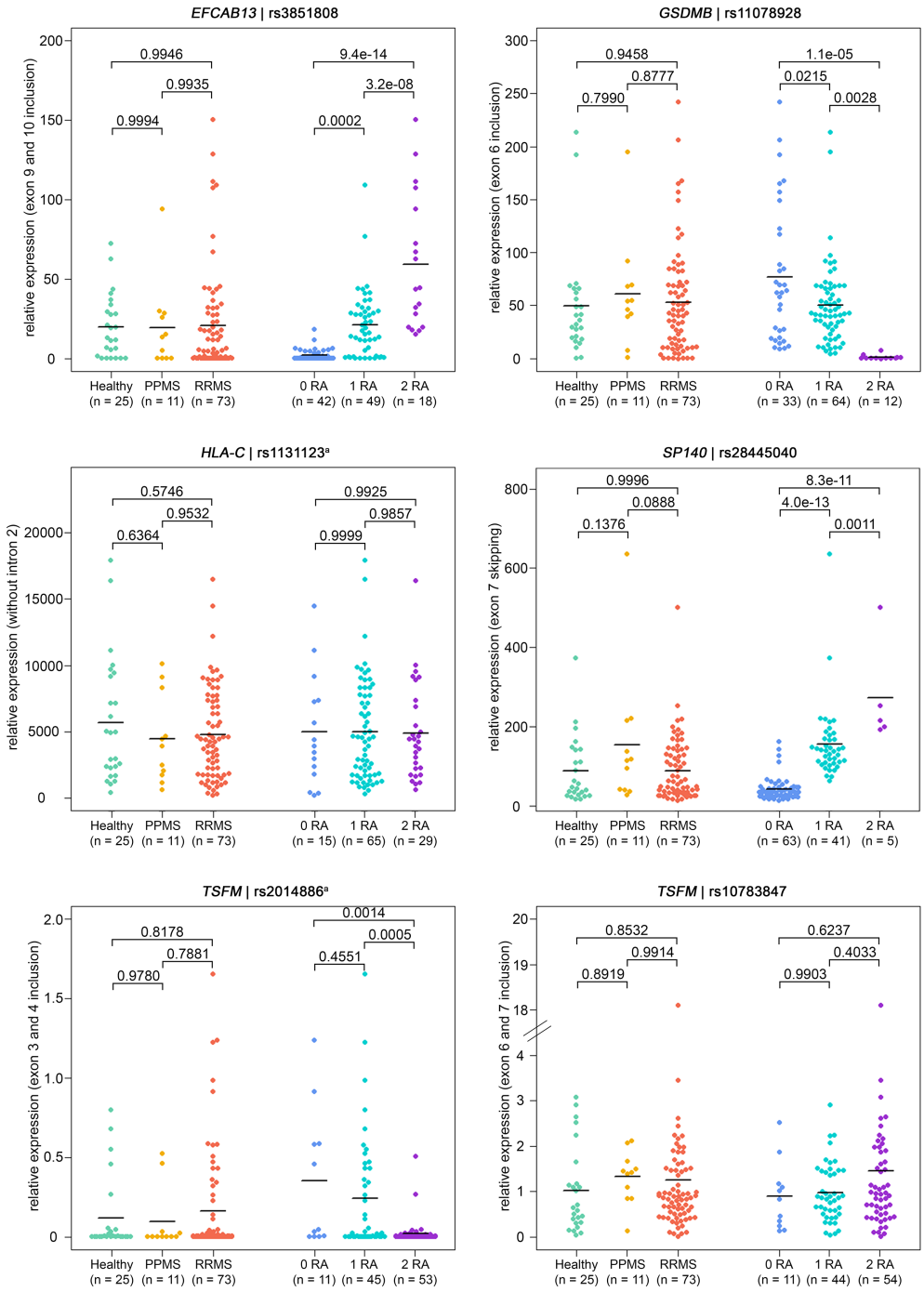

FIGURE 3
Verification of ASEs in MS patients *vs.* healthy controls and in relation to splice SNP genotypes. Relative expression as measured in B cells by qPCR (n = 109 samples). The same ASEs as in Figure 2 are visualized (but for *HLA-C* related to the isoform with intron 2 spliced out). Means per group are shown as horizontal black lines. Shown are the comparisons of mRNA isoform expression levels between the three study groups (on the left) and between the splice SNP genotypes (on the right). P-values from pairwise Tukey *post-hoc* analyses and the number of samples for each group are given. The numbering of exons and introns is as specified in Table 1. [a]For technical reasons, the designated splice SNP was tagged by a proximal SNP (Supplementary file). ASE, alternative splicing event; MS, multiple sclerosis; PPMS, primary progressive MS; qPCR, quantitative real-time PCR; RA, risk allele; RRMS, relapsing-remitting MS; SNP, single-nucleotide polymorphism.

proportion of nearly 100% exon skipping to a proportion of 61% exon skipping and 39% exon inclusion (p = 2.8e-09). We verified that the ASE of *TSFM* depends on splice SNP rs2014886 by sequencing (Figure 4D). These findings are in line with the results from the analyses of B cells with microarrays and qPCR assays (Tables 4, 5).

We also observed a preferential intron 2 retention for *HLA-C* related to the MS risk allele T of SNP rs1131123 (Figure S3). In the presence of the allele T, we saw a shift of the relative proportion of intron 2 retention from 67% to 87% (p = 3.6e-08). This is consistent with the observations from the microarray data analysis (Figure 2). However, for the other five SNP–gene pairs (*CLEC16A* | rs11074944, *CLEC16A* | rs3214361, *EFCAB13* | rs3851808, *NCAPH2* | rs2782 and *TSFM* | rs10783847), similar relative proportions of the different transcription products were obtained independently of the allelic variant, and the tests for interactions did not reach the significance level. Thus, we could not confirm that these ASEs are causally related to the splice SNP genotypes in the minigene assays (Figure S3).

Discussion

In this study, we combined *in silico* evaluations to identify SNPs that may alter pre-mRNA splicing with expression analyses of B cells and with cell culture experiments. We demonstrate that the genotype of SNPs in LD with MS-associated genetic variants can affect pre-mRNA splicing and thus the expression of splice isoforms. We observed an association of the splice SNP genotype with the expression of exons and exon–exon junctions for six SNP–gene pairs (*EFCAB13* | rs3851808, *GSDMB* | rs11078928, *HLA-C* | rs1131123, *SP140* | rs28445040, *TSFM* | rs10783847, and *TSFM* | rs2014886) in the microarray data. The differential alternative splicing could be verified by qPCR analyses for *EFCAB13*, *GSDMB*, *SP140*, and *TSFM*. With our findings for *GSDMB* and *SP140*, we could support previous results in the literature showing that the MS-associated SNPs affect alternative splicing (49).

As a starting point, we used various bioinformatic tools to prioritize genetic variants that are likely to alter the pre-mRNA splicing of MS risk genes. We here focused on SNPs located in an exon or within 400 bp of the adjacent intronic regions of these genes. According to previous studies, most splicing factor motifs can be found within this selected 400 bp window (59–61). For the prediction of splicing events due to genetic variants, different tools and databases are available (62–64). We used the Human Splicing Finder to investigate whether a SNP may affect a *cis*-element such as a branch point, a splice site, or an exonic/intronic splicing enhancer or silencer, and we used the POSTAR2 database to identify SNPs in experimentally determined RBP-binding sites. Finally, we determined 10 SNP–gene pairs (10 SNPs and 8 different genes) for the further event-focused investigations. The reliability of our splice SNP selection procedure was supported by the identification of ASEs for *GSDMB* | rs11078928, *IL7R* | rs6897932, and *SP140* | rs28445040 as an aberrant genotype-dependent splicing in MS has been previously described for these

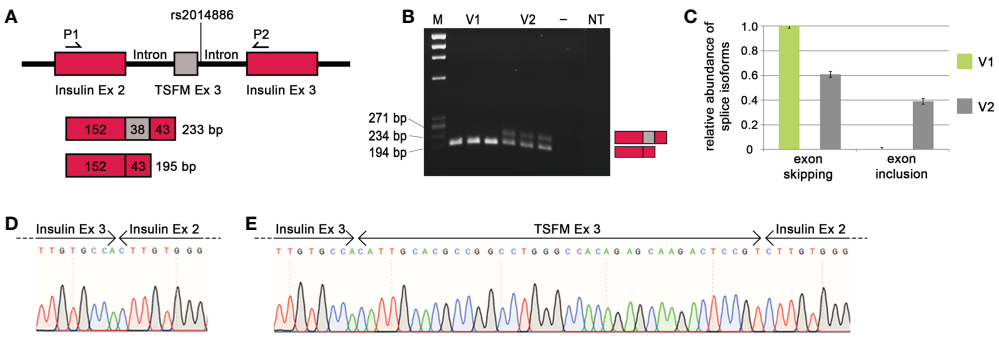

FIGURE 4
Effect of MS-associated splice SNP on *TSFM* exon 3 skipping. **(A)** Simplified depiction of the minigene assay for *TSFM*. The sequences of *TSFM* exon 3 (gray box) as well as 400 bp of the up- and downstream introns were cloned between rat insulin exon 2 and 3 (burgundy boxes) of the pDESTsplice vector. The splice SNP rs2014886 is located in the intronic region 2 nucleotides downstream of exon 3. The matching sequences for the PCR primers are located in the sequences of the rat insulin exons (P1 and P2). If *TSFM* exon 3 is included in the resulting transcript, the PCR product has a size of 233 bp. If exon 3 is skipped, the resulting PCR product has a size of 195 bp. **(B)** The PCR products for the variants V1 and V2 (from triplicate measurements) were visualized by gel electrophoresis. V1 represents the MS risk allele C and V2 represents the alternative allele T of splice SNP rs2014886. **(C)** The relative proportions of splice isoforms that resulted due to *TSFM* exon 3 skipping or *TSFM* exon 3 inclusion for the allele variants V1 (green) and V2 (gray). The MS risk allele C of splice SNP rs2014886 favors *TSFM* exon 3 skipping. The two splice isoforms were verified by reverse direction sequencing **(D, E)**. —, negative control; bp, base pairs; Ex, exon; M, size standard; MS, multiple sclerosis; NT, non-template control; P1, PCR_RatInsEx2; P2, PCR_RatInsEx3 (Supplementary Table S6, Supplementary file); SNP, single-nucleotide polymorphism.

three SNP–gene pairs (49), and the SNPs are also reported as sQTL SNPs for whole-blood and EBV-transformed lymphocyte samples in the GTEx portal (34). Most of the eight prioritized genes are expressed with a low immune cell type specificity according to the Human Protein Atlas (65). However, two of the genes are expressed more specifically in certain immune cell types: *IL7R* is expressed mainly in the subsets of T cells and natural killer cells, and *SP140* is expressed mainly in memory B cells (66).

Then, we examined the association between the genotype of splice SNPs with the expression of the genes as well as with the levels of individual exons/junctions of the distinct splice isoforms of these genes in B cells from MS patients and healthy controls. Apart from the fact that we did not include SPMS patients, the group of MS patients resembled the typical characteristics of MS patients in European MS registries in terms of age, disease status, and sex (67). In line with the literature (50, 68–70), we observed a significant differential expression of exon 6 of *IL7R* in MS patients as compared to healthy controls. In our data, the levels of transcripts containing exon 6 were lower in MS patients, but we could not find the previously described association to the MS risk allele C of the non-synonymous splice SNP rs6897932 (T244I). However, the latter might result from the fact that we studied the expression in B cells and not in T cells, in which *IL7R* is more strongly expressed (66). *IL7R* encodes for a cell surface receptor for interleukin-7, which plays an essential role for the development and survival of T cells (71). Gregory et al. reported that the C allele of SNP rs6897932 augments an exonic splicing silencer and thus promotes exon 6 skipping, leading to a splice isoform that encodes a soluble form of the protein (50). This is of relevance as increased levels of soluble interleukin-7 receptor have been shown to exacerbate the disease severity in an EAE mouse model, presumably by increasing the activity or bioavailability of interleukin-7 (72). Our analyses of B-cell RNA samples by microarrays and qPCR indicated a genotype-dependent skipping of *GSDMB* exon 6 and *SP140* exon 7. Consistent with our findings, Cardamone et al. (68), Garrido-Martín et al. (73), and Morrison et al. (74) found that the MS risk allele C of SNP rs11078928 affects the acceptor splice site of *GSDMB* exon 6, resulting in increased exon 6 skipping. The encoded protein Gasdermin-B mediates pyroptosis (75) and, in addition to MS, genetic variants in the *GSDMB* gene have also been associated with susceptibility to other multifactorial autoimmune diseases like rheumatoid arthritis (76) and ulcerative colitis (77). With regard to the genotype-dependent splicing of *SP140*, Cardamone et al. (78) and Matesanz et al. (79) could demonstrate *via* minigene assays that the MS risk allele T of SNP rs28445040 leads to the skipping of exon 7. The function of the protein encoded by *SP140* is only partially known. However, the presence of chromatin-related protein domains indicates a role in the chromatin-mediated regulation of gene expression (80). In addition, Karaky et al. reported that SP140 regulates the expression of immune-related genes that are associated with MS (81).

For four other SNP-gene pairs (*EFCAB13* | rs3851808, *HLA-C* | rs1131123, *TSFM* | rs2014886, and *TSFM* | rs10783847), we could detect differential alternative splicing in B cells in relation to the MS risk allele. We observed increased *EFCAB13* expression and preferential inclusion of exons 9 and 10 in the presence of the MS risk allele C of splice SNP rs3851808. The genotype dependency of this ASE is supported by an sQTL association that is reported for *EFCAB13* | rs3851808 for EBV-transformed lymphocytes and other cell types and tissues in the GTEx portal (34). The protein encoded by *EFCAB13* contains a calcium-binding domain that is shared by a variety of calcium sensor proteins, which play a role in neuronal function and plasticity (82, 83). Diseases implicated with calcium sensor proteins are, for instance, Alzheimer's disease (84) and various cancer types (85, 86).

For *HLA-C* | rs1131123, we observed a trend toward preferential *HLA-C* intron 2 retention in the presence of the MS risk allele T of the non-synonymous splice SNP rs1131123 (D114A). This genotype dependency was also observed with the minigene assay. *HLA-C* encodes a class I major histocompatibility complex antigen. Class I molecules play a central role in the immune system and have repeatedly been demonstrated to contribute to the genetic susceptibility to MS (87–89). However, there were challenges in examining the ASE of pre-mRNA from *HLA-C*: first, in the microarray data, we could only evaluate the expression of the transcript variant in which intron 2 is retained in the mRNA because there are no PSR/JUC probe sets for *HLA-C* intron 2 exclusion on the employed chip model. Second, only one of the two qPCR assays used to measure transcript splice isoforms of *HLA-C* provided valid data, which might be due to a sensitivity of the primer pair toward *HLA-C* subtypes. Since the SNP rs1131123 is not recorded in the GTEx portal (34), further investigations, e.g., with RNA sequencing, could be helpful to ascertain the presumed genotype-dependent splicing of *HLA-C* intron 2.

We found that exons 3 and 4 of the short transcript variant ENST00000417094 are more frequently skipped in the presence of the MS risk allele C of splice SNP rs2014886 and that this short transcript is only rarely expressed in B cells. In line with our B-cell transcriptome data and minigene assay data, an association of the C allele of SNP rs2014886 with *TSFM* exon 3 skipping was previously reported by Morrison et al. (74). However, they only studied a small study cohort of eight individuals per genotype. In a recently published report, which focused exclusively on the identification of potential cryptic exons based on literature reports and the dbSNP database, a genotype-dependent splicing of *TSFM* exon 3 was also postulated (90). Moreover, an sQTL that links the skipping of *TSFM* exon 3 and 4 with SNP rs2014886 is listed for EBV-transformed lymphocytes in the GTEx portal (34). For the second SNP–gene pair with *TSFM*, we found that the MS

risk allele G of splice SNP rs10783847 showed a strong trend toward *TSFM* exon 6 and 7 inclusion of the transcript isoform ENST00000550559. *TSFM* encodes for a mitochondrial translation elongation factor, which catalyzes the exchange of GDP to GTP (91, 92). As the respiratory chain function relies on proper mitochondrial gene expression, differential *TSFM* expression is associated with various diseases such as encephalomyopathy, hypertrophic cardiomyopathy, and MS (93–96). Noteworthy, Alcina et al. (96) described that SNP rs10877013 affects *TSFM* expression in MS by altering the enhancer activity of a regulatory element. This SNP is in almost perfect LD with the two splice SNPs rs10783847 and rs2014886 in the European population (33). Further studies are needed to better understand the functional role of the different splice isoforms of *TSFM* in relation to the pathogenesis of the multifactorial disease MS. According to the Ensembl database, ENST00000417094 codes for an 89 amino acid long protein sequence (UniProt F8WCK2) but it is likely a target of nonsense-mediated decay. However, experimental evidence remains to be established.

The following limitations should be considered when interpreting the data of this study. First, due to the stringent restrictions on the selection of experimentally screenable SNP–gene pairs, it is possible that we have missed some MS-specific ASEs. For instance, we did not include rare variants (minor allele frequency < 1%) because the sample size would be insufficient to study such variants. In addition, we focused only on SNPs in or near exons and thus did not capture the potential influence of deep intronic SNPs on splicing. Such variants have been described for other diseases (61, 97–100). Second, some genomic regions are characterized by long-range LD. Hence, the observed effects on splicing may not represent the only effects underlying the genetic associations with MS. Third, in the analyses of differences in gene expression and alternative splicing, we cannot exclude the possibility of confounding variables, e.g., medical treatment and comorbidities. Specifically, we observed a shift in the proportions of B-cell subsets in patients treated with alemtuzumab or cladribine (53). This contributed to the variance in the gene expression data. Fourth, we conducted our measurements in B cells and therefore may have missed or underestimated the differential alternative splicing of genes that are more abundantly expressed in other cell types (101). Even though genetic effects on splicing are usually highly shared across tissues and cell types (34), further insights into the effects of genetic risk variants could be obtained by studying other cell types, e.g., other peripheral immune cells such as T cells. Fifth, our analysis of the microarray data relied on transcript isoforms as annotated in the reference genome. Thus, we studied known splice isoforms and may have missed novel splicing patterns, which can potentially be identified by using RNA sequencing (102, 103). Fifth, as we have previously described (46), there are issues regarding the use of the minigene assay system, such as a possible interference by the Gateway cloning attachment sites, an insufficient amount of an important splicing factor in the used cell line, or the fact that only a small and specific part of the gene is examined. The latter may lead to the misinterpretation of ASEs as the splicing of exons can depend on the correct splicing of other exons of the gene that are not included in the minigene construct.

In conclusion, in this study, we focused on SNPs located in genetic risk loci for MS that presumably affect pre-mRNA splicing and thus may have an influential role in the pathogenesis of the disease. We were able to support findings from previous studies on MS-related ASEs for the pre-mRNAs of *GSDMB*, *IL7R*, and *SP140*. For four novel SNP–gene pairs, we found an association of the splice SNP genotypes with differential alternative splicing in the B-cell transcriptome data. Except for two SNP–gene pairs, we were able to validate the findings of the microarray data analysis with the qPCR assays. In addition, we were able to further substantiate our observations from the B-cell expression data on *TSFM* exon 3 skipping by using minigene assays. The MS risk allele C of the SNP rs2014886 almost always led to *TSFM* exon 3 skipping, whereas the alternative allele led to a low expression of ENST00000417094 transcripts. However, the potential functional impact of this ASE remains unclear. Further functional studies are needed to identify the disease-causing genetic variants and to explore their effects on splicing and the resulting consequences of an aberrant expression of splice isoforms to improve our understanding of the molecular pathomechanisms of MS.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics committee of the University of Rostock. The patients/participants provided their written informed consent to participate in this study.

Author contributions

EP performed the bioinformatic prioritization of SNP-gene pairs as well as the majority of the experiments. EP evaluated and interpreted the obtained data. Furthermore, EP drafted the manuscript and prepared the figures and tables. MH provided

support for the bioinformatic and statistical analyses of the data and critically revised the manuscript. MS coordinated the sample collection and gathered the clinical-demographic data. EP, NB and BF processed the blood samples. NB performed the SNP genotyping. DK was responsible for performing the microarray measurements. PL provided valuable advice concerning the splicing reporter minigene assays. MH, BF and UKZ conceptualized the study. MH, EP, NB and UKZ secured research funding. UKZ provided important intellectual insights and supervised the research. All authors contributed to the article and approved the submitted version.

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Conflict of interest

EP received travel funds from Novartis. MH received speaking fees and travel funds from Bayer HealthCare, Biogen, Merck, Novartis, and Teva. UKZ received research support as well as speaking fees and travel funds from Alexion, Almirall, Bayer HealthCare, Biogen, Bristol Myers Squibb, Janssen, Merck Serono, Novartis, Roche, Sanofi Genzyme, and Teva as well as EU, BMBF, BMWi, and DFG.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.931831/full#supplementary-material

References

1. Filippi M, Bar-Or A, Piehl F, Preziosa P, Solari A, Vukusic S, et al. Multiple sclerosis. *Nat Rev Dis Primers* (2018) 4:43. doi: 10.1038/s41572-018-0041-4

2. Lassmann H. Multiple sclerosis pathology. *Cold Spring Harb Perspect Med* (2018) 8:a028936. doi: 10.1101/cshperspect.a028936

3. Walton C, King R, Rechtman L, Kaye W, Leray E, Marrie RA, et al. Rising prevalence of multiple sclerosis worldwide: Insights from the atlas of MS, third edition. *Mult Scler* (2020) 26:1816–21. doi: 10.1177/1352458520970841

4. Blaschke SJ, Ellenberger D, Flachenecker P, Hellwig K, Paul F, Pöhlau D, et al. Time to diagnosis in multiple sclerosis: Epidemiological data from the German multiple sclerosis registry. *Mult Scler* (2022) 28:865-71. doi: 10.1177/13524585211039753

5. Lublin FD, Reingold SC, Cohen JA, Cutter GR, Sørensen PS, Thompson AJ, et al. Defining the clinical course of multiple sclerosis: the 2013 revisions. *Neurology.* (2014) 83:278–86. doi: 10.1212/WNL.0000000000000560

6. Zettl UK, Stüve O, Patejdl R. Immune-mediated CNS diseases: a review on nosological classification and clinical features. *Autoimmun Rev* (2012) 11:167–73. doi: 10.1016/j.autrev.2011.05.008

7. Reich DS, Lucchinetti CF, Calabresi PA. Multiple sclerosis. *N Engl J Med* (2018) 378:169–80. doi: 10.1056/NEJMra1401483

8. Dendrou CA, Fugger L, Friese MA. Immunopathology of multiple sclerosis. *Nat Rev Immunol* (2015) 15:545–58. doi: 10.1038/nri3871

9. Frahm N, Ellenberger D, Fneish F, Christoph K, Warnke C, Zettl UK, et al. Characteristics of secondary progressive multiple sclerosis: Disease activity and provision of care in Germany - a registry-based/multicentric cohort study. *Mult Scler Relat Disord* (2021) 56:103281. doi: 10.1016/j.msard.2021.103281

10. Rommer PS, Eichstädt K, Ellenberger D, Flachenecker P, Friede T, Haas J, et al. Symptomatology and symptomatic treatment in multiple sclerosis: Results from a nationwide MS registry. *Mult Scler* (2019) 25:1641–52. doi: 10.1177/1352458518799580

11. Kurtzke JF. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology.* (1983) 33:1444–52. doi: 10.1212/wnl.33.11.1444

12. Frischer JM, Bramow S, Dal-Bianco A, Lucchinetti CF, Rauschka H, Schmidbauer M, et al. The relation between inflammation and neurodegeneration in multiple sclerosis brains. *Brain.* (2009) 132:1175–89. doi: 10.1093/brain/awp070

13. Galea I, Ward-Abel N, Heesen C. Relapse in multiple sclerosis. *BMJ* (2015) 350:h1765. doi: 10.1136/bmj.h1765

14. Ellenberger D, Eichstädt K, Flachenecker P, Friede T, Haas J, Kleinschnitz C, et al. Decreasing longitudinal use of glucocorticosteroids in multiple sclerosis. *Mult Scler Relat Disord* (2018) 25:173–4. doi: 10.1016/j.msard.2018.07.040

15. Skierlo S, Rommer PS, Zettl UK. Symptomatic treatment in multiple sclerosis-interim analysis of a nationwide registry. *Acta Neurol Scand* (2017) 135:394–9. doi: 10.1111/ane.12612

16. Giovannoni G. Disease-modifying treatments for early and advanced multiple sclerosis: a new treatment paradigm. *Curr Opin Neurol* (2018) 31:233–43. doi: 10.1097/WCO.0000000000000561

17. Lünemann JD, Ruck T, Muraro PA, Bar-Or A, Wiendl H. Immune reconstitution therapies: concepts for durable remission in multiple sclerosis. *Nat Rev Neurol* (2020) 16:56–62. doi: 10.1038/s41582-019-0268-z

18. Rommer PS, Milo R, Han MH, Satyanarayan S, Sellner J, Hauer L, et al. Immunological aspects of approved MS therapeutics. *Front Immunol* (2019) 10:1564. doi: 10.3389/fimmu.2019.01564

19. Comi G, Bar-Or A, Lassmann H, Uccelli A, Hartung H-P, Montalban X, et al. Role of b cells in multiple sclerosis and related disorders. *Ann Neurol* (2021) 89:13–23. doi: 10.1002/ana.25927

20. Milo R. Therapies for multiple sclerosis targeting b cells. *Croat Med J* (2019) 60:87–98. doi: 10.3325/cmj.2019.60.87

21. Baker D, Herrod SS, Alvarez-Gonzalez C, Zalewski L, Albor C, Schmierer K. Both cladribine and alemtuzumab may effect MS *via* b-cell depletion. *Neurol Neuroimmunol Neuroinflamm* (2017) 4:e360. doi: 10.1212/NXI.0000000000000360

22. Hauser SL, Cree BAC. Treatment of multiple sclerosis: A review. *Am J Med* (2020) 133:1380–1390.e2. doi: 10.1016/j.amjmed.2020.05.049

23. Rommer P, Zettl UK. Treatment options in multiple sclerosis and neuromyelitis optica spectrum disorders. *Curr Pharm Des* (2022) 28:428-36. doi: 10.2174/1381612827666210920151231

24. Wiendl H, Gold R, Berger T, Derfuss T, Linker R, Mäurer M, et al. Multiple sclerosis therapy consensus group (MSTCG): position statement on disease-modifying therapies for multiple sclerosis (white paper). *Ther Adv Neurol Disord* (2021) 14:17562864211039648. doi: 10.1177/17562864211039648

25. Belbasis L, Bellou V, Evangelou E, Ioannidis JPA, Tzoulaki I. Environmental risk factors and multiple sclerosis: an umbrella review of systematic reviews and meta-analyses. *Lancet Neurology* (2015) 14:263–73. doi: 10.1016/S1474-4422(14)70267-4

26. Olsson T, Barcellos LF, Alfredsson L. Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis. *Nat Rev Neurol* (2017) 13:25–36. doi: 10.1038/nrneurol.2016.187

27. Bjornevik K, Cortese M, Healy BC, Kuhle J, Mina MJ, Leng Y, et al. Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. *Science* (2022) 375:296–301. doi: 10.1126/science.abj8222

28. Hecker M, Bühring J, Fitzner B, Rommer PS, Zettl UK. Genetic, environmental and lifestyle determinants of accelerated telomere attrition as contributors to risk and severity of multiple sclerosis. *Biomolecules.* (2021) 11:1510. doi: 10.3390/biom11101510

29. Rommer PS, Ellenberger D, Hellwig K, Haas J, Pöhlau D, Stahmann A, et al. Relapsing and progressive MS: the sex-specific perspective. *Ther Adv Neurol Disord* (2020) 13:1756286420956495. doi: 10.1177/1756286420956495

30. Sherry ST, Ward MH, Kholodov M, Baker J, Phan L, Smigielski EM, et al. dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res* (2001) 29:308–11. doi: 10.1093/nar/29.1.308

31. Brookes AJ. The essence of SNPs. *Gene.* (1999) 234:177–86. doi: 10.1016/s0378-1119(99)00219-x

32. International Multiple Sclerosis Genetics Consortium. Multiple sclerosis genomic map implicates peripheral immune cells and microglia in susceptibility. *Science* (2019) 365:eaav7188. doi: 10.1126/science.aav7188

33. Machiela MJ, Chanock SJ. LDlink: a web-based application for exploring population-specific haplotype structure and linking correlated alleles of possible functional variants. *Bioinformatics.* (2015) 31:3555–7. doi: 10.1093/bioinformatics/btv402

34. GTEx Consortium. The GTEx consortium atlas of genetic regulatory effects across human tissues. *Science* (2020) 369:1318–30. doi: 10.1126/science.aaz1776

35. James T, Lindén M, Morikawa H, Fernandes SJ, Ruhrmann S, Huss M, et al. Impact of genetic risk loci for multiple sclerosis on expression of proximal genes in patients. *Hum Mol Genet* (2018) 27:912–28. doi: 10.1093/hmg/ddy001

36. Vandiedonck C. Genetic association of molecular traits: A help to identify causative variants in complex diseases. *Clin Genet* (2018) 93:520–32. doi: 10.1111/cge.13187

37. Zhernakova DV, Deelen P, Vermaat M, van Iterson M, van Galen M, Arindrarto W, et al. Identification of context-dependent expression quantitative trait loci in whole blood. *Nat Genet* (2017) 49:139–45. doi: 10.1038/ng.3737

38. Chen L, Ge B, Casale FP, Vasquez L, Kwan T, Garrido-Martín D, et al. Genetic drivers of epigenetic and transcriptional variation in human immune cells. *Cell.* (2016) 167:1398–1414.e24. doi: 10.1016/j.cell.2016.10.026

39. Shi Y. Mechanistic insights into precursor messenger RNA splicing by the spliceosome. *Nat Rev Mol Cell Biol* (2017) 18:655–70. doi: 10.1038/nrm.2017.86

40. Lee Y, Rio DC. Mechanisms and regulation of alternative pre-mRNA splicing. *Annu Rev Biochem* (2015) 84:291–323. doi: 10.1146/annurev-biochem-060614-034316

41. Fu X-D, Ares M. Context-dependent control of alternative splicing by RNA-binding proteins. *Nat Rev Genet* (2014) 15:689–701. doi: 10.1038/nrg3778

42. Manning KS, Cooper TA. The roles of RNA processing in translating genotype to phenotype. *Nat Rev Mol Cell Biol* (2017) 18:102–14. doi: 10.1038/nrm.2016.139

43. Anna A, Monika G. Splicing mutations in human genetic disorders: examples, detection, and confirmation. *J Appl Genet* (2018) 59:253–68. doi: 10.1007/s13353-018-0444-7

44. Pineda JMB, Bradley RK. Most human introns are recognized *via* multiple and tissue-specific branchpoints. *Genes Dev* (2018) 32:577–91. doi: 10.1101/gad.312058.118

45. Yabas M, Elliott H, Hoyne GF. The role of alternative splicing in the control of immune homeostasis and cellular differentiation. *Int J Mol Sci* (2015) 17:3. doi: 10.3390/ijms17010003

46. Putscher E, Hecker M, Fitzner B, Lorenz P, Zettl UK. Principles and practical considerations for the analysis of disease-associated alternative splicing events using the gateway cloning-based minigene vectors pDESTsplice and pSpliceExpress. *Int J Mol Sci* (2021) 22:5154. doi: 10.3390/ijms22105154

47. Scotti MM, Swanson MS. RNA Mis-splicing in disease. *Nat Rev Genet* (2016) 17:19–32. doi: 10.1038/nrg.2015.3

48. Yeo GW, van Nostrand E, Holste D, Poggio T, Burge CB. Identification and analysis of alternative splicing events conserved in human and mouse. *Proc Natl Acad Sci U S A.* (2005) 102:2850–5. doi: 10.1073/pnas.0409742102

49. Hecker M, Rüge A, Putscher E, Boxberger N, Rommer PS, Fitzner B, et al. Aberrant expression of alternative splicing variants in multiple sclerosis - a systematic review. *Autoimmun Rev* (2019) 18:721–32. doi: 10.1016/j.autrev.2019.05.010

50. Gregory SG, Schmidt S, Seth P, Oksenberg JR, Hart J, Prokop A, et al. Interleukin 7 receptor alpha chain (IL7R) shows allelic and functional association with multiple sclerosis. *Nat Genet* (2007) 39:1083–91. doi: 10.1038/ng2103

51. Desmet F-O, Hamroun D, Lalande M, Collod-Béroud G, Claustres M, Béroud C. Human splicing finder: an online bioinformatics tool to predict splicing signals. *Nucleic Acids Res* (2009) 37:e67. doi: 10.1093/nar/gkp215

52. Zhu Y, Xu G, Yang YT, Xu Z, Chen X, Shi B, et al. POSTAR2: deciphering the post-transcriptional regulatory logics. *Nucleic Acids Res* (2019) 47:D203–11. doi: 10.1093/nar/gky830

53. Hecker M, Fitzner B, Putscher E, Schwartz M, Winkelmann A, Meister S, et al. Implication of genetic variants in primary microRNA processing sites in the risk of multiple sclerosis. *EBioMedicine.* (2022) 80:104052. doi: 10.1016/j.ebiom.2022.104052

54. Thompson AJ, Banwell BL, Barkhof F, Carroll WM, Coetzee T, Comi G, et al. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *Lancet Neurol* (2018) 17:162–73. doi: 10.1016/S1474-4422(17)30470-2

55. McCall MN, McMurray HR, Land H, Almudevar A. On non-detects in qPCR data. *Bioinformatics.* (2014) 30:2310–6. doi: 10.1093/bioinformatics/btu239

56. Kishore S, Khanna A, Stamm S. Rapid generation of splicing reporters with pSpliceExpress. *Gene.* (2008) 427:104–10. doi: 10.1016/j.gene.2008.09.021

57. Ritchie ME, Phipson B, Wu Di, Hu Y, Law CW, Shi W, et al. Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* (2015) 43:e47. doi: 10.1093/nar/gkv007

58. Fox J, Weisberg S. *An r companion to applied regression. 3rd ed.* Thousand Oaks: SAGE (2018).

59. Barash Y, Calarco JA, Gao W, Pan Q, Wang X, Shai O, et al. Deciphering the splicing code. *Nature.* (2010) 465:53–9. doi: 10.1038/nature09000

60. Carazo F, Romero JP, Rubio A. Upstream analysis of alternative splicing: a review of computational approaches to predict context-dependent splicing factors. *Brief Bioinform* (2019) 20:1358–75. doi: 10.1093/bib/bby005

61. Vaz-Drago R, Custódio N, Carmo-Fonseca M. Deep intronic mutations and human disease. *Hum Genet* (2017) 136:1093–111. doi: 10.1007/s00439-017-1809-4

62. Yi Y, Zhao Y, Huang Y, Wang D. A brief review of RNA-protein interaction database resources. *Noncoding RNA*. (2017) 3:6. doi: 10.3390/ncrna3010006

63. Ohno K, Takeda J-I, Masuda A. Rules and tools to predict the splicing effects of exonic and intronic mutations. *Wiley Interdiscip Rev RNA*. (2018) 9:e1451. doi: 10.1002/wrna.1451

64. Ptok J, Müller L, Theiss S, Schaal H. Context matters: Regulation of splice donor usage. *Biochim Biophys Acta Gene Regul Mech* (2019) 1862:194391. doi: 10.1016/j.bbagrm.2019.06.002

65. Uhlen M, Karlsson MJ, Zhong W, Tebani A, Pou C, Mikes J, et al. A genome-wide transcriptomic analysis of protein-coding genes in human blood cells. *Science* (2019) 366:eaax9198. doi: 10.1126/science.aax9198

66. Monaco G, Lee B, Xu W, Mustafah S, Hwang YY, Carré C, et al. RNA-Seq signatures normalized by mRNA abundance allow absolute deconvolution of human immune cell types. *Cell Rep* (2019) 26:1627–1640.e7. doi: 10.1016/j.celrep.2019.01.041

67. Ohle L-M, Ellenberger D, Flachenecker P, Friede T, Haas J, Hellwig K, et al. Chances and challenges of a long-term data repository in multiple sclerosis: 20th birthday of the German MS registry. *Sci Rep* (2021) 11:13340. doi: 10.1038/s41598-021-92722-x

68. Cardamone G, Paraboschi EM, Rimoldi V, Duga S, Soldà G, Asselta R. The characterization of GSDMB splicing and backsplicing profiles identifies novel isoforms and a circular RNA that are dysregulated in multiple sclerosis. *Int J Mol Sci* (2017) 18:576. doi: 10.3390/ijms18030576

69. Galarza-Muñoz G, Briggs FBS, Evsyukova I, Schott-Lerner G, Kennedy EM, Nyanhete T, et al. Human epistatic interaction controls IL7R splicing and increases multiple sclerosis risk. *Cell*. (2017) 169:72–84.e13. doi: 10.1016/j.cell.2017.03.007

70. Hoe E, McKay FC, Schibeci SD, Gandhi K, Heard RN, Stewart GJ, et al. Functionally significant differences in expression of disease-associated IL-7 receptor alpha haplotypes in CD4 T cells and dendritic cells. *J Immunol* (2010) 184:2512–7. doi: 10.4049/jimmunol.0902900

71. Mazzucchelli R, Durum SK. Interleukin-7 receptor expression: intelligent design. *Nat Rev Immunol* (2007) 7:144–54. doi: 10.1038/nri2023

72. Lundström W, Highfill S, Walsh STR, Beq S, Morse E, Kockum I, et al. Soluble IL7Rα potentiates IL-7 bioactivity and promotes autoimmunity. *Proc Natl Acad Sci U S A*. (2013) 110:E1761–70. doi: 10.1073/pnas.1222303110

73. Garrido-Martín D, Borsari B, Calvo M, Reverter F, Guigó R. Identification and analysis of splicing quantitative trait loci across multiple tissues in the human genome. *Nat Commun* (2021) 12:727. doi: 10.1038/s41467-020-20578-2

74. Morrison FS, Locke JM, Wood AR, Tuke M, Pasko D, Murray A, et al. The splice site variant rs11078928 may be associated with a genotype-dependent alteration in expression of GSDMB transcripts. *BMC Genomics* (2013) 14:627. doi: 10.1186/1471-2164-14-627

75. Zhou Z, He H, Wang K, Shi X, Wang Y, Su Y, et al. A from cytotoxic lymphocytes cleaves GSDMB to trigger pyroptosis in target cells. *Science* (2020) 368:aaz7548. doi: 10.1126/science.aaz7548

76. Eyre S, Bowes J, Diogo D, Lee A, Barton A, Martin P, et al. High-density genetic mapping identifies new susceptibility loci for rheumatoid arthritis. *Nat Genet* (2012) 44:1336–40. doi: 10.1038/ng.2462

77. McGovern DPB, Gardet A, Törkvist L, Goyette P, Essers J, Taylor KD, et al. Genome-wide association identifies multiple ulcerative colitis susceptibility loci. *Nat Genet* (2010) 42:332–7. doi: 10.1038/ng.549

78. Cardamone G, Paraboschi EM, Soldà G, Cantoni C, Supino D, Piccio L, et al. Not only cancer: the long non-coding RNA MALAT1 affects the repertoire of alternatively spliced transcripts and circular RNAs in multiple sclerosis. *Hum Mol Genet* (2019) 28:1414–28. doi: 10.1093/hmg/ddy438

79. Matesanz F, Potenciano V, Fedetz M, Ramos-Mozo P, Del Abad-Grau MM, Karaky M, et al. A functional variant that affects exon-skipping and protein expression of SP140 as genetic mechanism predisposing to multiple sclerosis. *Hum Mol Genet* (2015) 24:5619–27. doi: 10.1093/hmg/ddv256

80. Zucchelli C, Tamburri S, Quilici G, Palagano E, Berardi A, Saare M, et al. Structure of human Sp140 PHD finger: an atypical fold interacting with Pin1. *FEBS J* (2014) 281:216–31. doi: 10.1111/febs.12588

81. Karaky M, Fedetz M, Potenciano V, Andrés-León E, Codina AE, Barrionuevo C, et al. SP140 regulates the expression of immune-related genes associated with multiple sclerosis and other autoimmune diseases by NF-κB inhibition. *Hum Mol Genet* (2018) 27:4012–23. doi: 10.1093/hmg/ddy284

82. Burgoyne RD, Haynes LP. Understanding the physiological roles of the neuronal calcium sensor proteins. *Mol Brain* (2012) 5:2. doi: 10.1186/1756-6606-5-2

83. Heizmann CW. Ca2+-binding proteins of the EF-hand superfamily: Diagnostic and prognostic biomarkers and novel therapeutic targets. *Methods Mol Biol* (2019) 1929:157–86. doi: 10.1007/978-1-4939-9030-6_11

84. Braunewell K-H. The darker side of Ca2+ signaling by neuronal Ca2+-sensor proteins: from alzheimer's disease to cancer. *Trends Pharmacol Sci* (2005) 26:345–51. doi: 10.1016/j.tips.2005.04.008

85. Laitinen VH, Rantapero T, Fischer D, Vuorinen EM, Tammela TLJ, Wahlfors T, et al. Fine-mapping the 2q37 and 17q11.2-q22 loci for novel genes and sequence variants associated with a genetic predisposition to prostate cancer. *Int J Cancer* (2015) 136:2316–27. doi: 10.1002/ijc.29276

86. Natanzon Y, Earp M, Cunningham JM, Kalli KR, Wang C, Armasu SM, et al. Genomic analysis using regularized regression in high-grade serous ovarian cancer. *Cancer Inform* (2018) 17:1176935118755341. doi: 10.1177/1176935118755341

87. Hollenbach JA, Oksenberg JR. The immunogenetics of multiple sclerosis: A comprehensive review. *J Autoimmun* (2015) 64:13–25. doi: 10.1016/j.jaut.2015.06.010

88. Link J, Lorentzen AR, Kockum I, Duvefelt K, Lie BA, Celius EG, et al. Two HLA class I genes independently associated with multiple sclerosis. *J Neuroimmunol* (2010) 226:172–6. doi: 10.1016/j.jneuroim.2010.07.006

89. Yeo TW, de JPL, SG G, LF B, Walton A, Goris An, et al. A second major histocompatibility complex susceptibility locus for multiple sclerosis. *Ann Neurol* (2007) 61:228–36. doi: 10.1002/ana.21063

90. Keegan NP, Fletcher S. A spotter's guide to SNPtic exons: The common splice variants underlying some SNP-phenotype correlations. *Mol Genet Genomic Med* (2022) 10:e1840. doi: 10.1002/mgg3.1840

91. Cai YC, Bullard JM, Thompson NL, Spremulli LL. Interaction of mitochondrial elongation factor tu with aminoacyl-tRNA and elongation factor ts. *J Biol Chem* (2000) 275:20308–14. doi: 10.1074/jbc.M001899200

92. D'Souza AR, Minczuk M. Mitochondrial transcription and translation: overview. *Essays Biochem* (2018) 62:309–20. doi: 10.1042/EBC20170102

93. Boczonadi V, Ricci G, Horvath R. Mitochondrial DNA transcription and translation: clinical syndromes. *Essays Biochem* (2018) 62:321–40. doi: 10.1042/EBC20170103

94. Gil-Varea E, Fedetz M, Eixarch H, Spataro N, Villar LM, Urcelay E, et al. A new risk variant for multiple sclerosis at 11q23.3 locus is associated with expansion of CXCR5+ circulating regulatory T cells. *J Clin Med* (2020) 9:625. doi: 10.3390/jcm9030625

95. Handel AE, Handunnetthi L, Berlanga AJ, Watson CT, Morahan JM, Ramagopalan SV. The effect of single nucleotide polymorphisms from genome wide association studies in multiple sclerosis on gene expression. *PloS One* (2010) 5:e10142. doi: 10.1371/journal.pone.0010142

96. Alcina A, Fedetz M, Fernández O, Saiz A, Izquierdo G, Lucas M, et al. Identification of a functional variant in the KIF5A-CYP27B1-METTL1-FAM119B locus associated with multiple sclerosis. *J Med Genet* (2013) 50:25–33. doi: 10.1136/jmedgenet-2012-101085

97. Bergougnoux A, Délétang K, Pommier A, Varilh J, Houriez F, Altieri JP, et al. Functional characterization and phenotypic spectrum of three recurrent disease-causing deep intronic variants of the CFTR gene. *J Cyst Fibros* (2019) 18:468–75. doi: 10.1016/j.jcf.2018.10.012

98. Bauwens M, Garanto A, Sangermano R, Naessens S, Weisschuh N, de ZJ, et al. ABCA4-associated disease as a model for missing heritability in autosomal recessive disorders: novel noncoding splice, cis-regulatory, structural, and recurrent hypomorphic variants. *Genet Med* (2019) 21:1761–71. doi: 10.1038/s41436-018-0420-y

99. Jung H, Lee KS, Choi JK. Comprehensive characterisation of intronic mis-splicing mutations in human cancers. *Oncogene*. (2021) 40:1347–61. doi: 10.1038/s41388-020-01614-3

100. Moles-Fernández A, Domènech-Vivó J, Tenés A, Balmaña J, Diez O, Gutiérrez-Enríquez S. Role of splicing regulatory elements and in silico tools usage in the identification of deep intronic splicing variants in hereditary Breast/Ovarian cancer genes. *Cancers (Basel)* (2021) 13:3341. doi: 10.3390/cancers13133341

101. Kim-Hellmuth S, Aguet F, Oliva M, Muñoz-Aguirre M, Kasela S, Wucher V, et al. Cell type-specific genetic regulation of gene expression across human tissues. *Science* (2020) 369:eaaz8528. doi: 10.1126/science.aaz8528

102. Zhao S, Fung-Leung W-P, Bittner A, Ngo K, Liu X. Comparison of RNA-seq and microarray in transcriptome profiling of activated T cells. *PloS One* (2014) 9:e78644. doi: 10.1371/journal.pone.0078644

103. Rao MS, van Vleet TR, Ciurlionis R, Buck WR, Mittelstadt SW, Blomme EAG, et al. Comparison of RNA-seq and microarray gene expression platforms for the toxicogenomic evaluation of liver from short-term rat toxicity studies. *Front Genet* (2018) 9:636. doi: 10.3389/fgene.2018.00636

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Glossary

—	not available
Alt.	alternative
ANOVA	analysis of variance
ASE	alternative splicing event
bp	base pairs
CNS	central nervous system
C_T	threshold cycle
dist.	distance
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein–Barr virus
EDSS	Expanded Disability Status Scale
EDTA	ethylenediaminetetraacetic acid
eQTL	expression quantitative trait locus
ESE	exonic splicing enhancer
ESS	exonic splicing silencer
EUR	European population
GEO	Gene Expression Omnibus
GWAS	genome-wide association study
h	hours
HGNC	HUGO Gene Nomenclature Committee
ISE	intronic splicing enhancer
ISS	intronic splicing silencer
JUC	junction probe set
kb	kilobase
LD	linkage disequilibrium
mRNA	messenger RNA
MS	multiple sclerosis
MS RA	MS risk allele
MS SNP	MS-associated lead SNP according to the most recent GWAS (32)
MV	missing values
n	number
ng	nanogram
PBMC	peripheral blood mononuclear cells
PPMS	primary progressive multiple sclerosis
pre-mRNA	precursor messenger ribonucleic acid
PSR	probe selection region
qPCR	quantitative (real-time) polymerase chain reaction
RA	risk allele
RBP	RNA-binding protein
RRMS	relapsing–remitting multiple sclerosis
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SNP	single-nucleotide polymorphism
snRNPs	small nuclear ribonucleoproteins
SNP splice	potentially splice-altering SNP that is located in or near an exonic region of a gene and that is in LD with an MS SNP
SPMS	secondary progressive multiple sclerosis
sQTL	splicing quantitative trait locus
TAC	Transcriptome Analysis Console

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Dynamic changes in kynurenine pathway metabolites in multiple sclerosis: A systematic review

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Background: Multiple sclerosis (MS) is a debilitating neurodegenerative disorder characterized by axonal damage, demyelination, and perivascular inflammatory lesions in the white matter of the central nervous system (CNS). Kynurenine pathway (KP), which is the major route of tryptophan (TRP) metabolism, generates a variety of neurotoxic as well as neuroprotective compounds, affecting MS pathology and the severity of impairments. Alterations in KP have been described not only in MS, but also in various psychiatric and neurodegenerative diseases. The purpose of this systematic review is to investigate the previously reported dysregulation of KP and differences in its metabolites and enzymes in patients with MS compared to healthy control subjects.

Method: Electronic databases of PubMed, Scopus, Cochrane Database of Systematic Reviews, and Web of Science were searched to identify studies measuring concentrations of KP metabolites and enzymes in MS patients and control subjects. The following metabolites and enzymes implicated in the KP were investigated: TRP, kynurenine (KYN), kynurenic acid (KYNA), quinolinic acid (QUIN), picolinic acid (PIC), hydroxyindoleacetic acid (HIAA), indoleamine 2,3-dioxygenase (IDO), kynurenine aminotransferase (KAT), and their related ratios.

Result: Ten studies were included in our systematic review. Our review demonstrates that IDO expression is reduced in the peripheral blood mononuclear cells (PBMCs) of MS patients compared to healthy controls. Also, increased levels of QUIN and QUIN/KYNA in the serum and cerebrospinal fluid (CSF) of MS patients is observed. Differences in levels of other metabolites and enzymes of KP are also reported in some of the reviewed studies, however there are discrepancies among the included reports.

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Conclusion: The results of this investigation suggest a possible connection between alterations in the levels of KP metabolite or enzymes and MS. QUIN levels in CSF were higher in MS patients than in healthy controls, suggesting that QUIN may be involved in the pathogenesis of MS. The data indicate that differences in the serum/blood or CSF levels of certain KP metabolites and enzymes could potentially be used to differentiate between MS patients and control subjects.

KEYWORDS

multiple sclerosis, kynurenine pathway, kynurenine, tryptophan, picolinic acid, hydroxyindoleacetic acid, kynurenic acid, quinolinic acid

Introduction

Multiple sclerosis (MS) is one of the most prevalent neurological disorders worldwide, with an annual incidence rate of approximately 2 per 100,000 (1). MS is a disabling neurodegenerative, autoimmune, inflammatory, and demyelinating disease of the central nervous system (CNS) (2), predominantly affecting young adults during their most productive years which is from 20 to 50 (3, 4).

MS is characterized by axonal damage, demyelination, and perivascular inflammatory lesions in the CNS white matter. T lymphocytes autoreactive against CNS antigens may initiate MS pathogenesis (5). Numerous proinflammatory factors and cytokines have been found to be altered in the blood, brain tissues, and cerebrospinal fluid (CSF) of MS patients (6). The kynurenine pathway (KP) in MS is induced by proinflammatory cytokine cascades resulting in altered levels of KP metabolites (7, 8).

The KP is critical for providing cellular energy to the immune system under physiological conditions, by generating nicotinamide adenine dinucleotide (NAD^+). However, the metabolites of KP with neuroactive function, collectively referred to as "kynurenines" play an important role in chronic neuroinflammation. Under inflammatory conditions, these metabolites are typically considered neurotoxic and gliotoxic due to their adverse effects on glutamatergic neurotransmission and direct toxicity towards neurons and glial cells (9, 10). In addition, tryptophan (TRP) and some intermediate metabolites in the KP exhibit immunomodulatory properties. It is also well established that the indoleamine 2,3-dioxygenase (IDO) enzyme significantly contributes to immune regulation by depleting TRP and producing kynurenine (KYN) (11, 12). A link between the aryl hydrocarbon receptor and IDO is identified in the expansion of Th17 and regulatory T cells, which plays a significant role in various autoimmune disorders and cancer (13, 14). Kynurenic acid (KYNA), which is a metabolite produced through the KP, acts as a neuroprotective agent, while quinolinic acid (QUIN) is an established neurotoxic agent (15–20). Overall, alterations in the KP and in TRP metabolism are critical in MS pathogenesis, since abnormalities in TRP metabolism have been shown to impair regulation of T cell activity (21).

In the KP, TRP is the first substrate converted to KYN by two enzymes named IDO and tryptophan-2,3-dioxygenase (TDO). KYN is subsequently catalyzed by kynurenine aminotransferase (KAT) and kynurenine-3-monooxygenase (KMO) to produce two different metabolites, 3-hydroxykynurenine (3-HK) and KYNA, respectively. 3-HK is altered to 3-hydroxyanthranillic acid (3-HANA) by an enzyme called kynureninase, and the next metabolite produced from 3-HANA is QUIN. At last, NAD^+ is the ultimate metabolite of TRP produced through the KP (22).

While activation of some KP enzymes have short-term benefits, such as decreased T cell proliferation and immunosuppression, their chronic activation results in the production of neurotoxic metabolites and impairs the innate repair mechanism of remyelination (23). In MS patients, proinflammatory cytokine levels rise in the serum, resulting in IDO activation (7). TRP levels are decreased in the CSF and serum of patients with MS, suggesting the role of KP metabolism in MS pathogenesis (24–26). In all stages of MS, changes in the balance between neurotoxic and neuroprotective kynurenine metabolites have been observed (27). The CSF levels of HIAA are lower in MS patients compared to healthy control subjects (28). Finally, any alteration in each of the KP metabolites and

Abbreviations: CSF, cerebrospinal fluid; MS, multiple sclerosis; RBC, red blood cell; PBMC, peripheral blood mononuclear cell; TRP, tryptophan; KYN, kynurenine; KYNA, kynurenic acid; QUIN, quinolinic acid; PIC, picolinic acid; HIAA, hydroxyindoleacetic acid; LC, Liquid chromatography; GC, gas chromatography; IDO,Indolaeamine 2,3-Dioxygenase; HK,3-hydroxykynurenine; AA, anthranilic acid; XA, xanthurenic acid; KMO, Kynurenine 3 Monooxygenase; ROS, Reactive oxygen species; BBB, Blood brain barrier.

enzymes can affect neurons and contribute to the MS pathogenesis and neurodegeneration. Therefore, the purpose of this systematic review is to ascertain whether altered metabolites and enzymes of KP can be measured in MS.

Materials and methods

Search strategy

We searched the following four databases for relevant studies published up to March 2021: PubMed, Scopus, Cochrane Database of Systematic Reviews, and Web of Science. Two authors conducted an independent search using the following query: (tryptophan OR kynurenine OR kynurenate OR kynurenic OR anthranilic OR anthranilate OR quinolinate OR quinolinic OR picolinate OR picolinic OR xanthurenic OR xanthurenate) AND (multiple sclerosis OR disseminated sclerosis). Additionally, we searched the reference lists of related articles to avoid overlooking relevant studies. All 678 papers found during the search were inserted into the Endnote software for screening. Following that, 366 duplicate publications were deleted. Subsequently, the Newcastle-Ottawa scale was used to evaluate the included studies (Table 2).

Inclusion and exclusion criteria

We included all observational studies published in English that measured KP metabolites or enzymes in MS patients and corresponding control subjects. We excluded animal studies, those that lacked a control group, and those without randomized sampling. After excluding duplicates (366), two authors independently screened the initially identified articles based on their titles and abstracts (31 studies remained). They examined the full text of the selected studies and then shortlisted the studies that met the inclusion criteria (10). Potential disagreements were resolved by a third author (Figure 1).

Data extraction

All relevant data from eligible studies were extracted, including first author name, country of origin, publication date, metabolite measurement methods, patient and control group characteristics, and measured levels of TRP, KP metabolites and enzymes. To this end, two authors extracted data independently and then compared their results. The current study was approved by the Shahid Beheshti University of Medical Sciences ethics committee IR.SBMU.RETECH.REC.1400.919.

Results

The current study has been performed based on PRISMA checklist. After screening the titles and abstracts of the initially searched studies, 41 potentially relevant studies remained, of which 17 articles were included in the systematic review following full-text screening (3, 8, 16–18, 29–39) (Figure 1). It should be regarded that we also excluded some potentially relevant studies due to their designs. For instance, some studies assessed KP metabolites only in animals, and some did not compare KP metabolites levels with a healthy control group (19, 40, 41). All studies included were published in English and published up to March 2021. Two studies assessed the variables in two distinct populations (32, 39). One study utilized two cohorts, one of which met our inclusion criteria and was included in the systematic review (16). Table 1 summarizes the study characteristics and significant findings from the included studies.

Kynurenine

Eleven studies (3, 8, 16, 29, 31, 33, 35, 37–39, 42) involving 730 individuals provided data on KYN levels (468 MS patients and 262 healthy controls). Three studies (8, 16, 33) used CSF as the sample source, four studies utilized serum (3, 29, 37, 38), one study employed peripheral blood mononuclear cells (PBMCs) (35), one study used urine as the sample source (31), and two studies utilized both CSF and serum (39, 42). Negrotto et al. (35) reported that RRMS patients in the remission phase had remarkably lower KYN levels than controls in PBMCs ($P< 0.001$). Moreover, according to a study by Gaetani et al. (31), RRMS patients had significantly lower KYN levels than controls in the urine sample ($P= 0.010$). In contrast, a study conducted by Rajda et al. (8) illustrated that MS patients had significantly higher levels of kynurenine in their CSF than healthy controls ($P=0.049$). Moreover, Sadowska-bartosz et al. (38) and Adamczyk-sowa et al. (29) stated in their studies that RRMS patients without treatment have considerably increased levels of KYN than healthy controls in their serum ($P<0.05$). Additionally, Sadowska-bartosz et al. (38) and Adamczyk-sowa et al. (29) reported significantly elevated levels of KYN in the serum of RRMS patients without treatment compared with RRMS patients treated with IFN-β1b ($P<0.05$ and $P<0.01$, respectively). Besides, Herman et al. (33) illustrated that SPMS patients had remarkably higher KYN levels in their CSF in comparison to healthy controls and RRMS patients ($P<0.05$). Also, in studies by Aeinehband et al. (16) on CSF ($p>0.05$), Mancuso et al. (3) on serum ($P>0.05$), Lim et al. (42) on serum and CSF, Olsson et al. on serum, and Tomosi et al. (39) on both CSF and serum samples ($P=0.169$ and $P= 0.894$, respectively), no

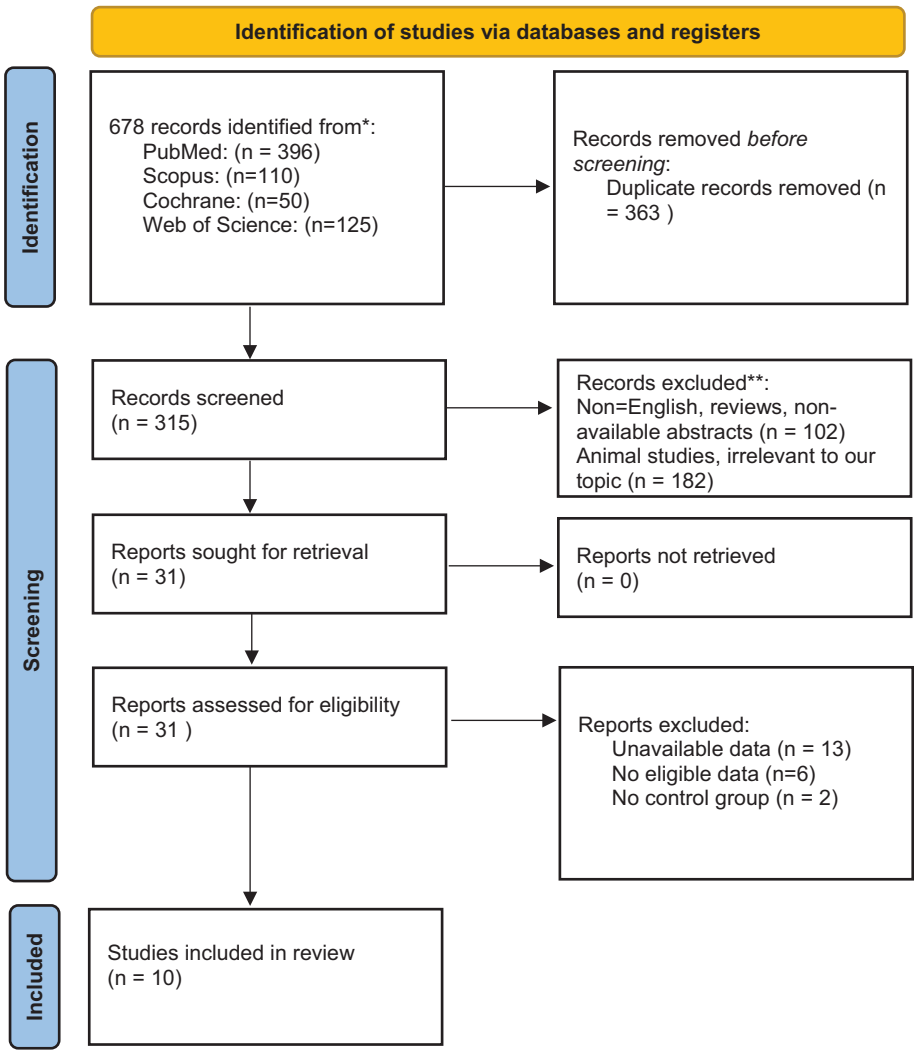

FIGURE 1
PRISMA 2020 flow diagram for new systematic reviews which included searches of databases.

significant difference in kynurenine levels was observed between MS patients and controls. ()

Tryptophan

Eight studies (3, 8, 16, 35, 37–39, 42) involving 559 individuals (361 MS patients and 198 healthy controls) provided data on TRP levels. Two studies (8, 16) used CSF as the sample source, three studies utilized serum (3, 37, 38), one study used PBMCs as the sample source (35), and two studies employed both CSF and serum (39, 42). Negrotto et al. (35) reported significantly higher TRP levels in the PBMCs of RRMS patients who are in the remission phase compared to controls (p=0.0007). However, in a study by Rajda et al. (8), CSF levels of TRP were insignificantly lower in MS patients than in healthy controls (p=0.12). Three other studies by Aeinehband et al. (16), Tomosi et al. (39), and Lim et al. (42) demonstrated no significant differences in the CSF levels of TRP in MS patients in comparison to controls (p>0.05, p=0.92, and P>0.05 respectively). Similarly, five studies conducted by Tomosi et al. (39), Mancuso et al. (3), Lim et al. (42), Olsson et al. (37), and Sadowska-bartosz et al. (38) reported that TRP levels in the serum samples of MS patients did not change significantly (p> 0.05).

Kynurenic acid

Eight studies (8, 16–18, 32, 37, 39, 42) collected comparative data on KYNA levels in 478 individuals (299 patients and 179

TABLE 1 Summary of the main findings from studies included in the systematic review.

Study (ref)	Place	Patients (No)			Controls (No)			Age (mean (SD))		Types of MS	Treatment	Patients phase	Materials	Methods	Metabolites	Key findings
		All	Male	Female	All	Male	Female	Patients	Controls							
Rajda C (8)	Hungary	37	18	19	22	11	11	34.10	38.60	?	None	–	CSF	Mass spectrometry	TRP, KYN, KYNA, QUIN, PIC, HIAA	No significant difference in TRP, KYNA, PIC, AND HIAA levels between the two groups, KYN and QUIN levels higher in patients
Gaetani L (31)	Italy	47	7	40	43	16	27	31.80 (9.70)	32.70 (10.60)	RRMS	11 (None), 15 (Interferons), 10 (Glatiramer acetate), 6 (Dimethylfumarate), 3 (Fingolimod), 1 (Natalizumab), 1 (Alemtuzumab)	–	urine	HPLC-Mass spectrometry/Mass spectrometry	KYN, KYN/TRP	Lower KYN levels and KYN/TRP in patients
Hartai Z (32)	Hungary	13	6	7	14	5	9	35.40 (13.10)	33.50 (11.70)	RRMS	None	1 to 3 days after the appearance of new neurological signs	plasma	Mass spectrometry	KYNA	Higher KYNA levels in patients
Hartai Z (32)	Hungary	13	6	7	14	5	9	35.40 (13.10)	33.50 (11.70)	RRMS	None		plasma	spectrophotometrically	KAT I, KAT II	No significant change in the activities of the KATs in the plasma
Hartai Z (32)	Hungary	13	6	7	14	5	9	35.40 (13.10)	33.50 (11.70)	RRMS	None		RBCs	Mass spectrometry	KYNA	No significant difference in KYNA levels between the two groups
Hartai Z (32)	Hungary	13	6	7	14	5	9	35.40 (13.10)	33.50 (11.70)	RRMS	None		RBCs	spectrophotometrically	KAT I, KAT II	Higher KAT I and KAT II activities in the RBC of the patients with MS than in the control group.
Tomosi F (39)	Hungary	20	0	20	14			33.84 (9.14)	37.57 (10.09)	RRMS	–		CSF	UHPLC– Mass spectrometry/Mass spectrometry	TRP, KYN, KYNA, QUIN, PIC, HIAA,	Lower levels of KYNA, PIC, and KYNA/KYN in

(Continued)

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TABLE 1 Continued

Study (ref)	Place	Patients (No)			Controls (No)			Age (mean (SD))		Types of MS	Treatment	Patients phase	Materials	Methods	Metabolites	Key findings
		All	Male	Female	All	Male	Female	Patients	Controls							
															QUIN/KYNA, KYNA/KYN, KYN/TRP	patients. No significant difference in KYN, TRP, HIAA levels, and KYN/TRP between the two groups. Higher levels of QUIN and QUIN/KYNA in patients
Tomosi F (39)	Hungary	20	0	20	14			33.84 (9.14)	37.57 (10.09)	RRMS	–		serum	UHPLC– Mass spectrometry/Mass spectrometry	TRP, KYN, KYNA, QUIN, PIC, HIAA, QUIN/KYNA, KYNA/KYN, KYN/TRP	Lower levels of HIAA in patients. No significant difference in TRP, KYN, KYNA, PIC, KYNA/KYN, and KYN/TRP between the two groups. Higher levels of QUIN and QUIN/KYNA in patients
Mancuso R (3)	Austria	36	13	23	15	5	10	37.94 (8.52)	37.83 (9.55)	RRMS	Glucocorticoid treatment for AMS patients	21 AMS 15 SMS	Serum	HPLC	TRP, KYN, KYN/TRP	No significant difference in TRP, KYN, and KYN/TRP between SMS patients and HCs. KYN levels and KYN/TRP were significantly higher in SMS patients, AMS patients before the initiation of glucocorticoids, and HCs compared with AMS patients after the initiation of glucocorticoids.
Mancuso R (3)	Austria	36	13	23	15	5	10	37.94 (8.52)	37.83 (9.55)	RRMS	Glucocorticoid treatment for AMS patients	21 AMS 15 SMS	PBMCs	spectrophotometrically	IDO mRNA	IDO expression was decreased in SMS patients compared to HCs and AMS patients before the

(Continued)

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TABLE 1 Continued

Study (ref)	Place	Patients (No)			Controls (No)			Age (mean (SD))		Types of MS	Treatment	Patients phase	Materials	Methods	Metabolites	Key findings
		All	Male	Female	All	Male	Female	Patients	Controls							
																initiation of glucocorticoids. IDO expression was higher in SMS patients than AMS patients after the initiation of glucocorticoids.
Nergotto L (35)	Argentina	40	13	27	30	10	20	32.00 (7.90)	32.00 (5.90)	RRMS	16 (IFN-β1a) and 9 (glatiramer acetate)	Remission	PBMCs	reversed phase HPLC	TRP, KYN	Higher levels of TRP in patients, Lower levels of KYN in patients
Nergotto L (35)	Argentina	40	13	27	30	10	20	32.00 (7.90)	32.00 (5.90)	RRMS	16 (IFN-β1a) and 9 (glatiramer acetate)	Remission	PBMCs	Real time PCR, ELISA	IDO mRNA, IDO protein	Reduced levels of IDO expression in patients both at mRNA and protein levels
Aeinehband S (16)	Sweden	86	34	52	20	8	12	43.30 (11.80)	36.50 (9.30)	72 RRMS, 5 PPMS, and 9 SPMS	77 (None), 7 (interferons), 1 (rituximab), 1 (glatiramer acetate)	8 AMS, 19 SMS	CSF	HPLC- Mass spectrometry/Mass spectrometry	TRP, KYN, KYNA, QUIN, QUIN/KYNA, KYNA/KYN, KYN/TRP	Significant lower levels of TRP, KYN, KYNA, QUIN, KYN/TRP, and KYNA/KYN in MS patients compared with iOND patients. No significant difference in QUIN/KYNA between MS and iOND patients. Significant lower levels of QUIN/KYNA in RRMS-relapse patients compared with OND patients. No significant difference in TRP, KYN, KYNA, QUIN, KYN/TRP, and KYNA/KYN between RRMS-relapse patients and

(Continued)

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TABLE 1 Continued

Study (ref)	Place	Patients (No)			Controls (No)			Age (mean (SD))		Types of MS	Treatment	Patients phase	Materials	Methods	Metabolites	Key findings
		All	Male	Female	All	Male	Female	Patients	Controls							
																OND patients. The levels of TRP and KYNA were significantly higher in PPMS patients than in SPMS and RRMS patients. The levels of QUIN and KYNA were significantly higher in PPMS patients than in OND patients.
Agliardi C (30)	Italy	675	235	440	680	271	409	50.0	64.17	596 RRMS, 79 PPMS	–	–	Blood	Spectrophotometrically	IDO2 mRNA expression	No significant differences in IDO2 activity between multiple sclerosis patients and HCs
Huang YM (34)	Sweden	37	0	37	37	0	37	31.59 (6.54)	31.19 (6.27)	RRMS	None	Remission	PBMCs	Spectrophotometrically	IDO mRNA expression	Lower levels of IDO mRNA expression in all the Multiple sclerosis patients compared to all HCs
Nejati A (36)	Iran	84	24	60	70	20	50	34.55 (8.83)	34.16 (8.26)	RRMS	7 (None), 74 (Interferon), 1 (Zidovudine), 1 (Mitoxantrone), 1 (Glatiramer acetate)	–	PBMCs	Spectrophotometrically	IDO mRNA expression	Lower levels of IDO mRNA expression in Multiple sclerosis patients compared to HCs
Lim (42)	Australia	87	29	58	49	14	35	47.44 (10.39)	45.29 (11.7)	50 RRMS, 20 SPMS, 17 PPMS	–		Serum, CSF	UHPLC	TRP, KYN, KYNA, KYN/TRP	Increased KYN/TRP in the serum of all the MS subtype groups, higher KYNA serum levels in the RRMS group relative to HCs and progressive MS groups
Olsson (37)	Denmark	58	14	44	50	16	34	34	33	RRMS	None	Before initiation of	Serum	Mass spectrometry	TRP, KYN, KYNA, IDO	Lower KYNA levels in MS patients, No

(Continued)

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TABLE 1 Continued

Study (ref)	Place	Patients (No)			Controls (No)			Age (mean (SD))		Types of MS	Treatment	Patients phase	Materials	Methods	Metabolites	Key findings
		All	Male	Female	All	Male	Female	Patients	Controls							
												the first disease modifying therapy				differences in IDO1 expressions between the two groups
Sadowska-bartosz (38)	poland	60			18			26-50	26-45	RRMS, SPMS	INF β1a, INF β1b, mitoxantrone		Serum	Fluorescence assessment	TRP, KYN	KYN increased in RRMS patients without treatment and RRMS patients treated with IFN-β1b
Rejdak (17)	poland	26								RRMS, SPMS	None	Remission	CSF	HPLC	KYNA	Lower KYNA levels in MS patients compared with patients with non-inflammatory neurological disorders
Rejdak (18)	poland	20	6	14	10	4	6	28	29	RRMS	None	Relapse	CSF	HPLC	KYNA	The CSF KYNA was higher in the RRMS group
Herman (33)	sweden	46			10			45.6 (13.6)	39	16 SPMS, 30 RRMS	–		CSF	HPLC	KYN	SPMS patients had higher KYN levels compared with healthy controls, and RRMS patients
Adamczyk-sowa (29)	poland	14	7	7	11	5	6	40.65 (10.01)	34.54 (9.6)	RRMS	IFNβ, melatonin		Serum	Fluorescence assessment	KYN	Levels of KYN were elevated in non-treated RRMS

CSF, cerebrospinal fluid; RBC, red blood cell; PBMCs, peripheral blood mononuclear cell; HPLC, High-performance liquid chromatography; UHPLC, Ultra-high performance liquid chromatography; TRP, tryptophan; KYN, kynurenine; KYNA, kynurenic acid; QUIN, quinolinic acid; PIC, picolinic acid; HIAA, hydroxyindoleacetic acid HCs, healthy controls; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; AMS, MS patients in acute phase; SMS, MS patients in stable phase.

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controls). Four studies (8, 16–18) used CSF as the source of the sample; two studies utilized both CSF and serum (39, 42), one study used serum samples (37), and one study employed both red blood cells (RBCs) and plasma as the source of the sample (32). Rejdak and his colleagues (17) reported in a study that KYNA levels in the CSF of MS patients in the remission phase are lower than in patients with non-inflammatory neurological disorders (P<0.01). However, in another study by Rejdak et al. (18), they reported significantly elevated KYNA levels in the CSF of RRMS patients who are in the relapse phase (P=0.01). Also, KYNA levels in the CSF were significantly lower in RRMS patients than in controls in the studies conducted by Tomosi et al. (39) (p=0.04). In a study done by Olsson et al. (37) it was shown that RRMS patients without any treatment had lower serum KYNA concentrations (P<0.05), but there were no significant differences between MS patients and controls in the studies performed by Aeinehband et al. (16) and Rajda et al. (8) (p>0.05 and p=0.42, respectively). On the other hand, Hartai et al. (32) discovered that MS patients had a remarkably higher level of KYNA in their plasma than controls (p<0.05). Additionally, Lim et al. revealed that RRMS patients have higher serum KYNA in comparison to healthy controls and progressive MS patients (P<0.0001). However, studies were done by Tomosi et al. (39) on serum, and Hartai et al. (32) on RBCs, reported no significant difference in KYNA levels between MS patients and healthy controls (p=0.16 and p>0.05, respectively).

Quinolinic acid

Three studies (8, 16, 39) collected data on QUIN levels in 151 individuals (95 MS patients and 56 healthy controls). In two studies (8, 16), the sample source was CSF; in one study, the sample source was both CSF and serum (39). Rajda et al. (8) and Tomosi et al. (39) showed that the QUIN levels in CSF were remarkably higher in MS patients than in healthy controls (p=0.001 and p<0.0001, respectively). Likewise, Tomosi et al. (39) reported a higher level of QUIN in serum samples of MS patients compared with controls (p=0.030). Conversely, there was no significant difference in the CSF levels of QUIN in a study conducted by Aeinehband et al. (16) (p>0.05).

Picolinic acid

Two studies (8, 39) collected data on the picolinic acid (PIC) levels of 93 individuals (75 MS patients and 18 controls). In one study (8), the sample was obtained from CSF, while in another study, the sample was obtained from both CSF and serum (39). Tomosi et al. found that PIC levels were significantly lower in the CSF sample of MS patients than in healthy controls (39) (p=0.02). However, according to Rajda et al. (8) and Tomosi et al. (39), there was no significant difference in PIC levels of CSF and serum samples, respectively, between MS patients and controls (p=0.59 and p=0.25, respectively).

Kynurenine, tryptophan, quinolinic acid ratio

Five studies measured KYN/TRP ratios (3, 16, 31, 39, 42) in 354 individuals (213 MS patients and 141 healthy controls). One study used urine as the sample source (31), one used CSF (16), two used both serum and CSF (39, 42), and the last study used serum samples (3). It has been elucidated by Lim et al. (42) that KYN/TRP was significantly increased in the serum samples of all the MS subtype groups (RRMS, PPMS, and SPMS) compared to healthy controls (P<0.0001). Also, Mancuso et al. (3) showed that KYN/TRP was higher in MS patients in the stable phase, MS patients in the acute phase before the initiation of glucocorticoids, and healthy controls compared with AMS patients after the initiation of glucocorticoids (P<0.05). Gaetani et al. (31) found that MS patients had remarkably lower KYN/TRP ratio in urine samples than the controls (p=0.04). In three studies were done by Aeinehband et al. (16), Mancuso et al. (3), and Tomosi et al. (39), no significant differences in KYN/TRP ratios were observed between MS patients and controls in CSF or serum samples (3, 16, 39).

Two studies (16, 39) assessed QUIN/KYNA ratios in 92 individuals (58 MS patients and 34 healthy controls). In one study, the sample source was CSF (16), while the other study used serum and CSF (39). According to the result of the study by Tomosi et al. (39), QUIN/KYNA ratios were significantly higher in MS patients compared to healthy controls in both CSF and serum samples (p=0.0015 and p=0.0183, respectively). However, Aeinehband et al. (16) reported that there was no significant difference in CSF QUIN/KYNA ratios between MS patients and controls (p>0.05).

Two studies (16, 39) involving 92 participants determined the KYNA/KYN ratio (58 MS patients and 34 healthy controls) in MS patients and controls. Tomosi et al. (39) discovered that MS patients had significantly lower KYNA/KYN ratios compared to controls when CSF samples were analyzed (p=0.0041), but there was no significant difference when serum samples were measured (p=0.0832). Aeinehband et al. (16) reported no statistically significant difference in KYNA/KYN ratios between MS patients and controls (p>0.05).

Indoleamine 2,3-dioxygenase

The expression of IDO mRNA was analyzed in six studies (3, 30, 34–37), including 1812 individuals (930 MS patients and 882 healthy controls). five studies (3, 30, 34–36) assessed IDO mRNA in mononuclear cells. One study (37) used whole blood for assessment. Four studies were done by Huang et al.

(34), Mancuso et al. (3), Negrotto et al. (35), and Nejati et al. (36) reported reduced levels of IDO mRNA expression in MS patients in comparison to controls ($p<0.05$, $p=0.01$, $p<0.001$, and $p<0.0001$ respectively). However, Agliardi et al. (30) and Olsson et al. (37) showed no difference in IDO mRNA expression between MS patients and healthy controls. Additionally, Negrotto et al. (35) measured IDO1 protein levels in PBMCs and detected reduced IDO1 protein expression in MS patients when compared to healthy controls ($p<0.001$).

Kynurenine aminotransferase

One study, including 27 individuals (13 MS patients and 14 healthy controls) measured the enzymatic activity of both KAT I and KAT II in plasma and RBCs (32). It showed that the activities of both KAT I and KAT II enzymes are significantly higher in RBCs of MS patients compared with healthy controls ($p<0.05$). However, no significant difference in KAT I and KAT II plasma enzymatic activity could be detected between MS patients and healthy controls.

Discussion

In recent years, there has been mounting evidence that KP plays a significant role in neurodegenerative diseases such as MS (27). Inflammation or degeneration of the CNS induces the metabolism of TRP primarily through the production of KYN and related breakdown products (43). As MS progresses, levels of inflammatory cytokines, including interferon-γ (IFN-γ) and Tumor necrosis factor-α (TNF-α), increase, activating KP (44, 45). This study systematically reviewed 10 published primary research articles investigating differences between MS patients and healthy controls in serum, CSF, and urine levels of six major metabolites and two enzymes associated with the KP. We focused on TRP, KYN, KYNA, QUIN, and PIC levels as well as IDO mRNA expression and KAT activity.

MS pathogenesis likely involves several different mechanisms. One of the most popular hypotheses is that the infiltration of immune-activated macrophages and T cells causes death of oligodendrocytes that are responsible for myelinating axons in a healthy CNS (46, 47). KP metabolites have been suggested to promote both immune tolerance and autoimmunity according to this model of MS pathogenesis. Studies have revealed significantly lower TRP levels in the serum and CSF of MS patients, suggesting that KP activation may play a role in the disease pathogenesis (24, 25). In the human CNS, TRP is mostly metabolized through KP. Nevertheless, there are cells that do not express the entire enzymatic pathway. Only reactive microglia, infiltrating macrophages, and active neurons contain the complete pathway (43, 48). A study using urine samples from 47 Relapsing Remitting Multiple Sclerosis (RRMS) patients and 43 healthy controls reported that women had lower levels of urinary TRP and KYN than men (31). After adjusting for age and gender, urine concentrations of TRP did not show a significant difference between the RRMS and control group. Although the expanded disability scale (EDSS) has shown significant correlation with TRP urinary concentrations, disease duration has not been associated with KP metabolite levels (31). In contrast, another study reported significantly higher levels of TRP in PBMCs of RRMS patients compared to healthy controls (35). Aeinehband et al. (16) investigated cross-sectional cell-free CSF samples from patients with RRMS in both the relapse and remission phases, Primary-Progressive Multiple Sclerosis (PPMS), Secondary-Progressive Multiple Sclerosis (SPMS), for KP metabolites, using patients living with other neurological diseases, including syringomyelia, vertigo, anxiety, postcommotio syndrome, alcohol-related spastic paraparesis, neurasthenia, and unspecific sensory symptoms, as controls. They found that although there was no absolute difference in CSF concentrations of KP metabolites between PPMS and SPMS patients, PPMS patients displayed increased levels of all metabolites except for TRP in comparison to SPMS patients (16). In addition, disparities in TRP concentrations could be associated with variable characteristics of the enrolled patients reflecting the correlation between disease activity as well as disease courses with changes in KP metabolites. Moreover, inflammatory processes that initiate KP metabolism are associated with fluctuations in cytokine concentrations throughout the various phases of MS (3), which may contribute to the controversial results reported in recent publications. Future studies should compare concentrations of KP metabolites and MS disease activity in order to find novel therapeutic targets and prognostic markers.

The conversion of TRP to KYN is the first and rate-limiting step in KP metabolism, and is regulated by IDO-1 in most human tissues and TDO in liver cells (49). There have been multiple studies indicating that KYN influences the proliferation of several T cell subtypes, including $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes (50–52). In addition, it has been demonstrated that KYN can compromise the function of natural killer cells while simultaneously showing pro-apoptotic effects (53, 54). Therefore, KYN levels have been measured in the serum, CSF, PBMCs, and urine of MS patients and compared to healthy controls. There have been substantial differences among published results. RRMS patients had considerably lower urinary KYN concentrations when compared to healthy controls (31); however, KYN concentrations in CSF did not differ significantly between MS and non-inflammatory neurological disorders patients (16). When RRMS patients treated with IFN-β were compared to untreated RRMS patients, an increase in KYN level was observed (29, 55, 56). In contrast, another study did not find

any alterations of KP activation resulting from IFN-β therapy in untreated MS patients (16).

The KYN/TRP ratio is indicative of the IDO activity as well as KP. IDO expression could potently be induced by several mediators including IFN-γ, TGF-β, toll-like receptor ligands, polyamines, TNF-α, platelet activating factor, and human immunodeficiency virus (HIV) proteins (51, 57–61). IDO mRNA expression was found to be lower in MS patients compared to healthy controls (3, 31, 34–36). Lower KYN along with a decreased urine KYN/TRP ratio in RRMS patients was found to be inversely related to the intensity of disability, suggesting a reduced TRP metabolism in the earliest stages of the disease (31, 62). Contrarily, some studies determined a significantly increased KYN/TRP ratio in MS patients compared to healthy controls (42), in addition to other studies demonstrating no meaningful difference in KYN/TRP ratios between MS patients and healthy subjects (3, 16, 39). These discrepancies could result from different biofluid samples analyzed, indicating different phases of TRP metabolism and variation in KP enzymes involved in each site. Moreover, the treatment that MS patients receive may affect the KP metabolites. For example, in the study conducted by Gaetani et al. (31), most MS patients received MS treatment, especially interferons. Thus, to elucidate the variation in KP metabolite concentrations in different organs and tissues, further controlled studies on different body fluids such as urine, blood, and CSF concurrently in the same subjects are needed.

Enzyme KAT converts KYN to KYNA (63). The KYNA/KYN ratio was increased in the CSF of MS patients compared to controls, while no difference was detected in serum ratios from the same subjects (39). Authors, in line with previous studies, also demonstrated lower CSF levels of KYNA among MS patients compared to healthy controls (17). Lim et al. demonstrated decreases in the levels of both enzymes that produce KYNA in the postmortem MS brain sections, correlating with lower levels of KYNA (64). In disagreement, KYNA concentrations were found to be significantly higher in the plasma and cerebrospinal fluid of patients with MS compared to healthy subjects. Additionally, researchers have stated that KYNA has a neuroprotective role in progressive MS (32, 65). PPMS patients are unique in having significantly increased levels of KYNA, which has been found to display neuroprotective effects both experimentally and clinically, decelerating disease progression (66, 67). Further research has revealed elevated KYNA levels during the acute relapse phase of MS (18). Conversely, SPMS patients show a decreased neuroprotection index (68), confirming the idea of altered KP activation among patients with different MS clinical courses.

In contrast to neuroprotective KYNA, QUIN is considered a neurotoxic metabolite of KP (6, 6, 19), thus, shifting KP toward KYNA instead of QUIN could be a potential therapeutic strategy. Although astrocytes do not utilize the full enzymatic pathway, they produce high levels of KYN that can be metabolized to QUIN by microglia, monocytes, or infiltrating macrophages and result in neurotoxicity (69–71). The higher QUIN/KYN ratio during the relapse phase of MS patients compared with remission phase, confirms that the QUIN-induced apoptosis of myelin producing oligodendrocytes as a sign of failed remyelination (16, 68, 69, 72). Furthermore, QUIN was found to be responsible for the impaired phosphorylation of tau protein in progressive MS (73). Indeed, QUIN could be a potential biomarker of active relapse and demyelinating phases of MS (Figure 2).

Very few studies have measured PIC in MS patients and controls. PIC induces inflammatory macrophage proteins in association with IFN-γ at low concentrations and acts as an activator of macrophages (74–76). This process of macrophages co-activation by PIC emphasizes the importance of PIC neuroprotection in neurodegenerative conditions (77). Decreased PIC levels in MS are consistent with its protective role in this and other degenerative disorders. Notably, another study demonstrated the inverse relationship between PIC and QUIN, with PIC being higher in RRMS groups but lower in PPMS groups (78).

In summary, the importance of KP metabolites as prognostic, diagnostic, and therapeutic biomarkers is commonly known. It is still unknown whether KP is beneficial in the pathogenesis of MS by acting as a protective pathway or whether its activation is a sign of deterioration; however, it is well established that prolonged KP metabolism and the accumulation of neurotoxic metabolites accelerate the progression of MS. More controlled studies on specific fluid samples from particular disease phases are needed to unravel the changes the KP undergoes during MS pathogenesis. Further research is necessary to evaluate the KP metabolism rate and its possible subtypes in patients with MS at all stages while also considering demographic data of patients, including age and sex. Moreover, due to changes in KP during different phases of MS and in different types of MS and the effect of MS treatment on KP, it is suggested to report data about the type and phase of MS in patients and the treatment that they have been received when measuring the KP metabolites. Moreover, given the effect of disease-modifying therapies such as IFN-β1 on KP metabolite levels and the effect of KP activation on treatment efficacy, additional research should focus on the effect of available therapies on KP metabolite concentrations and their effects on treatment efficacy.

Limitations

Our study has important limitations. First, reported details of patient characteristics were limited, consequently, findings could not be conclusively extrapolated to MS in general. Second, only a small number of selected articles met our criteria for covering all MS stages. This could be one of the reasons for the discrepancies mentioned above. Third, the studies that were investigated included samples collected from different tissues, which made it difficult to comprehensively compare the results.

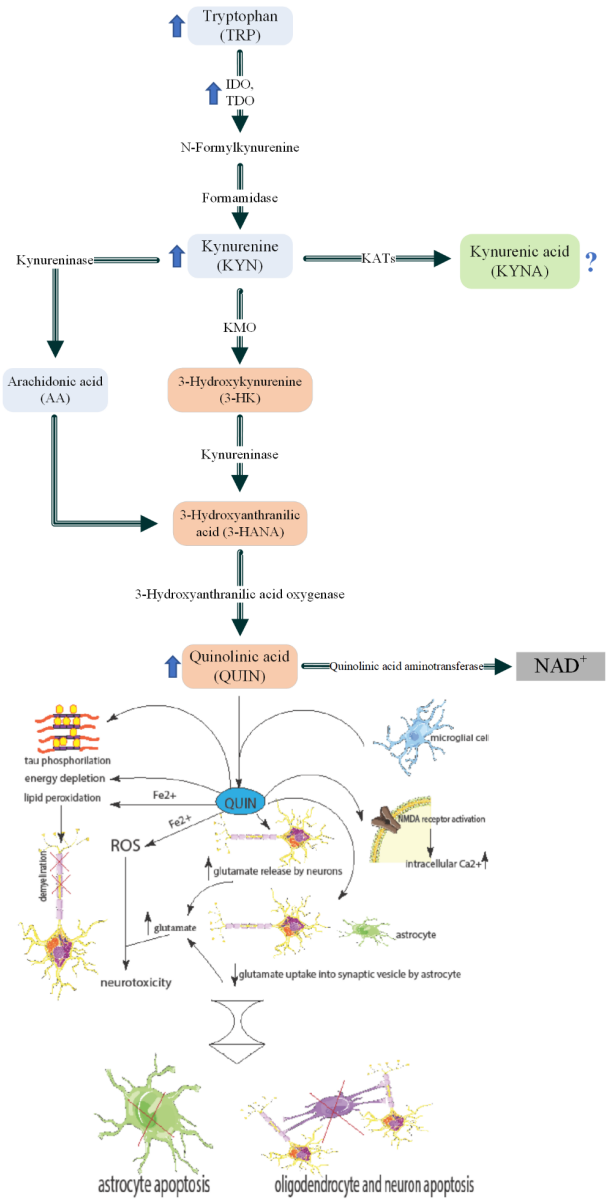

FIGURE 2
Kynurenine pathway and roles of QUIN in MS pathogenesis. Inflammatory cytokines such as TNF-α and INF-α which are increased in MS patients blood circulation. These induce increased activity and levels of IDO and KMO in macrophages. Higher activity of these enzymes leads to KP activation and thus high levels of QUIN is secreted to the blood. The increased QUIN can pass the BBB and enter the brain parenchyma. This QUIN is together with the QUIN secreted by microglia lead to several pathological mechanisms: 1) NMDA receptor activation in the cells and therefore higher intracellular calcium. 2) increased glutamate release by neurons and decreased glutamate uptake into synaptic vesicles by astrocytes. These cause higher glutamate levels in the micro-environment which cause neurotoxicity. 3) Increased ROS formation which causes neurotoxicity. 4) Lipid peroxidation that can lead to demyelination. 5) Energy depletion 6) Tau phosphorylation. The mentioned mechanisms, generally cause apoptosis of astrocytes, oligodendrocytes (an important cell in myelin production), and neurons. Parts of the figure were drawn by using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

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TABLE 2 Evaluation of quality of included studies using the QUADOMICS tool.

Study (ref)	1	2	3	4.1	4.2	5	6	7	8	9	10	11	12	13	14	15	16
Rajda C (8)	-	+	+	-	+	+	+	+	+	+	+	+	?	+	+	+	?
Gaetani L (31)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	?
Hartai Z (32)	-	+	-	+	+	+	+	+	+	+	+	?	?	+	+	+	?
Tomosi F (39)	-	+	+	+	-	+	+	?	+	+	+	-	+	+	+	+	+
Mancuso R (3)	-	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+	?
Nergotto L (35)	-	+	-	+	+	+	+	+	+	+	+	+	?	+	+	+	?
Aeinehband S (16)	-	+	?	+	+	+	+	+	+	+	+	+	?	+	+	+	?
Agliardi C (30)	+	+	+	+	-	?	+	+	+	+	+	+	?	+	+	+	?
Huang YM (34)	-	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+	?
Nejati A (36)	+	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+	?
Lim (42)	-	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	?
Olsson (37)	-	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+	+
Sadowska-bartosz (38)	+	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+	?
Rejdak (17)	-	+	+	+	+	+	+	+	+	+	+	?	?	+	+	+	?
Rejdak (18)	-	+	+	+	+	+	+	+	+	+	+	?	?	+	+	+	?
Herman (33)	+	+	+	+	-	+	+	+	+	+	+	+	?	+	+	+	?
Adamczyk-sowa (29)	-	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+	?

Conclusion

In conclusion, this study established a potential link between altered KP metabolite levels and MS disease progression. Based on our systematic review, different KYN metabolites can be measured in MS, highlighting the potential role of KP in the pathophysiology of MS. This finding is critical for future research, which would benefit from larger scale studies comparing KP metabolites in individuals with MS. QUIN has previously been suggested to contribute to neurodegeneration. In this review we found that QUIN levels in the CSF of MS patients was higher compared to healthy controls, indicating that QUIN may play a role in MS pathogenesis. Although it was suggested that KYNA is neuroprotective and have beneficial effects in MS, the difference of KYNA levels between MS patients and controls was not significant. Also, different levels of other KP metabolites, including KYN, TRP, PIC and their ratio were also found between MS patients and controls; however, there were discrepancies between studies. Further high-quality studies on peripheral and central KP metabolite concentrations are required to better understand the dynamics of these metabolite

levels in MS. Further research is also necessary to overcome our study limitations and to evaluate the rate of KP metabolism and its possible subtypes in patients with MS at all stages and ages. Moreover, given the effect of disease-modifying therapies such as IFN-β1 on KP metabolite levels and the effect of KP activation on treatment efficacy, additional research should focus on the effect of available therapies on KP metabolite concentrations and their likely effects on treatment efficacy.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Author contributions

MF, KV, and SY contributed to the conception and design of the study. MH and FS contributed to the supervision of the manuscript. SY organized the database. AK and AMc edited the paper scientifically. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Walton C, King R, Rechtman L, Kaye W, Leray E, Marrie RA, et al. Rising prevalence of multiple sclerosis worldwide: Insights from the atlas of MS, third edition. *Mult Scler* (2020) 26(14):1816–21. doi: 10.1177/1352458520970841

2. Thompson AJ, Banwell BL, Barkhof F, Carroll WM, Coetzee T, Comi G, et al. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *Lancet Neurol* (2018) 17(2):162–73. doi: 10.1016/S1474-4422(17)30470-2

3. Mancuso R, Hernis A, Agostini S, Rovaris M, Caputo D, Fuchs D, et al. Indoleamine 2, 3 dioxygenase (IDO) expression and activity in relapsing-remitting multiple sclerosis. *PloS One* (2015) 10(6):e0130715. doi: 10.1371/journal.pone.0130715

4. Stovner LJ, Zwart JA, Hagen K, Terwindt G, Pascual J. Epidemiology of headache in Europe. *Eur J Neurol* (2006) 13(4):333–45. doi: 10.1111/j.1468-1331.2006.01184.x

5. Stinissen P, Hellings N. Activation of myelin reactive T cells in multiple sclerosis: A possible role for T cell degeneracy? *Eur J Immunol* (2008) 38(5):1190–3. doi: 10.1002/eji.200838371

6. Biernacki T, Sandi D, Bencsik K, Vécsei L. Kynurenines in the pathogenesis of multiple sclerosis: Therapeutic perspectives. *Cells* (2020) 9(6):1564. doi: 10.3390/cells9061564

7. Beck J, Rondot P, Catinot L, Falcoff E, Kirchner H, Wietzerbin J. Increased production of interferon gamma and tumor necrosis factor precedes clinical manifestation in multiple sclerosis: do cytokines trigger off exacerbations? *Acta Neurol Scand* (1988) 78(4):318–23. doi: 10.1111/j.1600-0404.1988.tb03663.x

8. Rajda C, Galla Z, Polyák H, Maróti Z, Babarczy K, Pukoli D, et al. Cerebrospinal fluid neurofilament light chain is associated with kynurenine pathway metabolite changes in multiple sclerosis. *Int J Mol Sci* (2020) 21(8):2665. doi: 10.3390/ijms21082665

9. Penberthy WT, Tsunoda I. The importance of NAD in multiple sclerosis. *Curr Pharm design* (2009) 15(1):64–99. doi: 10.2174/138161209787185751

10. Tan VX, Guillemin GJ. Kynurenine pathway metabolites as biomarkers for amyotrophic lateral sclerosis. *Front Neurosci* (2019) 13. doi: 10.3389/fnins.2019.01013

11. Huang Y-S, Ogbechi J, Clanchy FI, Williams RO, Stone TW. IDO and kynurenine metabolites in peripheral and CNS disorders. *Front Immunol* (2020) 11:388. doi: 10.3389/fimmu.2020.00388

12. Majláth Z, Annus A, Vecsei L. Kynurenine system and multiple sclerosis, pathomechanism and drug targets with an emphasis on laquinimod. *Curr Drug Targets* (2018) 19(7):805–14. doi: 10.2174/1389450117666161223125417

13. Masoudi S, Nemati AH, Fazli HR, Beygi S, Moradzadeh M, Pourshams A, et al. An increased level of aryl hydrocarbon receptor in patients with pancreatic cancer. *Middle East J digestive Dis* (2019) 11(1):38. doi: 10.15171/mejdd.2018.126

14. Nguyen NT, Nakahama T, Le DH, Van Son L, Chu HH, Kishimoto T. Aryl hydrocarbon receptor and kynurenine: recent advances in autoimmune disease research. *Front Immunol* (2014) 5:551. doi: 10.3389/fimmu.2014.00551

15. Joisten N, Rademacher A, Warnke C, Proschinger S, Schenk A, Walzik D, et al. Exercise diminishes plasma neurofilament light chain and reroutes the kynurenine pathway in multiple sclerosis. *Neurol-Neuroimmunol Neuroinflamm* (2021) 8(3):1–11. doi: 10.1212/NXI.0000000000000982

16. Aeinehband S, Brenner P, Ståhl S, Bhat M, Fidock MD, Khademi M, et al. Cerebrospinal fluid kynurenines in multiple sclerosis; relation to disease course and neurocognitive symptoms. *Brain beh Immun* (2016) 51:47–55. doi: 10.1016/j.bbi.2015.07.016

17. Rejdak K, Bartosik-Psujek H, Dobosz B, Kocki T, Grieb P, Giovannoni G, et al. Decreased level of kynurenic acid in cerebrospinal fluid of relapsing-onset multiple sclerosis patients. *Neurosci Lett* (2002) 331(1):63–5. doi: 10.1016/S0304-3940(02)00710-3

18. Rejdak K, Petzold A, Kocki T, Kurzepa J, Grieb P, Turski W, et al. Astrocytic activation in relation to inflammatory markers during clinical exacerbation of relapsing-remitting multiple sclerosis. *J Neural Transm* (2007) 114(8):1011. doi: 10.1007/s00702-007-0667-y

19. Sundaram G, Brew BJ, Jones SP, Adams S, Lim CK, Guillemin GJ. Quinolinic acid toxicity on oligodendroglial cells: relevance for multiple sclerosis and therapeutic strategies. *J Neuroinflamm* (2014) 11(1):1–11. doi: 10.1186/s12974-014-0204-5

20. Addae JI, Stone TW. Quinolinic acid and related excitotoxins: Mechanisms of neurotoxicity and disease relevance. In: *Handbook of neurotoxicity*. Springer Nature (2021) 1–22.

21. Sakurai K, Zou J-P, Tschetter JR, Ward JM, Shearer GM. Effect of indoleamine 2, 3-dioxygenase on induction of experimental autoimmune encephalomyelitis. *J neuroimmunol* (2002) 129(1-2):186–96. doi: 10.1016/S0165-5728(02)00176-5

22. Maddison DC, Giorgini F. The kynurenine pathway and neurodegenerative disease. *Semin Cell Dev Biol* (2015) 40:134–41. doi: 10.1016/j.semcdb.2015.03.002

23. Vécsei L, Szalárdy L, Fülöp F, Toldi J. Kynurenines in the CNS: recent advances and new questions. *Nat Rev Drug Discovery* (2013) 12(1):64–82. doi: 10.1038/nrd3793

24. Monaco F, Fumero S, Mondino A, Mutani R. Plasma and cerebrospinal fluid tryptophan in multiple sclerosis and degenerative diseases. *J Neurol Neurosurg Psychiatry* (1979) 42(7):640–1. doi: 10.1136/jnnp.42.7.640

25. Rudzite V, Berzinsh J, Grivane I, Fuchs D, Baier-Bitterlich G, Wachter H. Serum tryptophan, kynurenine, and neopterin in patients with Guillain-barre-syndrome (GBS) and multiple sclerosis (MS). In: *Recent advances in tryptophan research*. Springer, Boston, MA: Springer (1996). p. 183–7.

26. Sandyk R. Tryptophan availability and the susceptibility to stress in multiple sclerosis: A hypothesis. *Int J Neurosci* (1996) 86(1-2):47–53. doi: 10.3109/00207459608986697

27. Füvesi J, Rajda C, Bencsik K, Toldi J, Vécsei L. The role of kynurenines in the pathomechanism of amyotrophic lateral sclerosis and multiple sclerosis: therapeutic implications. *J Neural Transm* (2012) 119(2):225–34. doi: 10.1007/s00702-012-0765-3

28. Dorszewska J, Florczak-Wyspianska J, Kowalska M, Stanski M, Kowalewska A, Kozubski W. Serotonin in neurological diseases. In: *Serotonin: A chemical messenger between all types of living cells*. InTech (2017) 219.

29. Adamczyk-Sowa M, Galiniak S, Żyracka E, Grzesik M, Naparło K, Sowa P, et al. Oxidative modification of blood serum proteins in multiple sclerosis after interferon beta and melatonin treatment. *Oxid Med Cell Longevity* (2017) 2017:7905148. doi: 10.1155/2017/7905148

30. Agliardi C, Guerini FR, Zanzottera M, Rovaris M, Caputo D, Clerici M. Indoleamine-2, 3-dioxygenase (IDO) 2 polymorphisms are not associated with multiple sclerosis in italians. *J Neurol Sci* (2017) 377:31–4. doi: 10.1016/j.jns.2017.03.048

31. Gaetani L, Boscaro F, Pieraccini G, Calabresi P, Romani L, Di Filippo M, et al. Host and microbial tryptophan metabolic profiling in multiple sclerosis. *Front Immunol* (2020) 11:157. doi: 10.3389/fimmu.2020.00157

32. Hartai Z, Klivenyi P, Janaky T, Penke B, Dux L, Vecsei L. Kynurenine metabolism in multiple sclerosis. *Acta Neurol Scand* (2005) 112(2):93–6. doi: 10.1111/j.1600-0404.2005.00442.x

33. Herman S, Åkerfeldt T, Spjuth O, Burman J, Kultima K. Biochemical differences in cerebrospinal fluid between secondary progressive and relapsing–remitting multiple sclerosis. *Cells* (2019) 8(2):84. doi: 10.3390/cells8020084

34. Huang Y-M, Liu X, Steffensen K, Sanna A, Arru G, Fois ML, et al. Immunological heterogeneity of multiple sclerosis in Sardinia and Sweden. *Multiple Sclerosis J* (2005) 11(1):16–23. doi: 10.1191/1352458505ms1127oa

35. Negrotto L, Correale J. Amino acid catabolism in multiple sclerosis affects immune homeostasis. *J Immunol (Baltimore Md 1950)* (2017) 198(5):1900–9. doi: 10.4049/jimmunol.1601139

36. Nejati A, Shoja Z, Shahmahmoodi S, Tafakhori A, Mollaei-Kandelous Y, Rezaei F, et al. EBV and vitamin d status in relapsing-remitting multiple sclerosis patients with a unique cytokine signature. *Med Microbiol Immunol* (2016) 205(2):143–54. doi: 10.1007/s00430-015-0437-7

37. Olsson A, Gustavsen S, Nguyen TD, Nyman M, Langkilde AR, Hansen TH, et al. Serum short-chain fatty acids and associations with inflammation in newly diagnosed patients with multiple sclerosis and healthy controls. *Front Immunol* (2021) 12:661493. doi: 10.3389/fimmu.2021.661493

38. Sadowska-Bartosz I, Adamczyk-Sowa M, Galiniak S, Mucha S, Pierzchala K, Bartosz G. Oxidative modification of serum proteins in multiple sclerosis. *Neurochem Int* (2013) 63(5):507–16. doi: 10.1016/j.neuint.2013.08.009

39. Tömösi F, Kecskeméti G, Cseh EK, Szabó E, Rajda C, Kormány R, et al. A validated UHPLC-MS method for tryptophan metabolites: Application in the diagnosis of multiple sclerosis. *J Pharm Biomed Anal* (2020) 185:113246. doi: 10.1016/j.jpba.2020.113246

40. Kayaoğlu MY, Girgin G, Solmaz I, Baydar T, Anlar B. Urine neopterin in childhood acute demyelinating diseases: Potential for differential diagnosis. *Multiple sclerosis related Disord* (2022) 59:103662. doi: 10.1016/j.msard.2022.103662

41. Sundaram G, Lim CK, Brew BJ, Guillemin GJ. Kynurenine pathway modulation reverses the experimental autoimmune encephalomyelitis mouse disease progression. *J Neuroinflamm* (2020) 17(1):1–14. doi: 10.1186/s12974-020-01844-y

42. Lim CK, Bilgin A, Lovejoy DB, Tan V, Bustamante S, Taylor BV, et al. Kynurenine pathway metabolomics predicts and provides mechanistic insight into multiple sclerosis progression. *Sci Rep* (2017) 7:41473. doi: 10.1038/srep41473

43. Lovelace MD, Varney B, Sundaram G, Franco NF, Ng ML, Pai S, et al. Current evidence for a role of the kynurenine pathway of tryptophan metabolism in multiple sclerosis. *Front Immunol* (2016) 7(246). doi: 10.3389/fimmu.2016.00246

44. Rajda C, Majláth Z, Pukoli D, Vécsei L. Kynurenines and multiple sclerosis: The dialogue between the immune system and the central nervous system. *Int J Mol Sci* (2015) 16(8):18270–82. doi: 10.3390/ijms160818270

45. Lovelace MD, Varney B, Sundaram G, Lennon MJ, Lim CK, Jacobs K, et al. Recent evidence for an expanded role of the kynurenine pathway of tryptophan metabolism in neurological diseases. *Neuropharmacology* (2017) 112(Pt B):373–88. doi: 10.1016/j.neuropharm.2016.03.024

46. Raine CS, Scheinberg LC. On the immunopathology of plaque development and repair in multiple sclerosis. *J neuroimmunol* (1988) 20(2-3):189–201. doi: 10.1016/0165-5728(88)90160-9

47. O'Connor RA, Prendergast CT, Sabatos CA, Lau CW, Leech MD, Wraith DC, et al. Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. *J Immunol* (2008) 181(6):3750–4. doi: 10.4049/jimmunol.181.6.3750

48. Guillemin GJ, Smith DG, Kerr SJ, Smythe GA, Kapoor V, Armati PJ, et al. Characterisation of kynurenine pathway metabolism in human astrocytes and implications in neuropathogenesis. *Redox Rep* (2000) 5(2-3):108–11. doi: 10.1179/135100000101535375

49. Sforzini L, Nettis MA, Mondelli V, Pariante CM. Inflammation in cancer and depression: A starring role for the kynurenine pathway. *Psychopharmacology* (2019) 236(10):2997–3011. doi: 10.1007/s00213-019-05200-8

50. Frumento G, Rotondo R, Tonetti M, Damonte G, Benatti U, Ferrara GB. Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2, 3-dioxygenase. *J Exp Med* (2002) 196(4):459–68. doi: 10.1084/jem.20020121

51. Grohmann U, Bronte V. Control of immune response by amino acid metabolism. *Immunol Rev* (2010) 236(1):243–64. doi: 10.1111/j.1600-065X.2010.00915.x

52. Fallarino F, Grohmann U, Vacca C, Bianchi R, Orabona C, Spreca A, et al. T Cell apoptosis by tryptophan catabolism. *Cell Death Differentiation* (2002) 9(10):1069–77. doi: 10.1038/sj.cdd.4401073

53. Chiesa MD, Carlomagno S, Frumento G, Balsamo M, Cantoni C, Conte R, et al. The tryptophan catabolite l-kynurenine inhibits the surface expression of NKp46-and NKG2D-activating receptors and regulates NK-cell function. *Blood* (2006) 108(13):4118–25. doi: 10.1182/blood-2006-03-006700

54. Terness P, Bauer TM, Roüse L, Dufter C, Watzlik A, Simon H, et al. Inhibition of allogeneic T cell proliferation by indoleamine 2, 3-dioxygenase–expressing dendritic cells: mediation of suppression by tryptophan metabolites. *J Exp Med* (2002) 196(4):447–57. doi: 10.1084/jem.20020052

55. Guillemin GJ, Kerr SJ, Pemberton LA, Smith DG, Smythe GA, Armati PJ, et al. IFN-β 1b induces kynurenine pathway metabolism in human macrophages: potential implications for multiple sclerosis treatment. *J Interferon Cytokine Res* (2001) 21(12):1097–101. doi: 10.1089/107999001317205231

56. Amirkhani A, Rajda C, Arvidsson B, Bencsik K, Boda K, Seres E, et al. Interferon-β affects the tryptophan metabolism in multiple sclerosis patients. *Eur J Neurol* (2005) 12(8):625–31. doi: 10.1111/j.1468-1331.2005.01041.x

57. Guillemin GJ, Smythe GA, Veas LA, Takikawa O, Brew BJ. Aβ1-42 induces production of quinolinic acid by human macrophages and microglia. *Neuroreport* (2003) 14(18):2311–5. doi: 10.1097/00001756-200312190-00005

58. O'Connor JC, André C, Wang Y, Lawson MA, Szegedi SS, Lestage J, et al. Interferon-γ and tumor necrosis factor-α mediate the upregulation of indoleamine 2, 3-dioxygenase and the induction of depressive-like behavior in mice in response to bacillus calmette-guerin. *J Neurosci* (2009) 29(13):4200–9. doi: 10.1523/JNEUROSCI.5032-08.2009

59. Smith DG, Guillemin GJ, Pemberton L, Kerr S, Nath A, Smythe GA, et al. Quinolinic acid is produced by macrophages stimulated by platelet activating factor, nef and tat. *J neurovirol* (2001) 7(1):56–60. doi: 10.1080/135502801300069692

60. Mondanelli G, Ugel S, Grohmann U, Bronte V. The immune regulation in cancer by the amino acid metabolizing enzymes ARG and IDO. *Curr Opin Pharmacol* (2017) 35:30–9. doi: 10.1016/j.coph.2017.05.002

61. Mondanelli G, Iacono A, Allegrucci M, Puccetti P, Grohmann U. Immunoregulatory interplay between arginine and tryptophan metabolism in health and disease. *Front Immunol* (2019) 10:1565. doi: 10.3389/fimmu.2019.01565

62. Puccetti P, Grohmann U. IDO and regulatory T cells: A role for reverse signalling and non-canonical NF-κB activation. *Nat Rev Immunol* (2007) 7(10):817–23. doi: 10.1038/nri2163

63. Han Q, Li J, Li J. pH dependence, substrate specificity and inhibition of human kynurenine aminotransferase I. *Eur J Biochem* (2004) 271(23-24):4804–14. doi: 10.1111/j.1432-1033.2004.04446.x

64. Lim CK, Brew BJ, Sundaram G, Guillemin GJ. Understanding the roles of the kynurenine pathway in multiple sclerosis progression. *Int J Tryptophan Res* (2010) 3:S4294. doi: 10.4137/IJTR.S4294

65. Kepplinger B, Baran H, Kainz A, Ferraz-Leite H, Newcombe J, Kalina P. Age-related increase of kynurenic acid in human cerebrospinal fluid–IgG and β2-microglobulin changes. *Neurosignals* (2005) 14(3):126–35. doi: 10.1159/000086295

66. Andiné P, Lehmann A, Ellrén K, Wennberg E, Kjellmer I, Nielsen T, et al. The excitatory amino acid antagonist kynurenic acid administered after hypoxic-ischemia in neonatal rats offers neuroprotection. *Neurosci Lett* (1988) 90(1-2):208–12. doi: 10.1016/0304-3940(88)90813-0

67. Atlas A, Franzen-Röhl E, Söderlund J, Jönsson EG, Samuelsson M, Schwieler L, et al. Sustained elevation of kynurenic acid in the cerebrospinal fluid of patients with herpes simplex virus type 1 encephalitis. *Int J Tryptophan Res* (2013) 6:S13256. doi: 10.4137/IJTR.S13256

68. Myint A-M, Kim YK, Verkerk R, Scharpé S, Steinbusch H, Leonard B. Kynurenine pathway in major depression: evidence of impaired neuroprotection. *J Affect Disord* (2007) 98(1-2):143–51. doi: 10.1016/j.jad.2006.07.013

69. Sas K, Robotka H, Toldi J, Vécsei L. Mitochondria, metabolic disturbances, oxidative stress and the kynurenine system, with focus on neurodegenerative disorders. *J neurol Sci* (2007) 257(1-2):221–39. doi: 10.1016/j.jns.2007.01.033

70. Guillemin GJ, Cullen KM, Lim CK, Smythe GA, Garner B, Kapoor V, et al. Characterization of the kynurenine pathway in human neurons. *J Neurosci* (2007) 27(47):12884–92. doi: 10.1523/JNEUROSCI.4101-07.2007

71. Lim CK, Smythe GA, Stocker R, Brew BJ, Guillemin GJ. Characterization of the kynurenine pathway in human oligodendrocytes. In: *International congress series*. Netherlands: Elsevier (2007).

72. Cammer W. Oligodendrocyte killing by quinolinic acid *in vitro*. *Brain Res* (2001) 896(1-2):157–60. doi: 10.1016/S0006-8993(01)02017-0

73. Anderson J, Hampton D, Patani R, Pryce G, Crowther R, Reynolds R, et al. Abnormally phosphorylated tau is associated with neuronal and axonal loss in experimental autoimmune encephalomyelitis and multiple sclerosis. *Brain* (2008) 131(7):1736–48. doi: 10.1093/brain/awn119

74. Bosco MC, Rapisarda A, Massazza S, Melillo G, Young H, Varesio L. The tryptophan catabolite picolinic acid selectively induces the chemokines macrophage inflammatory protein-1α and-1β in macrophages. *J Immunol* (2000) 164(6):3283–91. doi: 10.4049/jimmunol.164.6.3283

75. Melillo G, Cox GW, Radzioch D, Varesio L. Picolinic acid, a catabolite of l-tryptophan, is a costimulus for the induction of reactive nitrogen intermediate production in murine macrophages. *J Immunol* (1993) 150(9):4031–40.

76. Melillo G, Cox GW, Biragyn A, Sheffler LA, Varesio L. Regulation of nitric-oxide synthase mRNA expression by interferon-gamma and picolinic acid. *J Biol Chem* (1994) 269(11):8128–33. doi: 10.1016/S0021-9258(17)37169-7

77. Guillemin GJ, Kerr SJ, Smythe GA, Smith DG, Kapoor V, Armati PJ, et al. Kynurenine pathway metabolism in human astrocytes: A paradox for neuronal protection. *J neurochem* (2001) 78(4):842–53. doi: 10.1046/j.1471-4159.2001.00498.x

78. Grant R, Coggan S, Smythe G. The physiological action of picolinic acid in the human brain. *Int J Tryptophan Res* (2009) 2:S2469. doi: 10.4137/IJTR.S2469

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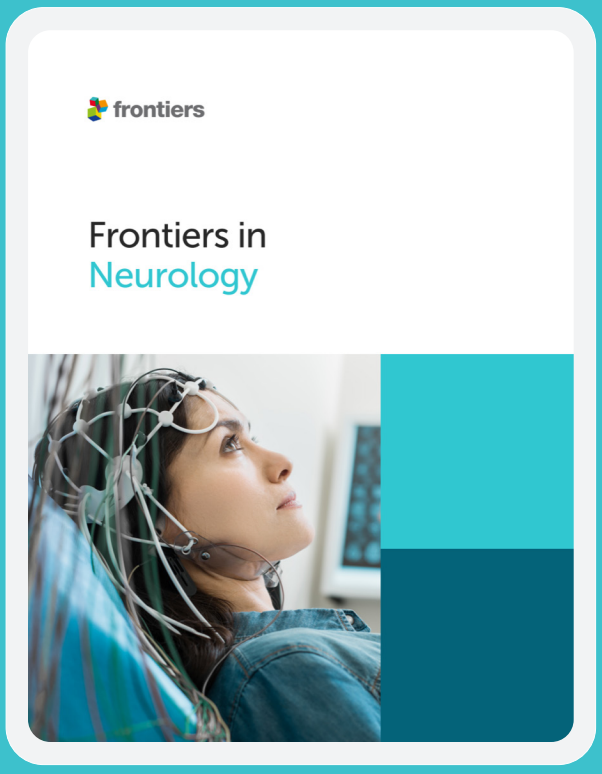

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