DISEASE MODIFYING THERAPIES IN MULTIPLE SCLEROSIS

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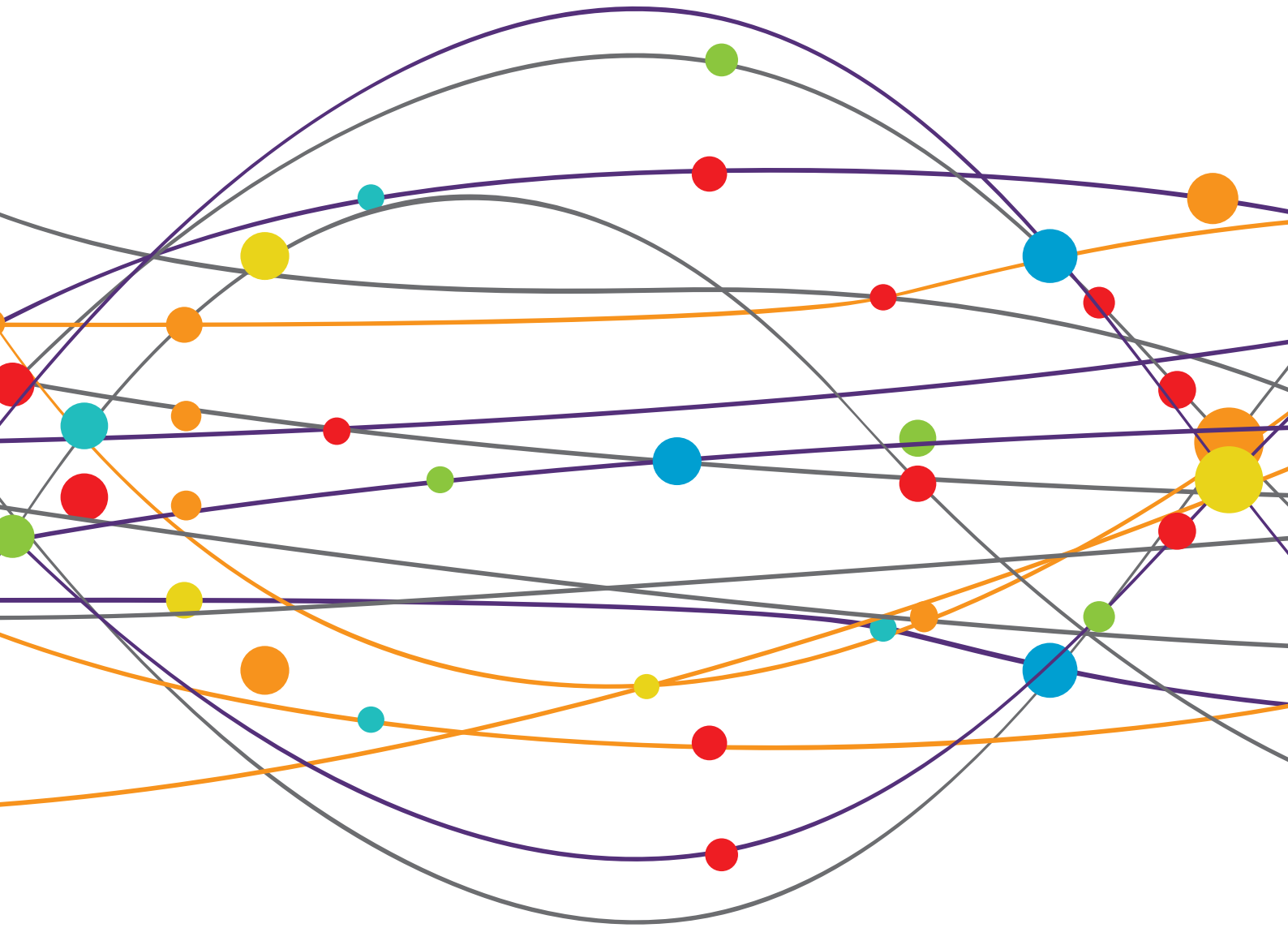

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DISEASE MODIFYING THERAPIES IN MULTIPLE SCLEROSIS

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Editorial: Disease modifying therapies in multiple sclerosis

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Editorial on the Research Topic
Disease modifying therapies in multiple sclerosis

Multiple sclerosis (MS) is a chronic autoimmune neuroinflammatory disorder of the central nervous system (CNS); it has an increasing prevalence worldwide and preferentially affects women of childbearing age.

Since the introduction of the first disease-modifying treatment (DMT), in the early 90's, numerous compounds have been developed, posing new challenges to the choice of the most appropriate therapeutic strategy for the individual patient with MS.

For this reason, there has been increasing efforts in developing decisional algorithms to stratify patients based on their clinical and radiological characteristics; more recently, with the Covid-19 pandemic, DMT choice has become even more difficult as clinicians attempt to balance the benefit with the infection risk potentially amplified by certain drugs.

In this Research Topic, we focused on potential drugs for MS, available DMTs, their efficacy and safety profiles, during the Covid-19 pandemic, in patients with different levels of disability, and particular conditions such as pediatric age and pregnancy.

Radandish et al. reviewed the pathogenetic role of microglia in MS and the potential effect of drugs targeting it. In the early stage of experimental autoimmune encephalomyelitis (EAE) and MS, the pro-inflammatory microglia (M1) has different roles in the promotion of inflammation through cytokine/chemokine release, and ROS and NO production lead to demyelination, thus the suppression of M1 can be useful in MS control. Several drugs (i.e., galectin-1, TQ, and Que) may act against the activated microglia, inhibiting the release of pro-inflammatory cytokines; others (i.e., FTY-720) suppress microglial activation and promote the switch from M1 to M2 (anti-inflammatory) phenotype. Conversely, M2 has anti-inflammatory functions and promotes remyelination *via* cytokines release; therefore, other potential drugs promoting M2 activity (IL-4, activin-A, IVM, rHIgM22, and rIFN-b, M-CSF, and progesterone) may potentially benefit EAE or MS.

Based on the results by Yang and Shi on experimental models of MS, other therapeutic targets, such as dendritic cells, could potentially prompt further studies on new molecules; indeed, these authors demonstrated a beneficial effect of silybin on EAE by inhibition of dendritic cell activation and Th17 cell differentiation. Silybin, blocking the migration of inflammatory cells into the CNS and remarkably inhibiting the demyelinating process, can relieve the disease development.

Ceylan et al. investigated *in vitro* the effects of iron on microglia and used the antipsychotic clozapine *in vitro* and chronic EAE to target features of progressive MS and identify protective medications. These authors found that iron impaired microglial function *in vitro*, while clozapine was able to regulate this effect by reducing the release of IL-6 and by normalizing neuronal phagocytosis. In chronic EAE, clozapine dose-dependently attenuated clinical signs and still had an effect if applied in the therapeutic setting. Histologically, demyelination was reduced by clozapine, and positive effects on inflammation strongly correlated with reduced iron deposition. These data deserve attention because they suggest that clozapine might be considered a possible add-on therapeutic for further development in progressive MS.

Moving on from EAE to MS, the pathogenetic role of intestinal permeability (IP) has been investigated by Buscarinu et al., also in relation to treatment with dimethyl fumarate (DMF). The authors focused on the gut triggers that may lower the threshold for disease development in susceptible individuals and investigated IP changes, the circulating CD161+CD8+ T-cell subset, and clinical/neuroradiological data in a cohort of relapsing-remitting (RR) MS patients before and after 9 months of DMF therapy. At baseline, 64% patients showed altered IP, while 56% had an active MRI. During DMF therapy they found a reduction in the percentage of CD161+CCR6+CD8+ T cells that significantly correlated with IP changes and a drop in MRI activity.

Tobin et al. reviewed the data supporting the role of gut microbiota and short-chain fatty acid (SCFA) metabolites, in particular propionate, in the pathophysiology of MS. Dysbiosis is responsible for a reduction in SCFA producing bacteria and in MS patients a reduction in stool and plasma levels of propionate has been shown. In particular, the action of propionate on T-cell activity results in decreased Th1 and Th17 pro-inflammatory profile and increased regulatory T cell and an overall anti-inflammatory profile, supporting the clinical benefit induced by supplementation of propionate in MS patients.

Treatment strategies are still a matter of debate; however, there is increasing evidence that the first choice in the clinical history of MS patients might deeply impact their future disability. This is the direction Simonsen et al. take, by using a real-world population-based registry to examine the impact of initial treatment in achieving no evidence of disease activity (NEDA) in patients treated with moderate or high efficacy DMTs. Their results showed that NEDA at year 1 and 2 is significantly more likely in patients on high-efficacy DMTs than on moderate efficacy therapies (68 vs. 36% year 1, 52.4 vs. 19.4% year 2), and the first choice of treatment is the most important.

Real-world studies on the efficacy and safety of DMTs are of great value to help MS neurologists in their clinical practice.

Boziki et al. reported the real-world experience of a Greek MS center about the efficacy and safety of natalizumab (NTZ) and fingolimod (FTY) in patients with long-term follow-up. In the matched analysis, NTZ was superior to FTY either for time to first relapse or for time to MRI activity under treatment and treatment discontinuation due to MRI activity. The safety profile of the two drugs confirmed the results from registration trials.

Ziemssen, Albrecht et al. investigated the effectiveness of FTY in young adults ($\leq$20 and $>$20 to $\leq$30 years) compared to older patients ($>$30 years) enrolled in the PANGAEA study. Although young adults had higher annual relapse rates (ARR) at study entry, the proportion of patients with no clinical disease activity in year 4 was significantly higher in young patients compared to older ones. Moreover, in the long-term follow-up, cognitive performances improved more in young adults than in older ones. These data suggest that young age is the best age frame for FTY treatment.

Ziemssen, Hoffmann et al. also reported the results of the interim analysis of the TREAT-MS study collecting data on the long-term effectiveness and safety of alemtuzumab in a large real-life cohort of MS patients. In non-naive patients, treatment sequences were documented, showing that patients with longer disease duration and higher EDSS had a higher number of previous DMTs. Compared to those enrolled in the registration trials, patients in the TREAT-MS study had a longer disease duration and a variety of previous DMTs. Effectiveness and safety data from this study, as well as patients' characteristics, might be useful to support future treatment decisions.

In clinical practice, safety concerns very often prompt the off-label use of DMTs, therefore real-life studies become relevant to understand whether drug effectiveness is preserved. In this regard, Riancho et al. reported the results of a 7-Year Retrospective Observational Study aimed to analyze the efficacy and safety of treatment with NTZ in MS patients initially treated with standard interval dosing (SID) who were then switched to extended interval dosing (EID) every 8 weeks. ARR, radiological activity, and disability progression did not significantly vary between the SID and EID groups. Furthermore, the proportion of patients maintaining the NEDA-3 status was slightly higher among naïve patients than among switchers, suggesting that earlier use of NTZ may benefit active patients.

Proschmann et al. characterized the pharmacokinetics and -dynamics and serum neurofilament light chain (sNfL) in correlation to clinical data in patients with RRMS and secondary progressive MS (SPMS) stopping NTZ. The authors measured free NTZ concentration, cell-bound NTZ, α4-integrin expression, and α4-integrin-receptor saturation as well as immune cell frequencies for up to 4 months after NTZ

withdrawal. Additionally, sNfL levels were observed for up to 12 months in RRMS and up to 4 months in SPMS patients. After stopping NTZ, disease activity returned in 38% of the RRMS and 33% of the SPMS patients within 12 and 7 months, respectively. The concentration of free and cell-bound NTZ, as well as α4-integrin-receptor saturation, decreased in the RRMS and SPMS patients whereas α4-integrin expression increased over time. In all RRMS during the follow-up period, sNfL levels peaked up to 16-fold and were linked to the return of disease activity in more than 50% of patients. This relation was observed also at the individual level; therefore, the authors suggest that they can also serve in clinical practice as an early marker to predict the recurrence of clinical or radiological disease activity.

Clinical response to DMTs varies among people with MS and within the same patient in different moments of their MS history. The identification of biomarkers to early identify responders to the different DMTs is a field of active research; Devi-Marulkar et al. investigated the cellular and molecular blood signatures associated with the efficacy of IFNb treatment by phenotyping regulatory CD4+ T cells and naïve/memory T cell subsets, by measuring the circulating IFNa/b proteins, and by analyzing ∼600 immune-related genes, including 159 interferon-stimulated genes. They also investigated the potential impact of HLA class II gene variation in treatment responsiveness by genotyping HLA-DRB1, -DRB3,4,5, -DQA1, and -DQB1. Non-responders had reduced circulating naïve regulatory T cells, enhanced effector memory CD4+ TEMRA cells, and altered expression of at least six genes with immunoregulatory function. Moreover, non-responders were enriched for HLA-DQB1 genotypes encoding DQ8 and DQ2 serotypes. All these data suggest that IFNb non-responders may suffer from pathogenic CD4+ T cells, likely restricted by DQ8 and DQ2, that may exert autoreactive and bystander inflammatory activities.

The study by Lorefice et al. aimed to characterize MS patients exposed to DMF to evaluate the predictors of therapeutic response. In this observational monocentric study, the authors examined the prescription flow of DMF in MS patients from 2015 to 2019 and analyzed clinical and MRI data and NEDA-3 status at 24 months of DMF treatment. Predictors of DMF response were lower ARR in 2 years pre-treatment and being naive patients; these parameters were associated with the NEDA-3 status at 24 months. A good efficacy profile of DMF was demonstrated in both naive patients and horizontal switchers although it did not eliminate the risk of MS reactivation in patients previously treated with NTZ.

Although siponimod was recently approved for secondary progressive MS, the treatment for patients with the progressive disease has been a challenge for a long time. Indeed, despite the development of highly efficient immunotherapies for MS, no treatment can completely suppress the compartmentalized and meningeal inflammation in the CNS that drives tissue injury and disability progression, and effectively promote regeneration–remyelination. Stem cells are strong immunomodulators that may potentially downregulate the localized and compartmentalized inflammation and may induce neuroprotection and enhance endogenous remyelination (as indicated by animal studies). In this Research Topic, we report the results by Petrou et al. who evaluated the safety and the long-term clinical and immunological effects of multiple intrathecal (IT) and intravenous (IV) injections (up to 8) of autologous mesenchymal stem cells (MSCs) in 24 patients with active-progressive MS at intervals of 6–12 months, followed up for 4 years. In general, there were no serious side effects and most of the patients were stable or improved at the last follow-up visit. Immunological follow-up showed a transient upregulation of CD4+CD25+FoxP3+ cells and downregulation of the proliferative ability of lymphocytes, sustaining the hypothesis that MSCs effects are mediated through peripheral immunomodulation. Since the authors recently demonstrated that the IT injection of MSC was superior to the IV at several parameters, they advocate that the neuroprotective and neurotrophic mechanisms play the most crucial role.

A further challenge in the treatment of MS is represented by pediatric patients (POMS) and pregnant women. Margoni et al. reviewed the state of the art in POMS therapy; observational and clinical studies on first-line and second-line immunomodulatory therapies in POMS have been reported. Since POMS is a severe form of MS, characterized by a high clinical and radiological activity and younger age at reaching cognitive and physical disability milestones, second-line treatment is preferred as demonstrated by the fact that the off-label use of newer DMTs is increasing in POMS and retrospective studies, case series, and phase II trials indicate that this approach appears to be highly effective and safe in children.

Lastly, Simone et al. collected the current evidence on the influence that pregnancy has on MS and the resulting impact of DMTs. Additionally, they discussed safety profiles for each drug and correlated them to both risks for the exposed fetus and risk for the mother interrupting treatments when seeking pregnancy. Based on current evidence, MS does not impact fertility or the women's ability to carry the fetus to term. The disease does not increase the risk of spontaneous abortion, malformations, and cesarean delivery. Pregnancy does not impact the long-term accumulation of disability, rather it appears to be protective against disease activity, particularly during the third trimester, but an increased risk of relapse is reported in the first 3 months postpartum. Exclusive breastfeeding may have a possible favorable effect. Since evidence suggest that some drugs could be safely used throughout the whole pregnancy course or, in specific cases, till the third trimester, neurologists should tailor the best therapy for any pregnant woman, without exposing the fetus to any possible risk and the mother to disease reactivation both in pregnancy and in the postpartum period.

Author contributions

SB wrote the editorial. MG and LL approved the draft. All authors contributed to the article and approved the submitted version.

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

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CASE REPORT
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Case Report: Borrelia-DNA Revealed the Cause of Arthritis and Dermatitis During Treatment With Rituximab

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Borrelia-specific antibodies in serum did not contribute to the diagnosis of Borrelia arthritis or Borrelia-associated dermatitis in a young woman with ongoing treatment with rituximab due to multiple sclerosis. The diagnosis was confirmed by the detection of Borrelia-DNA in a skin punch biopsy. The patient history did not reveal any tick exposure. She had suffered for several months from fluctuating pain and swelling of the right knee as well as skin involvement with redness and oedema around the ankle of the same leg. Monoarthritis was confirmed by a rheumatologist. Knee puncture was performed but the synovial fluid was only sufficient for microscopic examination of crystals. Neither monosodium urate crystals nor calcium pyrophosphate crystals were found. Borrelia serology in blood revealed borderline levels of immunoglobulin (Ig)M and IgG, respectively. Treatment with doxycycline resulted in resolution of the joint and skin manifestations within a month. This case highlights that Borrelia-specific antibody levels cannot be reliably interpreted in patients who have received B-cell depleting therapy. Under these circumstances, detection of the bacterial genome in different body fluids, such as in the skin, can be a useful complement to the diagnosis of Lyme disease. In this young female, the diagnosis would certainly have been further delayed without the detection of Borrelia-DNA in the skin.

Keywords: Lyme disease, Borrelia-DNA, arthritis, dermatitis, rituximab, Borrelia serology

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INTRODUCTION

An atypical clinical presentation and absence of an adequate immune response to infections are common phenomena in patients with primary immunodeficiency disorders (PID) (1) and multiple sclerosis (MS) (2), as well as in subjects receiving immunosuppressive agents (3). Furthermore, several immune modulating therapies may have similar effects, but this is less well-recognized among physicians lacking deeper knowledge of the impact of the immune system on the pathogenesis and the clinical manifestations of different infectious diseases. As immunomodulating therapies (IMT) are now widely used for a variety of autoimmune and inflammatory diseases,

specialists of many different disciplines may need to treat patients using this group of drugs. For instance, patients with MS are often diagnosed and started on treatment at young age and continue for years or decades (4). These circumstances emphasize the importance of physicians being aware of atypical reactions regarding both clinical symptoms and laboratory test results obscuring infections among these patients. This case illustrates that an ongoing infection can easily be overlooked or misinterpreted due to a weak serological response during treatment with a B-cell depleting drug.

CASE PRESENTATION

This case illustrates a 20-year-old female diagnosed with MS at the age of 17. She was initially treated with tocilizumab as *neuromyelitis optica* was suspected due to bilateral optical neuritis and the presence of spinal cord lesions. However, antibodies against aquaporin-4 and myelin oligodendrocyte glycoprotein were not detected and the magnetic resonance imaging (MRI) of the brain and spinal cord as well as cerebrospinal fluid (CSF) findings were supportive of MS. Apart from persistent bilateral severely reduced visual acuity she had no other signs of neurological dysfunction. She had previously been in good health and had no family history of PID, or other systemic inflammatory diseases.

Eighteen months prior to the episode of arthritis and skin symptoms reported here, she was started on off-label treatment with rituximab (RTX). RTX is the most frequently used immunomodulatory drug for MS in Sweden according to the Swedish MS registry (5). Initially, she received 1,000 mg of RTX followed by 500–1,000 mg every 6th month, resulting in depletion of circulating B-cells ($<0.001 \times 10^9$/L). During this period, there were no signs of neuroinflammatory activity of MS.

Clinical Episode

A rheumatologist confirmed the diagnosis of monoarthritis. The right knee had typical signs of inflammation with *rubor*, *tumor*, and *calor* accompanied by a discretely reduced range of motion. The general status was good without fever. The lower right leg was diffusely swollen and two circular erythematous areas around the ankle were seen (**Figures 1A,B**). A dermatologist interpreted the skin symptoms as possible panniculitis with atypical erythema nodosum as a potential alternative diagnosis. There were no other clinical or laboratory findings of sarcoidosis.

Timeline

Treatment with RTX had been ongoing for approximately one and a half year prior to the onset of arthritis and the cutaneous symptoms had been present for at least 6 months prior to the diagnosis. The last dose of RTX was given 1.5 months before the onset of symptoms related to Lyme disease.

Diagnostic Assessments

Aspiration of synovial fluid resulted in a limited volume, only sufficient for microscopic examination of crystals. Neither monosodium urate crystals nor calcium pyrophosphate crystals

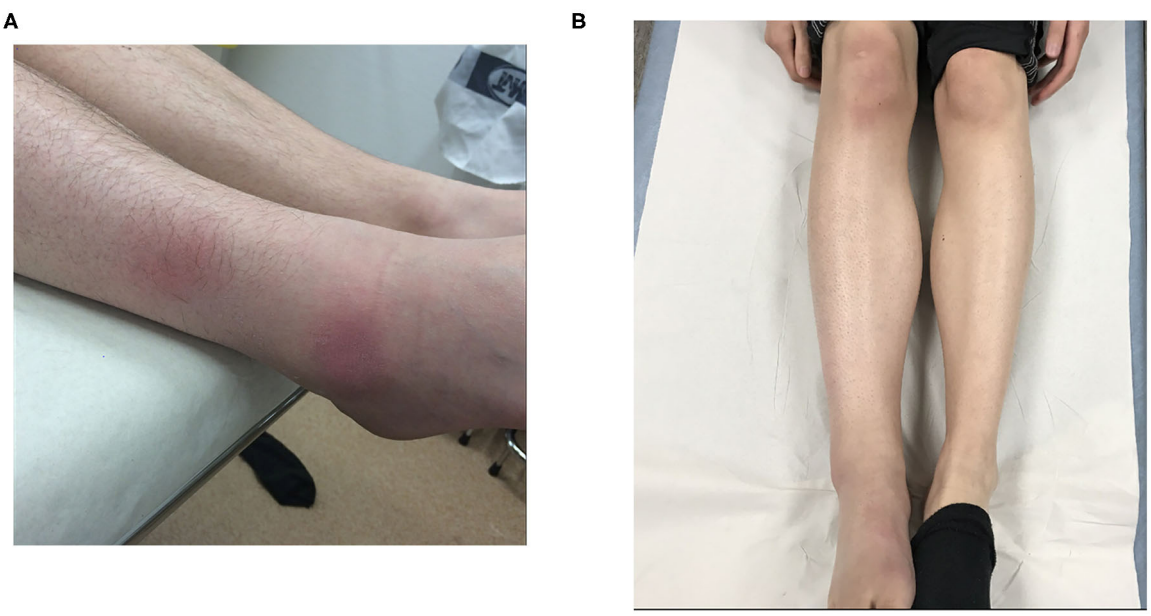

FIGURE 1 | Periarticular swelling of the right leg and ankle. The skin is slightly atrophic adjacent to the two erythematous circular areas seen on the lateral side. The blood vessels appear prominently over the apical part of the foot; a common phenomenon in late cutaneous borreliosis **(A)**. The right knee, calf, and ankle are swollen, without a distinct erythema. Fifteen to twenty degrees deficit in knee extension was observed. Note the dark discoloration of the medial and apical parts of the foot, typically seen in patients with late cutaneous borreliosis **(B)**.

were detected in the joint fluid. Duplex ultrasonography of the lower leg showed no signs of deep vein thrombosis and there were no laboratory signs of systemic inflammation. Serological analysis performed 5 months after the last dose of RTX showed borderline levels of immunoglobulin (Ig)M and IgG antibodies against recombinant Borrelia antigens (Liason®, Borrelia IgM detecting OspC and VlsE; Borrelia IgG detecting VlsE). The results were interpreted to be of uncertain clinical significance. Laboratory results are detailed in **Table 1**.

Despite the vague antibody results, there were an enduring clinical suspicion of Borrelia infection. Skin biopsies from one of the erythematous areas at the ankle were performed. Standard histopathology showed mild non-specific inflammation. Borrelia-DNA was detected in the biopsy analyzed by polymerase chain reaction (PCR). The method amplifies a 116 base-pair long fragment of the 16S rRNA gene. In addition, a lumbar puncture was done, and CSF was analyzed without presence of intrathecal Borrelia-specific antibodies or elevated levels of the B-cell chemokine CXCL13. Thus, the final diagnosis was Borrelia associated dermatitis and arthritis (Lyme disease).

TABLE 1 | Laboratory findings in blood.

Analyte	Results	Reference interval
Hemoglobin	151	117–153 g/L
Leukocyte count	7.2	3.5–8.8 × 10^9/L
Lymphocyte count	1.3	1.1–4.8 × 10^9/L
B-cells	<0.001	0.075–0.53 × 10^9/L
Platelet count	283	160–390 × 10^9/L
Erythrocyte sedimentation rate (ESR)	2	<21 mm/h
P-C-reactive protein (CRP)	<5	<10 mg/L
S-Creatinine kinase	1.5	<3.6 μkat/L
P-Alanine transaminase	0.35	<0.76 μkat/L
P-Creatinine	81	45–90 μmol/L
P-Urate	209	155–350 μmol/L
S-Angiotensin converting enzyme	0.51	<1.1 μkat/L
Anti-cyclic citrullinated peptide antibody (IgG)	1	<7 U/L
Borrelia antibody (IgM)	38.6	<30 AU/mL
Borrelia antibody (IgG)	16.6	<10 AU/mL

AU, arbitrary units; P-, analysis in plasma; S-, analysis in serum; U, units.

Therapeutic Intervention

Prior to the diagnosis of Lyme disease, the patient was prescribed topical steroids for the skin manifestations and the joint symptoms were managed with paracetamol. Once the diagnosis of Lyme disease was confirmed, doxycycline 200 mg once daily for 3 weeks was prescribed. The knee and skin symptoms dissipated during the following month.

Follow-Up and Outcome

At the last follow-up 1 year after the antibiotic treatment had been ended, there was still minor swelling of the lower leg but no signs of arthritis or dermatitis. An MRI of the lower leg showed mild oedema in musculus soleus and gastrocnemius. Creatinine kinase in plasma was within normal reference.

DISCUSSION

IMT in general, and particularly B-cell depleting therapies, may be associated with an increased risk of infections (2, 6, 7). Serological screening for IgG against several infectious agents is therefore routinely performed prior to initiation of IMT and vaccination should be considered when immunity is not detected. However, the fact that IMT can have an impact on the clinical picture and serological response to infectious agents is less well recognized among physicians outside the field of immunology and infectious diseases. B-cell depleting therapies are widely used in MS as well as in many other autoimmune diseases, often with a dramatic anti-inflammatory effect and symptom relief (6). In chronic inflammatory diseases like MS, the treatment is often continued for many years and results in undetectable or very low numbers of circulating B-cells. Although RTX, compared to other disease modifying drugs in MS, has been shown to be associated with an increased risk for serious infections it is widely used due its marked effect on the disease activity and disease progression (4). Another side effect is weak and non-protective responses to vaccinations, as long as the circulating B-cells are very low (8, 9).

In the patient described here, Borrelia caused late skin and joint manifestations that did not raise the clinical suspicion of Lyme disease, until the weak serological response was received. Still, the antibody results were interpreted to be of dubious significance. The correct diagnosis was not made until Borrelia-DNA was detected in the skin biopsy. This is consistent with previous and more recent findings of improved diagnostic accuracy using detection of the Borrelia genome in the skin of patients with *acrodermatitis chronica atrophicans*, the late cutaneous manifestation of borreliosis (10, 11). In addition, our case clearly illustrates that, during treatment with B-cell depleting therapies, infections may give rise to an atypical clinical picture as well as a weak serological response to specific pathogens. Awareness of these circumstances should be highlighted to clinicians serving patients on IMT.

Patient Perspective

The patient had suffered from knee pain and painful skin erythema for several months before the correct diagnosis was identified. She had been in contact with the primary health care several times before the correct diagnosis was made. Despite the fact that she was treated with IMT, her symptoms were initially interpreted as "non-specific findings of uncertain origin."

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patient provided her written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable image or data included in this article.

AUTHOR CONTRIBUTIONS

JS, CS, and CD had full access to all of the data and takes responsibility for the integrity, accuracy and interpretation of the data. All authors contributed to the article and approved the submitted version. All authors were involved in drafting the manuscript or revising it critically for important intellectual content and all authors approved the final version to be published.

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Descriptive Analysis of Real-World Data on Fingolimod Long-Term Treatment of Young Adult RRMS Patients

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Background: Fingolimod (Gilenya®) is approved for adult and pediatric patients with highly active relapsing–remitting multiple sclerosis (RRMS).

Objectives: The objective was to describe the effectiveness of fingolimod in young adults compared to older patients in clinical practice.

Methods: PANGAEA is the largest prospective, multi-center, non-interventional, long-term study evaluating fingolimod in RRMS. We descriptively analyzed demographics, MS characteristics, and severity in two subgroups of young adults (≤20 and >20 to ≤30 years) and older patients (>30 years).

Results: Young adults had lower Expanded Disability Status Scale (EDSS) scores compared to older patients (1.8 and 2.3 vs. 3.2) at baseline. The mean EDSS scores remained stable over 5 years in all subgroups. Young adults had higher annual relapse rates (2.0 and 1.7 vs. 1.4) at study entry, which were reduced by approximately 80% in all subgroups over 5 years. The proportion of patients with no clinical disease activity in year 4 was 52.6 and 73.4 vs. 66.9% in patients ≤20, >20 to ≤30 years and >30 years, respectively. The symbol digit modalities test score increased by 15.25 ± 8.3 and 8.3 ± 11.3 (mean ± SD) from baseline in patients >20 to ≤30 and >30 years.

Conclusions: Real-world evidence suggests a long-term treatment benefit of fingolimod in young RRMS patients.

Keywords: RRMS, fingolimod, young adults, real-world evidence, early treatment, long-term study

INTRODUCTION

Relapsing multiple sclerosis (MS) represents a continuous spectrum of disease ranging from clinically isolated syndrome over relapsing–remitting multiple sclerosis (RRMS) to secondary progressive MS (SPMS) (1). Most RRMS patients are diagnosed at an age of 30–40 years (2), but some patients show early onset of MS at a childhood age or as young adults (3). The disease characteristics in these patients differ from adult MS patients, e.g., in pediatric MS, the

relapse rate was shown to be two to three times higher, and pediatric MS patients often experience more severe relapses (4, 5). Despite increased relapse severity, pediatric patients often recover completely (5, 6). With respect to disability, cognitive dysfunction is typically more frequent in pediatric compared to adult patients, while locomotor disability is less pronounced (3, 7). Therefore, the time to disability milestones as measured by the Expanded Disability Status Scale (EDSS) might be longer in younger patients. Due to the early onset of the disease, these milestones are still reached at a younger age (3). Overall, these features suggest that MS in younger patients is even more characterized by inflammatory processes than in older patients. Despite differences in disease characteristics and limited efficacy data in younger MS patients, in general, the same treatment regimens should be used for adults, young adults, and even children. However, the treatment armamentarium for the latter group is limited, as not all MS drugs are approved for use in children.

Fingolimod (Gilenya®, Novartis Pharma AG) was first approved in 2011 as a once-daily oral treatment for adult patients with RRMS and since then has gained marketing authorization in over 80 countries. Approximately 296,700 patients have been treated with fingolimod in both the clinical trial and post-marketing settings, and the total patient exposure now exceeds 746,700 patient-years. In 2019, it has also gained approval for the treatment of children and adolescents with RRMS. Its efficacy and safety in pediatric patients had been investigated in the PARADIGMS study, in which fingolimod was shown to be more effective than treatment with interferon-beta 1a (8). The subgroup analyses of three pivotal studies in adults (FREEDOMS, FREEDOMS II, and TRANSFORMS) have shown benefits of fingolimod treatment over placebo and beta-interferons in terms of relapse prevention and MRI activity in young adults (9). However, data on the use of fingolimod in young adults is limited to a small number of patients in the respective study populations.

In the present analysis of the PANGAEA study (Post-Authorization Non-interventional German SAfety of GilEnyA in RRMS patients), the effectiveness of fingolimod in young adults in real-world settings was investigated. PANGAEA was a non-interventional study recruiting RRMS patients from 2011 to 2013 to assess long-term safety, tolerability, effectiveness, and patient-reported outcomes of fingolimod under real-life conditions (10–12) for an observational period of (maximum) 5 years.

Here we report the results of a descriptive analysis of fingolimod treatment in PANGAEA subgroups of young adult patients with RRMS (≤20 and >20–30 years of age) treated up to 5 years in daily clinical practice. A subgroup of the PANGAEA population with age above 30 years is used as the reference cohort.

PATIENTS AND METHODS

Study Design

PANGAEA was a prospective, multi-center, non-interventional, long-term study of fingolimod (0.5 mg) for the treatment of patients with RRMS (10). It was conducted in Germany, including office-based neurologists and neurology clinics. Patients who received fingolimod according to the summary of product characteristics were eligible. The treatment followed a common clinical routine, and the observation period was *a priori* set to up to 60 months. Follow-up visits were documented about every 3 months. Recruitment into the study started in April 2011 and finished in December 2013, with a total of 4,229 patients, of whom 4,032 were included in the analyses. Data included baseline characteristics (sex, age, body mass index) and MS characteristics (disease duration, number of relapses in the past year). Disease severity using EDSS, severity symbol digit modalities test (SDMT), multiple sclerosis severity score (MSSS), and annual relapse rate (ARR) was analyzed every year for the observational period of 60 months. Due to the non-interventional study design, assessments followed clinical practice routine and were optional. The present subgroup analysis of PANGAEA data comprises young adults, i.e., patients ≤20 years as well as patients >20 to ≤30 years of age in comparison to patients >30 years of age.

Administrative Procedures

The study was conducted according to the current recommendations for observational studies of the following institutions: the Voluntary Self-Control of Pharmaceutical Companies Codex (FSA-Codex), the Federal Institute for Drugs and Medical Devices and the Paul-Ehrlich-Institut (PEI), and the Research-Based Pharmaceutical Companies (vfa). Prior to study initiation, an ethics committee was consulted, and the study was notified to the competent higher federal authority, the Federal Association of Statutory Health Insurance Physicians and the Statutory Health Insurance. Patients were only included after providing written informed consent at the time of the baseline visit.

Statistical Methods

The presented data are part of analyses conducted in January 2020. All data were analyzed descriptively using SAS, version V9.4. Analysis of baseline characteristics included demographics, disease history, and prior treatment. The endpoints of interest were treatment interruptions, annual relapse rate, EDSS changes, SDMT changes, clinical disease activity as defined by relapses and disability development, as well as effectiveness and tolerability as reported by physicians and patients. In addition to a baseline score-referenced analysis of EDSS progression, a roving EDSS analysis approach was used (13). As this was a non-interventional study, no visit windows were defined and no rules for handling of missing or incomplete data were established. Therefore, instead of exact EDSS assessment dates, the follow-up visit schedule has been used for roving EDSS analysis. Assessments at month 1 follow-up visit were not included. Furthermore, missing EDSS values were not imputed and had no impact on the analysis.

A methodological limitation of this descriptive analysis is that correction for confounding factors like disease duration was not possible because of the strong correlation between this factor and

age. Due to the relatively low number of patients in the youngest age group, propensity score matching would have resulted in a comparison of individual cases instead of a representative group comparison. The present results therefore should be interpreted with caution, especially with respect to disease characteristics that depend on the disease duration.

All analyses were performed by age subgroups with the following cutoffs: <20 years, >20 to ≤30 years, and >30 years. Continuous data were analyzed as mean and standard deviation, while categorical data were analyzed as absolute and relative frequencies.

RESULTS

The present analysis included 81 patients younger than 20 years of age (2.0% of the total population), 819 patients aged 20–30 (20.3%) years, and 3,130 patients older than 30 years of age (77.6%). The gender distribution was similar between age groups. Young adults included in PANGAEA had a shorter disease duration on average (2.8 and 4.5 vs. 9.3 years) and lower EDSS and MSSS scores compared to patients older than 30 years (EDSS: 1.8 and 2.3 vs. 3.2; MSSS: 4.7 and 5.0 vs. 5.2). Although the EDSS scores were higher in older age groups, the SDMT scores were similar in patients aged 20–30 years and patients older than 30 years (SDMT: 45.5 vs. 45.6; SDMT was not assessed in patients <20 years). The annual relapse rate within 12 and 24 months prior to study inclusion was higher in younger patients (2.0 and 1.7 vs. 1.4 and 2.8 and 2.6 vs. 2.1), and the proportion of relapse-free patients within 12 months before study inclusion was smaller (6.3 and 15.1 vs. 21.5%). Time from first symptoms to diagnosis was 0.6 and 0.7 years in young adults, compared to 2.2 years in patients older than 30 years. The proportion of patients with concomitant diseases increases by age (16.0 and 24.1 vs. 34.8%). The most frequent diseases in all age groups were psychiatric disorders (3.7, 5.5, and 9.9%) and nervous system disorders (2.5, 6.3, and 8.9%), with a higher total frequency in older patients (**Table 1**).

About half of the patients in all three age groups completed the 5-year observational period on therapy (48.2 and 43.0 vs. 53.8%). Study discontinuations were more frequently related to a lack of effectiveness in the youngest group (9.5 and 6.2 vs. 5.6%), while disease progression or relapse (4.8 and 10.1 vs. 9.8%), patient wish (7.1 and 30.4 vs. 31.3%), and adverse events (7.1 and 13.3 vs. 26.0%) were more frequently reported as reason for discontinuation in the older subgroups of patients (numbers given for patients ≤20 years and >20 to ≤30 years vs. >30 years, respectively; multiple responses per patient included).

The mean annual relapse rate was reduced by ∼70% in the first year and over 80% in the fifth year in all patient subgroups. The proportion of relapse-free patients increased by ∼15% in all three age groups from year 1 to year 5 (**Table 2**).

The mean EDSS score remained almost stable in all subgroups over 5 years of treatment (**Figure 1**). In 23.4 and 19.1 vs. 17.2% of the patients, sustained EDSS improvement was documented in year 4 of the observation period, while 11.8 and 10.6 vs. 14.7% had 6 months of confirmed disability progression as measured

TABLE 1 | Patients' characteristics, disease history, and pretreatment at baseline.

Mean ± SD, unless otherwise specified	≤20 years *N* = 81	>20 to ≤30 years *N* = 900	>30 years *N* = 3,130
Female, *n* (%)	*N′* = 81 63 (77.8)	*N′* = 819 590 (72.0)	*N′* = 3,130 2,247 (71.8)
Age (years)	*N′* = 81 19.1 ± 1.0	*N′* = 819 26.2 ± 2.7	*N′* = 3,130 42.9 ± 7.6
Height (cm)	*N′* = 55 167.5 ± 9.5	*N′* = 608 171.7 ± 8.3	*N′* = 2,451 171.4 ± 8.8
Weight (kg)	*N′* = 54 65.2 ± 12.9	*N′* = 615 72.2 ± 17.7	*N′* = 2,446 75.0 ± 17.1
BMI	*N′* = 54 23.3 ± 4.1	*N′* = 602 24.3 ± 5.2	*N′* = 2,417 25.5 ± 5.2
Time since diagnosis (years)	*N′* = 77 2.8 ± 2.1	*N′* = 795 4.5 ± 3.1	*N′* = 2,911 9.3 ± 6.5
Time from first symptoms to diagnosis (years)	*N′* = 71 0.6 ± 1.9	*N′* = 686 0.7 ± 1.6	*N′* = 2,362 2.2 ± 4.1
Number of MS relapses within the last 12 months	*N′* = 79 2.0 ± 1.1	*N′* = 799 1.7 ± 1.3	*N′* = 3,079 1.4 ± 1.1
Number of MS relapses within the last 24 months	*N′* = 79 2.8 ± 1.4	*N′* = 800 2.6 ± 1.9	*N′* = 3,069 2.1 ± 1.6
Patients without relapse within the last 12 months, %	*N′* = 79 6.3	*N′* = 819 15.1	*N′* = 3,079 21.5
Patients with ≤1 relapse within the last 24 months, %	*N′* = 79 15.2	*N′* = 819 28.9	*N′* = 3,069 38.1
Total EDSS	*N′* = 74 1.8 ± 1.3	*N′* = 762 2.3 ± 1.5	*N′* = 2,875 3.2 ± 1.7
Total MSSS	*N′* = 71 4.7 ± 2.7	*N′* = 741 5.0 ± 2.6	*N′* = 2,691 5.2 ± 2.6
Total SDMT	Not assessed	*N′* = 26 45.5 ± 13.6	*N′* = 189 45.6 ± 13.6
Prior treatment, %	*N′* = 81	*N′* = 819	*N′* = 3,130
None	0.6	6.1	6.2
Beta interferons	63.0	53.1	47.4
Glatiramer acetate	18.5	22.8	23.6
Natalizumab	11.1	15.9	19.0
Mitoxantrone	0.0	0.7	1.5
Azathioprine	0.0	0.1	1.0
Missing	1.2	1.2	1.3
Most frequent (≥2% in any age group) concomitant diseases by SOC, %	*N′* = 81	*N′* = 819	*N′* = 3,130
Any concomitant disease	16.0	24.1	34.8
Psychiatric disorders	3.7	5.5	9.9
Investigations	2.5	1.2	2.3
Metabolism and nutrition disorders	2.5	2.6	4.6
Nervous system disorders	2.5	6.3	8.9
Vascular disorders	0.0	2.2	8.9
Endocrine disorders	0.0	1.8	4.1
Musculoskeletal and connective tissue disorders	0.0	1.6	3.5
General disorders and administration site conditions	1.2	1.5	2.9
Renal and urinary disorders	0.0	0.5	2.2
Respiratory, thoracic, and mediastinal disorders	1.2	1.3	2.0

BMI, body mass index; EDSS, Expanded Disability Status Scale; MSSS, Multiple Sclerosis Severity Score; n, number of patients in the category; N, number of patients in the total analysis population; N′, number of patients with available data; SD, standard deviation; SOC, system organ class.

TABLE 2 | Annual relapse rate and proportion of relapse-free patients.

	≤20 years *N* = 81	>20 to ≤ 30 years *N* = 819	>30 years *N* = 3,130
ANNUAL RELAPSE RATE, ARR ± SD (N')			
Baseline	2.0 ± 1.13 (79)	1.70 ± 1.33 (688)	1.40 ± 1.09 (3,067)
Year 1	0.65 ± 0.86 (68)	0.48 ± 0.80 (688)	0.41 ± 0.71 (2,658)
Year 2	0.63 ± 0.91 (52)	0.35 ± 0.64 (538)	0.31 ± 0.62 (2,207)
Year 3	0.48 ± 0.87 (42)	0.31 ± 0.65 (416)	0.25 ± 0.55 (1,834)
Year 4	0.47 ± 0.76 (32)	0.27 ± 0.65 (341)	0.20 ± 0.49 (1,575)
Year 5	0.38 ± 0.59 (21)	0.24 ± 0.55 (248)	0.20 ± 0.46 (1,134)
RELAPSE-FREE PATIENTS, % (N')			
Baseline (year−1)	6.3 (79)	15.1 (819)	21.5 (3,079)
Year 1	52.9 (68)	66.0 (688)	69.7 (2,658)
Year 2	57.7 (52)	72.5 (538)	75.4 (2,207)
Year 3	69.1 (42)	77.2 (416)	80.0 (1,834)
Year 4	65.6 (32)	80.9 (341)	83.1 (1,575)
Year 5	66.7 (21)	81.1 (248)	82.5 (1,134)

ARR, annual relapse rate; N, number of patients in the total analysis population; N', number of patients with available data.

by EDSS. In 64.7 and 70.2 vs. 68.1% of the patients, the EDSS score remained stable. In 4 years of treatment, the proportion of patients without clinical disease activity (defined as the absence of EDSS progression and relapse) increased to 52.6 to 73.4 vs. 66.9% (for patients ≤20 years and >20 to ≤30 years *vs.* >30 years, respectively; **Table 3**). The analysis using a roving EDSS approach shows a higher cumulative probability of EDSS worsening after 4 years in patients >30 years compared to younger patients (**Figure 2**). All age groups had a similar cumulative probability of EDSS progression unrelated to relapse activity after 4 years (**Figure 2**).

The mean MSSS decreased in all age groups, with the lowest MSSS seen in the youngest patients (2.4 points and 1.7 points vs. 1.2 points; **Figure 3**).

The SDMT total score increased from 45.5 points to 57.0 in patients ≤30 years of age and from 45.6 to 55.0 in patients >30 years of age. The mean change (± SD) in patients with available baseline and last visit data was 9.6 ± 12.8 ($n = 7$) and 8.1 ± 10.4 ($n = 74$) (**Figure 4**). No SDMT data were available in the subgroup of patients ≤20 years of age due to the small sample size of this subgroup, reflecting that SDMT is not a standard test in clinical practice.

Within 5 years of treatment, the effectiveness was deemed "good" or "very good" by ∼80%, and "good" or "very good" tolerability was attested by over 90% of patients and physicians in all three subgroups (data not shown). The nature of reported adverse events is consistent with previous findings from clinical trials. The risk for infections does not differ between age groups with similar frequencies of lymphopenia (19.8 and 20.4 vs. 16.9%)

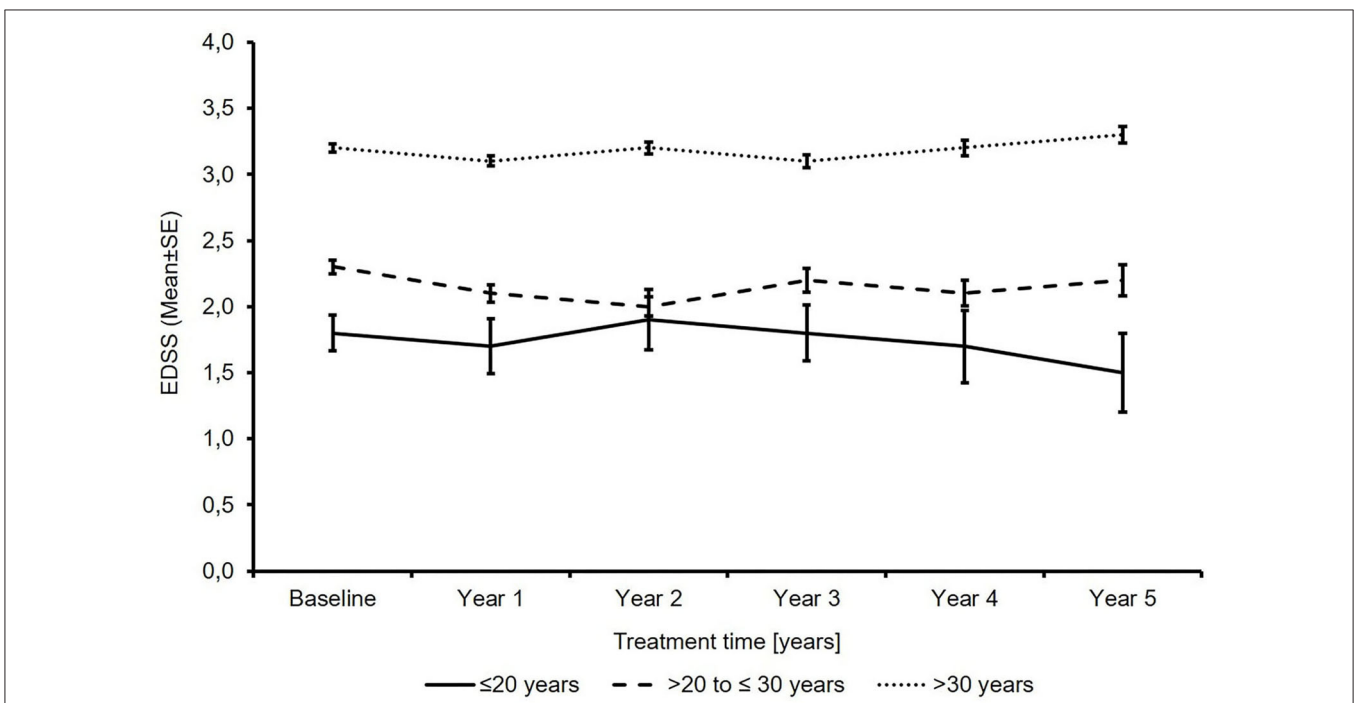

FIGURE 1 | Total expanded disability status scale score (*N'* ≤20 years: 74/53/37/27/18/13; *N'* >20 to ≤30 years: 762/538/409/297/243/184; *N'* >30 years: 2875/2076/1655/1295/1082/834; baseline/year 1/year 2/year 3/year 4/year 5).

TABLE 3 | EDSS change and clinical disease activity per year (not aggregated).

	≤20 years *N* = 81				>20 to ≤30 years *N* = 819				>30 years *N* = 3,130			
Year	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
EDSS change, *N'*	51	35	25	17	513	392	286	235	1,962	1,564	1,225	1,025
Stable EDSS, %	82.4	71.4	64.0	64.7	79.5	74.7	69.6	70.2	79.1	73.8	70.1	68.1
EDSS improvement, %	13.7	20.7	24.6	23.4	14.0	17.9	19.2	19.1	11.9	13.6	15.1	17.2
EDSS progression, %	3.9	8.6	12.0	11.8	6.4	7.4	11.2	10.6	9.0	12.7	14.8	14.7
Clinical disease activity, *N'*	57	41	30	19	546	415	301	244	2,127	1,661	1,306	1065
Patients without activity, %	43.9	53.7	56.7	52.6	61.0	68.7	68.1	73.4	61.5	65.1	66.5	66.9

EDSS, Expanded Disability Status Scale; N, number of patients in the total analysis population; N', number of patients with available data.

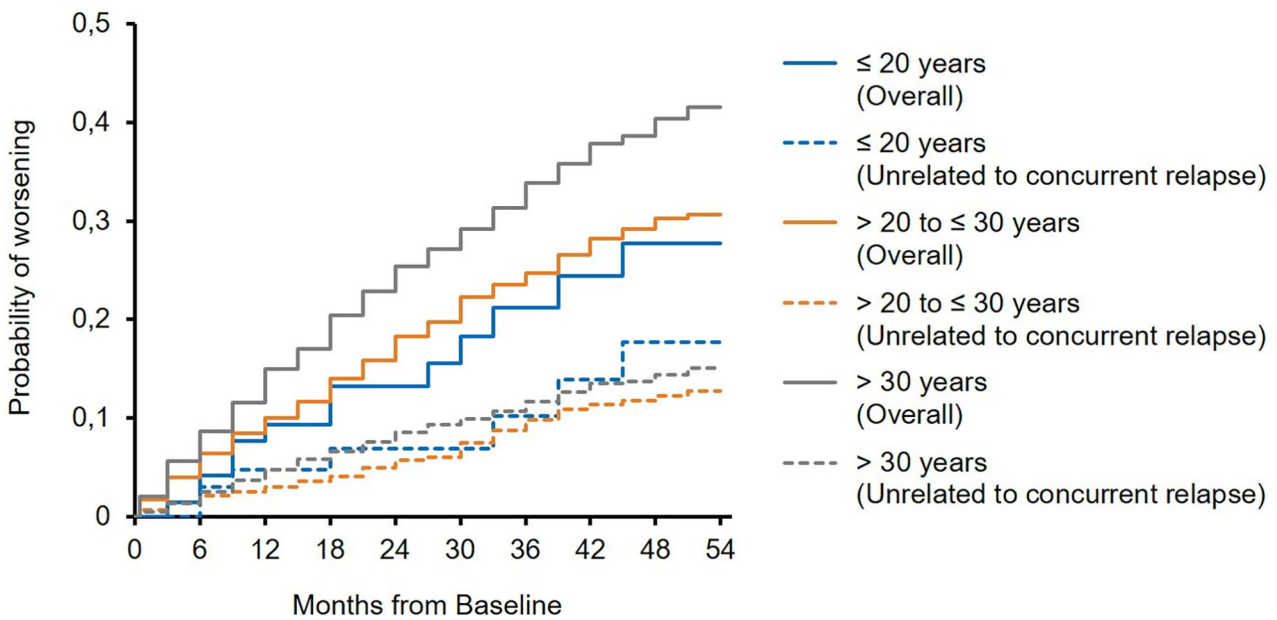

FIGURE 2 | Roving expanded disability status scale (EDSS) progression overall and unrelated to concurrent relapse (EDSS worsening is related to a relapse if at least one relapse occurred between 30 days before start of EDSS worsening and 30 days after confirmation of EDSS worsening).

and serious respiratory tract infections (1.2% and 2.5 vs. 2.86%) in all age groups.

DISCUSSION

The present descriptive analysis includes the final data of the PANGAEA study after the predefined maximum observation period of 5 years. It suggests that fingolimod provided long-term reduction of relapse rate, a stable or improved EDSS, and stable or improved cognitive function as assessed by SDMT in the majority of RRMS patients irrespective of age. The majority of patients in all age groups were free of any clinical disease activity, with the highest proportions reached in patients older than 20 years of age compared to younger patients. These results have to be interpreted in the context of age-dependent differences in patient characteristics, especially with respect to their baseline disease activity.

An analysis of the pivotal fingolimod trials by Gartner et al. has already assessed disease characteristics by age groups in patients from a clinical study setting (9). However, due to the inclusion and exclusion criteria in pivotal studies, the study populations do not cover the full range of patients treated in clinical practice. The present analysis of the PANGAEA study closes this gap and describes differences between age cohorts in a real-world setting. The same age cutoffs were used but with distinct groups for PANGAEA (≤20, >20 to ≤30 years, and >30 years), while Gartner *et al.* compared patients ≤20 and ≤30 years of age with the overall population. Consequently, patients in the oldest age group of PANGAEA were older on average and had a longer disease duration than the overall population of the pivotal studies. Despite these differences in the analyses, the PANGAEA results are very similar to what was found in the pivotal studies.

Over 90% of the young adults in the PANGAEA study showed relapse activity at baseline. In line with the present results, young

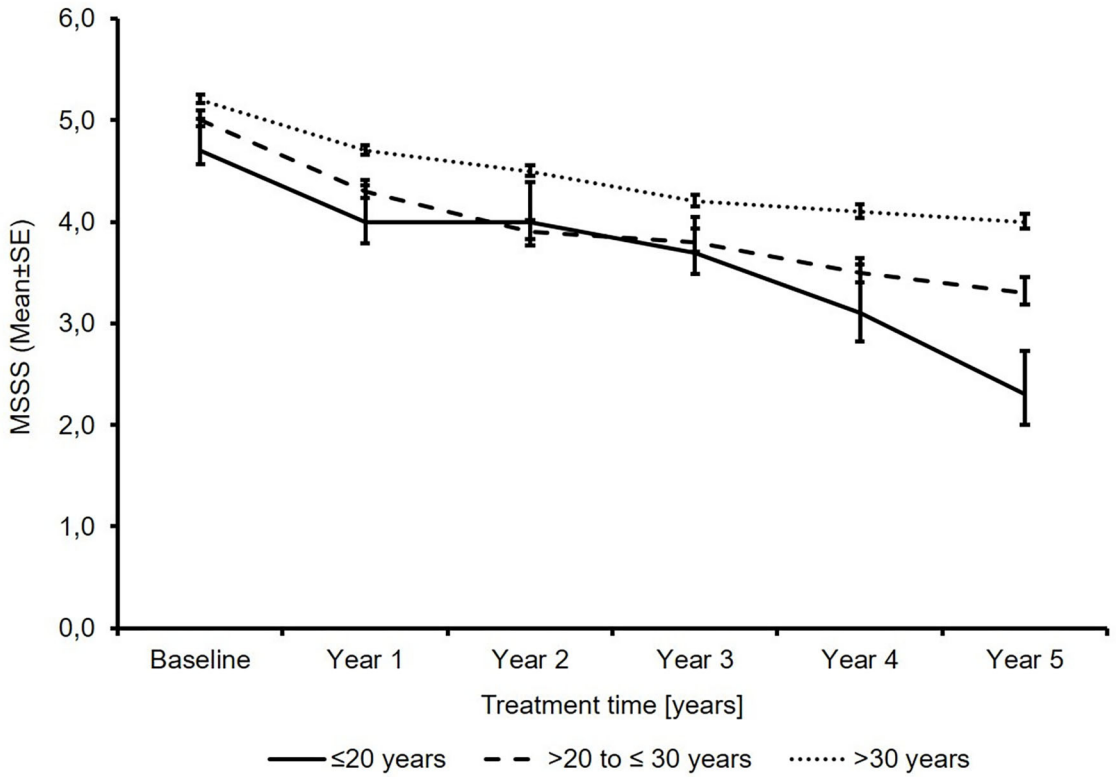

FIGURE 3 | Total multiple sclerosis severity score (*N*′ ≤20 years: 71/51/34/26/17/12; *N*′ >20 to ≤30 years: 741/525/397/292/239/179; *N*′ >30 years: 2691/1953/1552/1216/1016/775; baseline/year 1/year 2/year 3/year 4/year 5).

adults were also found to have the highest clinical activity in terms of relapses at baseline in the pivotal fingolimod trials (9). This was expected, as natural history data indicate that younger patients have more frequent and more severe relapses than older patients and that relapse activity declines with increasing disease duration (4, 5). It can be assumed that, at this age, patients show a more inflammatory disease course.

Due to their higher disease activity, young patients have an urgent need for a highly efficacious treatment. The level of clinical disease activity despite treatment observed in PANGAEA suggests that insufficient disease control is more frequent in young patients than in older patients. This might, on the one hand, be due to the higher background disease activity and, on the other hand, due to the lack of authorized treatments for adolescent patients. Interestingly, the proportion of patients treated with beta-interferons as their last documented DMT was highest in the youngest age group. As only the last DMT has been documented in PANGAEA, it remains unclear whether the patients have received other DMT before. However, as until recently only beta-interferons were authorized for the treatment of RRMS in adolescents, it can be assumed that the lack of alternatives for this special population might at least have contributed and prevented adequate treatment optimization. Since its label was extended to the use in children and adolescents in 2019, fingolimod can be used for early intervention in young patients with highly active RRMS. The previous analyses of study data of the pivotal trials have shown that fingolimod significantly reduced the ARR and the number of new T2 lesions compared to placebo and interferon-beta 1a in young adult patients (9) as well as in children and adolescents (8). According to the reduction of the ARR and lesion load in the clinical study setting, fingolimod adequately addresses these pathological processes in patients with early-onset MS. The present data of the PANGAEA study suggest that the effective relapse prevention observed in young adults in the clinical study setting translates into clinical praxis. As the proportion of relapse-free patients increased by ∼60 percentage points in each group, it can be assumed that RRMS patients benefit from fingolimod treatment to the same extent with respect to the reduction of relapse activity irrespective of their age. The higher underlying relapse activity in the younger group might be the reason for the lower overall proportion of relapse-free patients compared to the older groups.

Although younger patients have more frequent and more severe relapses, they often completely recover from their relapses. The present PANGAEA results on young adults indicate that a higher proportion of patients is able to reach disability improvement as measured by EDSS and that the positive treatment effect on cognitive function is more pronounced compared to older patients. This might be due to a higher compensatory capacity at this young age, which

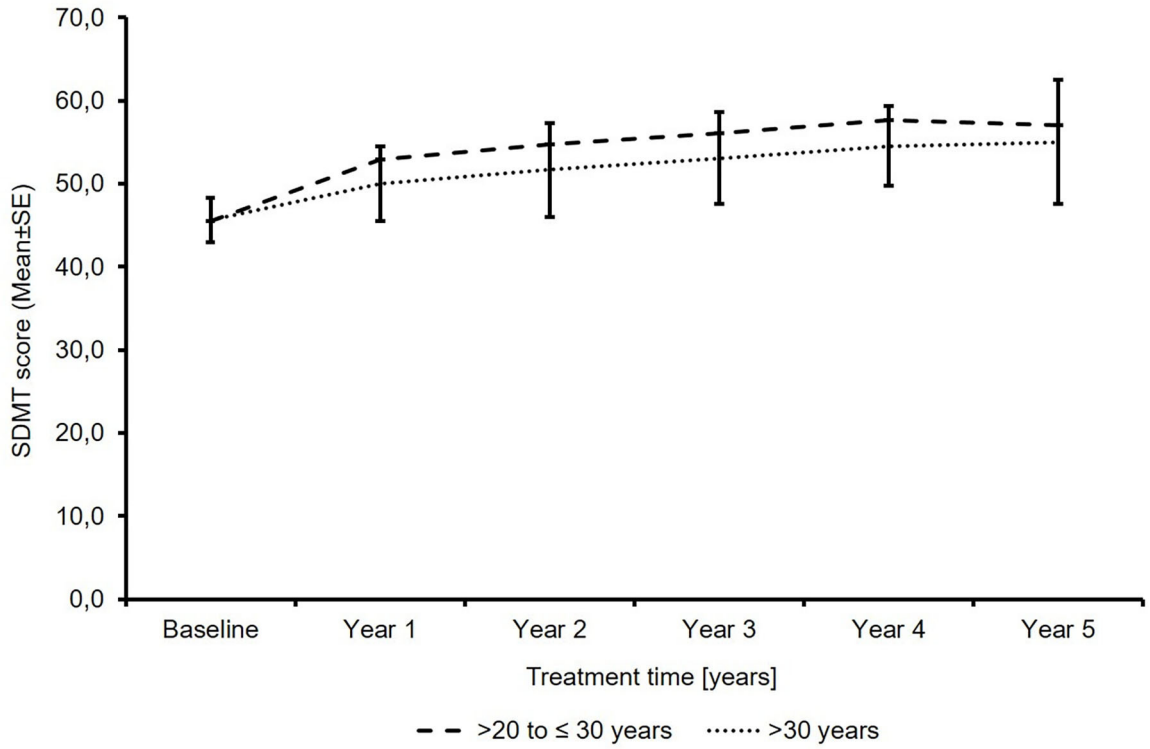

FIGURE 4 | Total symbol digit modalities test score (*N*' >20 to ≤30 years: 26/18/14/12/11/8; *N*' >30 years: 189/173/151/120/89/67; baseline/year 1/year 2/year 3/year 4/year 5).

then continuously declines with increasing age (14). In line with this, EDSS progression probability is higher in older patients, and although the relapse rate was not higher in these patients, relapse-related EDSS worsening was more frequent (15). It has to be pointed out that the higher EDSS progression probability might be confounded by a higher disease duration in older patients and is not solely age-driven. Nevertheless, it can be assumed that an increasingly incomplete recovery from relapses with older age and higher disease duration due to a decrease in compensatory activity hampered the effectiveness of fingolimod in older patients. The specific processes of such impairment in older MS patients are not fully understood yet, but cell senescence with oxidative stress, decreased intrinsic autophagy, and reduced neurotrophic support might play a role (16). Therefore, immunosenescence could have affected the effectiveness in older subjects.

The recovering capacities at a young age still do not allow for a delay in treatment initiation (17). Roving EDSS analysis from PANGAEA data showed that EDSS progression unrelated to relapses occurred to a similar extent in all age groups, supporting the concept that chronic disease progression is present already from disease onset and significantly contributes to overall disability progression (18). Therefore, undelayed treatment initiation and optimization are highly important in patients of any age, and the PANGAEA study data support the use of fingolimod in all age groups, including young adults. It is essential especially for young patients not only to prevent disability progression in terms of motor function but also to assure stable cognitive functionality. This phase of life is very demanding as, for example, academic studies, vocational education and training, and career entry and progression require full cognitive capacities. The SDMT is a strong predictor of vocational status (19), and an SDMT worsening of three points is clinically meaningful and results in reduced working capabilities and responsibilities (20). Hence, a slight deterioration can already have a marked impact. Early treatment intervention can help to prevent slight but meaningful deterioration at an early stage of the disease, and long-term treatment outcomes potentially benefit from the synergism of an effective disease activity control and a high compensatory activity. A higher effectiveness in terms of disability improvement in younger patients of the PANGAEA study might therefore reflect the benefits from early treatment initiation at a younger age and earlier diagnosis.

In line with this, recent analyses from pivotal fingolimod trials indicate that immediate treatment is superior to delayed treatment in young adults in terms of long-term benefits in disease activity and disability progression (21). High-efficacy treatment initiation within 2 years of MS onset compared to a start within 4–6 years after disease onset was associated with less disability (22). A propensity score-matched comparative analysis of PANGAEA and a non-interventional study on the use of beta-interferon or glatiramer acetate found that

switching to fingolimod early is more effective in patients with active disease than continuing beta-interferon or glatiramer acetate (23). Further analyses of real-world data, including the PANGAEA study data, provide good evidence of its effectiveness in the treatment of active MS (24). The results of a recent multicenter cohort study even support the preference of newer disease-modifying drugs, including dimethyl fumarate, fingolimod, teriflunomide, natalizumab, rituximab, ocrelizumab, and alemtuzumab, over beta-interferon or glatiramer acetate for the initial treatment of pediatric patients and clinically isolated syndrome (25). Furthermore, the risk of conversion to a secondary progressive disease course was found to be significantly reduced under initial treatment with fingolimod, natalizumab, or alemtuzumab (26). These findings may contribute to the current change in mindset toward an early intervention with efficacious drugs. An analysis of baseline characteristics in the PANGAEA study in comparison to the characteristics in a similar successor study, PANGAEA 2.0, indicate that patients were switched to fingolimod at an earlier stage of their disease (27).

Apart from the differences in their baseline disease activity, the age groups in the PANGAEA study also differed with respect to comorbidities, which, in general, were more frequent in older patients. The prevalence of psychiatric disorders, vascular disorders, nervous system disorders, endocrine disorders, as well as musculoskeletal and connective tissue disorders was at least twice as high in patients older than 30 years compared to young adults <20 years of age. The pattern of comorbidities observed in PANGAEA is in line with what has been previously reported for MS patients and what has been shown to be significantly associated with an increase in treatment switches due to intolerance and with a stronger EDSS increase (28). An increased cardiovascular risk, as estimated by the Framingham score, was significantly associated with a higher risk for relapse, for reaching EDSS 6.0 and for treatment escalation (29). Furthermore, psychiatric disorders are known to have a strong impact on the quality of life, fatigue, physical disability, and cognitive performance as well as medication adherence (30). In PANGAEA, older patients had more comorbidities and a higher risk for EDSS progression. With respect to the possible influence of comorbidities on MS symptoms and treatment outcomes, the impact of age-dependent comorbidity prevalence on the present comparison has to be considered. To what extent the comorbidities in older PANGAEA patients affected previous treatment switches and present treatment outcomes cannot be estimated.

From a safety point of view, fingolimod can be initiated immediately also in early-onset MS patients. According to the present PANGAEA analyses, physicians as well as patients rated the effectiveness and tolerability to be good or very good. This is in line with the results in young adults from the pivotal studies, which reported a safety profile similar to that of placebo and consistent with that observed in the overall adult population (9). It has been previously shown that patient-perceived good effectiveness and tolerability also translates into very low frequencies of treatment interruptions or discontinuations (31). In the PANGAEA study, about half of the patients discontinued study documentation prematurely, but only approximately one-third of these discontinuations were associated with a lack of effectiveness, disease progression, or lack of tolerability. Drop-outs due to a lack of effectiveness were more frequent in the youngest group compared to older patients. On the other hand, fewer patients <20 years reported disease progression or relapse as a reason for discontinuation. Taken together, the rate of patients who discontinued due to either a lack of effectiveness, disease progression, or relapse is similar. However, as multiple answers were possible, there might be an overlap of patients between both dropout categories.

Overall, this analysis of the PANGAEA study suggests disease- and non-disease-specific differences between younger and older patients, with higher disease activity in younger patients and higher levels of physical disability and more comorbidities in older patients. Despite these differences, fingolimod reduced the overall clinical disease activity as well as the relapse rate, slowed disability progression, and was well-tolerated irrespective of age. The present real-world data suggest that fingolimod can be used for treatment optimization in young patients already at an early stage of the disease.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TZ, HA, JH, LK, ML, CL, and SS contributed to data collection. TZ contributed to the study conception and design. Analyses were planned by TZ, US-T, and BE. The first draft of the manuscript was written by TZ and US-T with the assistance of a medical writer, and all authors commented on the previous versions of the manuscript. All authors contributed to the article and approved the submitted version.

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SYSTEMATIC REVIEW
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Incidence and Risk of Infection Associated With Fingolimod in Patients With Multiple Sclerosis: A Systematic Review and Meta-Analysis of 8,448 Patients From 12 Randomized Controlled Trials

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Background and Aims: There is a controversy regarding whether fingolimod is associated with an increased risk of infection in patients with multiple sclerosis (MS). We performed a systematic review and meta-analysis of data from randomized controlled trials (RCTs) to determine the risk of infection in these patients.

Methods: We systematically searched PubMed, EMBASE, the Cochrane Library, and clinicaltrials.gov from inception to April 8, 2020, to identify RCTs that reported the occurrence of infection in patients with MS treated with fingolimod. Relative risks (RRs) and 95% confidence intervals (95% CIs) were calculated using the random-effects model.

Results: Twelve RCTs including 8,448 patients were eligible. Compared with the control (placebo and other active treatments), fingolimod significantly increased the risk of infection (RR, 1.16; 95% CI, 1.07–1.27; I^2, 81%), regardless of whether the infection was a general infection (RR, 1.14; 95% CI, 1.05–1.25; I^2, 78%), or a serious infection (RR, 1.49; 95% CI, 1.06–2.10; I^2, 0%). Analyses of subgroups found that fingolimod significantly increased the risk of lower respiratory infection (RR, 1.48; 95% CI, 1.19–1.85; I^2, 0%) and herpes virus infection (RR, 1.34; 95% CI, 1.01–1.78; I^2, 9%). There appears to be no dose-dependent increase in the risk of infection associated with fingolimod (0.5 mg: RR, 1.15; 95% CI, 1.07–1.25; I^2, 91%; 1.25 mg: RR, 1.11; 95% CI, 0.97–1.28; I^2, 81%; $P_{interaction} = 0.66$).

Conclusions: Compared with a placebo and other active treatments, fingolimod was associated with a 16% increase in the risk of infection, especially lower respiratory infection and herpes virus infection. The risk of infection associated with fingolimod might not be dose related.

Keywords: fingolimod, multiple sclerosis, infection, dose-dependence, meta-analysis

INTRODUCTION

Multiple sclerosis (MS) is an immune-mediated chronic inflammatory demyelinating disease that mainly affects the central nervous system. Clinically, it is characterized by recurrent relapses, progression, or both, typically striking adults, primarily young adults, and ultimately leading to severe neurological disability (1, 2).

Disease-modifying therapy (DMT), which effectively reduces the recurrence rate and the accumulation of disability, is the preferred treatment in the remission period of MS. At present, there are 15 DMTs approved by the US Food and Drug Administration (FDA), including first-generation DMTs [such as interferon beta (IFN-β) and glatiramer acetate (GA)] and second-generation DMTs (such as fingolimod, teriflunomide, alemtuzumab, ocrelizumab, daclizumab, mitoxantrone, and natalizumab) (3). All DMTs target the immune system and interfere with the inflammatory process of the disease through immunomodulation or immunosuppression, which theoretically leads to a potential risk of infection in patients with MS (4). Therefore, the infection risk due to DMT has become one of the main considerations in the clinical decision-making process.

Among the second-generation DMTs, fingolimod is the first oral DMT approved by the FDA. With the dual functions of the regulation of immune inflammation and the protection of the central nervous system, fingolimod is one of the first-line DMTs for MS (5, 6). However, safety issues associated with fingolimod in randomized controlled trials (RCTs) have raised concerns about the risk of infection. More than 80% of the subjects who were included in three large phase III clinical trials of fingolimod experienced an infection of the event during the trial. FREEDOMS I (7) and II (8) showed that there was no significant difference in the incidence of infection between the fingolimod treatment group and the control group. In the TRANSFORMS study (9), the infection rate in the fingolimod treatment group was significantly higher than that in the control group. Given the contradictory results above, we therefore summarized all available evidence from RCTs for a comprehensive and rigorous meta-analysis of the risk of infection associated with fingolimod.

METHODS

Data Sources and Searches

We followed the standards of the Cochrane Collaboration and the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement for reporting systematic reviews (10). We searched PubMed, EMBASE, and the Cochrane Library databases (up to April 8, 2020) to identify published RCTs that focused on patients with MS treated with fingolimod. The search terms were as follows: ("Multiple sclerosis" OR "Sclerosis, Multiple" OR "Sclerosis, Disseminated" OR "Disseminated Sclerosis" OR "Multiple Sclerosis" OR "Multiple Sclerosis, Acute Fulminating" OR "related-limiting Multiple Sclerosis" OR "primary progressive Multiple Sclerosis" OR "secondary progressive Multiple Sclerosis") AND ("fingolimod" OR "FTY 720" OR "Gilenya" OR "Gilenia" OR Fingolimod) AND ("clinical trial" OR "controlled clinical trial" OR "randomized controlled trials"). We also identified potential studies from the clinicaltrials.gov platform (www.clinicaltrials.gov).

Study Selection and Outcomes

Studies were eligible if they met the following criteria: (1) RCTs reported in full-text publications; (2) comparison of fingolimod with a placebo or other DMTs (IFN-β, GA, teriflunomide, dimethyl pimarate-DMF, natalizumab, alemtuzumab, ocrelizumab, daclizumab, mitoxantrone, etc.) in patients with MS; and (3) the infection was reported as an adverse event. Two reviewers (ZZ and YL) independently screened all citations from the initial search. Any discrepancy was referred to a third reviewer (ZG) and resolved by discussion. The primary outcome of this study was the overall infection, and the secondary outcomes were general infection, serious infection, and other different types of infection. According to the definition of serious adverse events in clinical studies on the clinicaltrials.gov website, serious infection in studies included in this meta-analysis was defined as an adverse event with the following results: (1) life-threatening or resulting in death or (2) patient hospitalization or extension of a current hospital stay, resulting in an ongoing or significant incapacity or interfering substantially with normal life functions. An infection event that did not meet the definition above was considered a general infection.

Data Extraction

Two reviewers (ZZ and YL) independently extracted the data using a self-designed form, which included the first author (publication year), the National Clinical Trial (NCT) number, the sample size, duration of follow-up, the study design (intervention groups and control groups), the country of origin, patient characteristics (age and sex ratio), disease characteristics [MS subtype and expanded disability status scale (EDSS) criteria], DMTs used in 30 days prior to the start of the study, concomitant drugs, and data of infection events. Published data and supplementary data on the clinicaltrials.gov platform were collected for each of the studies, which included upper respiratory tract infection (nasopharyngitis, sinusitis, rhinitis, pharyngitis, etc.), lower respiratory tract or lung infection (bronchitis, pneumonia, etc.), influenza virus infection, herpes viral infection (herpes zoster infection, oral herpes infection, cerebral herpes infection, etc.), digestive system infection (appendicitis, gastroenteritis, diverticulitis, etc.), urinary system infection (urinary tract infection, cystitis, pyelonephritis, etc.), abscess (streptococcal abscess, knee abscess, abdominal abscess, etc.), and other infections, such as otitis media, urinary sepsis, cryptococcal infection, and vulvitis.

Quality Evaluation

The methodological quality of each included RCT was assessed according to the Cochrane Collaboration Risk of Bias Tool (11). The quality of trials was judged as low, unclear, or high in terms of the risk of bias based on the following domains: random sequence generation (selection bias), allocation concealment (selection bias), blinding (performance bias and detection bias), incomplete outcome data (attrition bias), and selective reporting (reporting bias).

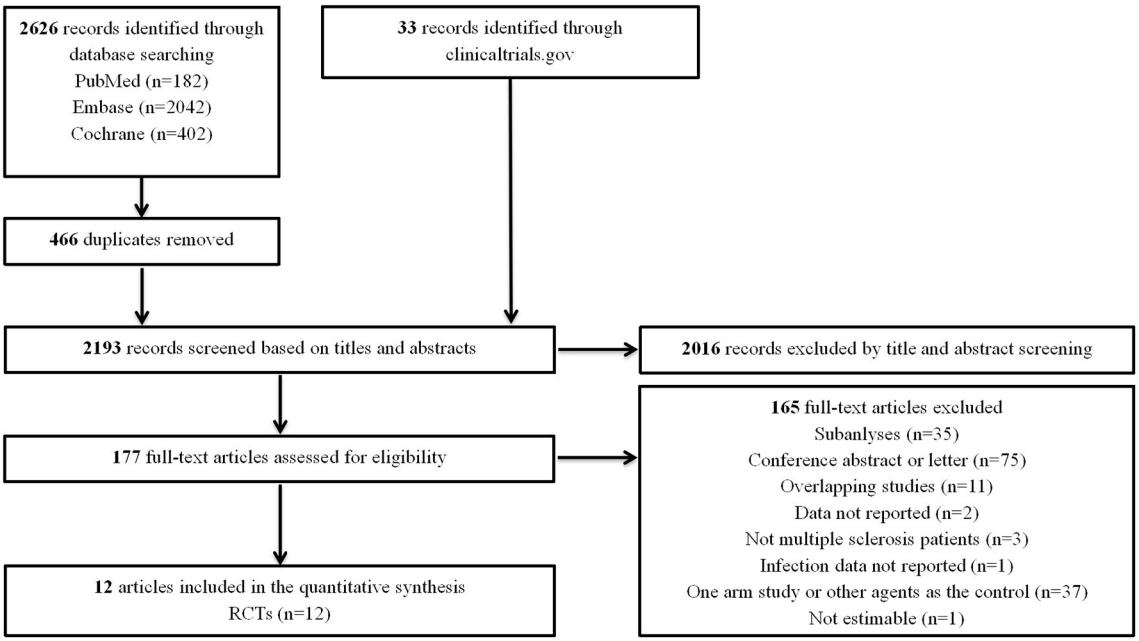

FIGURE 1 | Flow diagram for the selection of eligible randomized controlled trials. RCTs, randomized controlled trials.

Statistical Analysis

Statistical analyses were performed using RevMan 5.3 software (Nordic Cochrane Centre, The Cochrane Collaboration). Relative risks (RRs) and their 95% confidence intervals (95% CIs) were used to calculate the comparative effect sizes, with $p < 0.05$ indicating a statistically significant difference. Heterogeneity between studies was assessed and judged as low (< 25%), moderate (25–75%), and high (> 75%) by the I^2 statistic (12). Subgroup analyses were performed according to the severity of infection (general infection and serious infection), different types of infection events (upper respiratory tract infection, lower respiratory tract and lung infection, influenza, herpes virus infection, digestive system infection, urinary system infection, and abscess), and the dosage of fingolimod (0.5 mg daily and 1.25 mg daily). An interaction analysis (p for interaction) was performed to evaluate the estimated difference between a high dosage and low dosage of fingolimod. A leave-1-out sensitivity analysis was applied to explore whether a single study had an excessive influence on infection incidence. To detect the robustness of the results, further serial sensitivity analyses were conducted by excluding studies that were an open-label design, or excluding studies whose follow-up durations were < 12 months, or excluding studies that used an active agent as the control (IFN-β, GA, natalizumab) (13). Potential publication bias was evaluated by visually inspecting the funnel plots (12).

RESULTS

Search Results and Study Evaluation

Our initial search identified 2,626 records from databases and 33 records from the clinicaltrials.gov platform; 2,016 records were excluded by screening titles and abstracts. Then, we reviewed the full text of the remaining 177 articles and ultimately included 12 RCTs (7–9, 14–20) (**Figure 1**). The characteristics of the included RCTs and the detailed infection outcomes reported in each RCT are summarized in **Table 1**, **Supplementary Table 1**. The included studies were published from 2010 to 2019, with trial durations ranging from 6 weeks to 24 months. A total of 8,448 patients were enroled, among which 5,257 (62.2%) patients were treated with fingolimod and 3,191 (37.8%) patients were treated with a placebo or first-generation DMTs. Of these 12 trials, all studies (6,508 patients) involved low-dose fingolimod (0.5 mg daily), and four studies (1,940 patients) also involved high-dose fingolimod (1.25 mg daily). Details of the quality evaluation are summarized in **Supplementary Figure 1**. Of the 12 RCTs, 4 were non-double-blind clinical studies (16, 20); therefore, we considered the quality of the evidence to be moderate.

Overall Risk of Infection

The overall rate of infection was 55.13% (4,580/8,308) after pooling the data from the 12 RCTs: 56.78% (3,016/5,312) in the fingolimod-treated group and 52.20% (1,564/2,996) in the control group. Compared with the control, fingolimod significantly increased the overall risk of infection (RR, 1.16; 95% CI, 1.07–1.27; I^2, 81%). The subgroup analysis indicated that both general infection (RR, 1.14; 95% CI, 1.05–1.25; I^2, 78%) and serious infection (RR, 1.49; 95% CI, 1.06–2.10; I^2, 0%) were significantly more prevalent in the fingolimod treatment group than in the control group (**Figure 2**).

TABLE 1 | Characteristics of randomized clinical trials.

Source	Total number	Duration	Trial group		Control group		Participants		MS subtype	EDSS criteria	Previous DMT use	Combination medicine
			Treatment	*n*	Treatment	*n*	Age	Female,%				
Cohen et al. (9) (TRANSFORMS)	1,292	12 months	Fingolimod 0.5 mg or 1.25 mg po qd	748	IFNβ-1a 30 μg im qw	435	18–55	66.6 (fingolimod) 67.8 (placebo)	RRMS	0–5.5	NA	NA
Kappos et al. (7) (FREEDOMS)	1,272	24 months	Fingolimod 0.5 mg or 1.25 mg po qd	854	Placebo po qd	418	18–55	69.2 (fingolimod) 71.3 (placebo)	RRMS	0–5.5	NA	NA
Saida et al. (14)	171	6 months	Fingolimod 0.5 mg or 1.25 mg po qd	114	Placebo po qd	57	18–60	69.2 (fingolimod) 68.4 (placebo)	RRMS	0–6.0	NA	NA
Calabresi et al. (8) (FREEDOMS II)	1,083	24 months	Fingolimod 0.5 mg or 1.25 mg po qd	748	Placebo po qd	335	18–55	76.5 (fingolimod) 81 (placebo)	RRMS	0–5.5	NA	NA
Fox et al. (16) (EPOC)	1,053	6–9 months	Fingolimod 0.5 mg po qd	790	IFNβ-1b 0.25 mg sc qod or IFNβ-1a 30 μg im qw or IFNβ-1a 22/44 μg sc tiw or GA 20 mg sc qd	263	18–65	76.1 (fingolimod) 79.1 (iDMT)	RRMS	0–5.5	GA or IFNβ-1a or IFNβ-1b or natalizumab	NA
Kappos et al. (17)	138	12 weeks	Fingolimod 0.5 mg po qd	95	Placebo po qd	43	18–55	68.4 (fingolimod) 67.4 (placebo)	RRMS	0–6.5	NA	Seasonal influenza vaccination and tetanus booster vaccination
Lublin et al. (18) (INFORMS)	970	36 months to 2 years	Fingolimod 0.5 mg po qd	483	Placebo po qd	487	25–65	49 (fingolimod) 48 (placebo)	PPMS	0–5.0	NA	NA
Comi et al. (21) (GOLDEN)	198	18 months	Fingolimod 0.5 mg po qd	106	IFNβ-1b 0.25 mg sc every other day	51	18–60	71.25 (0.5 mg) 67.86 (IFN β-1b)	RRMS	0–5.0	NA	NA
Chitnis et al. (19) (PARADIGMS)	215	24 months	Fingolimod 0.5 mg po qd (0.25 mg po qd for patients with a body weight ≤40 kg)	107	IFNβ-1a 30 μg im qw	108	10–17	65.4 (fingolimod) 65.4 (IFN β-1a)	RRMS	0–5.0	NA	NA
Cree BAC et al. (20) (PREFERMS)	881	12 months	Fingolimod 0.5 mg po qd	436	GA 20 mg sc qd or IFNβ-1a 30 μg im qw	439	18–65	71.3 (fingolimod) 74.9 (iDMT)	RRMS	0–6.0	NA	NA
Biogen Study Medical Director (22) (REVEAL)	111	24 months	Fingolimod 0.5 mg po qd	54	Natalizumab 300 mg iv qw	54	18–65	70.4 (fingolimod) 68.5 (natalizumab)	RRMS	0–5.5	NA	NA
Novartis Pharmaceutical (23)	1,064	12 months	Fingolimod 0.25 mg or 0.5 mg po qd	722	GA 20 mg sc qd	342	18–65	74.8 (fingolimod) 73.7 (GA)	RRMS	0–6.0	NA	NA

GA, glatirameracetate; IFNβ, interferon β; iDMT, injected disease-modifying therapy; DMT, injected disease-modifying therapy; MS, multiple sclerosis; RRMS, relapsing-remitting multiple sclerosis; PPMS, primary progressive multiple sclerosis; EDSS, expanded disability status scale; po, peros; im, intramuscular injection; sc, subcutaneous; iv, intravenous injection; qd, quaque die; qw, quaque week; qod, quaque omni die; tiw, three times per week; NA, not available.

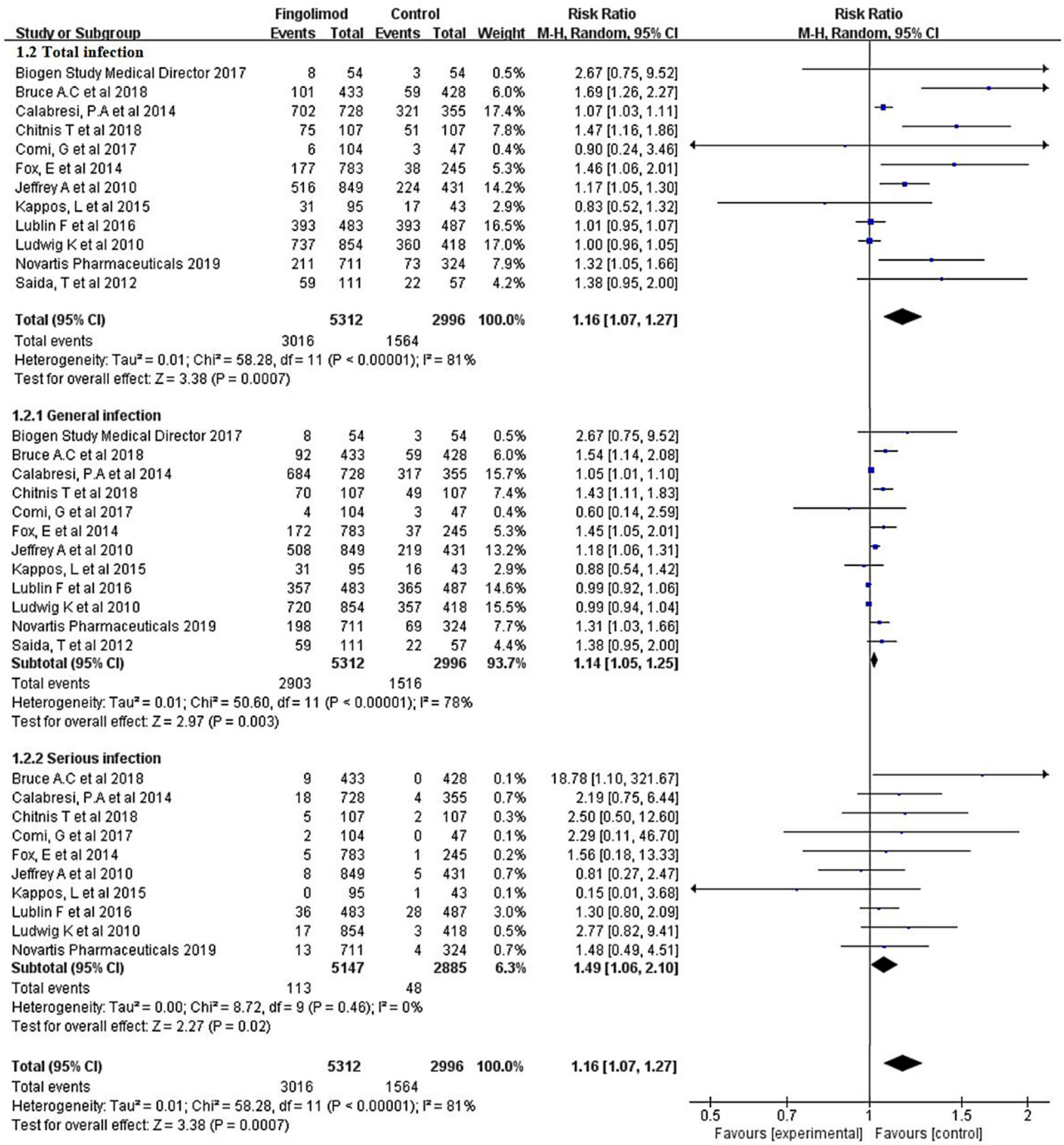

FIGURE 2 | Forest plot with meta-analysis of the overall risk of infection. RR, relative risk; 95% CI, 95% confidence interval.

Risk of Infection by Type of Infection

As shown in **Figure 3**, subgroup analyses were conducted for different infection types. Compared with the control, fingolimod significantly increased the risk of lower respiratory infection (RR, 1.48; 95% CI, 1.19–1.85; I^2, 0%) and herpes virus infection (RR, 1.34; 95% CI, 1.01–1.78; I^2, 9%). No significant risk difference was found between fingolimod and the control in terms of upper respiratory tract infection (RR, 1.05; 95% CI, 0.87–1.27; I^2, 86%), influenza virus infection (RR, 1.09; 95% CI, 0.90–1.33; I^2, 1%), digestive system infection (RR, 0.95; 95% CI, 0.65–1.39; I^2, 0%), urinary system infection (RR, 1.05; 95% CI, 0.84–1.33; I^2, 48%), and abscess (RR, 1.32; 95% CI, 0.45–3.91; I^2, 0%).

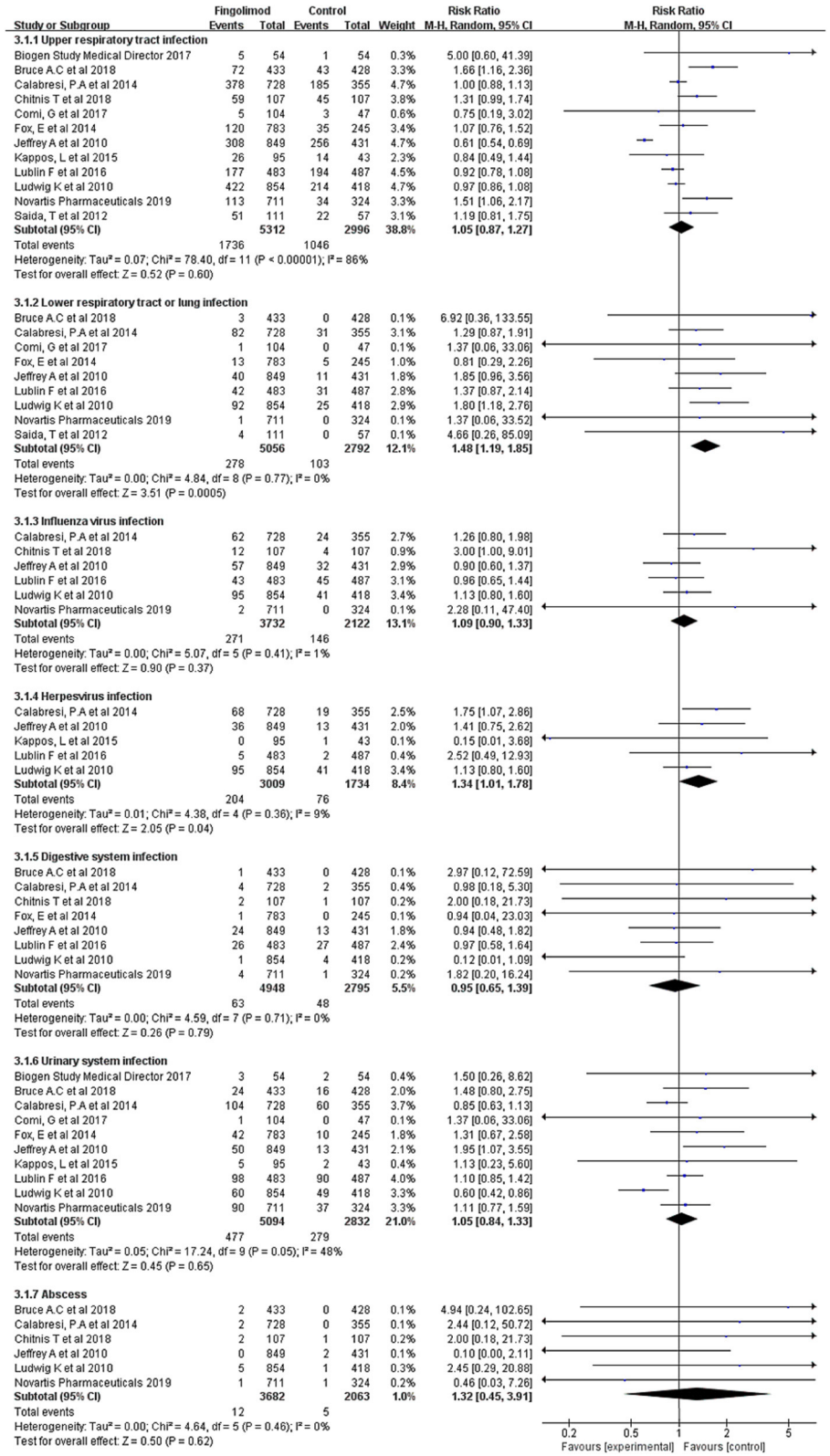

FIGURE 3 | Forest plot with subgroup analysis of different types of infection. RR, relative risk; 95% CI, 95% confidence interval.

Risk of Infection by Dose Size

Fingolimod was available in two doses: 0.5 mg daily and 1.25 mg daily. A total of 6,660 patients from 12 RCTs received fingolimod 0.5 mg daily, and compared with those in the control group, these patients had a significantly higher risk of infection (RR, 1.15; 95% CI, 1.07–1.25; I^2, 81%). A total of 2,521 patients from four RCTs received fingolimod 1.25 mg daily, and the incidence of infection was 80.40% (1,013/1,260) in the fingolimod-treated group and 73.51% (927/1,261) in the control group, indicating that there was no significant difference in the occurrence rate of infection between the fingolimod and control groups (RR, 1.11; 95% CI, 0.97–1.28; I^2, 91%) (**Figure 4**). However, we failed to find an estimated difference between the high dosage and low dosage of the fingolimod groups ($P_{interaction} = 0.66$), which indicated that the risk of infection associated with fingolimod might not be dose dependent.

Sensitivity Analyses

The leave-1-out sensitivity analysis failed to identify any individual trial as having influenced the primacy outcome. Besides, further sensitivity analyses by excluding studies that were an open-label design or whose follow-up durations were < 12 months or that used an active agent as the control (IFN-β, GA, natalizumab), which all confirmed the robustness of primacy results (**Supplementary Table 2**).

Publication Bias

Visual inspection of the funnel plots for the analyses showed that all plots exhibited fairly symmetrical inverted funnel shapes, suggesting that publication bias was not a concern (**Supplementary Figure 2**).

DISCUSSION

Major Findings

The risk of infection has been recognized as one of the main considerations when choosing appropriate DMT for patients with MS in the clinical setting (24). As a highly effective second-generation DMT, fingolimod has great clinical application in patients with MS, but whether fingolimod increases the risk of infection remains uncertain. This systematic review and meta-analysis firstly provided a comprehensive overview of fingolimod-associated infection risk based on 12 RCTs, including 8,448 patients with MS. The major findings were as follows: (1) fingolimod use increased the risk of overall infection by 16%, and the incidence of both general and serious infections increased significantly; (2) fingolimod use was associated with a higher risk of lower respiratory and herpes virus infection; and (3) the risk of infection associated with fingolimod might be dose independent.

Comparison With Previous Studies

There were three systematic reviews that focused on fingolimod, two of which systematically reviewed real-world data on fingolimod to determine its persistence and efficacy (25, 26). In these studies, the overall incidence of adverse events (AEs) was counted, and no systematic analysis for specific adverse events (such as infection) was performed. At present, only one study in 2019 evaluated the efficacy and safety of fingolimod using 10 RCTs (27). In that study, bronchitis, nasopharyngitis, sinusitis, and urinary tract infection were considered infection events, and the analysis indicated that fingolimod was associated with a significantly increased risk of bronchitis, which was consistent with our result that fingolimod increased the risk of lower respiratory infection. However, they did not find any significant difference between the fingolimod and control groups in terms of the overall incidence of infection. Considering an important limitation of that study, i.e., that only some AEs with a high incidence were retrieved, and their assessment of the risk of infection was not comprehensive. Therefore, considering the limitations of previous studies, this systematic review included all the infection events reported in RCTs, regardless of whether they were common or not, to systematically evaluate the risk of infection associated with fingolimod. Moreover, in addition to the currently published studies, we also included unpublished RCTs on the clinicaltrials.gov website to make the study more comprehensive.

We ultimately retrieved data from 12 RCTs, and we confirmed that fingolimod is associated with a relatively higher risk of infection than placebos and other active DMTs (IFN-β, GA, and natalizumab), which is consistent with two previous observational studies (28, 29). This study also highlighted the existence of different risk profiles for different types of infection associated with fingolimod. Subgroup analysis indicated that the incidence of lower respiratory infection and herpes virus infection increased significantly in patients treated with fingolimod. Since there were some studies suggesting that the occurrence of AEs associated with fingolimod might be dose dependent (30), we also assessed the correlation between infection risk and fingolimod dosage. However, the results showed that the risk of infection associated with fingolimod might be dose independent ($P_{\text{interaction}} = 0.66$).

Potential Mechanism

There were two possible explanations for why fingolimod was associated with a higher risk of infection in MS patients. First, as a sphingosine-1-phosphate (S1P) analog and a functional modulator of the S1P receptor, fingolimod-P causes the internalization of $S1P_1R$ from the cell membrane in lymph node T cells. As a result, the functional balance between $S1P_1R$ and lymph node-homing CC chemokine receptor (CCR7) is interrupted, and CCR7 + primitive T cells and central memory T cells (TCM) are unable to resist CCR7-mediated lymph node retention, thereby remaining in lymph nodes. Therefore, the number of peripheral T cells migrating to the CNS decreases, which may cause the occurrence of infection (5, 31, 32). A subanalysis of a phase III RCT for fingolimod indicated that the lymphocyte count dropped rapidly within 2 weeks after the start of treatment; however, no trend was found in the relationship between the incidence of infection and decreases in lymphocytes and the duration of treatment (33). Additionally, it is worth noting that the counts of peripheral lymphocytes reflect only 2% of the total lymphocytes in the body, and fingolimod mainly reduces circulating CD4 + T cells, retaining CCR7— effector T cells involved in controlling microbial infections (34).

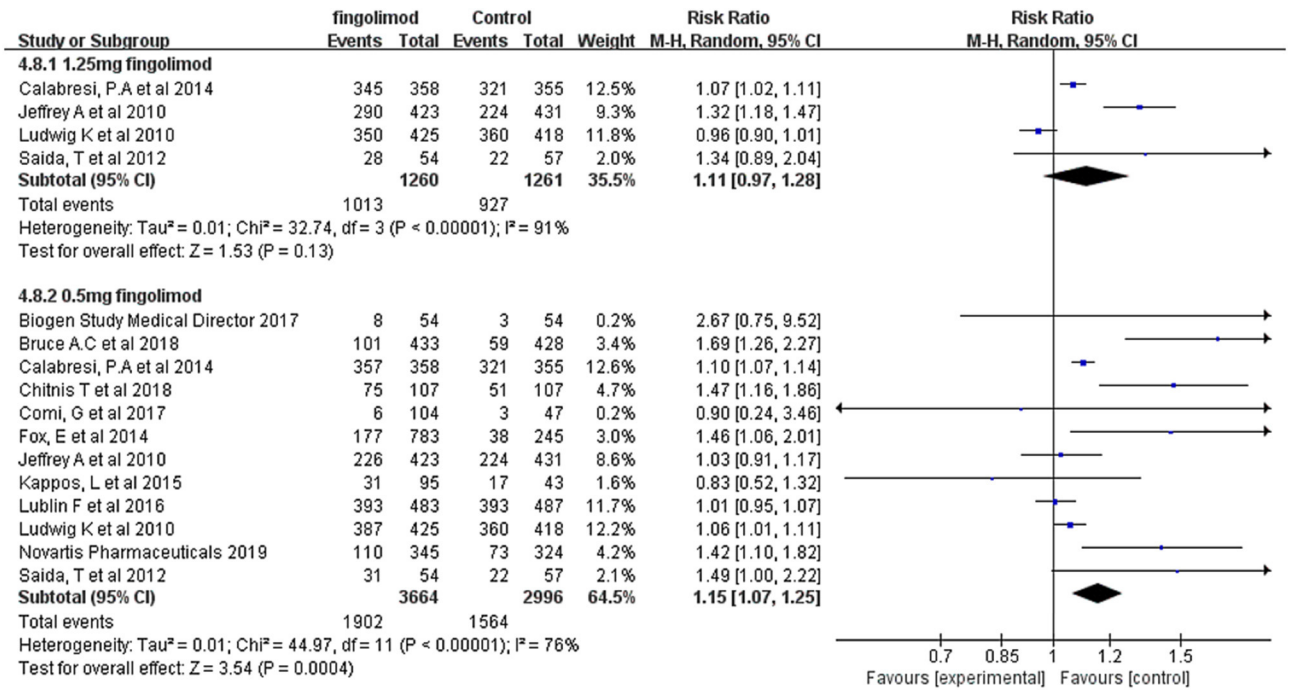

FIGURE 4 | Forest plot with subgroup analysis of different doses of fingolimod for infection risk. RR, relative risk; 95% CI, 95% confidence interval.

Accordingly, the relationship between the decrease in peripheral lymphocyte counts and the infection caused by fingolimod is still controversial. Second, studies have also argued that fingolimod induces some important functional changes in the immune system, which leads to an increased risk of infection. Under the effect of fingolimod, T cells decrease the production of cytokines, such as IFN-γ, IL-17, GM-CSF, and TNF-α, which can help to effectively kill congenital effector cells (such as neutrophils and macrophages) and promote the differentiation of T cells. Additionally, in the long term, the ratios of CD4 and CD8 in patients with MS taking fingolimod show a striking reversal of the normal 2:1 ratio, reminiscent of the changes associated with AIDS. Of course, the effect of fingolimod on the immune system is by no means comparable to that of AIDS-associated immune changes, but the effect of the reversal caused by fingolimod on the immune response is not fully understood (35). In short, the specific mechanism of fingolimod-associated infections is not yet clear, and further research and analyses are still needed.

Clinical Considerations

Given the higher incidence of infection in patients with MS treated with fingolimod, it might be reasonable to triage patients according to the following steps: First, clinicians should conduct a comprehensive assessment of patient conditions for the possible risk factors, such as their history of infection, history of immunosuppressive exposure, vaccination history, age, etc., to determine the best DMT for individual patients (30). Second, a higher risk of herpes virus infection associated with fingolimod was indicated in this study; thus, herpes virus serology should be performed before the start of fingolimod treatment, and flu vaccination can also be considered. Third, during the treatment with fingolimod, clinicians need to be alert to the occurrence of any infection with strict monitoring of clinical signs/symptoms, especially the lower respiratory infection and herpes virus infection (28). Adequate laboratory and instrumental tests are also necessary to make an early diagnosis and promptly start the treatment where appropriate. Although the current evidence indicates that the increased risk of infection caused by fingolimod is associated with its effect on the immune function to a certain degree, there is no well-established monitoring method in clinical practice. Monitoring of peripheral lymphocyte counts can be considered for the decreases in the lymphocyte count associated with fingolimod. Suppose the lymphocyte count drops below 0.2×10^9/L at any visit (at 2 weeks, 1, 2, and 3 months, and every 3 months after that). In that case, fingolimod should be temporarily discontinued for immune reconstruction (36), but this indicator has not been used as a standard for discontinuation in clinical practice. Moreover, for the potential immune downregulation of fingolimod, live attenuated vaccines should be avoided during treatment, if possible (34). Summarily, understanding the infectious effects of fingolimod, taking into account the prevention, is preferable to treatment.

Strengths and Limitations

The major strength of this study was that we comprehensively assessed the risk of infection of patients with MS treated with fingolimod on the basis of evidence from RCTs. Certainly, there are inherent limitations in this meta-analysis. First, four RCTs included were open-label studies with low quality, although the sensitivity analysis showed that their effect on the final result was

not significant. Second, the heterogeneity among the included RCTs was relatively high. To address this issue, the random-effects model was used for the meta-analyses. Besides, several subgroup analyses as well as serial sensitivity analyses were performed to strengthen the robustness of the results. All results were in line with the primacy results. Third, since the FDA only approved the clinical use of the 0.5 mg daily dose, clinical trials of 1.25 mg daily doses were limited. Hence, the analysis of different doses in our study might be limited by the small number of cases in the high-dose group; therefore, the results must be interpreted cautiously. Fourth, the clinical trials included in our study were performed at various international institutions, which might have varying expertise and ability to detect infection, making it possible that the reported incidence was biased. Fifth, the timing of infection occurrence might be related to the duration of treatment. We also conducted a sensitivity analysis by excluding short follow-up studies, and the result was consistent with primacy analyses. Sixth, due to the limited number of cases, certain infections (encephalitis viral, clostridial infection, mastoiditis, otitis media acute, urosepsis, tinea pedis, vulvitis, Lyme disease, labyrinthitis, hepatitis C, myelitis, septic shock, systemic mycosis, arthritis bacterial, clostridium difficile colitis, device-related sepsis, meningitis fungal, sepsis, etc.) were not included in the subgroup of different types of infection in the meta-analysis. Finally, this study only evaluated the infection risk of fingolimod based on the data from RCTs; to extend RCT findings to large patient populations in real-world clinical practice, further design of real-world studies on the evaluation of fingolimod safety is necessary.

CONCLUSION

In conclusion, by systematically evaluating evidence from RCTs, we confirmed that fingolimod significantly increased the risk of infection, especially lower respiratory infection and herpes virus infections, in patients with MS. Both general infection and serious infection increased to varying degrees. However, the risk of infection associated with fingolimod might not be dose related. These findings can help clinicians assess the risk of infection of patients treated with fingolimod.

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.

AUTHOR CONTRIBUTIONS

ZZ contributed to the review of published papers, the conception of the study, the data acquisition, analysis, and interpretation, and the writing of the manuscript. C-LM and Z-CG are the guarantors of the entire study, contributed to the conception of the study, the interpretation of the data, and review of the manuscript. YD contributed to the conception of the study and review of the manuscript. YL contributed to data acquisition for the study. M-KZ supervised the study. 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/fimmu.2021.611711/full#supplementary-material

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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.

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BRIEF RESEARCH REPORT
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Does Extended Interval Dosing Natalizumab Preserve Effectiveness in Multiple Sclerosis? A 7 Year-Retrospective Observational Study

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The extended interval dosing (EID) of natalizumab has been suggested to be associated with a reduced risk of progressive multifocal leukoencephalopathy (PML) and short-term preservation of efficacy but its long-term effectiveness remain unknown. We aimed to determine the long-term effectiveness and safety of natalizumab in an EID setting in a cohort of patients with multiple sclerosis (MS) treated for more than 7 years. We conducted an observational retrospective cohort study, including 39 (34 female, 5 male) patients with clinically definite relapsing-MS, initially treated with standard interval dosing (SID) of natalizumab (mean time 54 months [SD29]) who were then switched to EID, every 8 weeks (mean time 76 months [SD13]). The main outcome measures included the following: i) annualized relapse rate (ARR), ii) radiological activity, iii) disability progression, and iv) NEDA-3 no evidence of disease activity index. EID preserved ARR, radiological activity, and prevented disability worsening during follow-up. The proportion of patients maintaining their NEDA-3 status after 24, 48, and 72 months of natalizumab administration in EID was 94%, 73%, and 70%, respectively. Stratified analysis according to history of drug therapy showed that the EID of natalizumab was slightly more effective in naïve patients than in those previously treated with other immunosuppressive drugs. No cases of PML or other severe adverse reactions were reported. In conclusion, long-term therapy with natalizumab in an EID setting following an SID regimen maintained its disease-modifying activity, and was safe and well tolerated for over 7 years. These encouraging observational results need to be confirmed in controlled clinical trials.

Keywords: extended interval dosing, multiple sclerosis, natalizumab, disease modifying therapy, treatment

INTRODUCTION

The humanized monoclonal antibody natalizumab (Tysabri®; Biogen-Idec, Cambridge, MA, USA) is directed against the α4 subunit of both α4β1 and α4β7 integrins. The blockage of these integrins, which are expressed on the cellular surface of circulating mononuclear cells, prevents their entry into the central nervous system (CNS) through the blood-brain barrier (1). Natalizumab administered every 4 weeks reduces CNS inflammation, and thus it is a rapidly-acting and effective agent in reducing both clinical and radiological activity, as well as preventing disability progression in patients with multiple sclerosis (MS) (2, 3). As a consequence of its mechanism of action, natalizumab, albeit usually well tolerated, has been associated with an increased risk of progressive multifocal leukoencephalopathy (PML), a rare life-threatening infection caused by the John Cunningham virus (JCV) (4, 5). Presently, it is widely accepted that the risk of PML is particularly high in patients who have previously received immunosuppressive drugs, in JCV positive patients (index > 1.5), and in those treated with natalizumab for more than 24 months (5). In contrast, the discontinuation of natalizumab has been associated with MS reactivation and rebound (6). In this scenario, clinicians treating patients with MS who are at a high risk of PML must carefully consider either continuing treatment with natalizumab or switching to another highly-effective therapy (6, 7). For patients receiving long-term natalizumab treatment, several therapeutic strategies have been suggested to reduce the risk of PML. Among them, several investigators have suggested extended interval dosing (EID) schedules, most of them involving drug administration every 6 to 8 weeks (8–11). EID seems to result in a partial desaturation of drug receptors that might allow restoring some degree of anti-viral immune response (1, 12). On this basis, the natalizumab product information sheet has been recently amended to include the possibility of using EID (with dosing every 6 weeks) in patients at high risk of PML (https://www.ema.europa.eu/en/documents/product-information/tysabri-epar-product-information_en.pdf). Moreover, a few studies suggest that treatment with EID of natalizumab is associated with a lower PML risk, while preserving the effectiveness on the control of disease activity (8–11). However, these studies included small groups of patients who were followed-up for short periods (8–11). Confirmation of the effect of EID is critically important for clinicians to be able to discuss and help patients take informed decisions regarding a long-term therapeutic plan once the disease activity is controlled. Therefore, we aimed to analyze a quite unique cohort of patients with MS, followed-up for more than 7 years, to study the efficacy and safety of treatment with natalizumab in an EID setting.

PATIENTS AND METHODS

The present study was motivated by a recent organizational change at our hospital, in which one author (JR) was asked to take care of a cohort of patients with MS. This was an observational retrospective cohort study with analysis of data collected during routine clinical practice at Hospital Universitario Sierrallana, in Cantabria, Spain. The protocol was approved by the institutional review board [Comité de Ética de la Investigación con medicamentos de Cantabria (CEIm Cantabria), reference number: 2019.328] and the study was conducted in accordance to the relevant guidelines and regulations.

The inclusion criteria were as follows: i) a diagnosis of clinically definite relapsing-MS, according to the McDonald revised criteria (13); ii) age over 18 years; iii) previous treatment with SID of natalizumab (every 4 weeks) for at least 24 months; and iv) treatment switched to EID of natalizumab (every 8 weeks).

Clinical charts were reviewed to collect the following variables: sex, age at diagnosis, symptoms at onset, previous treatments, duration of treatment with natalizumab in SID, reason for natalizumab extension, duration of treatment with natalizumab in EID, clinical relapses during treatment, magnetic resonance imaging (MRI) lesion load, presence of gadolinium-enhanced lesions, and the Expanded Disability Status Scale (EDSS) score. In addition, we carefully checked for potential natalizumab-related adverse reactions, specifically PML. Serologic JCV status was monitored every 6 months.

The main outcome measures were as follows: i) the annualized relapse rate (ARR), ii) presence of brain MRI activity (considered as at least 2 new T2-hyperintense lesions and/or new gadolinium-enhancing lesions), iii) EDSS score, and iv) disability progression assessed by the EDSS and defined as an increase of 1.5, 1 or 0.5 points in patients with MS having a previous EDSS score of 0, < 5.5, and ≥ 5.5, respectively. As an outcome parameter of global disease control, we estimated the no evidence of disease activity (NEDA-3) status, which includes the combined absence of clinical relapses, radiological activity as well as disability progression.

In a complementary analysis, patients were stratified according to history of previous drug therapy. Thus, we divided patients into "switchers" if they had previously undertaken other disease modifying therapy (DMT) and "naïve" if natalizumab was the first DMT used.

Baseline characteristics were compared by the non-parametric Mann–Whitney U test and the Fisher exact test. Global differences in ARR and EDSS across groups were tested by the Kruskal-Wallis test. Subsequently, the Wilcoxon test was used for pairwise between-group comparisons. Kaplan-Meier analyses were used to assess the proportion of patients who maintained their NEDA-3 status and an EDSS score < 6. Differences were then tested by the Gehan-Breslow-Wilcoxon test. This procedure was also used to compare differences in the course of the NEDA-3 status between switchers and naïve patients. p-values < 0.05 were considered as significant. Prism software (GraphPad Software Inc., San Diego, California) was used for statistical analysis.

RESULTS

Patients' Characteristics

Thirty-nine patients (34 female and 5 male; mean age at diagnosis, 33 years) were included in the study. The patient characteristics have been summarized in **Table 1**. Among them, 26 patients had been previously treated with other DMTs (25, interferon; 1, azathioprine), while in 13 patients (33%) natalizumab had been chosen as the initial DMT. Regarding treatment with natalizumab, all patients included in the study followed the same therapeutic regimen; they were treated with natalizumab in an SID setting for at least 24 months. Subsequently, because of safety concerns, and after having evaluated other therapeutic options, the dosing schedule was switched to EID. The primary reason for extending the dosing interval of natalizumab was the concern of a high risk of PML. At the inception of this cohort there were very scarce data. Therefore, it was opted for a potentially safer 8-week scheduling.

In this context, at the initiation of EID of natalizumab, 32 out of 39 patients (82%) were seropositive for JCV (quantitative data concerning the evolution of the JCV index was not available for all patients). Of note, at the completion of this study, the JCV index was low (<0.9) in 6 patients, intermediate (0.9–1.5) in 4, and high (>1.5) in 22 patients. The mean age at the SID initiation of natalizumab was 39 years (SD, 11) and mean duration of treatment with SID of natalizumab was 51 months (SD, 20).

Regarding the EID of natalizumab, patients' mean age at initiation was 43 years (SD, 10) and the mean duration of treatment was 77 months (SD, 13).

Natalizumab administration in both, SID and EID regimens, was well tolerated. We did not find any case of PML or any other severe adverse reactions leading to natalizumab discontinuation during the administration of SID or EID regimens (**Table 1**). The most frequent adverse effects were respiratory and urinary tract infections.

ARR, Radiological Activity, and Disability Progression

Regarding the ARR, a significant difference was found between the study groups (p<0.0001) (**Figure 1A**). After initiating treatment with SID of natalizumab, the ARR significantly decreased from 0.54 (SD, 0.60) to 0.03 (SD, 0.09; p=0.0005) (**Figure 1A**). However, the ARR did not vary significantly between the SID and EID groups (SID-ARR, 0.025 [SD, 0.026]; EID-ARR, 0.02 [SD, 0.06]; p 0.72) (**Figure 1A**). Specifically, ARR remained low during the entire period of treatment with natalizumab in both SID and EID regimens, ranging from 0 to 0.036 and 0 to 0.035, respectively throughout the 7-year follow-up period (**Figure 1B**).

TABLE 1 | Main patients characteristics.

Pre-Natalizumab		
Number of patients	39	
Age at diagnosis, mean (SD)	33 (10.4)	
Females, n (%)	34 (87%)	
Previous DMTs	25 (64%)	
-IFNβ, n (%)	1 (2.5%)	
-AZA, n (%)	0.45 (0.53)	
Pre-Natalizumab AAR (patients treated with DMTs)		
Natalizumab		
	SID (4 weeks) (n=39)	**EID (8 weeks) (n=39)**
Age at the beginning, mean (SD)	38.97 (11.10)	43.41 (10.71)
Duration of treatment, mean (SD)	51.12 months (19.89)	76.68 months (13.31)
JCV +, n(%)	–	32 (82%)
EDSS at the beginning, median [IR]	2 [1-3.5]	2 [1-3.5]
ARR, mean (SD)	0.03 (0.09)	0.02 (0.06)
Radiological activity	0.05 (0.03)	0.04 (0.03)
EDSS at the end of the treatment, median [IR]	2 [1-3.5]	2 [1-3.5]
Adverse reactions (clinical) (n,[%])	Respiratory infection (5 [13%]) Urinary infection (4 [10%]) Pharyingitis (3 [8%]) Diarrhea (1 [3%]) Herpes labialis (1[3%]) Headache (1[3%])	Urinary infection (6 [15%]) Respiratory infection (2 [5%]) Pharyingitis (2 [5%]) Pneumonia (1[3%]) Diarrhea (1[3%]) Herpes labialis (1[3%]) Herpes zoster (1[3%]) External otitis (1[3%])
Adverse reaction (analytical) (n,[%])	Mild lymphocytosis (27 [70%]) Mild liver test alteration (4 [10%]) Mild granulocytosis (3 [8%]) Decreased mean platelet volume (3 [8%]) Anemia (1 [3%])	Mild lymphocytosis (21 [54%]) Mild granulocytosis (2 [5%]) Decreased mean platelet volume (2 [5%]) Anemia (1 [3%])

ARR, annualized relapse rate; AZA, azathioprine; DMT, disease modifying therapies; EID, expanded interval dosing; EDSS, expanded disability status scale; IFNβ, interferon beta; IR, interquartile range; JCV, John Cunningham virus; SD, standard deviation; SID, standard interval dosing. Radiological activity was defined as the appearance of at least 2 new T2-hyperintense lesions and/or new gadolinium-enhancing lesions.

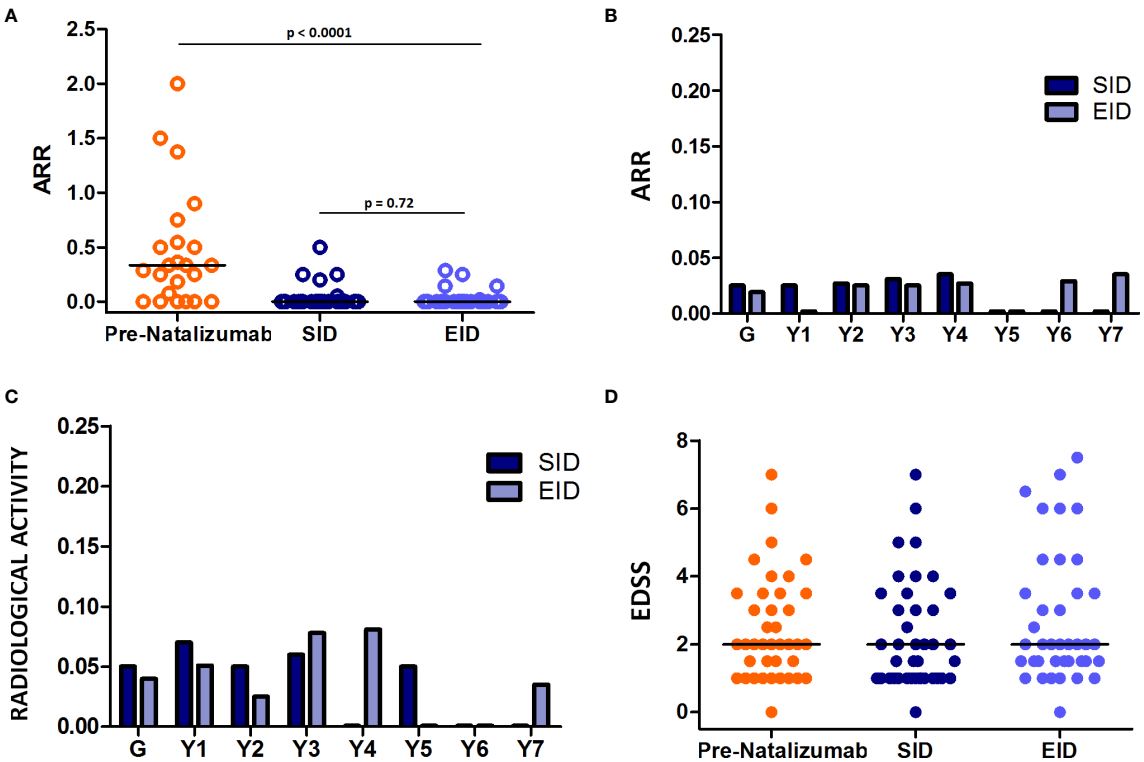

FIGURE 1 | Annualized relapse rate (ARR) and disability progression in patients treated with natalizumab in extended interval dosing (EID). **(A)** AAR before natalizumab treatment (Pre-Natalizumab, orange), during the standard interval dosing (SID, dark blue), and EID (light blue) of natalizumab. A significant difference was found between the studied groups (Kruskal-Wallis test, p<0.0001). ARR did not significantly vary between the SID and EID groups (Wilcoxon test, p=0.72). **(B)** Detailed ARR during the 7-year follow-up of patients treated with natalizumab in SID (dark blue) and EID (light blue). Y1-Y7: ARR during years 1 through 7 in patients on natalizumab in SID and EID. **(C)** Radiological activity during the follow-up of patients treated with natalizumab in SID (dark blue) and EID (light blue). Y1-Y7: radiological activity during years 1 through 7 in patients on natalizumab in SID and EID. Radiological activity was defined as the appearance of at least 2 new T2-hyperintense lesions and/or new gadolinium-enhancing lesions. **(D)** Expanded Disability Status Scale (EDSS) score before natalizumab treatment (pre-natalizumab, orange), during treatment with natalizumab in SID (dark blue) and EID (light blue) settings. No significant differences were noted among the three groups (Kruskal-Wallis test, p=0.46).

The radiological activity also remained low in both groups of patients with MS receiving the two natalizumab regimens throughout the follow-up period (SID, 0.050 [SD, 0.03]; EID, 0.040 [SD, 0.03]; p= 0.67). Specifically, it ranged from 0 to 0.076 and 0 to 0.081 in the SID and EID groups, respectively (**Figure 1C**). Analysis of ARR and radiological activity showed some discrepancies, and ARR did not always correlate well with radiological activity (for example, in year 1 of the EID regimen, radiological activity was relatively high whereas ARR remained very low). Of note, ARR represented clinical relapses alone and not radiological activity (**Figures 1B, C**). Concerning disability progression, no significant variations in EDSS scores were observed during the follow-up period (Pre-Natalizumab: median, 2; [interquartile range (IQ), 1–3.5]; Natalizumab-SID: median, 2; IQ, 1–3.5; Natalizumab-EID: median, 2; IQ, 1.5–3.5; p=0.46) (**Figure 1D**).

The beneficial effect of natalizumab-EID in maintaining functional status was confirmed by the Kaplan-Meier analysis. As shown in **Figure 2A**, the proportion of patients maintaining NEDA-3 status was 94%, 73%, and 70% after 24, 48, and 72 months of therapy with EID regimen, respectively. At month 72 of the EID regimen, 83% of patients showed no disability progression and 86% showed no clinical relapses (**Figure 2A**). In addition, after 84 months of treatment with EID regimen, more than 85% of patients maintained an EDSS score < 6 (**Figure 2B**).

Natalizumab-EID in Switchers and Naïve Patients

In a complementary analysis, patients were divided into two groups depending on whether they had been treated with other DMTs prior to natalizumab-SID ("switchers") or not ("naïve"). The cohort included 26 switchers and 13 naïve patients. No sex differences were evidenced between groups (switchers: female, 22; males 4; naïve: female, 12; male, 1; p=0.45). Of note, switcher patients were slightly older than naïve patients (mean age, 41 *vs.* 34 years; p=0.05), and exhibited a more advanced disease status (mean EDSS score, 2.75 *vs.* 1.50; p=0.006). No significant differences were observed in the mean duration of treatment with the SID regimen (switchers *vs.* naïve: 39 *vs.* 38 months;

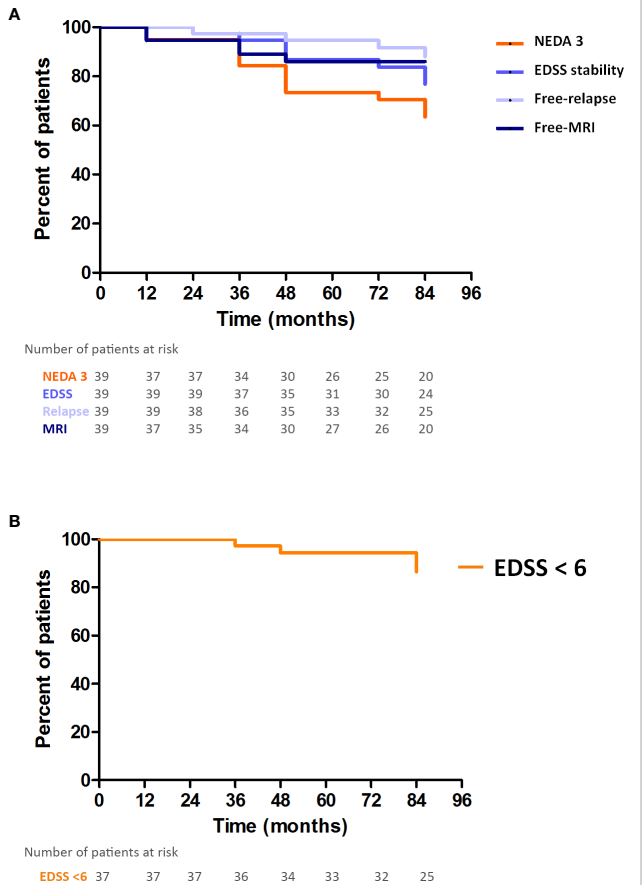

FIGURE 2 | Preservation of the no evidence of disease activity (NEDA-3) status and Expanded Disability Status Scale (EDSS) score < 6 in patients treated with natalizumab in extended interval dosing (EID). **(A)** Kaplan-Meyer plots representing the proportion of patients i) maintaining the NEDA-3 status (orange), ii) showing no worsening of the EDSS score (light blue), iii) showing no evidence of clinical relapse (purple), and iv) showing no evidence of radiological activity while on an EID of natalizumab (dark blue). **(B)** Kaplan-Meyer plot of the proportion of patients maintaining an EDSS score < 6 while on an EID of natalizumab.

p=0.11) and EID regimen (switchers *vs.* naïve: 76 *vs.* 78 months; p=0.24) between groups. Primary patient data are summarized in (**Table 2**). Among switchers, ARR significantly decreased after initiating SID of natalizumab (from 0.42 [SD, 0.53] to 0.026 [SD, 0.07]; p=0.0008) and remained at the same level when these patients were treated with the EID regimen (p > 0.99) (**Figure 3A**). In naïve patients, the ARR remained low with both SID (0.038 [SD 0.13]) and EID (0.010 [SD 0.03]) regimens, without significant differences between the two periods (p > 0.99) (**Figure 3A**). In concern to radiological activity, no significant differences were found after extending natalizumab administration from SID to EID in both switchers and naïve patients (switchers: 0.05 [0.04] vs 0.04 [0.04] p=0.94; naïve patients: 0.06 [0.05] vs 0.03 [0.04]; p = 0.20).

Regarding disability progression, although the baseline EDSS score at initiation of EID regimen was worse in switcher patients than in the naïve ones, the EDSS score was uniformly maintained during natalizumab-EID in both groups. In fact, among switchers, the median EDSS score was 2.75 pre-SID, 2.75 pre-EID, and 2 post-EID. Among the naïve patients, the EDSS score was maintained at 1.5 all through the three study time-points (**Figure 3B**).

Kaplan-Meier plots of NEDA-3 showed that naïve patients had a significantly more favorable control of disease activity, when compared to switchers (p=0.012). In this context, after 72 months of EID regimen 84 and 54% of naïve and switcher patients, respectively, maintained the NEDA-3 status (**Figure 3C**).

DISCUSSION

This study was conceived as an opportunity to assess the efficacy of administering natalizumab in an EID setting following a SID regimen in patients with MS who were at a high risk of PML. Monthly natalizumab is a highly effective regimen for the treatment of patients with MS (2, 3, 14). However, its long-term use is limited by an increased risk of PML, which is particularly high in patients seropositive for JCV, those previously treated with other immunosuppressant drugs, and in those receiving natalizumab for more than 2 years (5, 15–17). Based on its pharmacokinetics, it has been proposed that natalizumab in an EID setting might be associated with a lower risk of PML. Interestingly, cases of PML in patients with MS treated with natalizumab in an EID setting exhibit less severe disease course, characterized by a prolonged pre-symptomatic phase, pauci-symptomatic onset, low JCV load, less severe functional impairment during immune reconstitution, and a mild disability burden (18).

This is supported by several preclinical studies that reported that extending the dosing interval to 6–8 weeks resulted in a partial drug receptor desaturation, allowing a small proportion of lymphocytes to pass through the blood-brain-barrier, leading to some degree of viral protection (1, 12, 19).

However, there are no studies on the effectiveness of long-term EID regimen yet. The present study shows that a long-term EID regimen (up to 7 years) following an SID regimen exhibited a high effectiveness in controlling disease activity, as evidenced by parameters such as ARR, radiological activity, and disability progression. Although several previous studies involved larger sample size, these included patients with variable dosing intervals, ranging from 5 to 8 weeks (10, 11), thus complicating the analysis of effectiveness (8–11). In our study, all patients followed the same 8-week dosing schedule, which was well tolerated and safe, specifically concerning the risk of PML throughout the 7-year follow-up. Thus, our long-term results provide further support for natalizumab therapy in an EID setting, as suggested previously by a few studies with shorter follow-up (8–11). As expected, treatment with natalizumab in both SID and EID settings reduced both the clinical relapse rate and radiological activity. However, there were some discrepancies between ARR and radiological activity. At some time points of the EID period, there were no clinical relapses, despite some evidence of radiological activity, while at other time points, ARR was slightly higher than radiological activity. This has been described as the clinico-

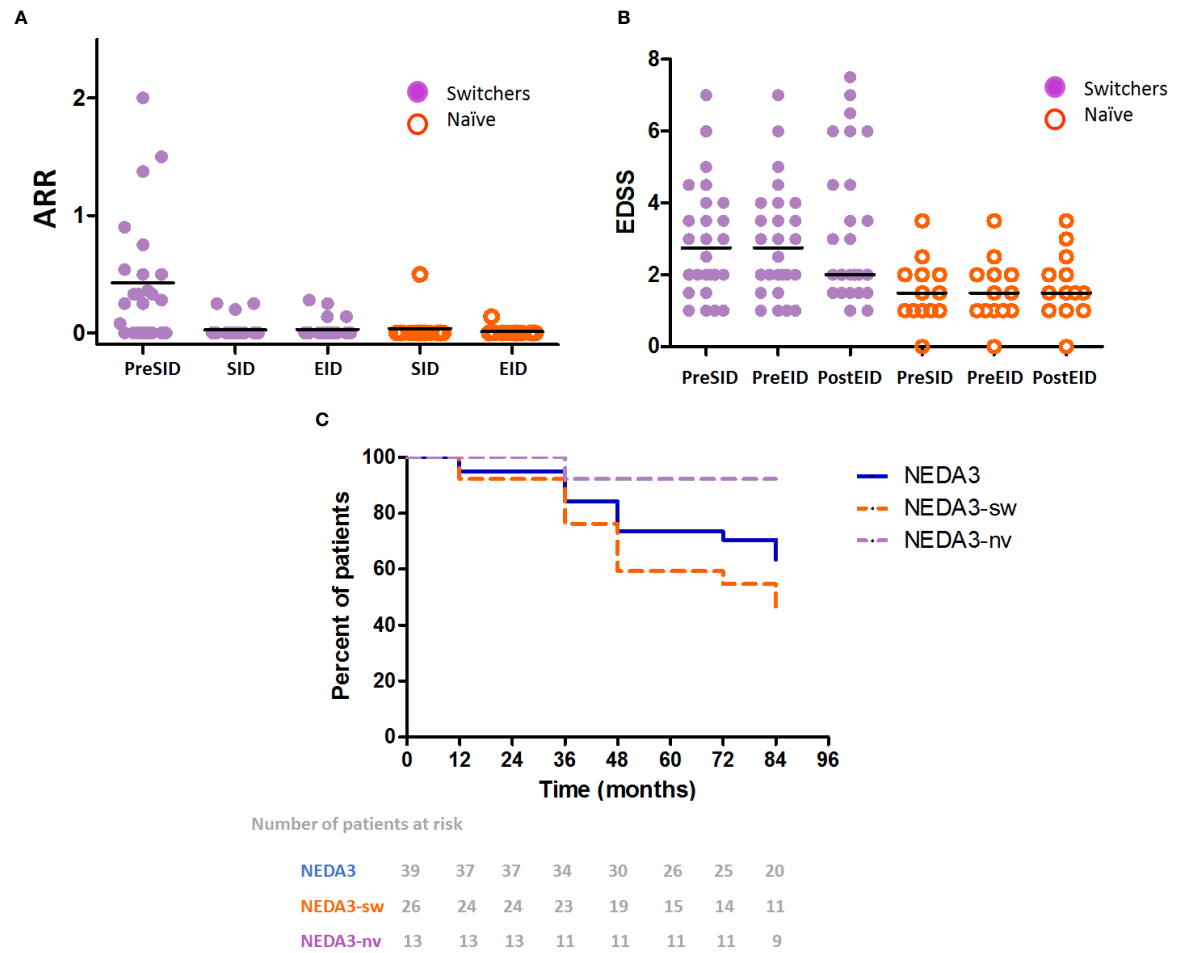

FIGURE 3 | Extended interval dosing (EID) of natalizumab in switchers and naïve patients. **(A)** The mean annualized relapse rate (AAR) before natalizumab treatment (Pre-SID), during treatment with natalizumab in standard interval dosing (SID), and in EID in switchers (purple) and naïve (orange) patients. A significant decrease was evidenced in switchers after initiating treatment with natalizumab (Wilcoxon test, p=0.0008). ARR remained low in both naïve patients and switchers treated with natalizumab in SID and EID. **(B)** The median Expanded Disability Status Scale (EDSS) scores before natalizumab treatment (Pre-SID), before EID (Pre-EID), and at the end of EID period (Post-EID) in switchers (purple) and naïve (orange) patients. Although switchers exhibited a significantly higher EDSS score, the score remained stable all through the follow-up period in both groups. **(C)** Kaplan-Meyer plots of the proportion of patients maintaining the no evidence of disease activity (NEDA-3) status (global data: blue line; switchers: orange dashed line; naïve: purple dashed line; Gehan-Breslow Wilcoxon test p=0.012).

radiological paradox (20). In fact, MRI may be more sensitive than clinical observation to detect some mild (subclinical) relapses. It has been suggested that this may be explained, at least in part, to cortical plasticity (21). Thus, it might be speculated that EID regimens might protect more profoundly from clinically evident inflammatory activity than from subclinical radiological flares. However, our data cannot give a clear answer and further randomized trials are needed to either confirm or disprove this contention.

Stratification of patients according to previous use of other DMTs showed that natalizumab-EID had a beneficial effect on both switchers and naïve patients, maintaining ARR at low levels and limiting disability progression as assessed by the EDSS scores. In contrast to that observed with ARR, disability analysis among switcher patients revealed that the EDSS scores did not decrease after initiating natalizumab administration in an SID setting, but decreased slightly after treatment with the EID regimen. We do not have a clear explanation for the lack of disability improvement among switchers after switching to SID of natalizumab, as has been commonly reported in routine clinical practice (14, 22). Intriguingly, the proportion of patients maintaining the NEDA-3 status was slightly higher among naïve patients than among switchers. This could be related to the fact that patients in the latter group were initiated on treatment with natalizumab-SID at an advanced age and with a more advanced disease status than naïve patients. We speculate that treatment with natalizumab at earlier stages of the disease, in a more severe inflammatory state, might exert a more pronounced immunomodulatory effect that possibly delays long-term disease progression (22, 23). Nevertheless, considering the small sample size of our study, the results of the subgroup analysis should be interpreted cautiously.

TABLE 2 | EID natalizumab in switchers/naïve patients.

	Switchers n=26	Naïve n=13	p
Gender	22F, 4M	12F, 1M	0.45
JCV +,n(%)	22 (85%)	10 (77%)	0.66
SID			
Age, mean (SD)	41 (12)	34 (7)	**0.050**
Duration, mean (SD)	39 (11)	39 (18)	0.109
EDSS, median [IR]	2.75 [1.875-4]	1.5 [1-2]	**0.006**
ARR, mean, (SD)	0.026 (0.07)	0.038 (0.13)	0.790
Radiological activity (SD)	0.05 (0.04)	0.06 (0.05)	0.92
EID			
Age, mean (SD)	46 (11)	38 (7)	**0.020**
Duration, mean (SD)	76 (16)	78 (6)	0.240
EDSS, median [IR]	2.75 [1.875-4]	1.5 [1-2]	**0.006**
ARR, mean, (SD)	0.031 (0.07)	0.010 (0.03)	0.480
Radiological activity (SD)	0.04 (0.04)	0.03 (0.04)	0.286

Main patient's characteristics. ARR, annualised relapse rate; EID, expanded interval dosing; EDSS, expanded disability status scale; IR, interquartile range; JCV, John Cunningham virus; SD, standard deviation; SID, standard interval dosing.

To the best of our knowledge, this is the first study reporting the long-term effects of treatment with natalizumab in an EID regimen following an SID regimen. Importantly, the present study has some limitations due to its observational approach, lack of a comparison control group, and limited sample size. Regarding the last concern, the small sample size impeded further subgroup analyses. Therefore, these encouraging results await to be confirmed by ongoing clinical trials (https://clinicaltrials.gov/ct2/show/NCT03689972). Pending the completion of these trials, our findings provide useful information on efficacy and safety that help decision making by clinicians and patients confronting therapeutic options after several years of therapy with SID of natalizumab.

In conclusion, the present study provides new real-world evidence that long-term administration of natalizumab in an EID setting with an 8-week dosing interval following an SID regimen is safe and maintains therapeutic efficacy in MS. Clinical trials are needed to confirm the benefits of this therapeutic regimen.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité de Ética de la Investigación con medicamentos de Cantabria (CEIm Cantabria). The ethics committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

JR: conception, data collection, analysis, and writing. SS: data collection and revision. JS data collection and revision. MT-B: radiological analysis. MM: data collection and revision. JP data collection. TC-T: critical revision. CM-G: radiological analysis. MD-A: data collection, writing and revision. All authors contributed to the article and approved the submitted version.

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Natalizumab Pharmacokinetics and -Dynamics and Serum Neurofilament in Patients With Multiple Sclerosis

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Background: Natalizumab (NAT) is a high-efficacy treatment for relapsing remitting multiple sclerosis (RRMS). However, it is associated with an increased risk of progressive multifocal leukoencephalopathy that sometimes requires treatment cessation with a risk of returning disease activity. The aim of this study was to characterize the pharmacokinetics and -dynamics as well as neurodestruction marker serum neurofilament light chain (sNfL) in patients with RRMS and secondary progressive MS (SPMS) stopping NAT in correlation to clinical data.

Methods: In this study, 50 RRMS and 9 SPMS patients after NAT cessation were included. Five RRMS patients on NAT treatment holiday were evaluated. Clinical and radiological disease activity were systemically assessed by frequent exams after NAT stop. Free NAT concentration, cell bound NAT, α4-integrin expression and α4-integrin-receptor saturation as well as immune cell frequencies were measured for up to 4 months after NAT withdrawal. Additionally, sNfL levels were observed up to 12 months in RRMS and up to 4 months in SPMS patients.

Results: NAT cessation was associated with a return of disease activity in 38% of the RRMS and 33% of the SPMS patients within 12 and 7 months, respectively. Concentration of free and cell bound NAT as well as α4-integrin-receptor saturation decreased in the RRMS and SPMS patients whereas α4-integrin expression increased over time. NAT induced increase of lymphocytes and its subsets normalized and a non-significant drop of NK and Th17 T-cells counts could be detected. All RRMS patients showed physiological sNfL levels <8pg/ml 1 month after last NAT infusion. During follow-up period sNfL levels peaked up to 16-fold and were linked to return of disease activity in 19 of the 37 RRMS patients. Treatment holiday was also associated with a return of disease activity in 4 of 5 patients and with an increase of sNfL at an individual level.

Conclusions: We demonstrate the reversibility of NAT pharmacodynamic and -kinetic

markers. sNfL levels are associated with the recurrence of disease activity and can also serve as an early marker to predict present before onset of clinical or radiological disease activity on the individual level.

Keywords: multiple sclerosis, natalizumab (TYSABRI), natalizumab concentration, neurofilament light (NFL) chain, recurrence of disease activity, cessation of natalizumab, alpha-4 integrin expression, alpha-4 integrin receptor saturation

INTRODUCTION

The recombinant humanized monoclonal antibody natalizumab (NAT) is one of the most effective treatments for relapsing remitting multiple sclerosis (RRMS). NAT binds to the α4 subunit of the α4β1-integrin on circulating mononuclear cells, thus limiting the entry of lymphocytes through the blood brain barrier (1). This mechanism of action impacts the central nervous system immunosurveillance, which is responsible for the development of progressive multifocal leukoencephalopathy (PML), a rare but potentially fatal brain infection caused by the John Cunningham Virus (JCV). A treatment duration of more than 2 years, JCV antibody seropositivity and the use of an immunosuppressive treatment before initiation of NAT therapy have been identified as risk factors for developing a PML (2). In case of treatment discontinuation due to increased PML risk, patients are faced with the possible recurrence or even rebound of disease activity even when switching to another disease modifying therapy (DMT) (3–6). High disease activity and a high level of disability prior to NAT therapy were identified as risk factors for reactivation of clinical disease activity after NAT withdrawal (7). Controlled treatment holidays, different dosing regimens as well as extended interval dosing (EID) were proposed as strategies to reduce PML risk while maintaining efficacy of NAT therapy (8–13). However, efficacy of EID vs. standard interval dosing (SID) is still going to be evaluated in a randomized controlled clinical trial (NCT03689972).

After NAT withdrawal the reversibility of NAT effects on immune cells seems to be linked with the recurrence of disease activity. Whereas, absolute lymphocyte counts increased during NAT therapy due to NAT's mode of action, a decrease of T helper (Th) 17 cells, CD4+ and CD8+ T-cells, CD19+ B-cells and CD56+ NK-cells was observed after NAT cessation (14–17). Regarding pharmacokinetics of NAT, Plavina et al. demonstrated a decrease of free NAT concentration and α4-integrin (CD49d) saturation and an increase of α4-integrin expression levels during washout period, which is in line with our previously published data (17, 18). Lohmann et al. revealed that the extent of NAT induced reduction on CD49d levels but not the kinetics of recovery might predict stable disease course during switching to another treatment (19).

The most promising biomarker of neuroaxonal injury as well as disease activity in multiple sclerosis (MS) is serum neurofilament light chain (sNfL) (20–27). Recently, it has been postulated that sNfL may also serve as a treatment response marker (28, 29). Additionally, sNfL levels have been found to be elevated early during NAT-associated PML and correlate with PML lesion volume (30, 31). Until now, data about sNfL dynamics during NAT washout and under subsequent treatment are missing.

In this study, we address the pharmacokinetics and -dynamics (PK, PD) in association to clinical and subclinical parameters during the washout period of NAT in RRMS and SPMS patients. We aim to identify immunological and serological biomarkers that could assist in individualized management of treatment switch after NAT treatment.

METHODS

Subjects

In our study, we included at least 64 MS patients on NAT treatment. Different approaches were chosen to answer our study questions: (1) 50 RRMS patients were evaluated that stopped NAT treatment primarily due to increased PML risk and switched to other DMTs (cohort 1). (2) Nine patients with SPMS were included that participated in the phase III study ASCEND and stopped NAT therapy (cohort 2) (32). (3) A third cohort of 5 patients with RRMS was evaluated for both effects of cessation and restart of NAT treatment (treatment holiday, cohort 3). All patients of cohort 3 participated in the phase II, randomized, placebo controlled RESTORE study observing disease activity in MS during a 24-week interruption of NAT therapy (33). Patient characteristics are reported in **Table 1**.

All patients were closely screened for the occurrence of clinical confirmed relapses and radiological disease activity, defined by new/enlarging and/or gadolinium enhancing (GdE) lesions in MRI scan. Clinical visits were performed every 4 weeks and patients were screened for relapses by a trained and experienced neurologist. Relapses were defined as new/worsening of neurologic symptoms persisting $\geq$ 24 h in the absence of fever or infection. MRI was performed at different timepoints within the first 12 months after discontinuation of NAT therapy in cohort 1. Patients in cohort 2 were screened for radiological disease activity with MRI as earliest as 3 weeks after cessation, after 4 and 7–8 months. Patients of cohort 3 were monitored with MRI every 4 weeks.

Abbreviations: DMT, disease modifying therapy; EID, extended interval dosing; FACS, fluorescence activated cell sorting; GdE, gadolinium enhancing; IQR, interquartile range; JCV, John Cunningham virus; MFI, Mean fluorescence intensity; NAT, natalizumab; NK cells, natural killer cells; PD, pharmocodynamic; PK, pharmacokinetic; PML, progressive multifocal leukoencephalopathy; RRMS, relapsing remitting multiple sclerosis; SD, standard deviation; SID, standard interval dosing; SIMOA, single molecule array technology; SPMS, secondary progressive multiple sclerosis; SS, steady state; sNfL, serum neurofilament light chain; Th17 cells, T-helper 17 cells.

TABLE 1 | Patient characteristics ($N = 64$).

	Cohort 1	Cohort 2	Cohort 3
Patients, n	50	9	5
Disease course	relapsing remitting	secondary progressive	relapsing remitting
Gender, female, n (%)	31 (62)	4 (44)	2 (40)
Age, years, Mean ± SD	39.6 ± 12.1	46.1 ± 8	28.2 ± 8.9
Range	21–62	34–57	20–40
Disease duration, years, Mean ± SD	8.7 ± 5.7	11.1 ± 5.2	6.6 ± 4.1
Range	1–28	4–18	3–11
EDSS, Mean ± SD	3.2 ± 1.7	5.6 ± 0.98	n.a.
Median (range)	3.0 (1.0–8.0)	6.0 (4.0–6.5)	
Previous use DMT, n (%)	33[a] (73)	5[b] (100)	5 (100)
Total number of NAT infusions, mean ± SD	37 ± 21	7 ± 3	39 ± 8
Range	6–104	3–10	2–13
Positive JCV antibody status, *n* (%)	45 (90)	7 (78)	n.a.

Baseline characteristics of evaluated patients. DMT, disease modifying treatment; EDSS, expanded disability status scale.
[a]Data available for 45 of 50 patients, [b]Data available for five of nine patients.

Blood samples for PK and PD evaluations were obtained every 4 weeks after NAT cessation up to 12 weeks in cohort 1 and up to 16 weeks in cohort 2 and 3. Additional blood samples were collected for up to 20 weeks after restarting NAT treatment in cohort 3.

Ethical Approval

The immunological substudy was performed according to the Declaration of Helsinki and the study protocol was approved by the Ethics Committee of the Faculty of Medicine of the Dresden University of Technology, Germany. All participants provided written informed consent.

Immune Cell Phenotyping Using Fluorescence Activated Cell Sorting (FACS)

After blood collection absolute cell counts of T-cells, B-cells and natural killer (NK) cells were measured at the Institute of Clinical Chemistry and Laboratory Medicine, University Hospital in Dresden, Germany. The institute complies with standards required by DIN-EN-ISO 15189:2014 for medical laboratories. Cells were characterized by surface staining with fluorescence labeled anti-CD3, anti-CD4, anti-CD8, anti CD-16, anti CD-14, anti CD-19 and anti CD-56 antibodies (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Negative controls included directly labeled or unlabeled isotype-matched irrelevant antibodies (BD Biosciences, San Jose, CA, USA). Cell subsets were measured using FACS Canto II flow cytometer (BD Bioscience, San Jose, CA, USA).

For further evaluation of immune cell subsets, peripheral blood mononuclear cells were isolated from the heparinised blood samples using Biocoll separating solution (Biochrom Ag, Berlin, Germany) and Ficoll-Paque (Amersham Biosciences, Amersham, United Kingdom) in LeucoSep tubes (Greiner Bio One, Frickenhausen, Germany). Subpopulations of T-cells were characterized by surface staining with fluorescence labeled anti-FoxP3 and intracellular staining with fluorescence labeled anti-Il17 antibodies (BD Bioscience, San Jose, CA, USA) according to the manufacturer's instructions. Cell frequencies were evaluated on LSR Fortessa cytometer (BD Bioscience, San Jose, CA, USA).

Measurement of Pharmacodynamic and –Kinetic Data Using a HL60 Cell Based FACS Assay

For analysis of cell bound NAT, CD49d expression and α4-integrin receptor saturation on CD3+ T-cells peripheral blood mononuclear cells were isolated from the heparinised blood samples using Biocoll separating solution (Biochrom Ag, Berlin, Germany) and Ficoll-Paque (Amersham Biosciences, Amersham, United Kingdom) in LeucoSep tubes (Greiner Bio One, Frickenhausen, Germany). Cells were stained with fluorescence-labeled anti-CD3 (BD Bioscience, San Jose, USA), anti-immunoglobulin (IG)-G4 (Southern Biotech, Birmingham, AL, USA), and anti-CD49d (BD Biosciences, San Jose, CA, USA) antibodies, isotype controls were used. Mean Fluorescence intensity (MFI) was analyzed using fluorescence activated cell sorting (FACS, FACS Calibur, BD Bioscience, San Jose, CA, USA). Plasma supernatants were collected and stored at −20°C for subsequent NAT concentration measurements which was performed using our previously described HL60 cell based FACS assay (18).

Evaluation of sNfL Dynamic Using Single Molecule Analysis (SIMOA)

Serum samples were stored at −80°C until after preparation. sNfL levels were determined using a Simoa HD-1 instrument (Quanterix, Lexington, MA, USA) (23, 34). The Advantage NF-Light singleplex Kit was used and samples were prepared as defined in the manufacturer's instructions (Quartered, Lexington, MA, USA). Sample dilution was calculated and done by the

instrument. The mean intra-assay coefficient of variation of duplicates was below 10%.

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Our longitudinal patient data were analyzed per cohort by generalized linear mixed models for repeated measures with gamma distribution and log link function due to right-skewed distribution pattern of the data and timepoint as the fixed effect of the model. Bonferroni correction for pairwise tests was used. Values of $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ were considered as statistically significant. Clinical parameters are depicted in a Kaplan-Meier survival curve for relapses and new and/or GdE lesions in MRI scan. Statistical analyses were performed using the IBM SPSS Software for Windows (Version 25.0; IBM Corporation, Armonk, NY, USA).

RESULTS

NAT Cessation in RRMS (Cohort 1) and SPMS (Cohort 2) Patients

Clinical and Radiological Data - Cohort 1

At the timepoint of NAT cessation, RRMS patients presented with a mean EDSS about 3.2 ± 1.7 (range 1–8). Patients received a mean number of 37 ± 21 (range 6–104) NAT infusions before treatment stop. Information about pre-treatment was available for 45 of the 50 RRMS patients. 73% of the patients with RRMS had received a DMT before NAT whereas 27% were treatment naïve. The majority of RRMS patients (91.8%) were free of disease activity during NAT treatment. NAT therapy was stopped because of JCV seropositivity (90% positive JCV serostatus), treatment duration over 24 months and/or previous immunosuppressive treatment with increased PML risk in 48 of 50 RRMS patients (**Table 1**). Only two patients discontinued treatment primarily due to adverse events (one patient with generalized pain, one patient without data available) and one due to pregnancy.

After NAT withdrawal, 37/50 of RRMS patients switched to fingolimod within 3.4 ± 1.1 months and 10/50 to alemtuzumab within 4.6 ± 2.9 months. Overall, the washout period between switching from NAT to another DMT was on average 3.7 ± 1.7 months (range 2–13 months). Two patients received no further treatment due to conversion to SPMS and one patient received no further treatment due to pregnancy.

The relapse-free survival rate was 70 % at 6 months and 62% at 12 months and survival rate without new/enlarging and/or GdE lesions was 74 % at 6 months and 62% at 12 months in RRMS patients (**Figure 1A**). Mean time to relapse was 5.2 ± 2.8 months and new/enlarging and/or GdE T2 lesions were revealed within 6 ± 2.2 months. The mean number of new cerebral T2 lesions was 2.2 ± 1.8 in RRMS patients, in 87.5 % of patients new T2 lesions or GdE were detected. A total of 10 out of 50 RRMS patients experienced a relapse and 10 patients presented radiological disease activity while 9 patients suffered both clinical and radiological disease activity. In 6/10 patients presenting with a relapse a new DMT was already started whereas this was the case in 7/10 patients with new/enlarging T2 lesions in cerebral MRI scan. In the 9 patients with both clinical and radiological disease activity, 6 had already started a new DMT before disease activity occurred.

Clinical and Radiological Data - Cohort 2

SPMS Patients presented with a mean EDSS about 5.6 ± 1.0 (range 4–6.5) and had received a mean number of 7 ± 3 (range 3–10) NAT infusions. For 5 of the nine SPMS patients information about pre-treatment was available, all of them received a DTM in their previous disease course. The majority of these patients was relapse free during NAT treatment (**Table 1**). About 78% of patients presented a positive anti-JCV serostatus (**Table 1**).

After NAT discontinuation, survival relapse free as well as survival without new/enlarging T2 and/or GdE lesions was 67% at 7 months (**Figure 1B**). In 2 SPMS patients, one resp. 2 new GdE lesions were detected whereas one patient presented with 9 new GdE lesions. Taking into account the clinical and radiological disease activity after NAT withdrawal in these 3 SPMS patients, they received subsequent DMT (1 Rituximab, 2 NAT).

Peripheral Immune Cell Subsets

For both cohorts, a reduction in absolute lymphocyte count was observed after cessation of NAT therapy. The decrease reached statistical significance at week 12 in both patient cohorts (**Figures 2A,B**). On average the absolute lymphocyte count remained within the normal range at all timepoints. Cell counts of CD4+ T-cells were not affected by NAT cessation in RRMS within first 12 weeks. A decrease of CD4+ T-cells was documented 16 weeks after NAT stop in SPMS patients (**Figures 2C,D**).

Frequencies of Foxp3 + Treg-cells were not affected by NAT withdrawal in both cohorts. Cell counts of Th17-cells decreased after the cessation without reaching statistical significance. The NK-cell count tended to decrease after NAT cessation in both patient cohorts, although it did not reached statistical significance in cohort 2 (**Figures 2E,F**). Absolute cell counts of NKT-cells were not affected by NAT discontinuation. The absolute B-cell count was found to be upper the normal limit in both patient cohorts 4 weeks after NAT withdrawal. After cessation, a decrease was observed in both cohorts reaching statistical significance after week 12 (**Figures 2G,H**).

Plasma NAT Levels

The mean free NAT plasma concentration observed 4 weeks after the last infusion was similar in cohort 1 and 2 (33.3 ± 17.5 μg/ml vs. 33.7 ± 9.4 μg/ml). At week 8, NAT concentration levels were significantly decreased in both patient cohorts. Twelve weeks after therapy cessation, NAT concentration was below 2.5 μg/ml or undetectable in the majority of the patients and after 16 weeks, no free NAT was detectable in any patient (**Figures 3A,B**).

Cell Bound NAT

For cell bound NAT on CD3+ T-cells, a mean MFI of 7,486 ± 731 in cohort 1 and of 5,729 ± 1,601 in cohort 2 was detected 4 weeks after last NAT infusion. A significant decrease was revealed 12 weeks after NAT withdrawal in cohort 1 and after 16 weeks in cohort 2 (**Figures 3C,D**).

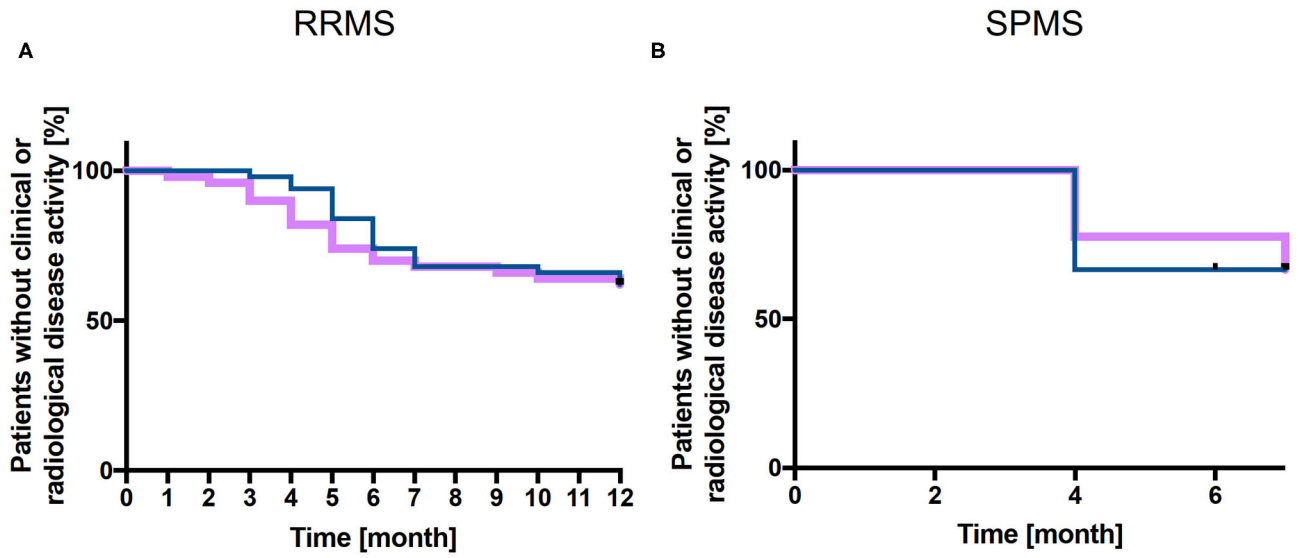

FIGURE 1 | Kaplan-Meier analysis of relapse-free survival and without new and/or enlarging T2 lesions. Relapse free (purple line) and without new/enlarging T2 lesions survival (blue line) is presented for cohort 1 (RRMS patients, $n = 50$) up to 12 months **(A)** and for cohort 2 (SPMS patients, $n = 9$) up to 7 months **(B)** after NAT withdrawal.

CD49d Expression and Saturation

A mean CD49d expression on CD3+ T-cells of 819 ± 65 MFI and of 305 ± 100 MFI was observed in cohort 1 and 2 four weeks after NAT cessation. An increase of CD49d expression at week 12 was detected for RRMS and SPMS patients, respectively (**Figures 3E,F**). In addition, CD49d saturation was analyzed in SPMS patients. The mean CD49d saturation on CD3+ T-cells was 77 ± 7.5% 4 weeks after the last NAT infusion. At week 16 CD49d saturation was decreased to 10% on CD3 + T-cells (**Figure 3G**). Mean CD49d expression measured 4 weeks after last NAT infusion tended to be higher in RRMS patients with clinical and/or radiological disease activity or with an increase of sNfL as compared to patients without any evidence of disease activity after NAT withdrawal without reaching statistical significance.

Serum NfL Levels

A mean sNfL level of 4.6 pg/ml ± 1.7 (IQR 1.5-11 pg/ml) was measured in 46 of the 50 RRMS patients (cohort 1) 4 weeks after NAT cessation. sNfL levels remained stable in the majority of patients within the first 8 weeks of the NAT washout period. During follow up of 12 months there was an increase up to 16-fold sNfL baseline level (range 5.2–101.0 pg/ml) in 37 of 46 patients. The earliest sNfL increase was seen 8 weeks after stopping NAT in 2 RRMS patients, respectively, after 12 weeks in 5 RRMS patients. To evaluate association of sNfL increase with disease activity, a steady state (SS) value of sNfL was defined for the measurement 4 weeks after NAT cessation. A relevant sNfL peak was defined as sNfL value ≥ SS + 2SD. A relevant sNfL peak was documented in 37 RRMS patients 8 weeks after NAT stop. Registered sNfL peaks were associated with clinical and/or radiological disease activity in 19 of the 37 patients. For 11 of this 19 patients an increase of individual sNfL levels, defined as sNfL value ≥ SS + 1 or 2SD was detected 3 ($n = 1$), 2 ($n = 4$) or 1($n = 6$) month's before first symptoms of relapse appeared and/or MRI activity was detected. For 3 patients with onset of clinical or radiological disease activity the following month, no increase of sNfL levels was detectable the month before. For 5/19 patients no serum sample for sNfL evaluation was available the month before disease activity occurred.

For 18 of the 37 patients, neither new nor worsening symptoms were documented and follow-up MRI showed no new/enlarging T2 or GdE lesions. At the timepoint of sNfL peak, 26 of the 37 patients had already started a new DMT for at least 1 month (**Figure 4A**).

In SPMS patients (cohort 2), sNfL levels were at 4.8 ± 2.7 (IQR 1.8 – 7.4 pg/ml) 4 weeks after NAT and remained stable at the individual level during the 16 weeks follow up period (**Figure 4B**).

NAT Treatment Holiday in RRMS Patients (Cohort 3)

Clinical and Radiological Data

In cohort 3, RRMS patients that stopped and restarted NAT were evaluated. A mean number of 39 ± 8 (range 27–48) NAT infusions were administered before patients entered the drug holiday (**Table 1**). None of the 5 patients experienced clinical disease activity while on NAT treatment. Individual disease course during drug holiday and after re-starting NAT is depicted (**Figure 5** patient 1–5). Four of 5 patients presented with new relapses within 20 ± 3.3 weeks after NAT cessation. For all patients with clinical disease activity, radiological disease activity was detected as well. Median time to recurrence of radiological disease activity was 18 ± 2.3 weeks and a median number of 2.5 ± 0.6 new T2 lesions were found. After restart of NAT therapy,

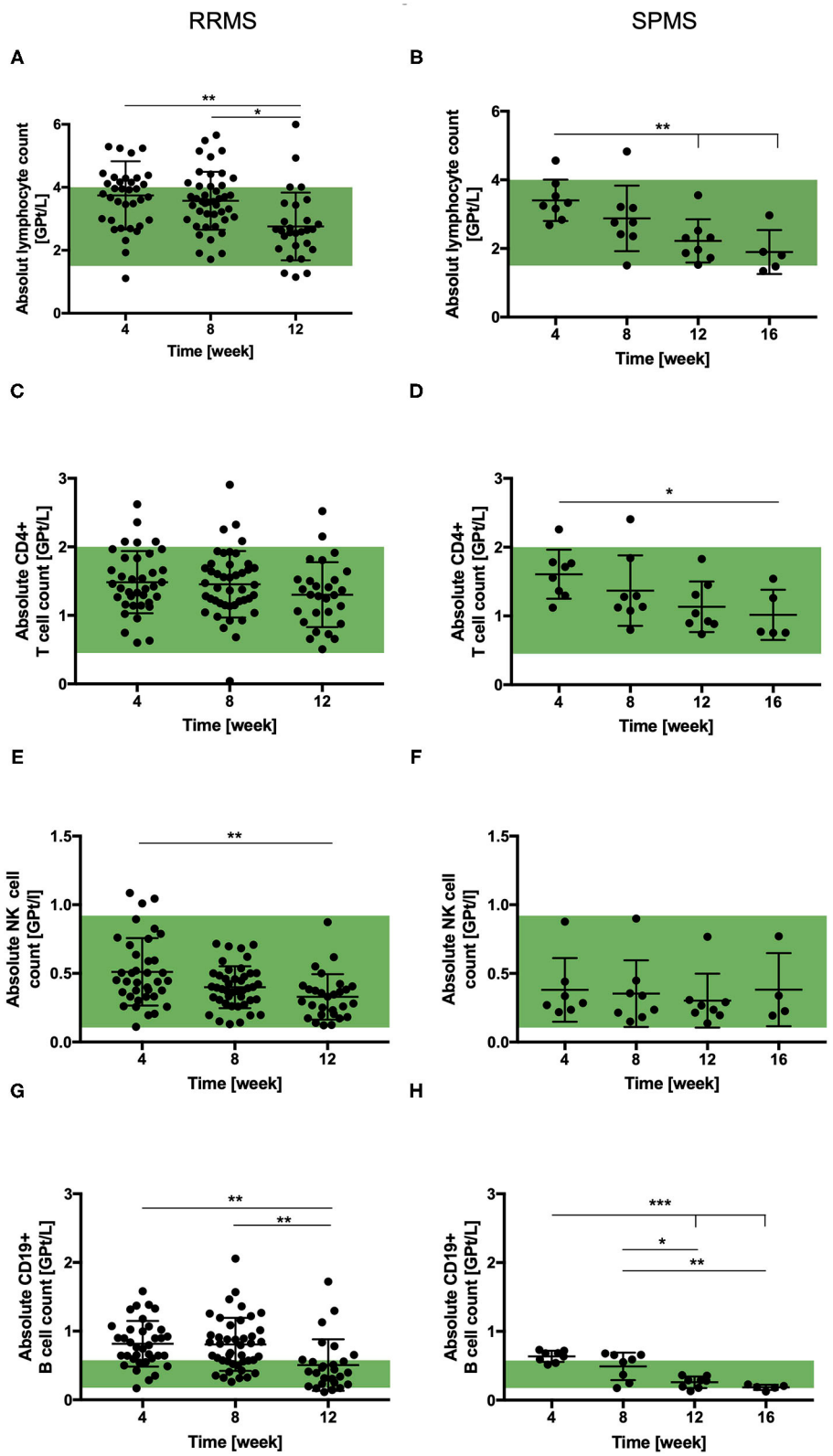

FIGURE 2 | Total lymphocyte and lymphocyte subset count in RRMS ($n = 50$) and SPMS ($n = 9$) patients after the cessation of NAT therapy. Mean absolute cell count ± SD of lymphocytes **(A,B)**, CD4+ T-cells **(C,D)**, NK cells **(E,F)** and CD19+ B-cells **(G,H)** are presented for RRMS (left) up to 12 weeks and for SPMS (right) patients up to 16 weeks after the cessation of NAT treatment. Reference range is green. Data were analyzed by generalized linear mixed models for repeated measures. Asterisks indicate a statistically significant difference (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

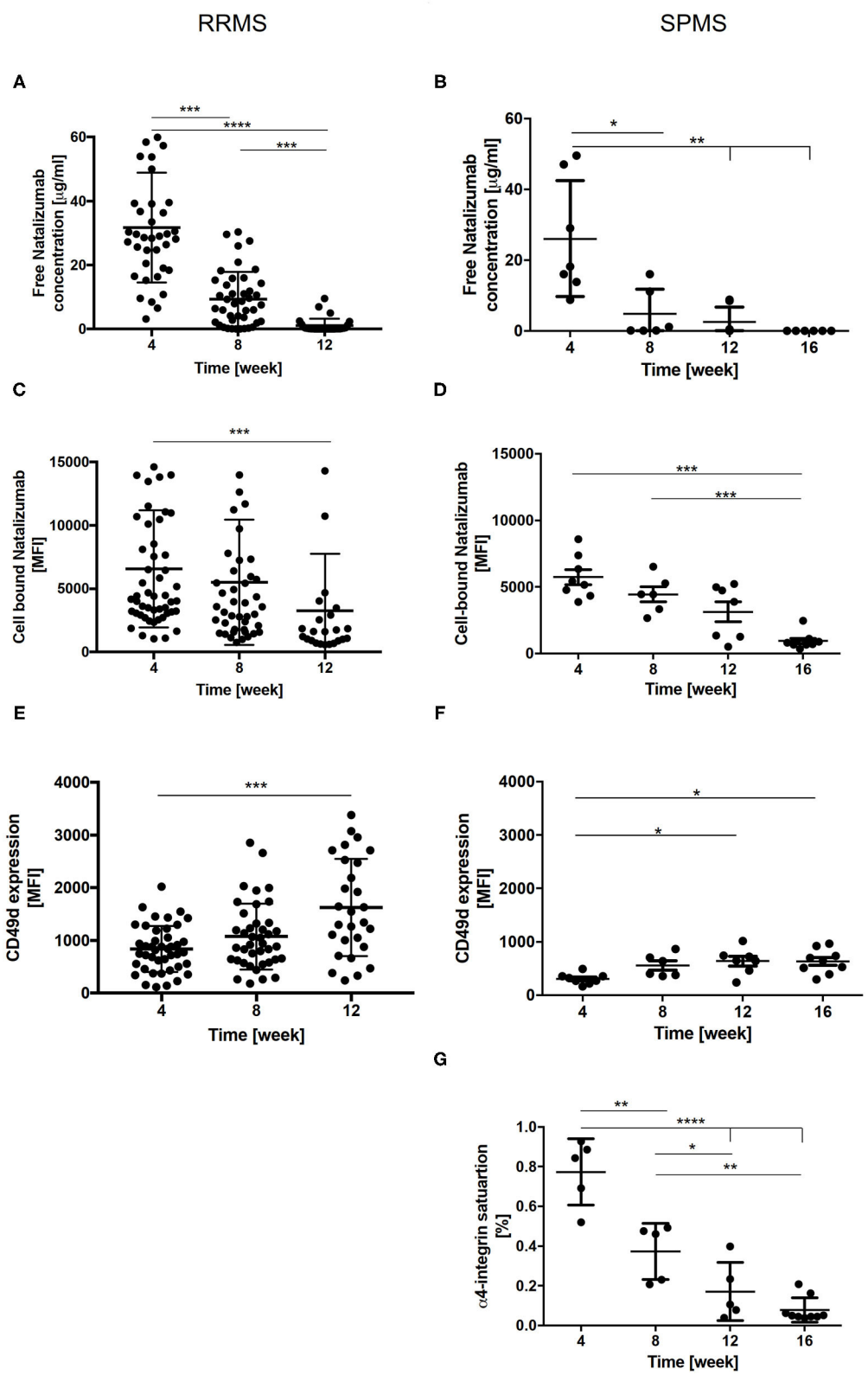

FIGURE 3 | Free NAT concentration, cell bound NAT, CD49d expression and α4-integrin saturation in RRMS ($n = 50$) and SPMS ($n = 9$) patients after the cessation of NAT therapy. Mean values ± SD of free NAT concentration in plasma **(A,B)**, cell bound NAT on CD3+ T-cells **(C,D)** and CD49d expression on CD3+ T-cells **(E,F)** were assessed during the washout of NAT up to 12 weeks in RRMS (left) and up to 16 weeks in SPMS (right) patients. For SPMS patients the mean α4-integrin saturation level on CD3+ T-cells after the cessation of NAT treatment is presented **(G)**. Data were analyzed by generalized linear mixed models for repeated measures. Asterisks indicate a statistically significant difference (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). MFI, mean fluorescence intensity.

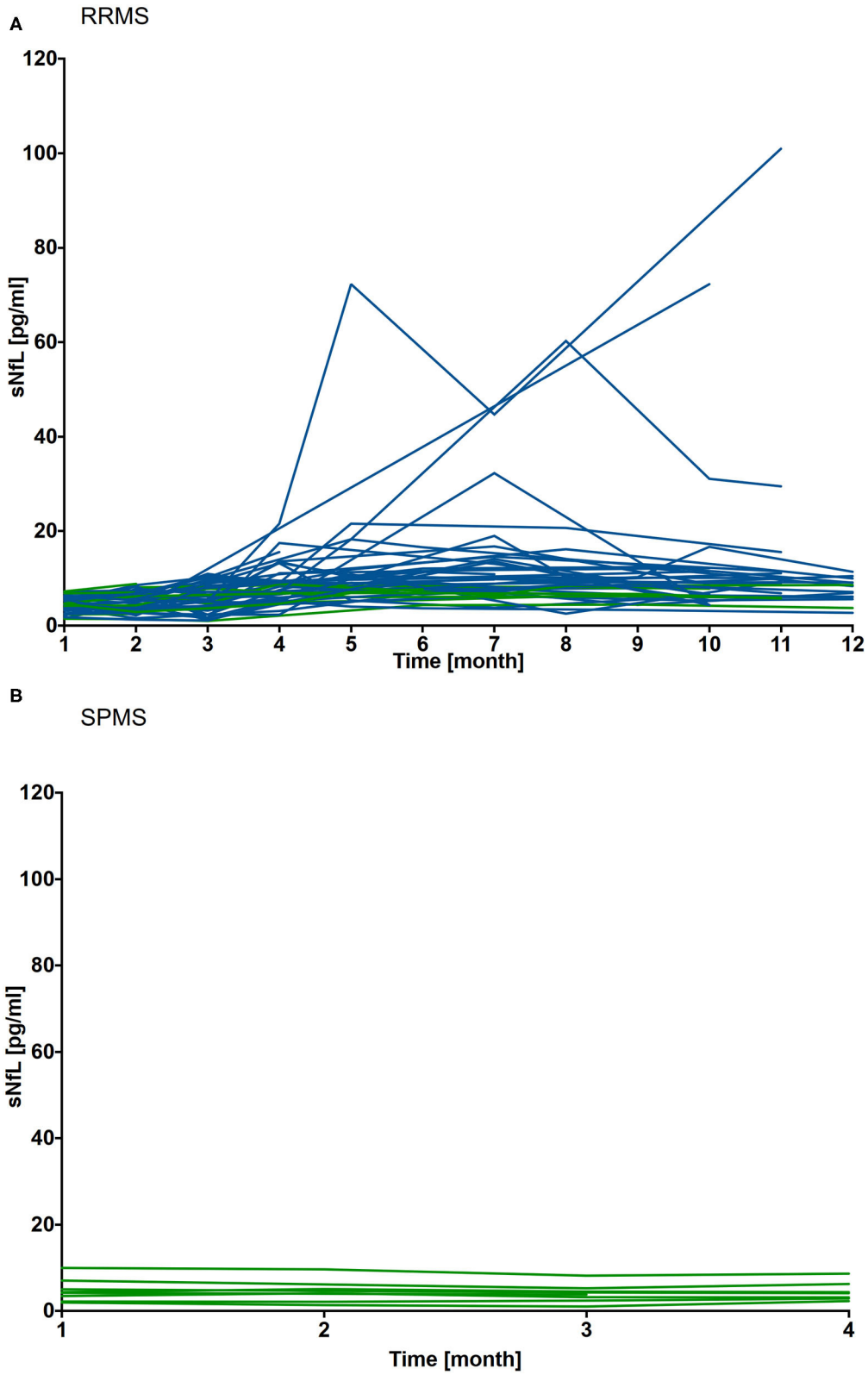

FIGURE 4 | Individual sNfL levels after cessation of NAT treatment in RRMS ($n = 46$) and SPMS ($n = 9$) patients. sNfL levels were assessed during washout period up to 12 months in RRMS patients **(A)** and for 4 months in SPMS patients **(B)**. sNfL value measured 4 weeks after NAT cessation was defined as individual steady state (SS) value. A relevant increase of sNfL was defined as sNfL values $\geq$ SS + 2SD. Individual sNfL courses are depicted, relevant sNfL increase are labeled blue, patients without an increase are depicted green.

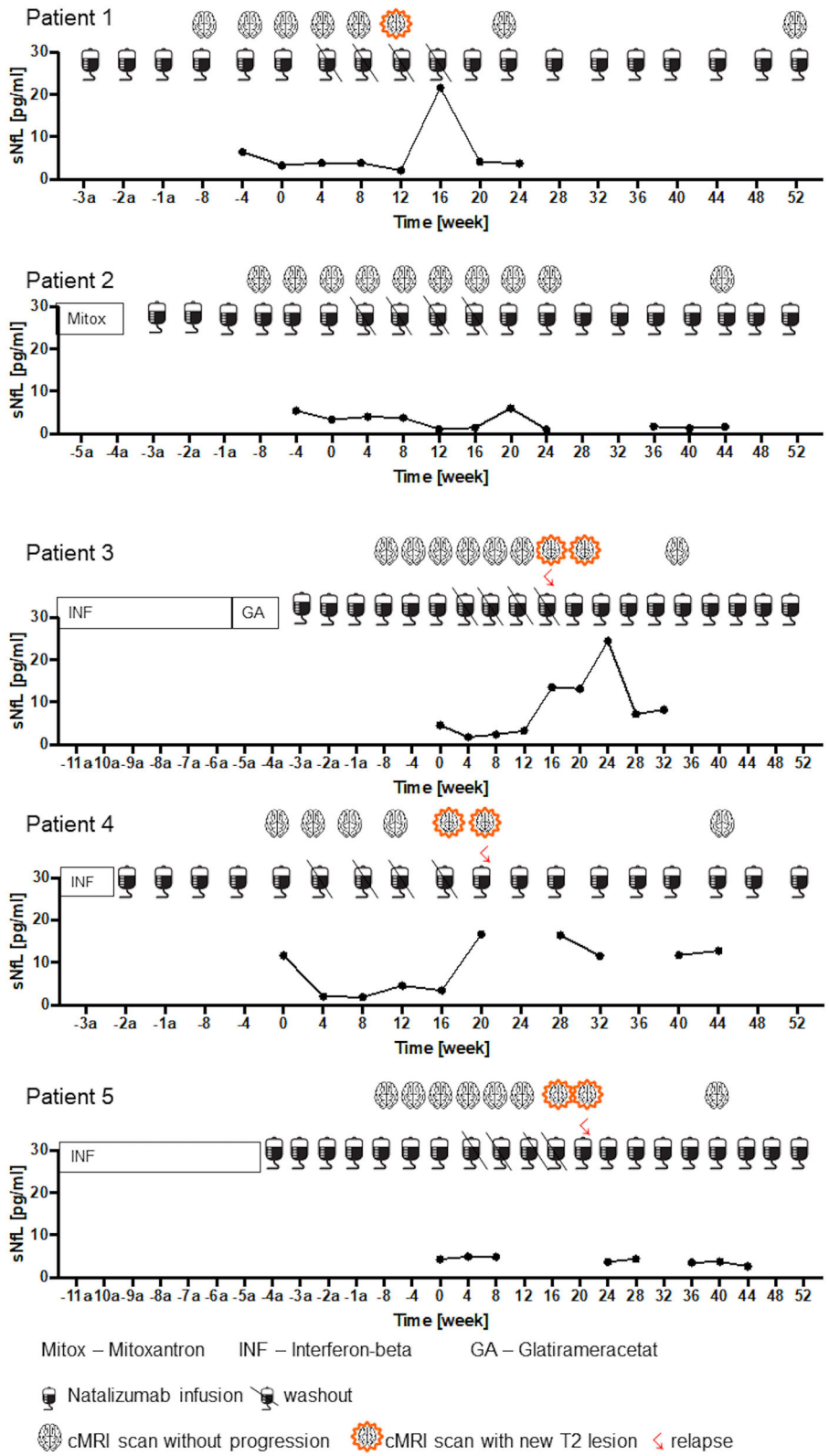

FIGURE 5 | Clinical and radiological disease activity and sNfL dynamics during NAT treatment interruption. sNfL levels during NAT treatment, during washout period and after restart of NAT infusions are presented ($n = 5$). Pre-treatment, timepoint since NAT therapy initiation and clinical confirmed relapses and radiological disease activity are shown up to week 52 = End of study.

regression of demyelinating lesions was documented for 3 of the 5 patients whereas three new lesions without GdE were detected in one patient. All 3 patients were free from clinical disease activity until end of the study (week 52) after re-initiating NAT treatment.

Plasma NAT Levels

A mean free NAT plasma concentration of 34.2 ± 10.3 µg/ml was measured at month 0. During treatment holiday, a decrease of free NAT plasma concentration was observed. The first significant reduction was presented 8 weeks after last infusion (7.6 ± 2.0 µg/ml, $p < 0.001$). Low free NAT plasma concentrations were detectable in all of the 5 patients 16 weeks after NAT interruption. After restart of NAT therapy, a mean free NAT plasma concentration of 18.5 ± 5.8 µg/ml and 27.5 ± 11.5 µg/ml was measured after the first and second infusion, respectively (**Figure 6A**).

Cell Bound NAT

A mean MFI of cell bound NAT on CD3+ T-cells of 1,720 ± 949 was detected at month 0. Compared to the rapid decrease of free NAT concentration, the decrease of cell bound NAT was much slower, the first significant decrease was observed at week 16 ($p < 0.05$). A mean MFI of cell bound NAT on T-cells of 1,647 ± 625 and of 1,127 ± 945 was measured after the first and second infusion after restart (**Figure 6B**).

CD49d Expression and Saturation

Mean CD49d expression on CD3+ T-cells was 477 ± 184 at month 0. After cessation a 2.5-fold increase after 16 weeks was detected. Mean CD49d expression of CD3+ T-cells was 625 ± 620 after the first and 443 ± 446 after the second NAT infusion after the restart (**Figure 6C**). At month 0, a mean CD49d saturation on CD3+ T-cells of 51.8 % was determined. At week 16 CD49d expression was decreased by 85.7 % ($p < 0.05$). CD49d saturation on CD3 + T-cells was lowest after restart with a mean of 8.8 ± 6.9 %, whereas a mean of 53.6 ± 10.2 was reached after 5 NAT infusions (**Figure 6D**).

Serum NfL Levels

Individual sNfL variation is depicted for each patient in **Figure 5**. Four patients presented sNfL values below 5 pg/ml at month 0, one patient with a sNfL value of 11.7 pg/ml before drug holiday (**Figure 5**. patient 4). During NAT treatment stop, an increase up to 24.4 pg/ml was seen in association to clinical confirmed relapse and/or new T2 lesions. After NAT re-initiation, sNfL decreased again in accordance with the stable disease course during the follow up period (**Figure 5**. patient 1-5).

DISCUSSION

The monoclonal antibody NAT is one of the most efficacious treatment options for patients with active RRMS. NAT is generally well tolerated, but has the highest risk for PML development among all approved MS treatments. In patients at high risk for developing PML, NAT discontinuation is frequently performed. However, NAT withdrawal remains challenging because it is associated with the recurrence or even rebound of

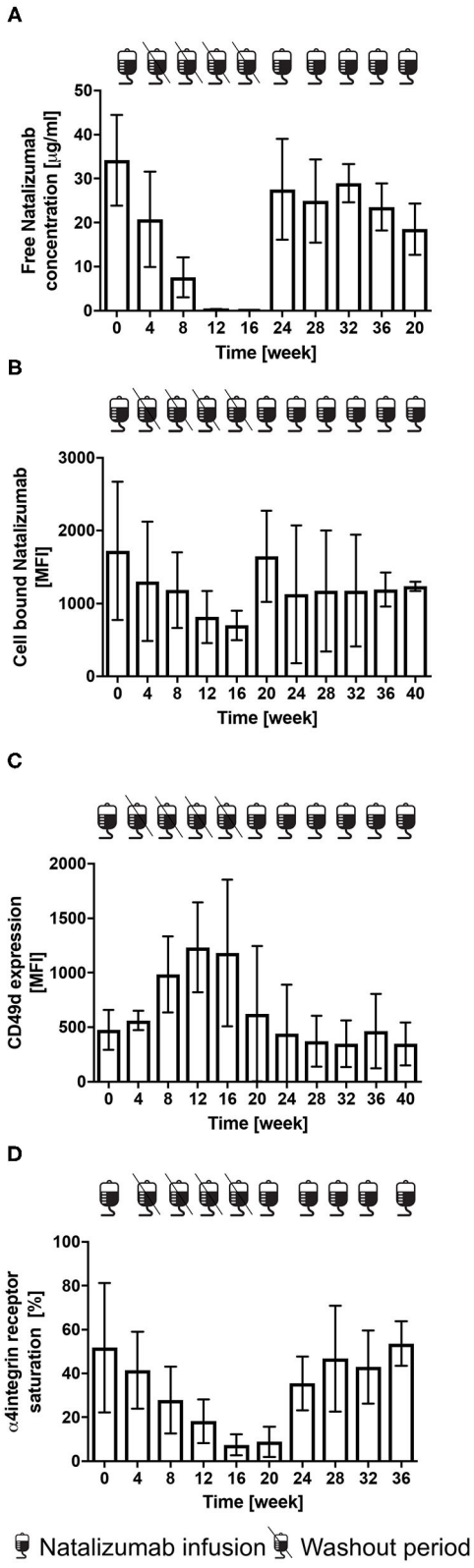

FIGURE 6 | Free NAT concentration, cell bound NAT, CD49 d expression and α4-integrin saturation after the cessation and restart of NAT therapy in RRMS patients ($n = 5$). Mean levels ± SD of free NAT concentration in plasma **(A)**,

(Continued)

FIGURE 6 | cell bound NAT on CD3+ T-cells **(B)**, CD49d expression **(C)** and α4-integrin saturation **(D)** on CD3+ T-cells are depicted for baseline = week 0, during washout period and after restart of NAT therapy up to week 40. Data were analyzed by generalized linear mixed models for repeated measures. MFI, mean fluorescence intensity.

disease activity as demonstrated by several studies and by a recent review of Prosperini et al. (3–6, 12, 33, 35–43). Even the length of NAT washout period and its association to disease activity reactivation remains a point of controversial discussions. A short washout period may decrease the risk of post NAT disease re-activation, but may also increase the risk for carry-over PML: a PML that develops few months after cessation of NAT therapy and after initiating an alternative DMT (44–47).

Indeed, consensus is still lacking in regard to DMT sequencing following NAT withdrawal. Studies addressing treatment switch from NAT to another DMT revealed a superiority of rituximab and alemtuzumab vs. fingolimod in controlling disease activity (48, 49). Recent studies suggested ocrelizumab as a possible choice to reduce the risk of MS disease activity reactivation in patients previously treated with NAT SID and EID (50). Considering the NAT associated elevation in peripheral total and memory B-cells together with the essential role of B-cells in MS pathogenesis, B-cell depleting agents might be a favorable post NAT DMT choice by effectively reducing this cells (51). However, further evaluations including comparisons between alemtuzumab and B-cell depleting therapies with careful observations regarding carry-over PML, are necessary.

Different studies already revealed that the recurrence of disease activity coincides with the decrease of NAT concentration and desaturation of NAT target on the surface of lymphocytes - α4-integrin. However, these markers are not yet well-established in clinical practice although they may be helpful to identify the right individualized timing for the start of an alternative treatment (3, 17). In this study, we assessed clinical and radiological disease activity after the cessation of NAT therapy as well as the reversibility of NAT PK and PD effects in RRMS and SPMS patients.

Clinical and radiological disease activity was detected earliest at 8 weeks after NAT cessation. In our study, 38% of the RRMS patients experienced clinical reactivation of the MS, 83% suffered from a relapse during the first 6 months after NAT withdrawal which confirms data from previous studies in which the proportion of patients with relapses post NAT has ranged from 9 to 80% (43). Even the initiation of a new DMT early after NAT cessation was not able to prevent disease activity following NAT withdrawal, which is in line with a recent published study from Mustonen et al. (7).

The reactivation of disease activity is closely related to reversal NAT effects on PK and PD. Earliest significant changes could be observed 8 and 12 weeks after the last NAT infusion with a decrease of free NAT concentration in plasma and cell bound NAT on CD3+ T-cells, respectively. CD49 expression observed 4 weeks after last NAT infusion tended to be lower in the patients with a stable disease course during follow up as compared to the patients presenting with disease activity. However, statistical significance was not reached. This discrepancy to the results from Lohmann et al. may be influenced by the fact that they compared patients with a stable and an exacerbated disease course defined by a relapse and $\geq$5 GdE lesions while we compared stable patients and patients with any evidence for disease activity (relapse, new/enlarging T2 lesion's and sNfL peak) (19).

NAT treatment has shown to be associated with increased absolute lymphocyte, CD3+ T-cell, CD4+ T-cell, CD8+ T-cell, CD19+ B-cell and NK-cell counts (14). Our findings are in line with a previous study, in which the effects of NAT on peripheral immune cell subsets were also reversible during washout period (17). In our study, we could observe that cessation of NAT has no effects on FoxP3+ T-regulatory cells which was previously discussed by Stenner et al. (52). Another T-cell subtype, the Th17-cell, is considered to be a critical mediator of disease activity in MS (53). Haas et al. monitored Th17-cell frequency in MS patients without, during and after NAT cessation and found increased frequencies in the immunology periphery during long term treatment as well as a decrease after NAT withdrawal. Additionally, they could observe that Th17-cells became almost undetectable in the blood of patients that presented relapses during the washout period (16). We could detect a Th17-cells drop after NAT cessation likewise, however the decrease was not statistically significant.

Although the immunological pattern may help to identify patients prone to develop clinical and/or radiological disease activity, there is a need for more directed biomarkers that could be implemented into clinical practice. Here, we first present data regarding sNfL dynamics after NAT withdrawal and after starting subsequent DMT during an up to 12 month follow up period. As reported by Gunnarson et al. and Kuhle et al., NFL levels in cerebrospinal fluid decreased during NAT treatment (54, 55). We detected low sNfL levels 4 weeks after last NAT infusion followed by sNfL peaks in 80.4% of the RRMS patients. In general, increases in sNfL levels were linked to reactivation of disease activity and seen up to 3 months before onset of disease activity in some patients. Nevertheless, in 18 of the 37 patients sNfL peaked without evidence of relapse disease or MRI activity. However, patients were only monitored by cerebral MRI as spinal cord MRI was not performed regularly. Other events (trauma, stroke, metabolic diseases) which could be associated with sNfL increase were not reported. As postulated in one of our studies investigating sNfL during alemtuzumab therapy, sNfL peaks without evidence of disease activity can indicate subclinical disease activity (29). For 2 patients, the suspicion of a relapse was reported. However, for both of them no significant variations of sNfL were found leading to the assumption that they do not have suffered from a clinical confirmed relapse. So, sNfL may be a potential tool to proof clinical disease activity and reappearance of disease activity in time-period of planed treatment switch.

To date, only limited data on the cessation of NAT therapy in SPMS patients are available (32). Miravalle et al.

investigated a 3- to 4- months drug holiday in 24 RRMS and 8 SPMS patients receiving NAT therapy for a period longer than 12 months. No other DMT was administered during drug holiday. Relapses occurred in 25% of the SPMS and in 38% of the RRMS patient group. This period was associated with new MRI disease activity in nearly all patients (40). Our data are in line with these data demonstrating that the cessation of NAT therapy is associated with a recurrence of disease activity in SPMS patients. PK and PD data of SPMS patients are comparable with RRMS patients stopping NAT. For the investigated time course of the α4-integrin receptor desaturation, our findings are comparable to the findings from Derfuss et al. and Plavina et al. (3, 17). Immune cell frequencies in SPMS patients showed similar patterns as RRMS patients. Frequencies of Th17-cells and NK-cell count decreased after the cessation, although statistical significance was not reached.

Mean sNfL in SPMS patients after NAT cessation did not show yet a significant increase. SPMS patients remained stable and presented without any clinical and radiological disease activity within 4 months after NAT stop. During long-term follow up, three patients presented with return of disease activity in the SPMS group. Unfortunately, no blood samples were available to correlate these clinical characteristics with additional sNfL levels beyond 4 months of follow up.

Our data confirm that drug holiday is not well tolerated and that the reversibility of NAT PK and PD effects coincides with a return of clinical and radiological disease activity. In line with previous findings from Fox et al. and Kaufmann et al., relapses occurred as early as 8 weeks and new or enlarging T2 lesions were detected as early as 12 weeks after NAT cessation. (33, 38). According to our observations from cohort 1 and 3, changes in PK and PD markers were observed as early as 8 weeks after interruption of NAT therapy with significantly reduced free plasma NAT concentrations as previously prescribed (17). 16 weeks after last NAT infusion cell bound NAT and CD49d saturation on CD3+ T-cells were also found to be significantly decreased. In this study, we correlated PK and PD parameters with sNfL measurements. We could show that disease activity reactivation is reflected by sNfL increase at an individual level. Furthermore, we could demonstrate that a drop of sNfL after re-initiation of NAT therapy was linked to a lesion and relapse free disease course. After restarting NAT therapy, CD49d receptor saturation on CD3+ T-cells was found to be above 50% even after the first NAT infusions.

Our data demonstrate that cessation and interruption of NAT therapy is associated with a high risk of recurrence of disease activity in both RRMS and SPMS patients. Although there are some limitations in our observations (limited patient number, different protocols for RRMS and SPMS patients), we present stable effects on clinical data, PK, PK and sNfL level within the first three months after stopping NAT. The return of disease activity is linked to the reversibility of NAT effects on PK and PD. Our observational data do not support the concept of drug holidays in patients with active RRMS treated with NAT. In this context, the concept of EID seems to be better in clinical practice (56).

Additionally, our data suggest that monitoring PD and PK parameters and sNfL may provide guidance to identify the optimal time window for switching to other highly efficacious treatments. sNfL has a high potential as a treatment response marker with regard to a subsequent DMT post-NAT. However, to define its role as a marker for upcoming radiological and clinical disease activity further investigations are required. Free NAT concentration may also serve as a basis for EID and could be the marker that is the easiest to establish in clinical pratice besides sNfL.

In conclusion, a combination of PK and PD parameters could contribute to the future development of individualized NAT treatment schedules. sNfL seems to be a promising biomarker to monitor clinical and subclinical disease activity as well as treatment response. Additional data have to be generated to support our findings and to establish these biomarker combination in daily clinical practice.

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/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Commitee of the Technical University Dresden. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual's for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

UP, KA, and TZ: study concept and design and drafting of the manuscript. UP: acquisition of data. UP and KA: analysis and interpretation of data. HI: critical revision of the manuscript for important intellectual content. UP: statistical analysis. KA and TZ: study supervision. All authors contributed to the article and approved the submitted version.

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Clozapine Regulates Microglia and Is Effective in Chronic Experimental Autoimmune Encephalomyelitis

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Objective: Progressive multiple sclerosis is characterized by chronic inflammation with microglial activation, oxidative stress, accumulation of iron and continuous neurodegeneration with inadequate effectiveness of medications used so far. We now investigated effects of iron on microglia and used the previously identified neuroprotective antipsychotic clozapine *in vitro* and in chronic experimental autoimmune encephalomyelitis (EAE).

Methods: Microglia were treated with iron and clozapine followed by analysis of cell death and response to oxidative stress, cytokine release and neuronal phagocytosis. Clozapine was investigated in chronic EAE regarding optimal dosing and therapeutic effectiveness in different treatment paradigms. Animals were scored clinically by blinded raters. Spinal cords were analyzed histologically for inflammation, demyelination, microglial activation and iron accumulation and for transcription changes of regulators of iron metabolism and inflammation. Effects on immune cells were analyzed using flow cytometry.

Results: Iron impaired microglial function *in vitro* regarding phagocytosis and markers of inflammation; this was regulated by clozapine, reflected in reduced release of IL-6 and normalization of neuronal phagocytosis. In chronic EAE, clozapine dose-dependently attenuated clinical signs and still had an effect if applied in a therapeutic setting. Early mild sedative effects habituated over time. Histologically, demyelination was reduced by clozapine and positive effects on inflammation strongly correlated with reduced iron deposition. This was accompanied by reduced expression of DMT-1, an iron transport protein.

Conclusions: Clozapine regulates microglial function and attenuates chronic EAE, even in a therapeutic treatment paradigm. This well-defined generic medication might therefore be considered as promising add-on therapeutic for further development in progressive MS.

Keywords: progressive multiple sclerosis, neuroprotection, microglia, iron, EAE (experimental autoimmune encephalomyelitis), clozapine

INTRODUCTION

Multiple Sclerosis (MS) is a multifactorial chronic-inflammatory disorder of the central nervous system, leading to neurodegeneration and chronic disability (1). While nowadays a broad spectrum of medications is available for the relapsing-remitting phase (RRMS) of the disease with differing efficacy and side effect profiles (2) it still remains challenging to tackle the progressive phase of the disease. Reasons for this are differing mechanisms of chronic inflammation with predominance of trapped inflammation behind the blood brain barrier (BBB) of cells of innate immunity such as microglia, release of iron, oxidative stress and cellular damage also including mitochondrial impairment – altogether fueling progressive neurodegeneration and clinically functional impairment (3, 4). Until now, only a limited number of medications have been FDA-approved for either (active) secondary progressive MS (SPMS; interferon-β1a or b, mitoxantrone, cladribine, siponimod) or primary progressive MS (PPMS; ocrelizumab) (5). Those medications are an important step to slow down progression but still have limited efficacy (interferons) or severe sideeffects (mitoxantrone); it therefore remains crucial to better understand and target pathomechanisms of progression to further improve therapy for those with progressive forms of MS.

To address this need, we and others have used systematic screening approaches to target features of progressive MS and identify protective medications. Approaches were directed to enhance remyelination (6) or reduce neurodegeneration by iron (7). Iron age-dependently accumulates in the CNS of progressive MS patients (8) and might amplify cellular damage by driving inflammation and generating reactive oxygen metabolites *via* the Fenton reaction (9). To address this mechanism, we conducted a high throughput screening and identified several orally available generic medications with presumably neuroprotective features (7). One of those medications was the atypic antipsychotic clozapine, which reduced iron-mediated neurotoxicity and prevented mitochondrial damage to neurons, reduced T cell proliferation, and showed antioxidative properties (7). We here set out to better understand effects of iron on microglia in culture and investigated clozapine both *in vitro* and in an animal model of progressive MS, chronic experimental autoimmune encephalomyelitis, in different therapeutic paradigms.

METHODS

Cell Culture

HMC3 Cells

Microglia of the human microglial cell line 3 (HMC3) (10) were used as previously described (11). HMC3 cells were cultured in T75 flasks in Minimum Essential Medium (MEM, no glutamine) supplemented with 1% 10,000 units/ml penicillin/streptomycin, 1% glutamine (GlutaMAX Supplement; all Gibco, Life Technologies, Carlsbad, CA, USA) and 10% fetal bovine serum (FBS) (FBS Standard, Pan Biotech, Aidenbach, Germany). Cells with a confluence of 90% were split using Accutase (Invitrogen, Life Technologies, Carlsbad, CA, USA). For experiments, cells were cultured for at least 24 h in 96 well plates in a density of 20,000 cells/well (200,000 cells/ml). Cells were treated with iron sulfate ($FeSO_4$, Iron(II)) sulfate heptahydrate; Sigma-Aldrich, St. Louis, MO, USA) as previously described (7) and diluted in medium to achieve desired concentrations. Clozapine was prepared fresh and dissolved in DMSO 0.025%. After 24 h the cells were stained with Hoechst 33342 (6 µg/ml, Invitrogen) for 90 min and Propidium iodide (PI, 400 ng/ml; Invitrogen) for 15 min. Thereafter, cells were washed and four double-images were taken per well (Olympus BX51, Tokyo, Japan, 10x). The images were analyzed for cell particle number and area with macro instructions for ImageJ (National Institutes of Health, Bethesda, MD, USA). The values of PI^+ images were divided by the corresponding values of Hoechst-images to determine relative cell death/apoptosis. The viability was measured with Calcein AM staining using a fluorescence plate reader at 530 nm (Infinite 200 Pro, Tecan Group AG, Männedorf, Switzerland).

N2a Cells

Neuro2a mouse neuroblastoma cells (N2a, Department of Neuroanatomy and Molecular Brain Research, Ruhr-University Bochum, Bochum, Germany) were cultured in T75 flasks in DMEM (DMEM, high glucose, GlutaMAX Supplement, Gibco) with 1% 10,000 units/ml penicillin/streptomycin and 5% FBS. Cells were split using trypsin-EDTA 0.5% (Gibco) at a confluency of 90%. For experiments, cells were detached, heated for 10 min in 90°C PBS and cooled down on ice for another 10 min to secure cell death.

Secretome Analysis

20,000 HMC3 cells/well were plated in a 96 well plate and incubated for 24 h, following medium change, treatment with clozapine for 1 h and stimulation with $FeSO_4$ for another 24 h. Supernatants were harvested and stored at -80°C. Cytokines were analyzed using the Cytokine Cytometric Bead Assay (BD Biosciences, Franklin Lakes, NJ, USA) on a FACS Canto II (BD Biosciences) as previously described (12). Data were analyzed using the software FACS Cap Array v.3.0.

Oxidative Stress

Tert-Butyl hydroperoxide (t-BHP) was used to induce oxidative stress. 20,000 HMC3 cells/well were plated in a 96 plate and incubated for 24 h. Clozapine was added 1 h prior to addition of t-BHP in different concentrations between 50 µM and 800 µM following analysis using the MTT after 2 and 4 h. The absorbance was measured at 570 nm using a plate reader.

Phagocytosis Assay

HMC3 cells were plated at a density of 20,000 cells/well in 96 well plates. Dead N2a cells following heat treatment as indicated above were stained with PI (400 ng/ml) for 15 min. 50,000 stained and dead N2a cells were added to each well and incubated for 1 h. After 2 wash steps with cold PBS fluorescence intensity was measured at 535 nm/617 nm with a plate reader (Synergy H1, BioTek Instruments, Winooski, VT, USA; Tecan).

Experimental Autoimmune Encephalomyelitis

All animal experiments were approved by the animal care committee of North Rhine-Westphalia, Germany (LANUV,

no. 84-02.04.2017.A132). For all experiments, seven-week-old female C57BL/6J mice were used (Janvier Lab, Le Genest-Saint-Isle, France). Mice were housed under environmentally controlled conditions with constant temperature and a 12:12 h dark-light cycle under pathogen free environmentally controlled conditions. Mice had free access to chow and water *ad libitum*. Prior experiment start, mice were adapted to the environment for at least one week. Experimental autoimmune encephalomyelitis (EAE) was induced upon injection of an emulsion containing 500 µg/ml Myelin Oligodendrocyte Glycoprotein$_{35-55}$ (MOG_{35-55}) in Complete Freund's adjuvant containing 2,000 µg/ml *Mycobacterium tuberculosis* as previously described (13). 50 µl emulsion was injected subcutaneously in each hind flank. Mice were injected with 200 ng Pertussis toxin dissolved in PBS on days 0 and 2 to induce blood-brain-barrier leakage. Animals were scored daily before administration of clozapine to rule out sedative effects according to a previously defined scoring scheme with the following scores: 0: no signs of disability; 1: tail paresis; 2: complete tail paralysis; 3: missing compensatory movements while walking; 4: ataxia; 5: moderate hind leg paresis; 6: complete paresis of one hind leg or stronger paresis of both hind legs; 7: paraplegia; 8: tetraparesis; 9: moribund; 10: death (13). Mice with a score of 7 were euthanized according to animal care guidelines. Before treatment initiation animals were randomized according to weight or according to the score in the therapeutic experiments. Animals were treated with clozapine prophylactically from day 0 or therapeutically by oral gavage as indicated in respective figure legends. Clozapine was solved in PBS.

Open-Field

The activity of animals was evaluated on a weekly basis using the open-field test. The test was performed in a quiet environment without disturbing stimuli. Activity was tracked for 15 min and analyzed regarding track, speed, activity time and rearing. To minimize the effect of habituation, two baseline measurements were performed before induction of EAE. The chamber was cleaned with 70% ethanol and water between each measurement to minimize disturbance by animal odor.

Explant

The EAE was terminated 12 h after the last administration of clozapine and mice were anesthetized with 120 mg/kg ketamine and 16 mg/kg xylazine. Blood samples were taken by intracardiac puncture and animals were subjected to PBS-perfusion. Spleens and lymph nodes (axillary, cervical and inguinal) were obtained for flow cytometry. Before fixation a small sample of the lumbar spinal cord was snap frozen for further PCR analysis. The remainder of spinal cords and brains were fixed in 4% buffered formalin. After fixation, the spinal cords were divided into cervical, thoracic and lumbar parts, put in cassettes, filled up with Frozen Section Medium NEG-50 (Sigma Aldrich, St. Louis, MO, USA) and placed immediately on dry ice following storage at a temperature of -20°C. Blood cells, lymph node cells and splenocytes were used for flow cytometry analysis.

Flow Cytometry

Cells from lymph nodes and spleens were obtained by pressing them through 100 µm and 70 µm strainers and washing with cold PBS. Splenocytes and blood cells were put in Erythrocytes Lysing Buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM Triplex III) to eliminate erythrocytes. Cells were stained with primary antibodies (**Additional Table 1A**) and analyzed by flow cytometry using FACS Canto II (BD Biosciences, Franklin Lakes, NJ, USA). Data were analyzed using FlowJo (FlowJo X 10.0.7r2, Becton, Dickinson and Company, Ashland, OR, USA).

Histology

Cryosections were stained with haematoxylin (Sigma Aldrich) and eosin 0.1% solution (Merck), anti-Iba-1 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan; **Additional Table 1B**) and goat anti-rabbit-immunoglobulin-Alexa Fluor 568 (abcam, Cambridge, UK) to stain microglia, FluoroMyelin (Invitrogen) to stain myelin and acidified 20% potassium ferricyanide solution (Laborladen.de, Hüfingen, Germany) with DAB intensification (Merck) to stain ferrous iron. All images were merged (Image Composite Editor, Microsoft Corporation, Redmond, WA, USA) and blinded (AntRenamer, Antoine Potten). HE-stains were evaluated following manual definition of infiltrates. The remainder of stains was evaluated using ImageJ after defining thresholds. The grey matter was excluded from the analysis.

qPCR Analysis

RNA was isolated from lumbar spinal cord samples with the Qiagen mini Kit according to the manufacturer instructions (Qiagen, Hilden, Germany). Amount and purity of isolated RNA was revealed through nanodrop measurements. Primers were designed using Primer Blast with refseq codes (National Center for Biotechnology Information, Bethesda, USA) for suitable targets, synthesized (microsynth, Balgach, CH) and analyzed for efficiency (**Additional Table 2**). Only primers with an efficiency between 85% and 115% were used. Tata Box protein (Tbp) and Hypoxanthine-guanine-phosphoribosyltransferase 1 (Hprt1) were used as housekeeping genes. Data were generated using a QuantStudio 3 RT-PCR System and analyzed using QuantStudio Design & Analysis Software v1.5.1 (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA).

RESULTS

Clozapine Regulates Iron-Mediated Effects of Microglia *In Vitro*

To understand the effect of iron release on microglial functions and whether those are altered by clozapine we performed extensive experiments *in vitro*. HMC3 cells were treated with iron in different concentrations and analyzed regarding cell death. Unexpectedly, iron treatment reduced cell death in HMC3 cells in all dosages and did not have a toxic effect up to 100 µM (**Figure 1A**). Clozapine increased microglial viability at a dosage of 1 µM ($p < 0.05$; **Figure 1B**), while concentrations of 100 µM were toxic and reduced microglial viability ($p < 0.0001$; **Figure 1B**). We investigated the release of the chemokine CCL5 and inflammatory cytokine IL-6 to understand effects regarding markers of inflammation. While 25 µM iron supplementation did not alter the cytokine release, pre-treatment with clozapine in a dosage of 10 µM reduced the

release of IL-6 following iron treatment by 23% compared to the iron treated control condition ($p < 0.05$; **Figures 1C–F**). Since oxidative stress is a driver of progression, leading to an altered function of both microglia and neurons (4, 14) and since we have shown that clozapine is a potent anti-oxidative compound with a gallic-acid equivalent of 4.6 ($p < 0.05$) (7), we set out to analyze the effect of oxidative stress on microglia and investigated microglial viability upon t-BHP treatment at different time points. Microglial viability was dose-dependently reduced with a reduction of 22% upon treatment with 800 µM after 2 h (**Figure 1G**). Clozapine had no effect after 2 h. Toxic effects of t-BHP treatment were even more pronounced after 4 h (**Figure 1H**). Of note, after 4 h clozapine rescued microglia if treated with 50 µM t-BHP ($p < 0.01$; **Figure 1H**). To analyze whether microglial function is also attenuated we set out to investigate effects on phagocytosis. While iron administration in low concentrations (10 µM) increased phagocytosis, higher concentrations of 100 µM impaired microglial function as indicated by a reduction of phagocytosis of 28% which however lacked significance (**Figure 1I**). Clozapine normalized those effects. Altogether, those data show that clozapine can moderately regulate microglial inflammatory responses and function elicited by iron treatment.

Clozapine Positively Attenuates Chronic Experimental Autoimmune Encephalomyelitis in a Dose-Dependent Manner

We then set out to investigate the effect of clozapine in chronic EAE. We investigated different dosages of clozapine applied by

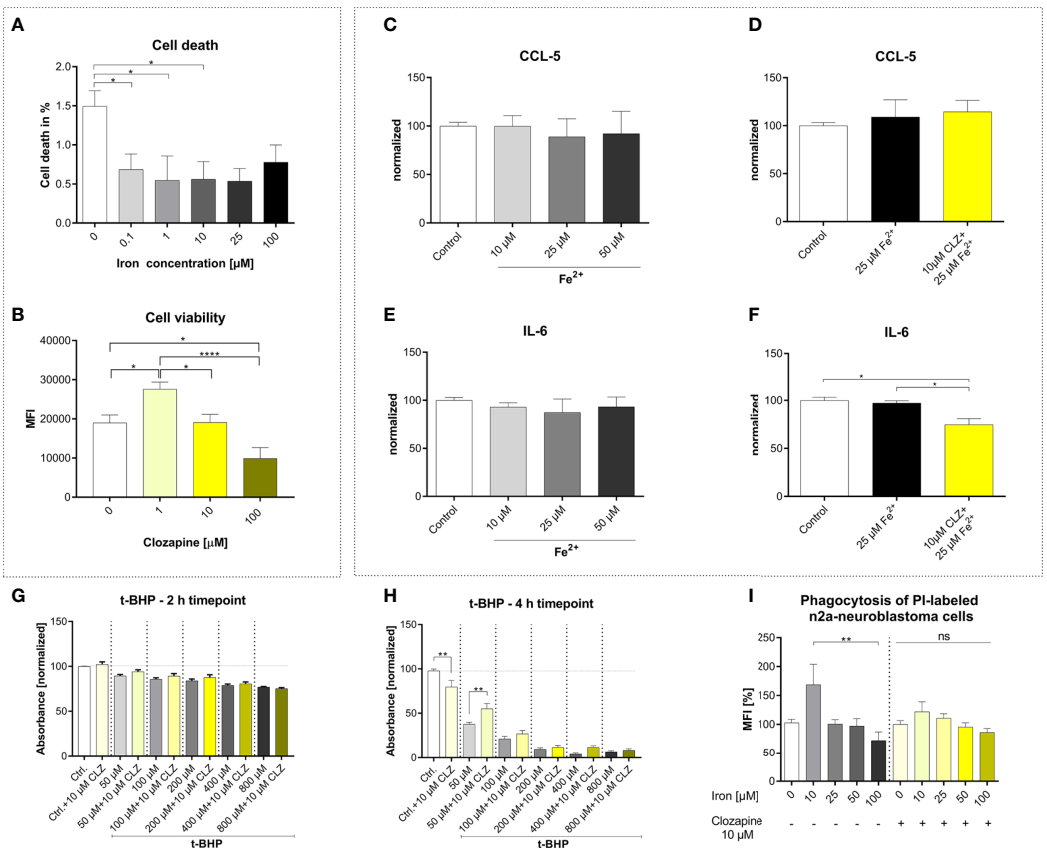

FIGURE 1 | Clozapine enhances microglial viability, reduces the release of IL-6, protects microglia against oxidative stress and normalizes microglial phagocytosis. **(A)** Iron treatment reduced cell death of microglia (cell line HMC3) in low concentrations. **(B)** Viability of microglia was increased upon treatment with clozapine in a concentration of 1 µM ($p < 0.05$), while concentrations of 100 µM were toxic ($p < 0.05$). Release of CCL5 **(C)** was not altered after iron treatment (25 µM) **(D)**. Clozapine, however reduced IL-6 release in iron treated microglia **(F)**, while iron itself did not have any effect on IL-6 release **(E)**. **(G)** Oxidative stress, induced by the addition of t-BHP, led to a dose-dependent reduction of cell viability after 2 h; clozapine had no effect after 2 h. **(H)** After 4 h, toxic effects of t-BHP were more pronounced and clozapine was able to attenuate cell death in t-BHP treated cells treated in a dosage of 50 µM ($p < 0.05$). **(I)** Microglia were treated with iron following addition of dead neurons. Iron in a concentration of 10 µM trended towards enhanced phagocytosis, 100 µM trended towards the opposite. The effects were normalized after clozapine treatment in a dosage of 10 µM. **(A, B)** Data are shown as mean ± SEM of 3 **(A)** and 4 **(B)** independent experiments performed in triplicates **(A)** and quadruplicates **(B)**, **(C–F)** mean ± SEM of 2 independent experiments performed in triplicates, **(G, H)** 3 (2) independent experiments performed in quadruplicates and **(I)** 8 independent experiments for control and 3 independent experiments with clozapine in triplicates and quadruplicates. Data were analyzed using a one-way ANOVA with Tukey's (A-B, I), non-parametric Kruskal-Wallis with Dunn's **(C–F)** and Sidak's **(G–H)** multiple comparison as *post hoc* analysis. **(I)** Outliers were eliminated with ROUT method Q = 1. * = $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$.

oral gavage from the day of MOG-immunization (**Figure 2A**) and treated animals over the whole period of the experiment. To assure that mice received equivalent concentrations of clozapine, animals were treated by oral gavage. Pilot experiments showed that the administration of 30 mg/kg clozapine led to exuberant sedation resulting even in death of some animals (not shown). We therefore performed a dose-finding study to establish the effective and safe dose of 15 mg/kg. While control animals treated with vehicle showed marked signs of EAE with a mean score of 4.3 ± 0.7 at the peak of EAE and chronification over more than 50 d, clozapine led to a dose-dependent reduction of clinical signs (**Figure 2B**). Disease incidence declined dose-dependently following clozapine administration (**Additional Table 3**). Moreover, the onset of clinical signs was delayed by 3 d in 15 mg/kg treated mice. While the clinical scores of mice treated with 2.5 mg/kg clozapine increased ($p = 0.0228$) we observed an improvement in mice treated with 7.5 mg/kg ($p = 0.0059$) and 15 mg/kg clozapine ($p=0.0016$) compared to the control condition. This was reflected in a higher body weight as marker of general health with the exception that we could not observe a significant weight change in 15 mg/kg clozapine treated animals (7.5 mg/kg vs. control $p < 0.0001$, 2.5 mg/kg vs. control $p = 0.0357$; **Figure 2C**). Positive effects of clozapine on the clinical course was reflected in sum-of-scores. While sum-of-scores from day 10 to the end of the experiments only trended towards positive effects of higher clozapine concentrations (**Figure 2D**), analysis of sum-of-scores during the chronic phase from day 35 showed a significant effect of 7.5 mg/kg (28.1 ± 12.1; $p < 0.05$) and 15 mg/kg (22.8 ± 10.6; $p<0.05$) compared to 2.5 mg/kg (**Figures 2E, F**).

Since clozapine has sedative effects, also documented in our pilot experiments, we wanted to rule out that those might influence the neurological phenotype. We therefore performed an open-field analysis and investigated the overall distance, the active time, rearing and the speed. While we could document a reduction of all aforementioned tests, presumably due to habituation, clozapine treated groups and the control group did not differ (**Figures 2G–J**).

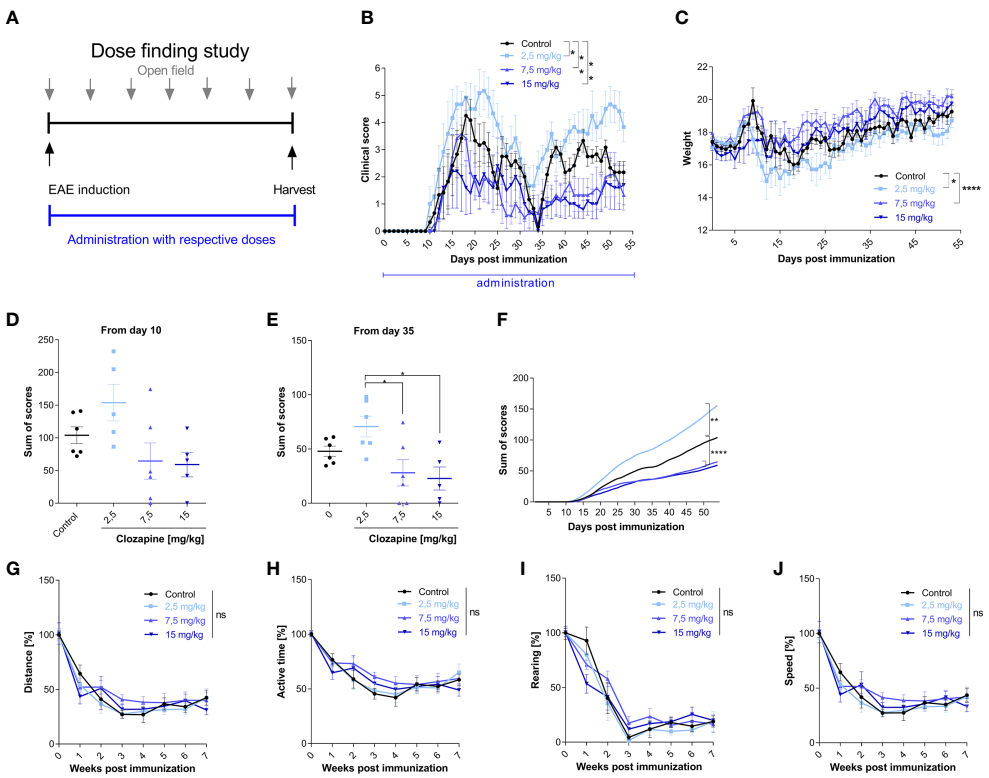

FIGURE 2 | Treatment with clozapine ameliorates chronic EAE dose-dependently in a prophylactic treatment paradigm. **(A)** MOG-immunized C57BL6/J mice (female, 8 weeks old) were treated with different clozapine concentrations (2.5 mg/kg, 7.5 mg/kg, 15 mg/kg) or 5% DMSO/0.0025% acetic acid in saline (control) once a day from day 0 and evaluated weekly using the open field analysis. **(B)** The clinical scores and **(C)** weight of mice treated with 2.5 mg/kg clozapine declined ($p = 0.0228$ for score, $p = 0.0357$ for weight) whereas the groups treated with 7.5 mg/kg ($p=0.0059$ for score, $p = < 0.0001$ for weight) and 15 mg/kg clozapine ($p = 0.0016$ for score) improved compared to the control group. **(D)** Differences in sum of scores were not significant regarding the timespan of clinical signs from day 10. **(E)** Sum of scores during the chronic phase of EAE (from day 35) differed significantly for 7.5 mg/kg ($p = 0.0222$) and 15 mg/kg ($p = 0.0136$) treated groups compared to the 2.5 mg/kg treated group. **(G–J)** Open field experiments were conducted to measure the sedative effect of clozapine. Groups did not differ regarding distance, active time, rearing and speed. Control group, 2.5 mg/kg group and 7.5 mg/kg group n=6, 15 mg/kg group n=5. Data are shown as mean ± SEM. Non-parametric Kruskal-Wallis test **(B–E, G–J)** and ordinary one-way ANOVA **(F)** with 95% confidence interval. Significances are depicted as *$p < 0.05$, **$p < 0.01$, ****$p < 0.001$, ns: not significant ($p>0.05$).

Therapeutic Administration of Clozapine Also Ameliorates Chronic EAE

Having identified the effective dose (15 mg/kg body weight), we set out to investigate whether clozapine also attenuates MOG-EAE if applied in a therapeutic treatment paradigm and compared this setting to a prophylactic paradigm with treatment initiation from the day of immunization. We chose a time-point when about 50% of animals had developed clinical signs of EAE after 11 d (**Figure 3A**) and used the effective dosage of 15 mg/kg clozapine once daily, identified in the dose-finding study. In this experiment, the onset of clinical signs in the prophylactic group was even later compared to the dose-finding study (8 d delay, p <0.0001; **Additional Table 4**). While the control group displayed robust chronification after the initial relapse, both treated groups had reduced signs of disease (prophylactic administration 1.6 ± 0.5 (mean ± SEM); therapeutic administration 2.5 ± 1.8) in contrast to the control group at the end of the experiment (4.2 ± 1.5; p <0.0001 vs. therapeutic administration; p <0.0001 vs. prophylactic treatment) (**Figure 3B**). As expected, the prophylactic treatment paradigm had stronger positive effects and delayed the onset of disease by 8 d compared to the control group. Of note, the weight increase was notably lower in treated mice at the end of the experiment (prophylactic treatment 20.5 ± 1.5 g; therapeutic treatment 19.5 ± 1.2 g) in contrast to the control group (21.2 ± 1.7 g; p=0.02 vs. prophylactic treatment; p <0.0001 vs. therapeutic treatment; **Figure 3C**). Positive treatment effects were again reflected in total sums of scores (day 16: control group 170.7 ± 18.2 vs. prophylactic treatment 58.1 ± 7.2, p = 0.0116; day 35: control group 113.0 ± 13.3 vs. prophylactic treatment 38.0 ± 6.0 p = 0.0098; **Figures 3D–F**). Open field experiments did not show differences between the control group and the two therapeutic regimen. To rule out sedative effects of clozapine, we also investigated non-immunized mice with

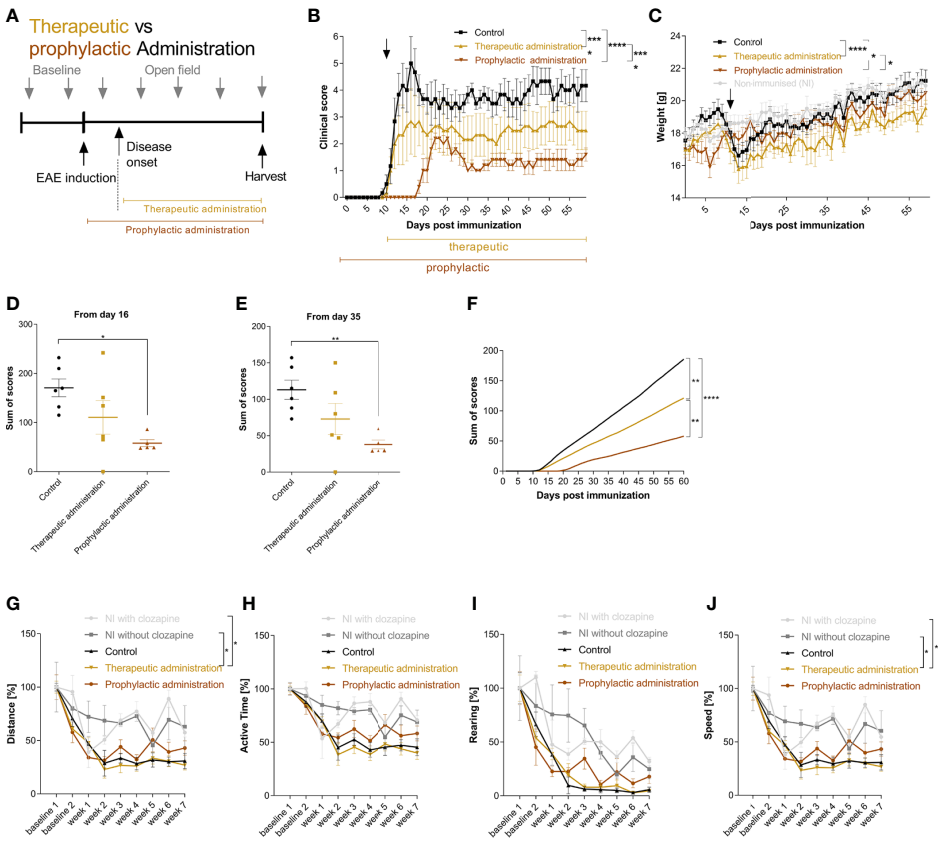

FIGURE 3 | Therapeutic and prophylactic administration ameliorate chronic EAE. **(A)** MOG-immunized C57BL6/J mice (female, 8 weeks old) were treated with 15 mg/kg from day 0 (prophylactic administration) or from day 11 (therapeutic administration, 50% of mice showed clinical signs at this timepoint) once a day. The control group was treated with 5% DMSO/0.0025% acetic acid in saline (vehicle) from day 0. **(B)** The clinical condition and **(C)** weight of the control mice declined compared to the prophylactically (p < 0.0001 for score, p = 0.0202 for weight) and therapeutically treated (p < 0.0001 for score, p < 0.0001 for weight) mice. **(D)** The sums of scores for the timespan of clinical symptoms after peak disease (from day 16) were higher in the control group compared to the prophylactically treated group (p=0.0116). **(E)** This was mirrored during the chronic phase of EAE (p=0.0098) and **(F)** regarding the analysis of the overall sum of scores. **(G–J)** Open field experiments were conducted to measure the sedative effect of clozapine. Speed and distance were significantly higher in non-immunized groups compared to therapeutic treatment. The remainder did not differ regarding distance, active time, rearing and speed. Control group n= 6, therapeutic treatment group n = 6 and prophylactic treatment group n=5. Data are shown as mean ± SEM. Non-parametric Kruskal-Wallis test **(B–E, G–J)** and ordinary one-way ANOVA **(F)** with 95% confidence interval. Significances are depicted as *p < 0.05, **p < 0.01, ****p < 0.001.

or without clozapine. While clozapine treated mice were less active in the first measurements, the effect was gone at week 3, arguing for habituation (**Figures 3G–J**).

Treatment During Late Chronic EAE

Having established that clozapine is effective also in a therapeutic treatment paradigm we asked whether late treatment might still be effective and therefore performed analyses of clozapine treatment in late chronic-EAE. To investigate this phase, we performed an experiment with therapy induction from day 29 (**Figure 4A** and **Additional Table 5**). Here, we could document mild beneficial effects (mean score at day 60: control group 4.3 ± 0.7, clozapine group 2.8 ± 0.7; p <0.05, **Figure 4B**). Of note, especially mice with a higher disease activity (top 50% of the scores) profited from the medication (**Figure 4D**, p <0.001), whereas mildly impaired animals did not show a response (**Figure 4E**). Clozapine had no effect on weight. Sum of scores during the chronic phase therefore did not differ (control 129.8 ± 19.7; clozapine treatment 100.3 ± 20.9; p = 0.2229).

Histological Analysis Shows Reduced Infiltration and Demyelination

Histological analysis of the spinal cord revealed that general infiltration in all parts of the spinal cord trended towards a reduction in the prophylactic group compared to the control condition (thoracic cord p <0.05; **Figures 5A, B**). Demyelination was significantly reduced both in the symptomatic and prophylactic treatment group compared to the control (p <0.05, **Figures 5C, D**). Microglia were also reduced in the cervical and thoracic cord (p <0.05), which, however, lacked significance upon analysis of the whole spinal cord (**Figures 5E, F**). We then evaluated the effects of clozapine on iron deposition *in vivo*. Again, we saw a reduction, mostly in the prophylactic group, which however lacked significance (**Figures 5G, H**). Correlations of histological data showed that general infiltration and demyelination, iron deposition and infiltration of Iba1$^+$ cells as well as iron deposition and demyelination did not correlate (**Figures 5I, L, M**), while general infiltration and iron deposition (r = 0.74, p = 0.001) as well as general infiltration

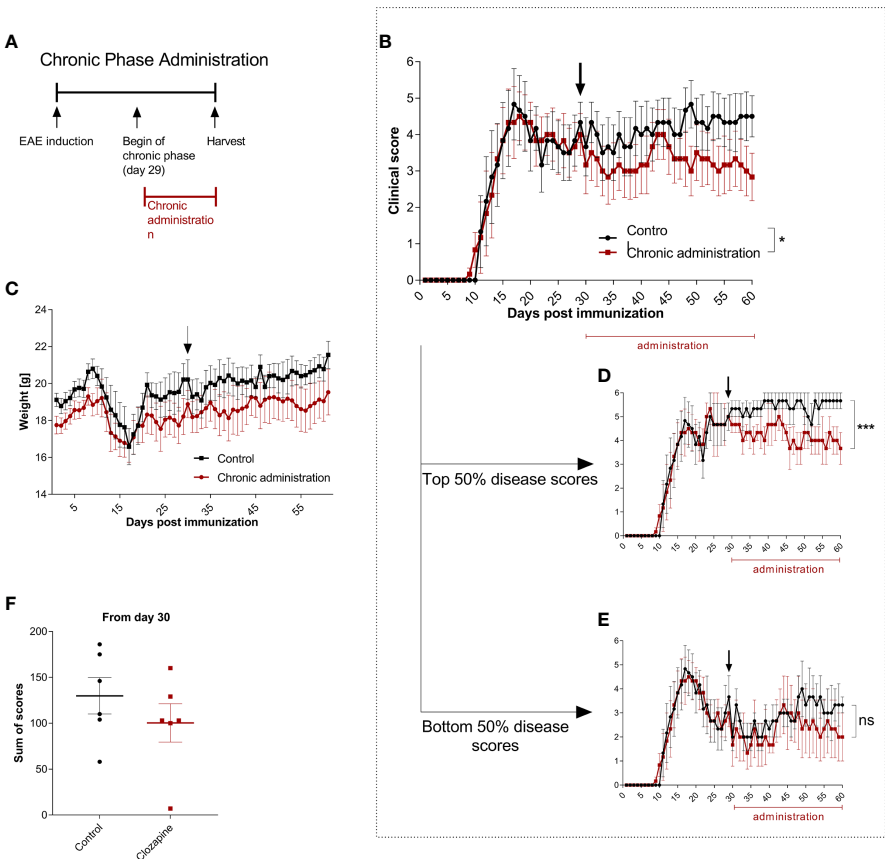

FIGURE 4 | Treatment with clozapine during chronic EAE ameliorates clinical signs with higher benefit in animals with higher disability. **(A)** MOG-immunized C57BL6/J mice (female, 8 weeks old) were treated with 5% DMSO/0.0025% acetic acid in saline (vehicle) from day 0. Animals were randomized from the chronic phase (day 29) and one group was treated with 15 mg/kg clozapine once a day. **(B)** The clinical condition of treated mice improved in the chronic phase compared to the control group (p = 0.0388) while **(C)** weight did not differ. **(D)** This effect was mediated by mice with higher disability (top median) which showed a decline of signs (p = 0.0005) whereas **(E)** the lower median did not profit during the chronic phase (p = 0.1204). **(F)** Sums of scores in the chronic phase did not differ. Control group n = 6, chronic phase treatment group n = 6. Data are shown as mean ± SEM. Area under the curve (AUC) with unpaired t-test **(B–E)** and Mann-Whitney test **(F)** with 95% confidence interval. Significances are depicted as *p < 0.05, ***p < 0.001, ns: not significant (p>0.05).

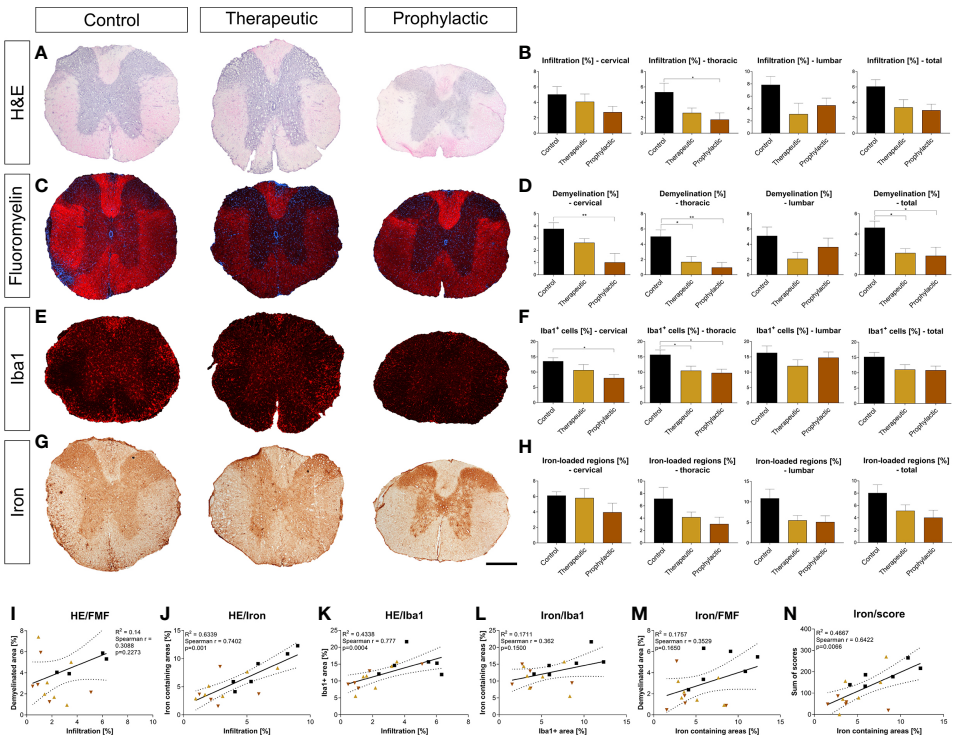

FIGURE 5 | Prophylactic and therapeutic clozapine administration reduce infiltration of inflammatory cells, iron loaded regions, demyelination and microglia activation in spinal cord sections. **(A)** Representative images of H&E stained spinal cord sections. **(B)** Clozapine administration led to a trend towards decreased infiltration in all spinal cord segments, reaching significance in thoracic segments of the spinal cord in prophylactically treated mice (p=0.0427). **(C)** Representative images of fluoromyelin stained spinal cord sections. **(D)** Demyelination of total spinal cord sections was significantly reduced after both symptomatic ($p < 0.05$) and therapeutic administration ($p < 0.05$). **(E)** Infiltration of macrophages/microglia, as assessed using Iba1 staining. **(F)** Less infiltration in cervical and thoracic cord in prophylactic treated mice ($p < 0.05$), lacking significance upon analysis of the whole spinal cord. **(G)** Representative images of iron stained sections. **(H)** Trend towards less iron deposition in all sections, lacking significance. **(I)** While infiltration and demyelination as well as **(L)** iron deposition and macrophage/microglial infiltration and **(M)** iron deposition and demyelination did not correlate, **(J)** there was a strong correlation of infiltration and iron deposition (Spearman r = 0.74; p = 0.001), **(K)** general infiltration and microglial/macrophage infiltration (Spearman r = 0.77; p = 0.0004) as well as (N) iron deposition and individual animal score (Spearman r = 0.64; p = 0.0066). **(B, D, F, H)** Ordinary one-way ANOVA and Dunnett's multiple comparison with a single pooled variance and 95% confidence interval. **(I-N)** Correlation using Spearman r and R^2. Data are shown as mean ± SEM. Significance is shown as *p < 0.05, **p < 0.01. Scale bar for a, c, e and g is 400 µm.

and infiltration of Iba^+ cells (r = 0.78, p = 0.0004) strongly correlated (**Figures 5J, K**). Moreover, iron deposition and sum of scores strongly correlated (r = 0.64, p = 0.0066) (**Figure 5N**).

Transcription of Iron Metabolism Proteins and Markers of Inflammation Are Regulated by Clozapine

Since clozapine reduces iron load upon clozapine treatment as evidenced using histological analyses, we further elucidated regulation of proteins involved in iron metabolism (**Figure 6**). H-ferritin was unaffected in EAE mice and upon clozapine treatment. L-ferritin was significantly upregulated in EAE mice (p <0.05), but not affected by clozapine treatment. Treatment with clozapine during the acute phase led to decreased DMT-1 transcription (vehicle vs. prophylactic p=0.0031). Ferroportin 1 was downregulated upon therapeutic administration (p <0.05). Treatment with clozapine during the chronic phase did not affect transcription of aforementioned proteins. TNF-α as inflammatory marker was significantly upregulated upon prophylactic therapy (p <0.01) compared to both the vehicle group and therapeutic therapy. CD86 trended towards an increase following clozapine therapy compared to vehicle treated EAE mice. CD206 was significantly upregulated in vehicle EAE mice compared to non-EAE mice and trended towards a downregulation following clozapine therapy which lacked significance.

Clozapine Induced Modest Effects on Peripheral Immune Cells

To understand effects of clozapine on peripheral immune cell subsets we investigated immune cells changes in blood, spleen and lymph node cells. Clozapine significantly reduced the frequency of $CD4^+$ T cells in all compartments with strongest and dose-dependent effects in lymph nodes (15 mg/kg 29% reduction, p <0.05; Figure e1). Th17 ($CD4^+IL17^+$) cells were reduced in the spleen (p<0.05). Th1 ($CD4^+IFN\gamma^+$) cells were not considerably affected, in lymph nodes a slight increase was seen in 15 mg/kg clozapine treated mice which lacked significance. Clozapine interestingly induced a profound

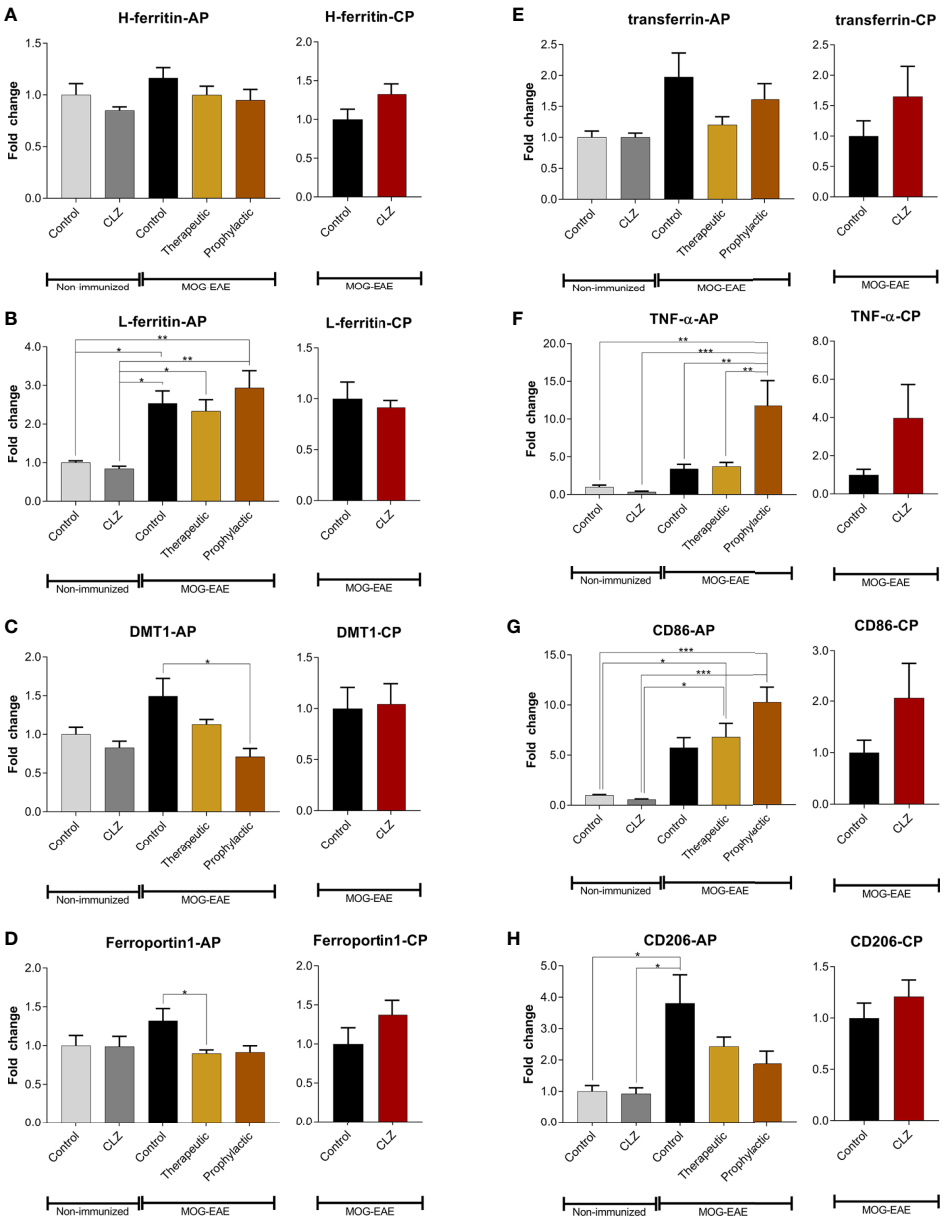

FIGURE 6 | Clozapine administration alters iron metabolism of the spinal cord. **(A)** While H-ferritin was not affected, **(B)** L-ferritin was upregulated in EAE mice ($p < 0.05$) but not altered following clozapine administration. **(C)** DMT-1 was downregulated following prophylactic clozapine administration compared to untreated EAE mice ($p < 0.05$). **(D)** Ferroportin-1 was downregulated following therapeutic treatment with clozapine ($p < 0.05$). **(E)** Transferrin was not affected. **(F)** Prophylactic clozapine administration led to significant upregulation of TNF-α compared to non-immunized mice or therapeutic treatment ($p < 0.01$). **(G)** CD86 was significantly upregulated compared to non-immunized mice ($p < 0.05$), but lacked significance compared to EAE mice. **(H)** CD206 was upregulated in vehicle EAE mice, clozapine had no effect. Non-immunized groups n = 3 each, acute phase treatment groups n = 6 each (prophylactic administration n = 5) and chronic phase treatment groups n = 6 each. Data were normalized to non-immunized and untreated mice for acute phase treatment and to vehicle group for chronic phase treatment. Tbp and Hprt1 were used as housekeeping genes. Data are shown as mean ± SEM. One-way ANOVA with Tukey's multiple comparison as *post hoc* analysis (AP) and two tailed unpaired t-test (CP) were used for analysis. Significances are depicted as *$p < 0.05$, **$p < 0.01$ ***$p < 0.001$.

reduction of regulatory ($CD4^+CD25^+FoxP3^+$) T cells in the blood in 2.5 mg/kg clozapine treated mice ($p < 0.01$) as well as dose-dependently in lymph nodes ($p < 0.05$). $CD86^+$ antigen-presenting cells were reduced in the spleen ($p < 0.05$), while activated $CD86^+F4/80^+$ cells did not differ. Clozapine had no effects on $CD8^+$ cytotoxic T cells or $CD45R^+$ B cells.

DISCUSSION

Therapeutic approaches to target aspects of progressive MS are still not effective enough to halt disease progression in most patients. This can in part be explained by the plethora of mechanisms being involved in progression, amplifying themselves *vice versa* (4).

By employing biochemical assays, we identified a group of neuroprotective generic medications with a well-known safety profile and potential for therapeutic development in progressive MS. We now investigated one of the medications identified, the antipsychotic clozapine, using *in vitro* experiments regarding its ability to modify microglial activation and foster neuroprotection in an animal model of progressive disease. In culture, clozapine moderately reduced the release of IL-6 of iron treated microglia and increased microglial viability in low concentrations. Moreover, iron impaired microglial phagocytosis was regulated using clozapine. *In vivo*, clozapine reduced disability progression in chronic EAE dose-dependently both in a prophylactic and therapeutic scenario.

Until now, the effectiveness of therapeutics for progressive MS is not overwhelming. A different approach than developing and designing new therapeutics against progression is tackling disease pathomechanisms with already approved generic medications. An advantage of this avenue is that medications are authorized for another indication, they have a well-known safety profile due to years of clinical practice and therefore a fast translation into clinical trials is potentially feasible. Moreover, those medications are also affordable for countries with poor healthcare systems. Clozapine is a low molecular weight atypical antipsychotic and follows Lipinski's rule, providing its exceptional ability to penetrate the CNS (15). It binds to different receptors such as dopamine, serotonin and alpha- and muscarinic acetylcholine receptors (15).

Since clozapine elicits sedative effects, we aimed to rule out that those might interfere with general health in treated mice. We therefore performed extensive open field analyses, which showed that initially observed sedative effects vanish within two weeks of treatment. For translation into human it is essential that dosages applied in mice can also realistically be achieved in human without inducing side effects. The concentrations of 2.5 – 15 mg/kg in mice used in our experiment are equivalent to human dosages between 0.2 and 1.2 mg/kg clozapine per day (16), equivalent to 14–84 mg for a 70-kg individual. Most patients treated for schizophrenia receive dosages ranging from 200-450 mg/day with a maximum of up to 900 mg/kg. Adverse effects occur especially at dosages above 450 mg/kg (15). Since we already observed positive effects in equivalent dosages of 84 mg, reaching a fraction of concentrations usually used for schizophrenia, we assume that the concentrations used in our experiments would be both clinically feasible and effective in patients with progressive MS; even if it might be suggested that side effects already occur in patients with progressive MS using lower dosages.

We provided evidence that clozapine is neuroprotective against iron mediated neurotoxicity, leading to the preservation of about 100% of neurons after a 24 h treatment period with iron in culture (7). Clozapine moreover is mitochondrioprotective ($p < 0.0001$) and has antioxidative effects with an gallic acid equivalent of 4.6 ($p < 0.05$), a potent anti-oxidative compound (7). Of note, we did not observe effects on T lymphocyte proliferative activity. Clozapine has been investigated in EAE previously (17, 18). Green et al. showed that clozapine has greater efficacy in halting EAE than risperidone, quetiapine or olanzapine (18). The administration of clozapine was, however, achieved by addition to the chow. The strong initial sedative effects of clozapine, documented in our experiments presented here, suggest, that the way of application might have led to incongruency due to reduced uptake. Clozapine does not reduce demyelination in the toxic demyelination cuprizone model but enhances the rate of functional recovery therein, associated with reduced astrocytic and microglial activation (19). Microglial activation is a key contributor to chronic inflammation in progressive MS and is therefore target for therapeutic development. Since microglia elicit not only negative but also protective effects, microglial function should be altered and not arrested. Our findings indicate that clozapine modulates microglial activity by regulating the inflammatory effect of free iron regarding phagocytosis of dead neurons, release of the inflammatory cytokine IL-6 and viability after oxidative stress. HMC3-microglia express NOX4 which leads to a constitutive generation of ROS, inducing an expression of IL-6 mRNA (20). While we could not observe an increase of IL-6 with ferrous iron, the decrease of IL-6 release following clozapine treatment might be due to a downregulation of the NOX4 system with reduced ROS-decrease. Those data are in line with reports showing that clozapine reduces the release of NO in LPS-treated microglia (21). Effects of clozapine on microglia might in part be mediated by calcium/calmodulin dependent Akt activation (22). It cannot be ruled out that the strong effects in EAE might also in part be mediated by immunomodulatory effects of clozapine. While we did not observe effects on the proliferation of T cells in our systematic screening (7), it is known that clozapine has strong effects on immune cells with reduction in class-switched memory B cells and secondary antibody deficiency (23), which might have been a contributing factor in our experiments.

Iron overload is a hallmark of the ageing CNS and is associated with several neurodegenerative disorders (24). In MS, iron has both beneficial and detrimental effects (8). Iron deposition in the basal ganglia correlates with progression and excess iron is toxic since it drives oxidative stress *via* the Fenton reaction (8). On the other hand, iron is important for the viability of oligodendrocytes and those receive their trophic support of iron in the form of H-ferritin through microglia (25). Iron metabolism is tightly regulated through a number of mechanisms and proteins. The upregulation of iron importer DMT-1 and downregulation of iron exporter ferroportin1 is a consequence of inflammatory stimuli and *vice versa* (26, 27). Transferrin is able to buffer iron (28) but can also be rapidly effluxed from the brain to the blood (29). H-ferritin has a ferroxidase activity and can catalyze the oxidation of ferrous iron to ferric iron by consuming the substrates for the Fenton's reaction; L-ferritin mediates and acclererates its storage (28, 30). Our data suggest that clozapine attenuates the uptake of iron by downregulation of DMT-1, leading to reduced iron in the spinal cord, evidenced by the histological analyses. Downregulation of ferroportin1 suggests a compensatory mechanism to prevent further iron loss. Late treatment during the chronic phase did not have an effect on DMT-1. Since we could also document reduced demyelination in early clozapine treated EAE mice we assume that the dosage used in our experiments did not elicit deleterious effects on oligodendrocytes. Of note, we could document an upregulation of TNF-α, an unexpected finding in light of the strong anti-inflammatory properties of clozapine. TNF exists as transmembrane form with signaling through TNFR2 and TNFR1 and a soluble form which acts *via* TNFR1 (31). Oligodendroglial TNFR2 is a key mediator of

transmembrane TNF dependent protection in EAE, crucial for oligodendrocyte differentiation (31). TNF-α also exhibits anti-inflammatory effects on TGF-β treated APCs, mediated by the TNF-R2 and thereby regulating immune responses (32). Those data altogether suggest, that the upregulation of TNF-α by clozapine mirrors the anti-inflammatory effects of the medication. To understand effects on innate immune cells we also investigated transcriptional changes of CD206 and CD86. CD206 peaks in late active and inactive MS lesions (33); the downregulation mediated by clozapine could thus in part be due to reduced lesion load following therapy. This is supported by data from the chronic experiment showing slight (but not significant) upregulation of CD206 following clozapine treatment, indicating enhanced regeneration.

There are some limitations of the data presented here. First, there is not an optimal model mimicking all aspects of progressive MS (34) including chronic EAE in C57Bl6 mice used here. Other models, previously used by us and others include the Biozzi Abh mouse model (35) which suffers from inconsistent EAE induction (36), the NOD model which is difficult to induce in our hand, or models using the Theiler murine virus. To address this question we performed extensive initial cell culture screening and addressed specific quesions using cell culture models (7). The initial screening also has limitations such as the usage of a circumscribed number of generic medications, used in a single screening concentration of 10 μM (7). Another drawback is the use of cell lines, which, however, enables performing complex experiments with several conditions, as done by us. Moreover we examined transcription changes of the whole spinal chord and did not perform single-cell RNA sequencing, which would have helped to better evaluate alterations induced by clozapine on different cell types, a question worthwhile to adress in future experiments. While the effect of the prophylactic and therapeutic treatment paradigm was strong, the effect of a treatment during the chronic phase was, although significant, less robust, and driven by highly impaired mice as identified following a post-hoc analysis. Moreoever, clozapine as substance has drawbacks such as (initial) sedative effects and agranulocytosis; hence, patients would have to be monitored closely to minimize the risks of the medication.

CONCLUSION

In summary, the work presented here shows that clozapine regulates microglial function upon iron stimulation, reflected in reduced release of inflammatory cytokines and normalization of neuronal phagocytosis, a scenario relevant in patients with progressive MS. Clozapine moreover dose-dependently attenuates clinical signs in chronic EAE, even if applied late during the chronic stage of the disease, with positive effects on histological markers such as demyelination. Dosages applied *in vivo* reflect low dosages readily achievable in human. We therefore consider clozapine as interesting target molecule for further development as add-on therapy in progressive MS.

GLOSSARY

EAE: Experimental autoimmune encephalomyelitis; DMT-1 : Divalent metal transporter 1; MS: Multiple sclerosis; RRMS: Relapsing-remitting multiple sclerosis; SPMS: Secondary progressive multiple sclerosis; PPMS: Primary progressive multiple sclerosis; BBB: Blood-Brain-Barrier; CNS: Central nervous system; HMC3: human microglial clone 3 cell line; PI: Propidium iodide; t-BHP: *tert*-Butyl hydroperoxide; ns: not significant; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MOG: Myelin oligodendrocyte glycoprotein; TNF-R1/R2: Tumor necrosis factor receptor 1/2.

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 animal study was reviewed and approved by animal care committee of North Rhine-Westphalia, Germany (LANUV, no. 84-02.04.2017.A132).

AUTHOR CONTRIBUTIONS

UC: Investigation and acquisition of data, analysis and interpretation of data, visualization, study concept or design, and original draft of the manuscript. SH: Investigation and acquisition of data, analysis and interpretation of data, and revising the manuscript. LK: Investigation and acquisition of data, analysis and interpretation of data, and visualization. JD: Investigation and acquisition of data, analysis and interpretation of data, and revising the manuscript. BA: Investigation and acquisition of data, analysis and interpretation of data, and revising the manuscript. RG: Analysis and interpretation of data, revising the manuscript, and funding acquisition. SF: Analysis and interpretation of data, visualization, original draft and reviewing of the manuscript, funding acquisition, and study supervision. 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/fimmu.2021.656941/full#supplementary-material

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Conflict of Interest: SF filed a United States Patent Application relating and entitled to "Treatment for Progressive Multiple Sclerosis", No. 62/412,534, filed October 25, 2016.

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.

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MINI REVIEW
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Therapy of Pediatric-Onset Multiple Sclerosis: State of the Art, Challenges, and Opportunities

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Treatment of pediatric-onset multiple sclerosis (POMS) has been tailored after observational studies and data obtained from clinical trials in adult-onset multiple sclerosis (AOMS) patients. There are an increasing number of new therapeutic agents for AOMS, and many will be formally studied for use also in POMS. However, there are important efficacy and safety concerns regarding the use of these therapies in children and young adults. This review will discuss the current state of the art of POMS therapy and will focus on the newer therapies (oral and infusion disease-modifying drugs) and on those still currently under investigation.

Keywords: pediatric onset multiple sclerosis, first-line therapies, second-line therapies, efficacy, safety

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INTRODUCTION

High relapse rate, rapid accumulation of white (WM) and gray matter (GM) damage, and worse long-term physical and cognitive disability are typical features of pediatric-onset multiple sclerosis (POMS) (1–6). Despite neuroplasticity, POMS patients reach similar levels of disability at a younger age than adult-onset MS (AOMS), and their quality of life (QoL) is frequently significantly compromised, with negative impacts on school, social, and physical activities (5, 7). Accordingly, POMS has to be considered a severe, highly disabling disease, with extremely high social costs. Approximately, POMS accounts for 2–10% of all MS cases (5), but incidence of MS in children and adolescents is increasing, and it has become relatively frequent to face the diagnosis and the treatment of this peculiar population.

Since no definite guideline exists on the management of POMS, treatment strategies often reflect the center-specific experience as well as the neurologist's therapeutic attitude and knowledge that derive from the application of adult-tailored MS therapeutic protocols. Despite heterogeneity, data on efficacy and safety of disease-modifying drugs (DMDs) [e.g., interferon beta (IFN β), glatiramer acetate (GA), natalizumab (NTZ), and rituximab] in POMS collected from single- of multi-center open-label observational studies indicate a marked effect on clinical and magnetic resonance imaging (MRI) parameters of inflammation (8–11), especially when therapy is initiated very early (12), as also pointed out by the 2012 International Pediatric MS Study Group (IPMSSG) consensus (13).

Recently, the US Network of Pediatric MS Centers reported data on 741 POMS patients, 197 treated with newer therapies [fingolimod, dimethyl fumarate (DMF), teriflunomide, NTZ, rituximab, and ocrelizumab] and 544 treated with IFN β or GA. As expected, those on newer DMDs had significant lower annualized relapse rate (ARR) than those with IFN β or GA ($p < 0.001$) (14). Moreover, a high rate of IFN β and GA treatment failure has been reported in POMS, ranging from 25 to 64% across studies (15). It is noteworthy that many of these drugs are still used off-label;

thus, the recent approval of fingolimod for POMS by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) constitutes a significant step forward in treating these patients (16).

Finally, the QoL must be strongly taken into consideration when treating POMS with DMDs. Therapies that induce symptoms (fever, headache, myalgias, etc.) that negatively and persistently impact school and sport performances, and therefore substantially modify the QoL, must be avoided or interrupted early.

Here, we review the state of the art of POMS therapy and focus on the newer therapies (oral and infusion DMDs) and on those currently under trial.

FIRST-LINE THERAPIES

Injectables

IFN β and GA (hereafter called injectables) are the most widely used DMDs in POMS (17–19). Both drugs showed a high-efficacy profile in the short term (see **Table 1** for a comprehensive overview) (18, 20–23, 28, 29) but also a consistent rate of treatment failure in the medium/long term. The US and Italian Network of the MS Centers collected longitudinal data on injectable-treated POMS, summarized in two reports: (1) after a mean follow-up of 3.9 years, 114 (44.2%) of 258 patients had their therapy changed to a second DMDs owing to refractory disease (27.9%) or poor tolerability of a first-line DMDs (16.3%) (30); (2) after a follow-up of 12.5 years, 82/97 (84.5%) patients needed a therapy switch, that in up to 58% of cases, was an immunosuppressive/second-line drug (9).

The US Network of Pediatric MS Centers analyzed 618 DMD-treated patients and reported that 147/483 (30.4%) of those treated with injectables switched to other therapies in a mean follow-up of 3.5 years (17). More recently, in a cohort of 741 patients, the 197 who were commenced on newer therapies (DMF, fingolimod, teriflunomide, NTZ, rituximab, and ocrelizumab) had significantly lower ARR than the 544 on injectable, confirming the higher efficacy of the newer therapies (14).

Although injectables are not associated with increased risk of infections or malignancies and the most reported side effects are injection site reactions for GA and flu-like symptoms for IFN β (25–35%) (19, 31), the loss of adherence (i.e., missing > 20% of doses) is high and is more frequently reported by patients (up to 41%) than by parents (14%) or pharmacist (7%) (15). Expert opinion suggests that IFN β is better tolerated if initiated at 25–50% of the standard dose followed by a gradual escalation to full dose over 1 to 3 months (32).

Orals

Dimethyl Fumarate

A phase II multicenter study (FOCUS) (25) with DMF (120 mg twice daily on days 1–7, 240 mg twice a day thereafter) on 22 POMS (20 of which completed the study) showed a median change in number of new/enlarged T2 hyperintense lesions of −2.0 at week 24 compared with baseline (−1.5 vs. −8.0, $p = 0.009$). The unadjusted ARR was 1.5 in the year prior to study and 0.8 at week 24. Adverse events (AEs) (most commonly gastrointestinal disorders and flushing) and pharmacokinetic (PK) were consistent with those observed in adults (25). The good safety and tolerability profiles of DMF were confirmed, in agreement with a previous small retrospective study on nine patients (24).

In the CONNECTED study (26), extension of FOCUS, a long-lasting benefit of therapy, was observed: 12/17 participants (71%) had no new/enlarged T2 hyperintense lesions from weeks 16 to 24. Over a mean treatment period of 120 weeks, a significant reduction of ARR compared with the year before DMF initiation was observed (from 1.5 to 0.2, $p < 0.0001$). AEs were reported in 18 patients (90%) during the 24-week follow-up (the most frequent being flushing, observed in 25% of the patients). However, no patient experienced severe AEs (SAEs) leading to DMF discontinuation.

A phase III, double-blind, placebo-controlled, three-arm randomized controlled trial (RCT) aiming on evaluating safety and efficacy of DMF compared with placebo and pegylated IFN β-1a is currently recruiting patients (ClinicalTrials.gov Identifier: NCT03870763).

Teriflunomide

The results of a 96-week, double-blind, randomized, placebo-controlled phase III RCT evaluating efficacy, safety, and PK of teriflunomide in 109 POMS aged 10–17 years (TERIKIDS) have been presented at ECTRIMS 2020 (27).

Teriflunomide numerically reduced the risk of clinical relapse by 34% relative to placebo, but this did not reach statistical significance ($p = 0.29$). Conversely, teriflunomide reduced the risk of the time of clinical relapse or switch due to high MRI activity by 43% ($p = 0.041$) and the appearance of Gd-enhancing and new/enlarged T2 hyperintense lesions compared with placebo (1.9 vs. 7.5, $p < 0.0001$ and 4.7 vs. 10.5, $p = 0.0006$, respectively). Three SAEs were observed [pulmonary tuberculosis, acute pancreatitis, and alanine aminotransferase (ALT) increase].

The open-label extension of this RCT is currently in progress. An interim analysis on 100 patients demonstrated that the time to first confirmed relapse and the 24-week sustained disability progression were numerically lower for the teriflunomide/teriflunomide (T/T) arm compared with the placebo/teriflunomide (P/T) arm but did not reach the significance (46.0 vs. 64.0%, $p = 0.098$ and 17.4 vs. 29.3%, respectively).

Furthermore, new/enlarged T2 hyperintense and Gd-enhancing lesions were significantly reduced in the T/T arm (6.3 vs. 13.0, $p = 0.0006$, and 1.9 vs. 4.2, $p = 0.0106$, respectively). The incidence of AEs was higher in the P/T arm compared with the T/T arm during the open-label period (82.7 vs. 68.0%, respectively). Two SAEs were recorded (acute pancreatitis; increased amylase and lipase). Although teriflunomide proved to be well-tolerated and disclosed a manageable safety profile, the SAEs mentioned above suggest that if this medication is ultimately approved, an adequate surveillance of biological

TABLE 1 | Observational and clinical studies on first-line immunomodulatory therapies in pediatric multiple sclerosis.

Treatment	First author	Year	Trial design	Number of patients	Clinical findings	MRI findings	Adverse effects
IFN β	Mikaeloff et al. (20)	2001	Prospective study (median FU 1 year)	16 RRMS	Stable EDSS	Stable T2 lesions in 3 patients ↑ T2 lesions in 6 patients	Fever: 50% Headache: 28% Myalgia: 17% Fatigue: 5%
	Ghezzi et al. (21)	2005	Prospective study (mean FU 34.4 ± 25.0 months in Rebif-Betaferon, 23.3 ± 13.4 months in Avonex)	18 RRMS Rebif-Betaferon 38 RRMS Avonex	↓ ARR (3.29 ± 2.3 at b vs. 0.86 ± 0.8 at FU in Rebif-Betaferon) ↓ ARR (2.49 ± 1.4 at b vs. 0.49 ± 0.5 at FU in Avonex)	–	<10% of patients
	Banwell et al. (19)	2006	Retrospective study (mean FU 29.2 months)	43 RRMS	–	–	Flu-like syndrome (35%), abnormal liver function test (26%), and injection site reaction (21%) No SAE
	Tenambaum et al. (18)	2006	Open-label, prospective, single-center study (6 years)	24 RRMS	↓ ARR (1.7 at b vs. 0.04 at y5)	–	96% of patients (58% flu-like syndrome, 17% myalgia/arthralgia)
	Mikaeloff et al. (22)	2008	Prospective study (mean FU 5.5 years)	197 RRMS	↓ Rate of the first attack during the first year of treatment (hazard ratio: 0.31, 95% confidence interval: 0.13–0.72) and 2 years (0.40, 0.20–0.83)	–	–
GA	Kornek et al. (23)	2003	Prospective study (24 months)	7 RRMS	2/7 relapse free EDSS stable	↓T2 lesions in 2/7 ↑ T2 lesions in 3/7	–
	Ghezzi et al. (21)	2005	Prospective study (mean FU 33.3 ± 27.6 months)	9 RRMS	↓ ARR (2.89 ± 1.3 at b vs. 0.26 ± 0.36 at FU)	–	–
Dimethyl fumarate	Makhani et al. (24)	2016	Retrospective study (median FU 15 months)	13 RRMS	↓ ARR in 8/13 children	New T2 lesions in 33%, one of whom had been non-adherent to treatment	8/13 (62%) flushing 7/13 (54%) GI discomfort, 3/13 rash (23%), 2/13 malaise (15%)
	Alroughani et al. (25)	2018	Phase II, single arm, multicenter, open label (FOCUS) (24 weeks)	22 RRMS	↓ ARR (1.5 at b vs. 0.8 at 24 weeks)	↓ New/enlarged T2 hyperintense lesions (−1.5 at b vs. −8.0 at 24 weeks, p = 0.009)	73% of patients (abdominal pain, nausea, vomiting, flushing) No SAE
	Alroughani et al. (26)	2020	Extension study of FOCUS (CONNECTED) (96 weeks)	20 RRMS	↓ ARR (1.5 at b vs. 0.2 at 120 weeks, $p < 0.0001$)	12/17 (71%) no new/enlarged T2 hyperintense lesions from w16 to w24	90% AEs (flushing in 25%) No SAE
Teriflunomide	Chitnis et al. (27)	2020	Double-blind, randomized, placebo-controlled Phase III (TERIKIDS) (96 weeks)	109 RRMS	↓ Clinical relapse by 34% ($p = 0.29$) ↓ Time of relapse or switch due to high MRI activity by 43% ($p = 0.041$)	↓ Gd+ and new/enlarged T2 hyperintense lesions (1.9 vs. 7.5, $p < 0.0001$ and 4.7 vs. 10.5, $p = 0.0006$, respectively) compared with Pbo	88.1% AEs 3 SAEs (pulmonary tuberculosis, acute pancreatitis, ALT increase)
	Chitnis et al. (27)	2020	Open label extension (TERIKIDS) (96 weeks)	100 RRMS	↓ First confirmed relapse and 24-week sustained disability progression compared with Pbo/Ter (46.0 vs. 64.0% and 17.4 vs. 29.3%).	↓ Gd+ and new/enlarged T2 hyperintense lesions in the Ter-treated group (1.9 vs. 4.2, $p = 0.0106$, 6.3 vs. 13.0, $p = 0.0006$)	↑AEs in the Pbo/Ter compared with the Ter/Ter (82.7% vs. 68.0%) 2 SAEs (acute pancreatitis, amylase and lipase increased)

B, baseline; EDSS, Expanded Disability Status Scale; FU, follow-up; GA, glatiramer acetate; IFN β, interferon β; Pbo, placebo; RR, relapse rate; RRMS, relapsing–remitting multiple sclerosis; Ter, teriflunomide.

parameters in treated patients is necessary. Finally, the placebo-controlled design for TERIKIDS raised some concerns on its inherent limiting ability to compare clinical trials with one another (33). Further studies with an active comparator and other MRI endpoints (e.g., annualized rate of brain atrophy) are warranted to further define the efficacy and safety profile of teriflunomide.

SECOND-LINE THERAPIES

Orals

Fingolimod

The randomized, double-blind, phase III RCT PARADIG*MS* (34) compared fingolimod with intramuscular (i.m.) IFN β-1a in a cohort of 215 patients (34). This study demonstrated a significant reduction of the adjusted ARR in the 107 patients treated with fingolimod compared with those treated with IFN β-1a (0.12 vs. 0.67, $p < 0.001$). Furthermore, new/enlarged T2 hyperintense lesions were reduced in fingolimod patients compared with IFN β-1a (4.39 vs. 9.27, $p < 0.001$). AEs occurred in 88.8% of patients who received fingolimod and in 95.3% of those who received IFN β-1a. SAEs occurred in 18 patients (16.8%) in the fingolimod group and included four cases of infections (appendicitis, cellulitis, gastrointestinal infection, oral abscess, viral infection, and viral pharyngitis) and six (5.6%) cases of convulsions [vs. 1 (0.9%) in the IFN β-1a arm]. Other SAEs in the fingolimod group included single cases (0.9%) of agranulocytosis, arthralgia, autoimmune uveitis, bladder spasm, dyspepsia, dysuria, elevated alanine aminotransferase level, elevated γ-glutamyl transferase level, gastrointestinal necrosis (intussusception or necrotic bowel), head injury, humerus fracture, hypersensitivity vasculitis, migraine, migraine without aura, muscular weakness, rectal tenesmus, second-degree atrioventricular block, and small-intestinal obstruction and two cases of leukopenia (1.9%).

In a secondary analysis on MRI parameters (16), fingolimod demonstrated a reduction in the annualized rate of formation of new/enlarged T2 hyperintense lesions (52.6%, $p < 0.001$), number and annualized rate of T1 hypointense lesions (66% and $p < 0.001$; 62.8% and $p < 0.001$, respectively), and combined unique active lesions (60.7%, $p < 0.001$) vs. IFN β-1a. Furthermore, the percent increase in T2 (18.4 vs. 32.4%, $p < 0.001$) and Gd-enhancing T1 lesion (−72.3 vs. 4.9%, $p < 0.001$) volumes and the annualized rate of brain atrophy (−0.48 vs. −0.80%, $p = 0.014$) were lower with fingolimod vs. IFN β-1a.

Prior to PARADIG*MS*, data from two small observational studies were available. In a study on 23 highly active POMS patients treated with fingolimod (35), a significant decrease in ARR (75%), new T2 hyperintense (81%), and Gd-enhancing (93%) lesions compared with pretreatment was reported. Noteworthy, seven patients with very high disease activity at clinical presentation experienced disease re-activation when switching from NTZ to fingolimod after a 2-month washout period. Six of them were further switched to alemtuzumab during the follow-up. These data suggested that very active POMS does not probably respond to fingolimod and needs to be treated with more potent immunosuppressive drugs. No SAE was reported in this study. In a second study conducted on 17 POMS treated with fingolimod for an average of 8.6 months, the majority of the patients remained free of clinical or radiological activity. An improvement in Expanded Disability Status Scale (EDSS) score compared with pretreatment was also observed (range of change −3 to −0.5) (36).

All together, these observations suggest fingolimod to be effective and well-tolerated in most POMS.

Cladribine

No data are currently available on cladribine-treated POMS.

Infusion Therapies

Natalizumab

Following the approval of fingolimod, in Italy, NTZ has been approved for POMS aged 12–17, having active and rapidly evolving MS not responsive to fingolimod, or in the presence of contraindications or persistent side effects due to fingolimod. No RCT has been conducted on POMS to date, but several observational studies have focused on NTZ efficacy and safety in these patients (see **Table 2** for a comprehensive overview).

The largest cohort of NTZ-treated POMS included 101 patients, having a mean age at onset of 12.9 ± 2.7 years and a mean EDSS of 2.6. Sixty-six percent had been previously treated with first-line DMDs. Patients were treated with NTZ for a mean period of 34.2 ± 18.3 months (40). Compared with baseline, a significant reduction in the mean ARR (from 2.3 ± 1.3 to 0.1 ± 0.3, $p < 0.001$) and new Gd-enhancing lesions (82.8 vs. 10.6%, $p < 0.001$) were observed at the end of the follow-up (40). Moreover, the no evidence of disease activity (NEDA)-3 status (i.e., no clinical relapses, no increase in disability, and no MRI activity) was achieved in 58% patients. These observations were confirmed in other observational studies (37–39, 41, 47). Recently, we studied the achievement of the NEDA-3 plus status, which includes cognition in the NEDA-3 (the cognitive decline was defined as a decrease of at least four points in the Symbol Digit Modality Test), in 20 naïve POMS treated with NTZ. We observed that 17/20 (85%) and 18/20 (80%) of patients achieved NEDA-3 plus at months 12 and 24, respectively (41).

NTZ was found to be well-tolerated and safe in POMS patients. In some studies, no clinical AE was experienced (39, 47). An open-label, multiple-dose, multicenter prospective study aimed to evaluate the PK/pharmacodynamic (PK/PD) profile, safety, and tolerability of NTZ in POMS, aged 10–18 years, demonstrated similar profiles in adults and pediatric patients in the short term (48). Longer studies, also including a larger number of younger subjects (aged 10–12 years), are required to further inform about long-term PK and PD parameters in POMS. Recently, some concerns about immunosuppression in MS were raised during the coronavirus disease 2019 (COVID-19) pandemic. In our experience, NTZ did not expose POMS to a higher risk of SARS-CoV-2 infection or to a clinically overt/severe disease (49).

At present, no cases of progressive multifocal leukoencephalopathy (PML) have been reported in POMS patients treated with NTZ. The prevalence of JCV

TABLE 2 | Observational and clinical studies on second-line immunomodulatory therapies in pediatric multiple sclerosis.

Treatment	First author	Year	Trial design	Number of patients	Clinical findings	MRI findings	Adverse effects
Natalizumab	Kornek et al. (37)	2013	Retrospective study (mean FU 11 months)	20 RRMS	↓ARR (3.7 at b vs. 0.04, $p < 0.001$) ↓ EDSS (2 at b vs. 1; $p < 0.02$)	↓ T2 lesions (7.8 at b vs. 0.5; $p < 0.001$)	50% (headache, asthenia, infections, and hypersensitivity)
	Arnal-Garcia et al. (38)	2013	Retrospective study (mean FU 17 months)	8 RRMS	↓ARR (3 at b vs. 0) ↓ EDSS (3 at b vs. 1)	No Gd+ lesion at FU	3 AEs
	Ghezzi et al. (39)	2013	Retrospective study (mean FU 26 months)	55 RRMS	3 relapses ↓ EDSS (2.7 at b vs. 1.9, $p < 0.001$)	88% free from radiological disease	Transitory AEs in 22/55 patients (headache, upper respiratory disorders, vertigo)
	Ghezzi et al. (40)	2015	Retrospective study (mean FU 26 months)	101 RRMS	↓ARR (2.3 ± 1.0 at b vs. 0.1 ± 0.3, $p < 0.001$) ↓ EDSS (2.6 ± 1.3 at b vs. 1.8 ± 1.2, $p < 0.001$)	T2/Gd+ lesions were observed in 10/91 (10.9 %) patients at 6 months, 6/87 (6.9 %) at 12 months, 2/61 (3.3 %) at 18 months, 2/68 (2.9 %) at 24 months, 3/62 (4.8 %) after 30 months	AEs in 36/101 (headache, upper respiratory disorders, vertigo)
	Margoni et al. (41)	2020	Retrospective study (24 months)	20 RRMS	↓ EDSS (2.6 ± 0.7 at b vs. 1.5 ± 0.5, $p < 0.0001$)	2 patients new T2 lesions	No AE
Alemtuzumab	Margoni et al. (42)	2019	Case series (mean FU 3.9 years)	5 RRMS	No relapse 3 patients had clinical improvement	No MRI activity	No SAE
	Jure Hunt et al. (43)	2020	Case series	2 RRMS	No relapse EDSS improvement	No MRI activity	No SAE
Rituximab	Dale et al. (44)	2014	Multicenter retrospective study (mean FU 3.3)	4 RRMS	Benefit: 1 definite, 0 probable, 1 possible, 1 none, 1 worsening	–	12.5% AEs (anaphylaxis in 3, 11 7.6% infections, 2 deaths)
	Salzer et al. (45)	2016	Retrospective study (median FU 23.6 months)	14 RRMS	EDSS stable in 93% of patients	1 lesion detected on MRI	No AE
Cyclophosphamide	Makhani et al. (46)	2009	Retrospective study (mean FU 2.7 years)		↓ARR (from 3.8 to 1.1, >70%) ↓ or stable EDSS in 83%	↓ T2 and gad+ lesions (>75%)	Nausea and vomiting: 88%
Fingolimod	Chitnis et al. (34)	2018	Randomized, double-blind, phase III trial (PARADIG*MS*) (24 months)	215 RRMS	↓ ARR (0.12 FTY vs. 0.67 IFN β, $p < 0.001$)	↓ T2 lesions (4.39 FTY vs. 9.27 IFN β, $p < 0.001$)	SAEs in 16.8% (infection, leukopenia, 6 patients had convulsions)
	Huppke et al. (35)	2019	Retrospective study (mean FU 31 months)	23 RRMS	↓ 75% ARR	↓ 81% T2 lesions	-
	Arnold et al. (16)	2020	Randomized, double-blind, phase III trial (PARADIG*MS*) (24 months)	215 RRMS	–	↓52.6% T2 lesions in FTY vs. IFN β ($p < 0.001$) ↓ 66% T1 lesions in FTY vs. IFN β ($p < 0.001$)	–

B, baseline; EDSS, Expanded Disability Status Scale; FTY, fingolimod; FU, follow-up; IFN β, interferon β; Pbo, placebo; RR, relapse rate.

seropositivity in POMS was reported to be higher [ranging from 39 to 51.6% (39, 40, 50)] than in the general healthy pediatric population [21% (51)] but lower than in AOMS patients (52).

While tolerability and safety data are reassuring and clearly indicated that NTZ can be considered the treatment of choice for very active POMS, long-term safety data on larger cohort of patients are needed, especially for evaluating the risk of PML.

Anti-CD20 Therapies: Rituximab and Ocrelizumab

The first anti-CD20 monoclonal antibody (MAb) used in MS is the chimeric antibody rituximab, currently prescribed off-label in case of a highly active disease. In POMS, rituximab is one of the most used second-line immunosuppressive therapies.

In a case series of 14 POMS treated with rituximab for a median period of 23.6 months, a stable disease was observed in 13/14 patients (93%) with no SAE reported (45). However, in a cohort of 144 pediatric patients with autoimmune and inflammatory central nervous system (CNS) disorders (among which four POMS), infusion AEs were recorded in 18/144 (12.5%), including grade 4 (anaphylaxis) in three; 11 patients (7.6%) had infections, including two with grade 5 (death) and two with grade 4 (disabling) (44). Furthermore, in a cohort of 1,019 pediatric patients with MS and clinically isolated syndromes, side effects and tolerability were similar to those reported in adults (14, 17). No rituximab-related PML cases have been reported in POMS. In the position paper of the International Pediatric MS Study Group, the potential benefit of rituximab was highlighted, but the need for a better evaluation of the optimal dosing, and the safety and efficacy profile were also stressed (13, 53).

Currently, there are no published reports of ocrelizumab use in POMS. An RCT evaluating the PK/PD and the efficacy of ocrelizumab 600 mg i.v. (300 mg i.v. if body weight $<$ 40 kg) in POMS is in progress (https://clinicaltrials.gov/ct2/show/NCT04075266).

Alemtuzumab

A phase III RCT aimed to evaluate safety and efficacy of alemtuzumab in POMS patients who have failed at least two DMDs is in progress (NCT03368664). Some observational reports showed that alemtuzumab was relatively well-tolerated and effective in POMS; indeed, no serious infusion reactions, infections, or relapses were recorded during the follow-up (42, 43).

Cyclophosphamide

Several studies have suggested that cyclophosphamide treatment may be most beneficial in younger adult MS patients (54–56). A single, multicenter retrospective study of 17 cyclophosphamide-treated POMS with a mean follow-up of 2.7 years showed a reduction in ARR (from 3.8 to 1.1), and stabilization of disability scores assessed 1 year after treatment initiation in most patients (83%) compared with baseline. Furthermore, a reduction in new/enlarged T2 hyperintense lesions and Gd-enhancing lesions was observed (100 vs. 75% and 91 vs. 67%, respectively) (46). The most frequent AEs included vomiting, transient alopecia, osteoporosis, and amenorrhea. One patient developed bladder carcinoma that was successfully treated (46).

Mitoxantrone

Given the risk of cardiotoxicity and acute myeloid leukemia (57), the use of mitoxantrone in POMS is discouraged.

FUTURE PERSPECTIVES AND CONCLUSIONS

POMS is a rare but severe form of MS, characterized by a more prominent clinical and radiological activity and younger age at reaching cognitive and physical disability milestones, even when treated with first-line DMDs. Furthermore, adherence to injectable DMDs is an important determinant of treatment efficacy in real-world clinical settings. Off-label use of newer DMDs is increasing in POMS and retrospective studies, case series, and phase II trials, indicate that this approach appears to be highly effective and safe in children. However, great efforts should be devoted to design RCTs in POMS. The low number of patients and the potentially severe long-term prognosis strongly indicate the necessity of identifying new and adequately powered MRI targets (e.g., annualized rate of brain atrophy) of treatment as well as more specific clinical (especially cognitive) endpoints for this peculiar MS population. Moreover, the harmonization of regulatory requirements for testing of new treatment should be prioritized to compare clinical trials with one another (33).

Although more data are needed before standardizing the use of first- and second-line newer therapies in POMS, the treatment paradigm implies to design therapeutic strategies based on highly effective drugs. Thus, the approval of fingolimod and the availability of high-efficacy Mab constitute a real step-forward in POMS management.

AUTHOR CONTRIBUTIONS

MM, PG, FR, and PP: design and drafting of the manuscript. All authors contributed to the article and approved the submitted version.

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Altered Immune Phenotypes and *HLA-DQB1* Gene Variation in Multiple Sclerosis Patients Failing Interferon β Treatment

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Background: Interferon beta (IFNβ) has been prescribed as a first-line disease-modifying therapy for relapsing-remitting multiple sclerosis (RRMS) for nearly three decades. However, there is still a lack of treatment response markers that correlate with the clinical outcome of patients.

Aim: To determine a combination of cellular and molecular blood signatures associated with the efficacy of IFNβ treatment using an integrated approach.

Methods: The immune status of 40 RRMS patients, 15 of whom were untreated and 25 that received IFNβ1a treatment (15 responders, 10 non-responders), was investigated by phenotyping regulatory $CD4^+$ T cells and naïve/memory T cell subsets, by measurement of circulating IFNα/β proteins with digital ELISA (Simoa) and analysis of ~600 immune related genes including 159 interferon-stimulated genes (ISGs) with the Nanostring technology. The potential impact of HLA class II gene variation in treatment responsiveness was investigated by genotyping *HLA-DRB1, -DRB3,4,5, -DQA1*, and *-DQB1*, using as a control population the *Milieu Interieur* cohort of 1,000 French healthy donors.

Results: Clinical responders and non-responders displayed similar plasma levels of IFNβ and similar ISG profiles. However, non-responders mainly differed from other subject groups with reduced circulating naïve regulatory T cells, enhanced terminally differentiated effector memory $CD4^+$ T_{EMRA} cells, and altered expression of at least six genes with immunoregulatory function. Moreover, non-responders were enriched for *HLA-DQB1*

genotypes encoding DQ8 and DQ2 serotypes. Interestingly, these two serotypes are associated with type 1 diabetes and celiac disease. Overall, the immune signatures of non-responders suggest an active disease that is resistant to therapeutic IFNβ, and in which $CD4^+$ T cells, likely restricted by DQ8 and/or DQ2, exert enhanced autoreactive and bystander inflammatory activities.

Keywords: multiple sclerosis, type I interferon, T cells, interferon-stimulated genes, HLA class II genes, immune phenotypes, blood biomarkers

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune and inflammatory disease of the central nervous system (CNS), leading to axonal demyelination, neuronal dysfunction, and neurodegeneration. These damages result from repeated attacks of several innate and adaptive immune cell types which have crossed blood–CNS barriers and exert a pathogenic activity together with resident activated microglia and macrophages (1, 2). Relapsing-remitting MS (RRMS), the most common form of the disease mainly affecting young adults with a female to male ratio of ~2.5–3 (3, 4), has been treated by type I interferon beta (IFNβ) for nearly three decades. To date, IFNβ and its highly stable pegylated form remain widely prescribed as a first-line disease-modifying therapy. Recent meta-analyses performed in a 'real-world' setting have confirmed the long-term efficacy of IFNβ in delaying disability and disease progression, and decreasing mortality risk (5, 6). However, ~30% of patients are or become non-responsive to the treatment while still being potentially subject to side effects (7). Given the increasing number of novel and targeted therapeutic options for RRMS, including injectable or oral first-line therapies (8, 9), it is critical to identify IFN treatment response biomarkers and better understand the mechanisms of disease onset and pathogenesis.

Susceptibility to MS is under the influence of genetic heritability [~50% of overall risk (10)] as well as of environmental and lifestyle factors (11). Non-genetic factors such as gender, Epstein–Barr virus infection, smoking, low vitamin D, or adolescent obesity are considered to contribute to, or to potentially trigger, disease onset. Recent large-scale genome-wide association studies uncovered up to 233 independent genetic associations with MS, 30 of these mapping across the MHC region (10, 12, 13). Most variants are related to the adaptive immunity, with a group of HLA class II allelic risk variants, dominated by *HLA DRB1*15:01* (OR~3.9) (13–15). Moreover, the interaction between the latter variant and non-genetic risk factors leads to a much higher susceptibility to develop MS (11).

Immune dysregulation is a hallmark of MS pathogenesis. Key players are $CD4^+$ and $CD8^+$ T cells that drive autoreactive and deleterious responses within the CNS while also promoting activation of myeloid and B cells. Notably, the association between specific HLA class II variants and MS points not only to the critical role of autoreactive $CD4^+$ T cells whose T cell receptor is restricted by these variants, but also to antigen-presenting cells expressing the variants and among them B cells. In fact, B cells were reported to drive T cell autoproliferation in RRMS patients bearing the HLA-DR15 haplotype and to contribute to autoimmune and pro-inflammatory cytokine responses (16–18). Their key role in MS pathophysiology is demonstrated by the impressive therapeutic effect of anti-CD20-based treatments (19).

Autoreactive cells can be activated by CNS and non-CNS derived antigens through various mechanisms such as molecular mimicry following viral reactivation, recognition of neo-autoantigens and/or bystander activation (1, 17, 20). In addition, autoreactivity and pro-inflammatory T cell responses can be promoted by dysfunctional regulatory mechanisms, such as those involving peripheral $CD4^+$ regulatory T cells (Tregs) and type 1 regulatory T cells (Tr1s) (21–23). Reduced thymic output of naïve Treg cells in RRMS patients may also indicate a defect of central tolerance mechanisms or an alteration of Treg homeostasis (24, 25). Conversely, effector $CD4^+$ T helper subsets such as Th17 and Th1/Th17 are increased in the periphery, display enhanced pro-inflammatory cytokine and gene expression programs, and may be more resistant to Treg activity (26–28). Circulating cytotoxic $CD28^-$ $CD4^+$ T cells were also found to be expanded and to correlate with disease activity (29–31). Finally, $CD8^+$ T cells are considered to play an important role in MS pathogenesis. These cells are enriched in cerebrospinal fluid (CSF) and CNS lesions and can be detected in the periphery with an activated effector/migratory phenotype (2, 32).

The therapeutic activity of IFNβ is largely attributed to the induction of a global anti-inflammatory program although its direct antiviral and pro-apoptotic activities may also contribute (20, 33, 34). Various mechanisms of action involving almost all immune cell types have been proposed. Among the major immunomodulatory effects of IFNβ treatment are the restoration or induction of regulatory T and B cell responses (22, 35–37), the reduced differentiation of inflammatory Th17 and B cells, and the attenuation of monocyte activation (33, 36, 38, 39). Many studies have also shown the strong promoting activity of IFNα/β on IL-10 expression (33, 40, 41).

Different findings have been reported on the frequency of circulating Treg, naïve and memory $CD4^+$ and $CD8^+$ T cell subsets in untreated and IFN-treated RRMS. The rapid evolution of phenotyping procedures, heterogeneous clinical features of patients, and the duration of IFN treatment may account for data variability. To date, no single blood biomarker can predict the therapeutic efficacy of IFNβ nor disease activity (42). On this basis, we have explored the possibility that a combination of

cellular and molecular blood biomarkers may prove valuable for patient stratification. We compared the immune status of IFN responders and non-responders with that of untreated RRMS patients and healthy donors. We used an integrated approach by analyzing circulating $CD4^+$ and $CD8^+$ T cell subsets, mRNA expression of IFN-stimulated genes (ISGs) and other immune genes, and allelic variation of HLA class II genes. Results of this exploratory study show converging immune signatures in non-responders, suggesting dysregulation of the immune response and higher disease activity in these patients.

MATERIALS AND METHODS

MS Patients and Healthy Controls

RRMS patients of Caucasian ethnicity were diagnosed according to the 2010 McDonald criteria (43) and were recruited at the hospital Pitié-Salpêtrière, Paris. Untreated patients did not receive any immunomodulatory or immunosuppressive treatment at least 3 months prior to blood collection. Patients treated with IFNβ1a Avonex (30 µg, IM, once a week) were considered as non-responders if they experienced one or more relapses during the last year of treatment. Blood was collected at least two days after IFN administration on lithium heparin-tubes for flow cytometry and gene expression assays and on EDTA-tubes for genomic DNA extraction and plasma cytokine analysis. Exclusion criteria were disease activity, steroidal anti-inflammatory or immunosuppressive drugs, antibiotics, acute or chronic infectious diseases, autoimmune and inflammatory diseases other than MS, and cancer. The study was approved by the CPP 2014/17NICB and the CNIL MMS/CWR/AR1411558. Healthy controls were from the CoSImmGEn cohort of the ICAReB platform (Clinical Investigation and Access to BioResources, Institut Pasteur) and EFS (Etablissement Français du Sang, Paris). For HLA class II genotyping, controls were from the *Milieu Interieur* (MI) cohort composed of 1,000 healthy donors, French citizens with metropolitan French origin for three generations (https://clinicaltrials.gov; NCT01699893 and NCT03905993, ANR-10-LABX-69-01). MI healthy controls and RRMS patients provided written informed consent including genetic analyses. Clinical and demographic characteristics of participants are shown in **Table 1**.

Flow Cytometry

Blood (200 µl, sampling <6 h) was washed with PBS at 1,500 rpm, 5 min, room temperature (RT). Cell pellet was incubated with antibodies premix for 20 min at RT then with viability dye (500 µl, 1/1,000) at 4°C for 30 min (eF506, eBioscience). After washing cells with cold PBS, red cells were lysed and leukocytes were fixed in 2 ml of lysis buffer (BD biosciences) for 15 min at RT in the dark. Stained cells were acquired in 200 µl PBS using MACSQuant® Analyzer 10 (Miltenyi Biotech). $CD4^+$ and $CD8^+$ T cell subsets were analyzed using FlowJo™10 by gating on $CD3^+$ cells after exclusion of dead cells and doublets. The following antibodies were used in two eight-color panels: anti-CD3-Vioblue (BW264/5), anti-CD4-APC-vio770 (VIT4), anti-CD45RA-FITC (T6D1, anti-CD8b-PE-Cy7 (SIDI8BEE), anti-CD25-PerCPeF710 (CD25-4E3), anti-CD27-PerCPvio700 (M-T271), anti-HLADR-PE (clone AC122), anti-CD127-APC (MB15-18C9) (Miltenyi Biotec, eBioscience). Treg, $CD4^+$ and $CD8^+$ T cell subsets were gated using the appropriate FMO control.

IFNα/β Measurement

Plasma was obtained by centrifugation of blood at 1,500 rpm, 5 min, RT, and frozen at −80°C. IFNα and IFNβ plasma levels were measured in duplicate by single molecule array (Simoa, Quanterix) digital ELISA using homebrew assays in which capture and detection monoclonal antibodies were 8H1 and 12H5 (Immunoqure AG) for IFNα (41), and 710322-9 and 710323-9 IgG1 for IFNβ (PBL Assay Science) (44).

mRNA Gene Expression

Prewarmed blood (1 ml) was incubated into TruCulture tubes (Myriad RBM) under a final volume of 3 ml, at 37°C, for 22 h. Trizol LS (Qiagen)-lyzed cell pellets were thawed on ice at least 1 h, vortexed twice at 2,250 rpm for 5 min and centrifuged at 3,500 g for 5 min at 4°C. Total RNA was extracted using nucleospin miRNA kit (Macherey-Nagel), eluted in 30 µl RNase-free water and aliquots were stored at −80°C. RNA quality was measured with NanoDrop™2000 (ThermoFisher) and the Agilent 2100 bioanalyzer (RNA 6000 Nano kit). mRNAs were quantified using the Nanostring (nCounter) technology. For that, RNA (100 ng, 20 ng/µl) was hybridized on 12-sample strips at 65°C for 16 h using the Human immunology_V2 (579 genes) codeset and a custom 9 gene codeset *(ADAR1, HERC5,*

TABLE 1 | Clinical and demographic characteristics of RRMS patients and healthy controls.

	RRMS patient groups			Control
	Untreated	**IFN responders**	**IFN non-responders**	**Healthy donors**
Number	15	15	10	14
Female sex (%)	73	86	80	64
Age (years)	40 (29–55)	41 (19–57)	34 (19–55)	37.5 (22–53)
Disease duration (years)	11 (2–24)	12 (2–22)	7.5 (2–25)	–
EDSS	1 (0–3)	0 (0–2.5) *[a]	1.8 (0–4) *[b]	–
Treatment duration (years)	–	7 (2–13)	2.5 (0.7–14)	

Values shown as median and (range).
*[a]Responders vs Untreated, [b]Non-responders vs Responders. *Mann–Whitney test, $p < 0.05$.*

ISG15, IRF2, IRF9, RIG-I, HLA-DQA1, HLA-DQB1, HLA-DRB4). RNA/probe complexes were immobilized on a cartridge with the 'Prep station' and quantified by the 'nCounter system' within 555 fields of views. Data were normalized to internal positive and negative controls. *TBP, POLR2A, SDHA, G6PD* housekeeping genes were determined with the algorithm 'gNorm' (nSolver software V4). Data were analyzed using a background threshold of 15 counts and were log2-transformed for Qlucore Omics Explorer analysis (V3.4). Geomean scores of ISG were determined for each patient according to (45) after normalization of mRNA counts from human_V2 and custom codesets.

HLA Class II Genotyping

Genomic DNA was extracted from 2 ml EDTA-blood of RRMS patients using the Nucleon BACC3 kit (GE-Healthcare) according to the manufacturer's instructions. Briefly, blood cells were lysed at RT, and pellet was stored at −80°C. Precipitated DNA was airdried for 10 min, resuspended in DNAse/RNAse free water overnight at 4°C and quantified using Qubit dsDNA HS Assay Kit and Qubit 4 Fluorometer (Invitrogen). Class II *HLA-DQA1*, *DQB1*, *DRB1*, and *DRB3/4/5* genotypes were determined by single molecule real time sequencing of exons 2–6 and introns 2–5 at 8× resolution (Histogenetics, USA). Genotypes were converted to serotypes according to the 2010 nomenclature of HLA system (46). *HLA-DRB1, -DQA1* and *-DQB1* typing data of 1,000 healthy donors were a resource of the *Milieu Interieur* consortium. Alleles were imputed at four-digit resolution from the analysis of 5,699,237 SNPs using SNP2HLA v1.0370 (47).

Statistical Analyses

One-way ANOVA Kruskal–Wallis with Dunn's correction for multiple comparisons or Mann–Whitney test was utilized for scatter bar plots showing flow cytometry, gene expression and IFNα/β level data (GraphPAD Prism 8). ANOVA multigroup comparison F-test was used for analysis of gene expression shown as heatmaps (Qlucore Omics Explorer V3.4). The distribution of HLA binding and non-binding probes (Nanostring assays) was analyzed using a generalized linear model of the binomial family followed by pairwise comparisons among groups using Tukey-like correction. HLA typing data were analyzed by pairwise comparisons of frequencies between the *Milieu Interieur* data set (n = 1,000 healthy donors) and other groups using a Fisher's test. P-values were adjusted to account for multiple testing (Center of Bioinformatics, Biostatistics and Integrative Biology (C3BI, Institut Pasteur).

RESULTS

Peripheral Blood T Cell Phenotypes in IFNβ-Treated RRMS Patients

We investigated cellular and molecular immune phenotypes in IFNβ-treated RRMS patients who were clinically defined as responders (Resp, n = 15) and non-responders (NR, n = 10). All patients received IFNβ1a IM (Avonex, 30 μg, once a week), which minimized possible variation of the treatment response due to dose, frequency, and type of IFNβ. Controls were untreated patients (UT, n = 15) and healthy controls (HC, n = 14) of similar age and sex ratio. All patients presented with a mild disease score (medians EDSS, 0–1.8) and were of Caucasian ethnicity (**Table 1** and *Materials and Methods*).

Number and frequency of regulatory T cells (Tregs), conventional $CD4^+$ T cells (Tconvs), and naïve/memory $CD4^+$ and $CD8^+$ T cell subsets were monitored in whole blood by flow cytometry in the four subject groups (**Figures 1A, B** for gating strategies and **Supplementary Tables 1**, **2**). Based on differential expression of CD25 and CD127, we found that IFN responders displayed a significantly higher number of Treg and Tconv cells as compared to healthy donors ($p < 0.05$ and $p < 0.005$, respectively) with unchanged Treg/Tconv ratio (**Figure 1A**). In line with this, responders showed an increased number and frequency of total $CD4^+$ T cells while the frequency of $CD8^+$ T cells tended to be decreased in all patient groups (**Supplementary Figure 1**). Additional gating on Treg cells using CD45RA and HLA-DR identified naïve, memory and activated/terminally differentiated subsets, respectively equipped with enhanced suppressive activity potential (48). Non-responders showed a significant reduction in the number and frequency of naïve Tregs as compared with responders or healthy controls ($p < 0.05$) and decreased Treg/Tconv ratio (**Figure 1A**), while responders showed a trend towards increased number and frequency of memory and activated Treg subsets (**Supplementary Figure 2D**). Finally, untreated patients tended to have decreased number and frequency of activated Tregs (**Supplementary Figure 2D**).

Naïve/memory $CD4^+$ and $CD8^+$ T cells were analyzed based on differential expression of CD45RA and CD27 (**Figure 1B**). Alterations in numbers and frequencies of naïve, central (CM) and effector memory (EM) T cell subsets were moderate among subject groups (**Supplementary Figures 2A, B** and **Supplementary Tables 1**, **2**). However and notably, non-responders displayed a higher number and frequency of terminally differentiated effector memory cells ($CD4^+$ T_{EMRA}) than other subject groups, in particular with age as compared to responders ($p < 0.05$ for cell number, **Figure 1C**). This was not the case for $CD8^+$ T_{EMRA}, which suggests a selective accumulation of $CD4^+$ T_{EMRA} cells in non-responders. Of note, given the strong association between $CD4^+$ T_{EMRA} cells and CMV seroprevalence in healthy donors (47), we measured CMV-specific IgG in non-responders and responders but found no significant difference between the two groups (not shown).

Altogether, alterations of circulating T cell subsets were mainly observed in non-responders within the Treg and $CD4^+$ T_{EMRA} compartments although we have to point out that differences were statistically significant only without correcting for multiple comparisons between subject groups.

Circulating IFNα/β Proteins and ISG Expression in IFNβ-Treated Patients

Next, we measured the plasma level of IFNβ and IFNα proteins and ISG expression in blood cells of IFN-treated patients. We first assessed patient adherence to the treatment by measuring

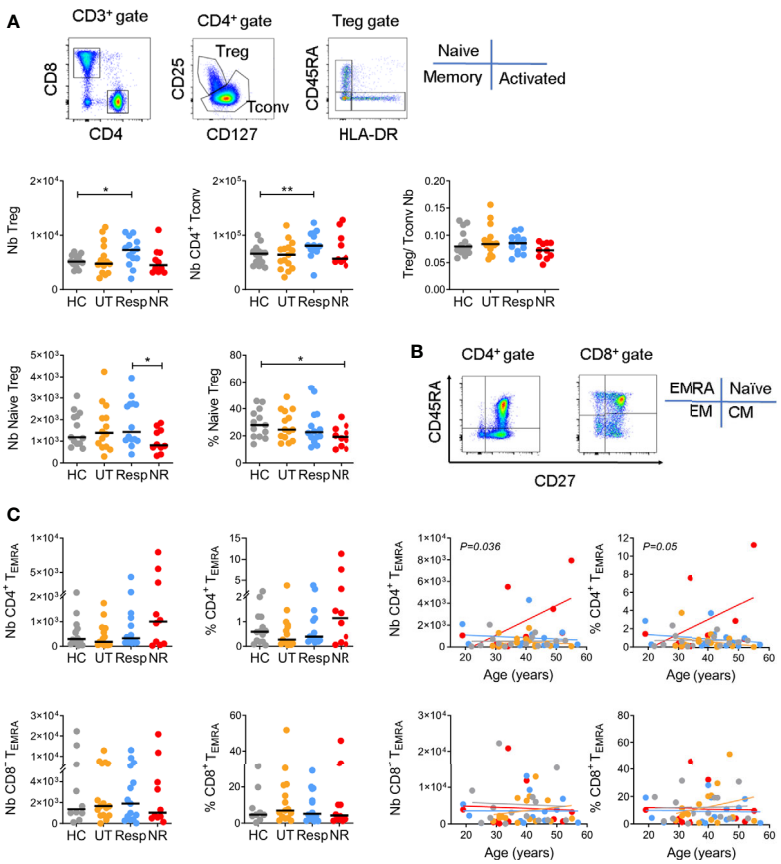

FIGURE 1 | $CD4^+$ T cell phenotypes in IFN responders and non-responders. **(A)** Gating strategy for CD4+ Treg and Tconv subsets. Upper panels: number (Nb) and Treg/Tconv ratios. Lower panels: number and frequency (%) of naive (CD45RA+ HLA-DR-) Tregs. Controls (HC = 13), untreated (UT = 14), responders (Resp = 14), non-responders (NR = 10). **(B)** Gating strategy for naïve (T_{Naive}, $CD45RA^+$ $CD27^+$), central memory (T_{CM}, $CD45RA^-$ $CD27^+$), effector memory (T_{EM}, $CD45RA^-$ $CD27^-$), and terminally differentiated effector memory (T_{EMRA}, $CD45RA^+$ $CD27^-$) subsets. **(C)** Left panels: number and frequency of $CD4^+$ and $CD8^+$ T_{EMRA}. HC = 9–14, UT = 15, Resp = 14, NR = 10. Right panels: linear regressions between T_{EMRA} cells and age. Indicated *p* values were obtained from NR and Resp comparison. **(A, B)** Horizontal lines represent medians. Mann–Whitney test, $^*p < 0.05$, $^{**}p < 0.005$.

circulating IFNβ using a Simoa digital ELISA. The level of IFNβ was similar, not statistically different, between responders and non-responders (median 120 and 75 pg/ml, respectively) and was undetectable in most untreated patients (**Figure 2A**). Circulating endogenous IFNα was also measured using anti-pan-IFNα antibodies of very high affinity (49). Interestingly, non-responders exhibited a moderate but significant increase in IFNα (2.2 fg/ml, 0.47–101) as compared to untreated patients (0.47 fg/ml, 0.47–18.6, $p < 0.05$), and healthy donors (0.47 fg/ml, 0.47–2.9, $p < 0.005$).

ISG induction was investigated in responders and non-responders with the Nanostring digital technology that allows direct mRNA counting by probe hybridization using the human immunology V2 codeset (579 genes) and a custom codeset (nine genes). Baseline mRNA expression of 159 ISGs was compared between the two groups and untreated patients. Among these, 153 ISGs were selected from previous Nanostring data obtained with *in vitro* IFNβ-stimulated blood of 25 healthy donors of the *Milieu Interieur* (MI) cohort (50). Analysis of ISG expression by hierarchical gene clustering and multiple comparison showed the upregulation of nine genes in IFN-treated patients (**Figure 2B**). Among these, three (*MX1*, *ISG15*, and *HERC5*) are canonical type I IFN-induced genes. Consistent with similar levels of circulating IFNβ in responders and non-responders, the three gene ISG scores did not significantly differ in the two groups but was higher than in the untreated patients (**Figure 2C**). A different analytical strategy, based on the high fold change of ISG induction (FC > 10) in MI healthy donors, revealed a 17 gene signature in IFN-treated patients. However, a large proportion of these genes were not canonical ISGs (**Supplementary Figure 3**). Altogether, these results point to *MX1*, *ISG15*, and *HERC5* as good ISG markers for monitoring the IFN biological response in treated patients.

Altered Gene Expression in IFNβ Non-Responders, Including HLA Class II Genes

The expression of genes other than ISGs was analyzed by hierarchical clustering in patient groups (**Figure 3A**). Non-responders differed from responders and untreated

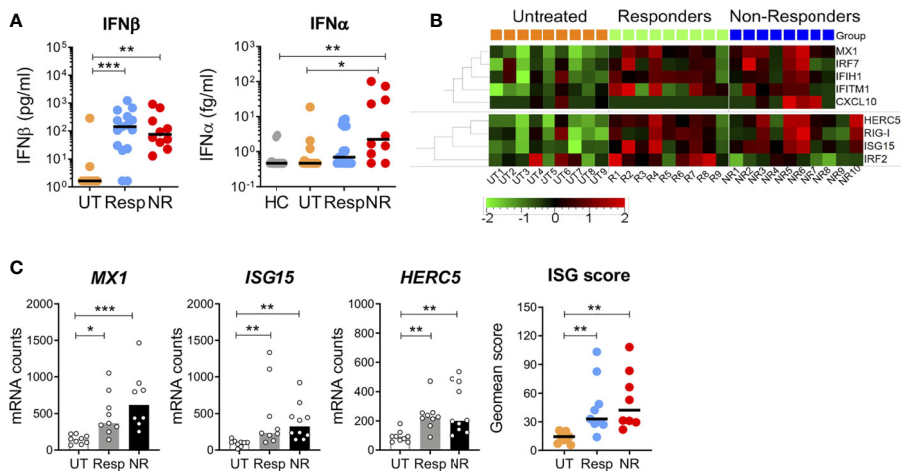

FIGURE 2 | Circulating IFNα/β proteins and interferon-stimulated gene expression in blood cells. **(A)** The plasma levels of IFNα and IFNβ were measured by digital ELISA (Simoa). Lower limits of detection were 0.47 fg/ml and 1.64 pg/ml for IFNα and IFNβ, respectively. UT = 13–15, Resp = 15, NR = 10, HC = 14. **(B)** ISG mRNA expression in blood cells of untreated, responders and non-responders was measured with the Nanostring technology. Heatmap depicts relative mRNA counts after one-way Anova F-test, $p < 0.05$. Upper panel: human V2 codeset, UT = 9, Resp = 9, NR = 8. Lower panel: custom codeset, NR = 10. **(C)** Canonical ISGs (*MX1, ISG15, HERC5*) and geomean scores in patient groups. Kruskal–Wallis test with Dunn's correction for multiple comparisons, *p < 0.05, **p < 0.005, ***p < 0.0005.

patients by 15 downregulated genes (cluster A) and two upregulated genes. Of interest, significant reductions ($p < 0.05$) were observed for genes encoding cell surface receptors involved in the regulation of adaptive and innate immune responses (right scatter bar graphs). For instance, CD46 and TGFBR1 control peripheral induction of Foxp3$^-$ Tr1 and Foxp3$^+$ Treg cells, respectively (23, 48). Interestingly, CD46-mediated induction of Tr1 cells has been reported to be altered in MS (23). IL-4R plays a crucial role in type 2 immunity, notably by promoting Th2 and B cell differentiation and by restraining neutrophil inflammatory function (51). IL-4R was also reported to dampen IL-1 response by upregulating the decoy receptor IL-1R2 (52). Consistently, a lower expression of *IL4R* and *IL1R2* was observed in non-responders. Conversely, the two cytokine encoding genes, *CSF1* and *SPP1*, were upregulated in non-responders. Interestingly, *SPP1* (*OPN*) was found to be more expressed in MS patients and to correlate with disease activity (53). Most genes contained in cluster B were upregulated in both responders and non-responders as compared to untreated patients and thus were mainly indicative of a response to the IFN treatment. Yet, a few genes were significantly more expressed in responders (**Supplementary Figure 4B**).

HLA gene expression was analyzed in patient groups, in particular classical HLA class II (*HLA-DRA, DRB1, DRB3, DRB4, DQA1, DQB1, DPA1, DPB1*) and class I (*HLA-A, B, C*) genes and non-classical class II genes (*HLA-DMA, DMB, DOB*). Based on sequence alignments, most probes were gene but not allele specific. However, *DQA1* and *DRB4* probes were preferentially directed against the **01* allele group, and the *DQB1* probe was mainly directed against the **05* and **06* allele groups. We observed in the three patient groups a binary (all or none) mode of binding of these probes (**Figure 3B**) as well as in healthy controls (not shown). Notably, non-responders strongly differed from responders by a significantly lower frequency of *DQA1*01, DQB1*05,*06* probe binding and a higher frequency of *DRB4*01* probe binding ($p < 0.05$, **Figure 3B**, upper panels). Of note, the lack of binding of both *DQA1* and *DQB1* probes was observed in the same subjects (not shown). We designed additional probes targeting all *DQA1* and *DQB1* allele groups and confirmed mRNA expression in non-responders, even if fewer *DQB1* mRNA counts were observed (**Figure 3B**, lower panels). Altogether, these data indicated differential allelic variation of *HLA-DQA1*, *DQB1*, and *DRB4* in non-responders. To further examine this possibility, IFN-treated patients were genotyped for a series of HLA class II genes.

Increased Carriage of *HLA-DQB1* Variants Encoding DQ8 and DQ2 Serotypes in IFNβ Non-Responders

Typing of *HLA-DQA1*, *DQB1*, *DRB1*, and *DRB3,4,5* was achieved by sequencing at high resolution the available gDNA from responders (n = 10) and non-responders (n = 9). To increase statistical power, HLA typing data from the 1,000 MI healthy donors were utilized as a control reference population. First, the profile of *HLA-DQA1*, *DQB1*, and *DRB1* four-digit allelic variants, obtained by imputation from a genome-wide SNP study of the MI cohort (47), was compared to that reported in a European-American reference cohort of healthy donors [n = 1,899 (54)]. The profiles of allele frequencies were similar between the two cohorts for *DQB1* and *DRB1*, but not for *DQA1*, with the notable absence of *DQA1*01:04* and *DQA1*03:02* variants in the MI study (**Figure 4A**). Further analyses were focused on *DQB1*, *DRB1*, and *DRB3,4,5* that encode DQβ and DRβ chains of the HLA α/β heterodimer. HLA-DQ and -DR serotypes were assigned from genotypes, and frequencies of serotype pairs were compared between

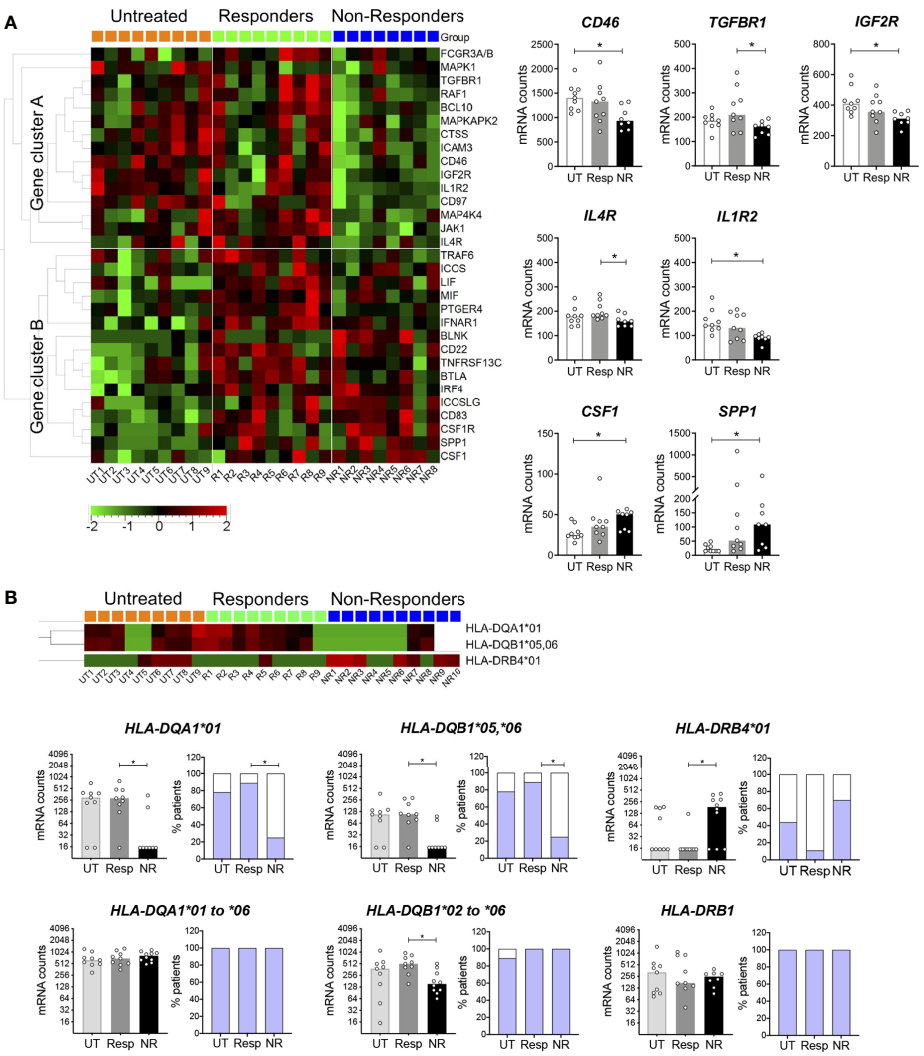

FIGURE 3 | Differentially expressed genes other than ISGs in IFN non-responders. **(A)** Heatmap of mRNA expression in blood cells of patients measured with the Nanostring technology as in **Figure 2**. One-way Anova F-test, $p < 0.05$. UT = 9, Resp = 9, NR = 8–10. Right panels: genes encoding cell surface receptors and cytokine in non-responders. Kruskal–Wallis test with Dunn's multiple comparisons, $^*p < 0.05$. **(B)** Differential HLA-*DQA1*, *DQB1*, and *DRB4* mRNA expression in non-responders. Kruskal–Wallis test with Dunn's multiple comparisons, $^*p < 0.05$. UT = 9, Resp = 9, NR = 8. Two-colored bar graphs show the proportion of patients for which HLA probe binding was observed (blue) or not (white). Pairwise comparison test followed by Tukey-like adjustment for multiple comparisons, $^*p < 0.05$.

responders, non-responders, and the MI 1,000 healthy controls. Strikingly, non-responders showed a marked and significant enrichment of genotypes corresponding to DQ2/DQ8 serotypes ($p < 0.05$, **Figure 4B**) and tended to carry *DQA1* genotypes including *DQA1*03* and *DQA1*02:01* variants (**Supplementary Table 3**). Non-responders were also more frequently positive for DQ2/DQ7 serotypes though this appeared to be driven by the increased usage of DQ2 and not DQ7 (**Figure 4C**). In accordance with known genetic linkage between specific HLA class II gene variants (55), non-responders carrying DR4/DR7 serotypes also carried the DR53 serotype (**Figure 4B**). As opposed to these findings, responders and MI healthy controls displayed a high diversity in the usage of DQβ and DRβ chains. Overall, this genotyping analysis strongly suggests that non-responders utilize a distinct repertoire of HLA-DQ and possibly HLA-DR molecules.

DISCUSSION

By using an integrated approach and sensitive technologies, we have made new observations related to the therapeutic efficacy of IFNβ in RRMS. Untreated patients and healthy controls globally showed modest differences, possibly explained by the mild MS disease score. On the other hand, non-responders and responders displayed distinct cellular and molecular immune phenotypes. Non-responders were characterized by a reduced number and frequency of naïve Tregs and higher number of

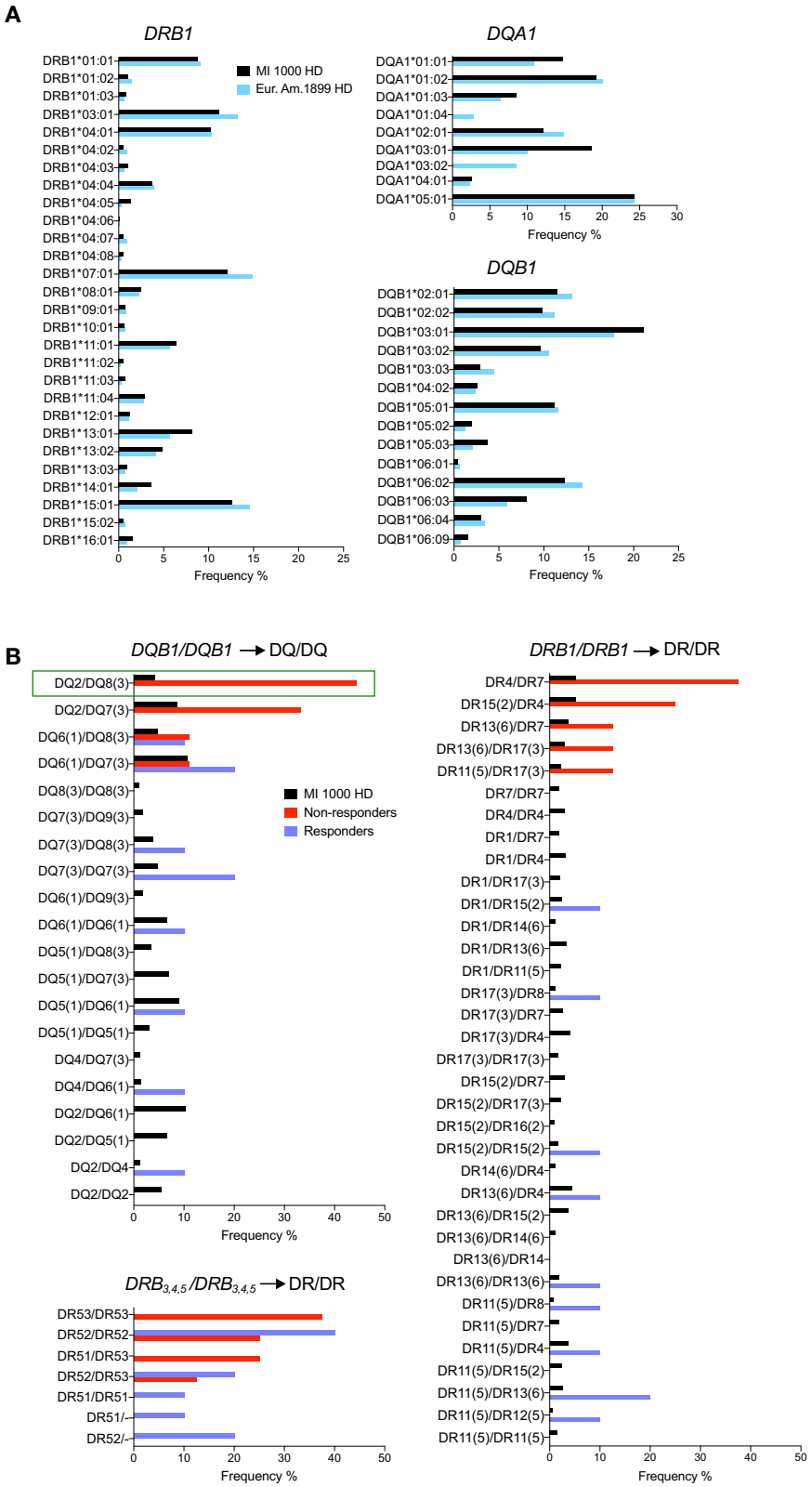

FIGURE 4 | Continued

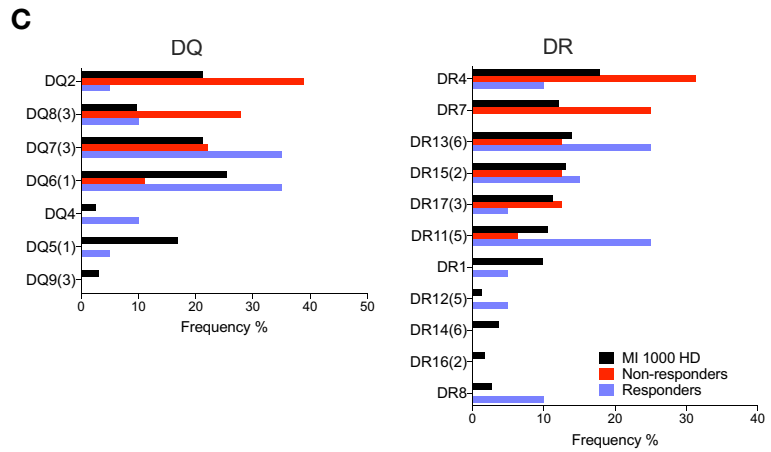

FIGURE 4 | Increased carriage of *HLA-DQB1*genotypes encoding DQ2/DQ8 serotypes in non-responders. **(A)** Comparison of *HLA-DRB1, DQA1*, and *DQB1* allele frequencies in the French *Milieu Interieur* (MI) cohort (n = 1,000) and in a European-American reference cohort [n = 1,899 (54)]. **(B)** Frequencies of DQ and DR serotype pairs in MI healthy controls, IFN non-responders and responders. (Resp = 10, NR = 9 and 8 for DR53). Pairwise comparison test between groups showed statistically significant difference of the DQ2/DQ8 frequency in NR *vs* MI HD (green rectangle) with *p < 0.05, adjusted for multiple comparisons. **(C)** Frequencies of DQ and DR serotypes in MI controls, IFN non-responders and responders.

$CD4^+$ T_{EMRA} cells, suggesting immune dysregulation in these patients. The mechanism underlying the reduction of naïve Tregs remains to be understood. These cells may have acquired an effector-like phenotype, as for the Th1-like Treg cells described in MS patients (22). Alternatively, the decreased thymic output of naive Tregs reported in untreated patients (24, 25) or an alteration of peripheral Treg homeostasis may be more pronounced in our non-responder patients. In contrast, responders showed a higher number and frequency of total Tregs, which was mainly reflected at the level of memory and activated Treg numbers. In line with this, therapeutic IFNβ has been proposed to promote redistribution within the Treg compartment towards memory Tregs (37, 56). Unexpectedly, we found an increase in the number of total $CD4^+$ T cells in responders. This finding is consistent with studies showing that IFNβ restores thymic function or T cell homeostasis that is altered in untreated patients (35, 57). However, other studies reported fewer circulating T cells in IFN-treated patients (37, 58). Treatment duration and time of blood collection may explain this difference. Indeed, therapeutic IFNβ is known to induce a cytopenia depending on the dose and administration frequency (59). This effect is transient since it was observed during the first 6–12 months and resolved thereafter (60). In our study, responders were long-term treated (median 7 years) once a week, and blood was collected at least two days after IFN administration. In other studies, blood was collected earlier (<24 h) and IFNβ was administered several times per week.

Another T cell phenotype observed in non-responders was the increase in $CD4^+$ T_{EMRA} cells, in particular with age. This accumulation may be cytokine- (61) or HLA class II/antigen-driven, and it would be interesting to know whether some of these cells are autoreactive (17, 20, 30). In humans, most $CD4^+$ T_{EMRA} cells are $CD28^-$ (62). Interestingly, memory $CD28^-$ $CD4^+$ T cells with a cytotoxic and pro-inflammatory potential were reported to be clonally expanded and to associate with MS progression (29–31). This suggests that $CD4^+$ T_{EMRA} cells enriched in non-responders may exert a deleterious activity.

Many studies have investigated gene expression in blood cells of IFN-treated patients, searching for a signature of IFN bioactivity and treatment response markers. However, no unified view has emerged, possibly due to variability of experimental settings (63–68). One correlate was proposed between elevated baseline ISGs, serum IFNβ prior to treatment and poor clinical outcome (65, 67, 69). In line with this, we found that non-responders displayed an increased expression of some ISGs, in particular *MX1*, a marker previously used to study IFN non-responders (56, 70, 71). Yet, a three canonical ISG score (*MX1*, *ISG15*, *HERC5*) or other scores based on several ISGs (**Supplementary Figure 3**) did not allow for distinguishing non-responders from responders but were consistent with similar levels of circulating IFNβ levels and IFNβ bioactivity in the two patient groups. Among other predictive markers associated with poor IFN bioactivity and therapeutic effect is the induction of neutralizing antibodies. Of interest for our study, Avonex was reported to be the least immunogenic preparation, affecting around less than 10% patients during the first 1–3 years of treatment (70, 72–74). Thus, it is likely that a significant impact of neutralizing antibodies was not well appreciated due to the limited number of studied patients (n = 10–15/group).

The analysis of immune related genes other than ISGs led to novel observations. Non-responders were characterized by a cluster of 15 downregulated genes, including cell surface receptors (*e.g. CD46*, *TGFR1*, *IL4R*, *IL1R2*) and two upregulated cytokines (*CSF1*, *SPP1*) with an immunomodulatory function. Among these genes, *CD46* and *SPP1* have been documented to be involved in MS pathogenesis (23, 53). Together with the alteration of circulating Treg and $CD4^+$ T_{EMRA} cell subsets, these results indicate some level of immune dysregulation in non-responders.

HLA allelic gene variation is known to mainly impact the antigen-binding groove formed by the α/β HLA class II heterodimer, which can result in modifications of the affinity or stability of the peptide-HLA complex and, potentially, the $CD4^+$ T cell repertoire (75). Importantly, we found remarkable differences between patient groups for HLA class II gene variation. First, the binary pattern of *HLA-DQA1*, *DQB1*, and *DRB4* mRNA expression was clearly altered in non-responders as compared to untreated and responder patients. Second, the profile of HLA class II genotypes markedly differed between non-responders and 1,000 healthy donors (MI cohort).

In European populations, at least six HLA class II risk variants have been reported for MS (14, 15). The strongest one, *HLA-DRB1*15:01*, is part of the extended haplotype *DRB5*01:01-DRB1*15:01-DQA1*01:02-DQB1*06:02* that corresponds to DR51–DR15–DQ6 serotypes. In our study, non-responders did not significantly differ from MI controls and responders for the carriage of *DRB1*15:01* encoding the DR15 serotype, but they tended to be enriched for DR4/DR7 and DR53 serotypes.

The most striking result was obtained for *DQB1* in non-responders who showed a significant enrichment of DQ8/DQ2 serotypes and mainly carried *DQA1* genotypes including *DQA1*03* and *DQA1*02:01* allelic variants. The enrichment of DQ8 in non-responders is consistent with two recent studies (13, 76). In a meta-analysis of HLA allelic variation performed with multiple MS cohorts of European ancestry, *DQB1*03:02*, the single allele encoding DQ8, was identified as a dominant risk for MS. *DQB1*03:02* was also found to be counteracted by the interaction with *DQB1*03:01* (encoding DQ7) in this study (13). Of note, none of our non-responders carried both *DQB1*03:02* and *DQB1*03:01* alleles (**Supplementary Table 3**). Using next-generation sequencing, the other study associated two extended haplotypes with MS in European-American patients. The haplotype encoding DR53–DR4–DQ8 serotypes was linked with MS risk, while the other haplotype encoding DR53–DR4–DQ7 serotypes was protective (76). Hence, DQ8 and DQ7, each encoded by a single allele, appear to influence MS susceptibility in an opposite manner. In line with this, our findings support the notion that DQ8 and DQ2 may represent predictive markers of poor MS outcome in IFN-treated patients. In addition, DQ8, DQ2, and DR4–DQ8 serotypes have been strongly associated with type 1 diabetes (77) and celiac disease (78), which suggests some sharing of pathogenic mechanisms between MS and these autoimmune diseases.

Overall, our findings suggest that the disease activity in our IFN non-responder patients is such that it cannot be counteracted by IFNβ bioactivity. Our non-responder patients may suffer from pathogenic $CD4^+$ T cells, likely restricted by DQ8 and DQ2, that may exert autoreactive and bystander inflammatory activities. These findings may be of interest towards improved patient follow-up but warrant further validation with larger cohorts of patients.

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 received administrative and ethical clearance in France from the "Comité de Protection des Personnes Ile de France IV (CPP)" 2014/17NICB and the "Commission Nationale de l'Informatique et des Libertés (CNIL)" MMS/CWR/AR1411558. Studies with healthy controls were approved for the CoSImmGEn cohort, ICAReB platform, Institut Pasteur (CPP 2010-dec.12483, CNIL 1161456), the Etablissement Français du Sang, Paris (CPSL UNT-18/EFS/04), and the Milieu Interieur (https://clinicaltrials.gov; NCT01699893 and NCT03905993, ANR-10-LABX-69-01). The patients provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PD-M performed experiments, analyzed the data, and contributed to the writing. CM-C performed experiments and analyzed the data. PC performed statistical analyses of HLA typing. BC and AM-K contributed to flow cytometry assays. VB, AL, and DD were involved in IFN measurement. CP and EM recruited patients. SP revised the manuscript. FM supervised the work and wrote the manuscript. 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/fimmu.2021.628375/full#supplementary-material

Supplementary Figure 1 | Numbers and frequencies of $CD3^+$, $CD4^+$, and $CD8^+$ T cells. Mann-Whitney test, $^*p < 0.05$.

Supplementary Figure 2 | Numbers and frequencies of **(A)** $CD4^+$ and **(B)** $CD8^+$ TNaive, TCM and TEM cells in $CD4^+$ and $CD8^+$ $CD3^+$ T cells. **(C)** Frequencies of Treg and Tconv cells in $CD4^+$ $CD3^+$ cells and ratio. **(D)** Numbers and frequencies of memory and activated subsets in Treg cells. Mann-Whitney test, $^*p < 0.05$.

Supplementary Figure 3 | ISG signatures in IFN-treated patients. A list of 153 ISG (FC) > 1.5) was determined from previous MI data obtained with 25 healthy controls (50). Baseline ISG geomean scores were calculated in patient groups for strong (FC > 10, n=17 genes), moderate (FC 2.5-7.5) and low ISG induction (FC 1.5–2.5) in UT=9, Resp= 9 and NR=8. Strong ISGs (n=17) were CCL2, CCL8, CXCL10, HERC5, IFI35, IFIH1, IFIT2, IFITM1, IL1RN, IRF7, ISG15, LAMP3, MX1, RIG-I, SERPING1, TNFSF10, TNFSF13B; moderate ISG were ADAR1, BST2, CCR5, CCRL2, CDKN1A, CEACAM1, GBP1, IFI16, IRF5, IRF9, LAG3, LILRB1, MSR1, SLAMF7, SOCS1, STAT1, TAP2; and low ISGs were BLNK, CCND3, CD53, CFB, CTSC, FCER1G, HLA-C, ICAM2, LAIR1, MCL1, PLAU, PLAUR, PSMB10, PSMB8, TAPBP. Kruskal-Wallis test with Dunn's multiple comparisons. $^*p < 0.05$.

Supplementary Figure 4 | Expression of genes other than ISGs in IFN-treated patients. Additional genes from cluster B in **Figure 3** with **(A)** lower expression in non-responders, **(B)** higher expression in responders or non-responders. Kruskal-Wallis test with Dunn's multiple comparisons. $^*p < 0.05$, $^{**}p < 0.005$.

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Long-Term Clinical and Immunological Effects of Repeated Mesenchymal Stem Cell Injections in Patients With Progressive Forms of Multiple Sclerosis

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Background: Mesenchymal stem cells (MSC) were shown to possess immunomodulatory and neurotrophic effects. Our previous trials, have shown that intrathecal (IT) and intravenous (IV) administration of MSCs were safe and provided indications of beneficial clinical effects.

Methods: This is an open prospective study to evaluate the safety and the long-term clinical and immunological effects of multiple injections of autologous MSCs in 24 patients with active-progressive MS. At inclusion, the mean age of the patients was 47.0 ± 9.22, and the mean EDSS score was 6.75 ± 0.68 (range: 5.5–7.5). Patients were initially treated with 1×10^6 MSCS/kg of body weight (IT + IV) and subsequently with up to additional eight courses of MSCs, at intervals of 6–12 months. The duration of the trial was 4 years.

Results: No serious, treatment-related adverse events were observed during the follow-up period. Twenty-two of the 24 patients were either stable or improved at the last follow-up visit. Ten patients had a lower than baseline EDSS at the last follow-up (nine were among those who received >2 treatments and one in the subgroup of ≤2 treatments, $p = 0.04$). The mean EDSS score reduced from 6.75 ± 0.68 at baseline to 6.42 ± 0.84 at the last visit, during a median follow-up period of 27.8 months ($p = 0.028$). Immunological follow-up showed a transient upregulation of CD4+CD25+FoxP3+ cells and downregulation of the proliferative ability of lymphocytes.

Conclusions: Repeated MSC treatments in patients with progressive MS were shown safe at the short/intermediate term and induced clinical benefits (especially in patients treated with >2 injections) that lasted for up to 4 years, paralleled by short-term immunomodulatory effects.

Clinical Trial Registration: www.ClinicalTrials.gov, identifier: NCT04823000.

Keywords: multiple sclerosis, stem cell, mesenchymal stem cell, progressive MS, clinical trial

INTRODUCTION

Mesenchymal stem cells (MSCs) are non-hematopoietic stromal cells, which reside mainly in the bone marrow compartment, and also in fat and other tissues. Their classical role is to support hematopoiesis and produce cells of the mesodermal lineage (1). Studies have described additional MSC properties, including immunomodulatory and neurotrophic effects (2–7). In preclinical studies, intravenous (IV) and intrathecal (IT) administration of MSCs has been shown to suppress experimental autoimmune encephalomyelitis (EAE) (3, 7, 8) and support remyelination following spinal trauma, brain ischemia, or induced demyelination (9).

A few small, mostly open-label, clinical trials have reported indications of favorable effects of MSC treatment in stroke, multisystem atrophy, multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS) (10–18). Whether the observed benefits were mediated by immunomodulatory mechanisms or by neurotrophic and neuroprotective effects remains controversial. Overall, MSCs given intravenously or intrathecally in MS were well-tolerated, with preliminary indications of clinical beneficial effects (12, 13).

In the latter trial (13), based on the data in EAE models (indicating probably two distinct mechanisms of action by the two different routes of MSC administration), a combined intrathecal and intravenous administration was used to maximize the potential therapeutic benefit by accessing the CNS both through the cerebrospinal fluid and the systemic circulation. The injected MSC, labeled with the superparamagnetic iron oxide ferumoxides (Feridex) could be visualized by MRI in the occipital horns of the ventricles, the meninges, subarachnoid space, and spinal cord, indicating a possible migration of the injected MSC to these areas.

In our recently published—first of its kind—phase II double-blind controlled study we examined the efficacy of MSC transplantation in progressive MS (19) and showed that autologous intrathecal MSC transplantation was safe and induced robust clinical beneficial effects. The intrathecal administration was found superior to the intravenous one. In most of the previously reported studies (17, 20, 21), there were signs of fading-off of the beneficial effects by time, with a peak benefit within 1–3 months following the administration of the stem cells.

We report here the results of an open prospective study with multiple intrathecal injections of autologous MSC in 24 patients with progressive forms of MS (secondary progressive, primary progressive, or relapsing progressive), who failed to respond to first and second lines of immunomodulatory treatments.

METHODS/STUDY PROTOCOL

Patients

Twenty-four patients, 12 males and 12 females [14 from those who participated in our previous clinical trial (13)] were included in this open-label trial, which was originally designed to represent an extension phase of our 2010 study (see study flowchart in **Figure 1**). In order to formulate a group with at least 24 patients (which would be borderly sufficient to detect significant clinical changes), we received a new license from Hadassah Hospital Ethics committee and the Israel Minsitry of Health to include 10 additional patients. The main aim of the current study was to evaluate the safety (and as secondary aim to detect signals of clinical and immunological effects) of repeated (up to eight) injections of autologous MSC during a period of up to 4 years. The participants suffered from progressive forms of MS (22 with secondary progressive MS and two with primary progressive MS) and were failures to first and second lines of immunomodulatory treatments (as defined in the inclusion criteria). All patients had either deteriorated (by at least 0.5 degree in the EDSS scale for baseline EDSS of >5.0 or 1 degree for lower EDSS scores) during the year preceding their inclusion to our study, or suffered from at least one major relapse accompanied by MRI activity (new lesions, expanding lesions, or gadolinium-enhancing lesions), or two clinical relapses. At inclusion, the mean age of our patients was 47.0 ± 9.22, the mean EDSS score was 6.75 ± 0.68 (range 5.5–7.5), and the mean duration of the disease was 13.4 ± 6.6 years (**Table 1**: Demographics of the patients). The patients did not receive any immunomodulatory treatment during the period that remained in the trial.

Seven patients received only two treatments, whereas the rest ones were treated with a variable number (3-8) of MSC injections. All 24 patients had a 1 year follow-up, 20 patients remained at follow-up at 2 years, 12 patients at 3 years, and seven patients at 4 years. The patients who stopped the follow-up did so because they expressed their will to start one of the new, disease-modifying drugs for MS that evolve during the time of the trial (**Tables 1**, **3**). In total, in the whole group of patients, 86 IV and 64 IT MSC injections were performed. Sixty-one out of the total 89 treatments included a combined IT + IV injection of MSC. Less than a third of the injections were not combined ones (IT + IV), either due to unwillingness of the patients to undergo additional lumbar punctures or due to insufficient number of cells.

Study Design

Inclusion Criteria

1. Consenting patients fulfilling the Poser's criteria for definite MS.
2. Age 18–70.
3. Male and female.
4. EDSS 5.5–7.5 (moderate to high disability).
5. Failure to two lines of the currently available registered immunomodulatory treatments [disease-modifying drugs (DMD)] for MS. The lack of response to the treatment was determined by either an increase in EDSS (0.5 degree for EDSS equal or above 5.5 and 1 degree for lower EDSS, confirmed by two evaluations 6 months apart) or the appearance of at least one relapse of MS accompanied by the appearance of new, enlarging or enhancing lesions in the MRI or two relapses, during the year prior to inclusion, under continuous DMD use.

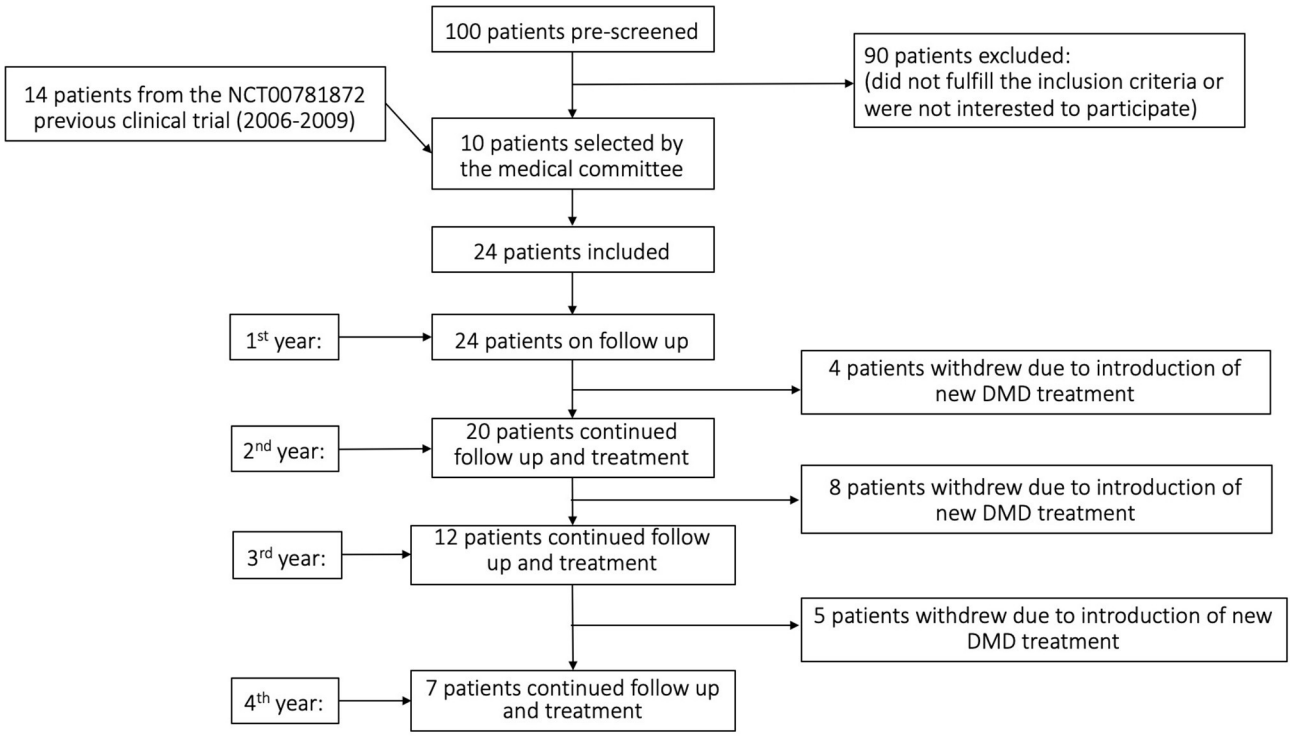

FIGURE 1 | Study flowchart.

Exclusion Criteria

1. Patients who were treated with cytotoxic medications during the last 3 months prior to inclusion (12 months for mitoxanthrone).
2. Patients suffering from significant cardiac, renal, or hepatic failure or any other disease that may risk the patient or interfere with the ability to interpret the results.
3. Patients with active infections.
4. Patients with cognitive decline or inability to understand and sign the informed consent.

Treatment Procedures

Bone marrow (BM) was aspirated according to the routine medical center procedure from the patient's iliac crest under local anesthesia and sedation, following testing negativity for HBV, HCV, and HIV. The aspirated BM was transferred immediately to the GMP facility and labeled by the physician or by the attending technical assistant. BM aspirates were transferred from the heparin-containing bone marrow aspiration bags into sterile 50-ml conical tubes (Corning, USA) using two spike tubing sets (Macopharma, USA) and diluted 1:1 (v:v) in Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich), and mono-nuclear cells (MNC) were separated from the total BM inoculum by Ficoll density gradient (1.073 g/ml) centrifugation (GE Healthcare, USA). Diluted BM was transferred to barrier-containing 50-ml tubes (LEUCOSEP™, Greiner-bio one, Germany) prefilled with 15 ml of Ficoll and centrifuged for 10 min, 1,000 × *g*, at 24°C. The MNC layer was removed using sterile pasture pipette (Greiner-bio one, Germany) and transferred to 50-ml sterile tubes and diluted with 30 ml of PBS. Cells were centrifuged twice for 10 min, at 1,000 rpm, 24°C and re-seeded into "complete culture media" containing Nutristem™ XF Basal Media (Biological Industries, Israel) supplemented with supplement media for further processing. MNCs were counted using a hematocytometer, and cell viability was evaluated using trypan-blue dye staining (Sigma-Aldrich, Israel). MNCs were washed and re-suspended with Nutristem XF™ complete media and seeded on 175-cm^2 culture flasks precoated with Attachment Solution XF. The culture supernatant containing the non-adherent mononuclear cells was removed, and the adherent cells were gently washed with 100 ml of DPBS. The medium was replaced twice a week, with fresh complete NutriStem™ XF growth medium until the culture reached 80–90% confluency but for no more than 12 days. Cells were subcultured at regular intervals, when the culture reached 80–90% confluence. Each subculture cycle was counted as a new passage. The cultures were cultured and subcultivated until reaching desired cell numbers (usually not more than three passages until cryopreservation).

A few days before cryopreservation, cells were characterized by FACS for human MSC markers and a biopotency test of mixed lymphocyte reaction (MLR). At the end of the process before cryopreservation, cells were tested for sterility, mycoplasma, and endotoxins. Cell were released for treatment upon receiving the results of the tests and according to the release criteria. Each cell batch was released with a certificate of analysis document (CoA).

TABLE 1 | Demographics of the patients.

Patients (Gender)	Age	Years of MS	EDSS 1 year before	Relapse or MRI activity during last year	EDSS at baseline	MS type	Previous DMDs
001 (M)	60	21	6.5	R*, M**	6.5	SPMS	Interferon, glatiramer acetate, mitoxatrone, natalizumab
002 (F)	45	13	5.5		6	SPMS	Interferon, natalizumab
003 (F)	49	17	6	R, M	6	SPMS	Interferon, natalizumab
004 (F)	54	30	6		6.5	SPMS	Glatiramer acetate, fingolimod
005 (F)	47	14	6.5	R, M	6.5	SPMS	Interferon, azathioprine, glatiramer acetate, natalizumab, mycophenolate
006 (F)	48	17	6.5		7	SPMS	Plasmapheresis, rituximab mitoxanthrone, interferon, glatiramer acetate
007 (F)	48	14	7	R, M	7	SPMS	Rituximab, azathioprine, natalizumab, interferon, plasmapheresis
008 (M)	47	11	7		7.5	PPMS	Mitoxathrone
009 (M)	43	10	7.5	R, M	7.5	SPMS	Interferon, natalizumab, mitoxanthrone
010 (M)	47	12	7	R, M	7	SPMS	Mycophenolate, interferon, IVIG
011 (F)	52	8	7	R, M	7	SPMS	HSCT, glatiramer acetate, interferon, natalizumab
012 (M)	27	9	6	R(2), M	6	SPMS	Plasmapheresis, mycophenolate
013 (M)	45	12	7		7.5	SPMS	Interferon, glatiramer acetate, natalizumab
014 (F)	53	7	5.5	R, M	5.5	SPMS	Azathioprine, natalizumab, methyprednisolone monthly pulses
015 (M)	70	6.5	7		7.5	PPMS	Mycophenolate, cyclophosphamide, rituximab
016 (M)	40	7	7	R	7.5	SPMS	Natalizumab, fingolimod
017 (F)	48	7	7		7.5	SPMS	Interferon, natalizumab
018 (M)	48	11	6	R, M	6	SPMS	Mycophenolate, azathioprine, natalizumab
019 (M)	30	9	5.5	R(2), M	5.5	SPMS	Glatiramer acetate, fingolimod, natalizumab
020 (F)	45	19	6		6.5	SPMS	Interferon, natalizumab
021 (M)	56	30	6		6.5	SPMS	Plasmapheresis, interferon, dimethyl fumarate, teriflunomide
022 (M)	30	8	7.0	R, M	7.5	SPMS	Interferon, fingolimod, natalizumab
023 (F)	49	10	6.0	R, M	6.5	SPMS	Interferon, natalizumab
024 (F)	46	19	7		7.5	SPMS	Interferon, azathioprine, mitoxathrone

R, relapse of MS during the year prior to inclusion.

***M, MRI activity (appearance of new, expanding, or enhancing lesions during the year prior to inclusion).*

Patients were initially treated (first treatment cycle) with 1×10^6 MSCs per kg of body weight, intrathecally (via a standard lumbar puncture), and with the same number of MSCs intravenously. The scheduled treatment protocol was intended to include additional combined IT + IV injections every 6 months for up to 4 years. However, due to limitations in the number of cultured cells or the unwillingness of the patients to undergo repeated lumbar punctures (and additional bone marrow harvesting), the treatment was modified in most of the cases to single IV injections, or the time intervals between the injections were extended. An additional reason for this extension of the time intervals between the injections (up to 12 months) was related to the difficulties in traveling arrangements for many of the included patients who came from abroad. The duration of the study was 4 years and the median follow-up period was 27.8 months.

Immunological Evaluation

Immunological analysis of peripheral blood mononuclear cells (PBMC) obtained from the treated patients was performed at baseline (before first treatment), after 4 h, at 1 day, and at 1, 3, and 6 months posttreatment, during the 6 month period following the first MSC transplantation. Specifically, the following tests were performed:

FACS Analysis of Lymphocyte Subsets

PBMCs were isolated by Histopaque-1077 (Sigma Aldrich, USA) density gradient centrifugation and, after gating for CD3 positivity, were stained with anti-CD4 PE, anti-CD25FITC (BD Biosciences, USA), anti-CD69 and anti-FoxP3 for FACS fluorescence cytometry. After gating for Lin-negativity, the isolated PBMCs were also stained for the myeloid dendritic markers CD11c and CD86PE (eBioscience,

TABLE 2 | Safety (adverse events).

Pts	No of tx	Route of administration	Intervals (months)	Adverse events	Severity	Outcome
001	3	1—IV+IT	0	1—none		
		2—IV+IT	6	2—none		
		3—IV+IT	12	3—none		
002	3	1—IV+IT	0	1—headache, fever	1—mild	1—resolved, 24 h
		2—IV+IT	12	2—headache	2—moderate	2—resolved, 3 days
		3—IV+IT	24	3—none		
003	4	1—IV+IT	0	1—fever, headache,	1—mild	1—resolved, 3 days
		2—IV+IT	12	general weakness		
		3—IV	24	2—headache, general	2—mild	2—resolved, 3 days
		4—IV	36	weakness		
				3—none		
				4—none		
004	8	1—IV+IT	0	1—headache, back pain	1—moderate	1—resolved, 2 days
		2—IV+IT	6	2—none		
		3—IV+IT	12	3—headache	3—mild	3—resolved, 24 h
		4—IV+IT	18	4—back pain	4—mild	4—resolved, 3 days
		5—IV+IT	24	5—none		
		6—IV+IT	30	6—none		
		7—IV	36	7—none		
		8—IV+IT	42	8—headache	8—mild	8—resolved 2 days
005	8	1—IV + IT	0	1—none		
		2—IV	6	2—none		
		3—IV	12	3—none		
		4—IV	18	4—none		
		5—IV	24	5—none		
		6—IV	30	6—none		
		7—IV	36	7—none		
		8—IV	42	8—none		
006	6	1—IV + IT	0	1—neck rigidity,	1—severe	1—resolved, 3 days
		2—IV + IT	6	headache, back pain,		
		3—IV + IT	12	leukocytosis		
		4—IV	18	2—headache, back pain	2—moderate	2—resolved, 4 days
		5—IV + IT	24	3—none		
		6—IV + IT	36	4—none		
				5—headache	5—mild	5—resolved 24 h
				6—headache	6—mild	6—resolved, 24 h
007	6	1—IV + IT	0	1—fever	1—moderate	1—resolved 24 h
		2—IV	12	2—none		
		3—IV	18	3—none		
		4—IV	24	4—none		
		5—IV	36	5—none		
		6—IV + IT	42	6—headache	6—mild	6—resolved 24 h
008	3	1—IV + IT	0	1—none		
		2—IV	12	2—none		
		3—IV	24	3—none		
009	4	1—IV + IT	0	1—headache	1—mild	1—resolved 24 h
		2—IV + IT	6	2—fever, headache	2—moderate	2—resolved 24 h
		3—IV + IT	18	3—none		
		4—IT	24	4—none		
010	3	1—IV + IT	0	1—headache	1—mild	1—resolved 24 h
		2—IV	12	2—none		
		3—IV	24	3—none		
011	2	1—IV + IT	0	1—none		
		2—IV	12	2—none		
012	5	1—IV + IT	0	1—none		
		2—IV + IT	6	2—fever	2—mild	2—resolved 24 h
		3—IV + IT	18	3—none		
		4—IV + IT	30	4—headache	4—mild	4—resolved 3 days
		5—IV + IT	42	5—none		

(Continued)

TABLE 2 | Continued

Pts	No of tx	Route of administration	Intervals (months)	Adverse events	Severity	Outcome
013	5	1—IV + IT 2—IV + IT 3—IV + IT 4—IV + IT 5—IT	0 6 12 24 36	1—urinary retention, fever, headache 2—none 3—none 4—none 5—none	1—moderate	1—resolved, 24 h
014	2	1—IV + IT 2—IV	0 12	1—none 2—none		
015	3	1—IV + IT 2—IV + IT 3—IV + IT	0 12 24	1—none 2—headache 3—none	2—mild	2—resolved 24 h
016	3	1—IV + IT 2—IV + IT 3—IV	0 12 24	1—none 2—none 3—none		
017	2	1—IV + IT 2—IV + IT	0 6	1—fever 2—headache	1—mild 2—mild	1—resolved 24 h 2—resolved 24 h
018	2	1—IV + IT 2—IV	0 6	1—none 2—none		
019	2	1—IV + IT 2—IT	0 6	1—none 2—none		
020	6	1—IV + IT 2—IV + IT 3—IV 4—IV + IT 5—IV + IT 6—IV + IT	0 6 18 24 36 42	1—none 2—back pain 3—none 4—headache, fever 5—none 6—headache	2—mild 4—mild 6—mild	2—resolved, 3 days 4—resolved 24 h 6—resolved 24 h
021	3	1—IV + IT 2—IV + IT 3—IV + IT	0 12 24	1—none 2—none 3—none		
022	2	1—IV + IT 2—IV + IT	0 6	1—none 2—none		
023	2	1—IV + IT 2—IV + IT	0 12	1—back pain 2—back pain, sciatic pain	1—mild 2—moderate	1—resolved, 3 days 2—resolved 7 days
024	2	1—IV + IT 2—IV	0 6	1—none 2—none		
	Total: 89	**IV = 86 injections IT = 64 injections IT + IV: 61**		**41 adverse events**	**1 severe, 13 of moderate severity, others: mild**	**All resolved between 1 and 7 days**

Tx = treatments with MSC; Pts = patients.

USA). The data were analyzed with a Beckman Coulter flow cytometer.

Lymphocyte Proliferation in Response to Phytohemaglutinin

The assay was carried out in 96-well, flat-bottomed Nunc plates (Daniel Biotech, USA). Lymphocytes were isolated from whole blood by Histopaque-1077 (Sigma Aldrich, USA) density gradient centrifugation and seeded at 2×10^5/well in RPMI/10% FCS, 1 mM glutamine, and a penicillin–streptomycin mixture (Biological Industries) and stimulated with the lectin phytohemagglutinin (PHA) 1 mg/ml (Sigma Aldrich). Cultures were incubated for 48 h in a humidified atmosphere of 5% CO_2 at 37°C, and then proliferation was assayed by 1 μCi/well of 3H thymidine (Amersham, UK) uptake. After 18 h of incubation with ^{3}H thymidine, cells were frozen in −20°C and then harvested on fiberglass filters using a cell harvester (Skatron, Norway); radioactivity was measured by standard scintillation technique. The "Stimulation index" was calculated as the ratio between activated and non-activated cells.

RESULTS

Safety

In general, there were no serious side effects during the whole 4 year duration of the study. Forty-one adverse events were

TABLE 3 | Long-term clinical effect of multiple MSC transplantations.

Patient	No. of Tx	1 year before	EDSS Baseline	12 months	24 months	36 months	48 months
001	3	6.5	6.5	6.5	6.5		
002	3	5.5	6.0	6.5	6.5	6.5	
003	4	6.0	6.0	6.0	6.0	6.0	
004	8	6.0	6.5	4.5	4.0	4.5	4.5
005	8	6.5	6.5	5.0	5.0	5.0	5.0
006	6	6.5	7.0	6.5	6.5	6.5	6.5
007	6	7.0	7.0	6.5	6.0	6.0	6.0
008	3	7.0	7.5	7.5	7.5	7.5	7.5
009	4	7.5	7.5	7.5	7.5		
010	3	7.0	7.0	6.5	6.5	6.5	
011	2	7.0	7.0	7.0	7.0		
012	5	6.0	6.0	5.5	6.5, R*	6.0	6.0
013	5	7.0	7.5	7.0	7.0	7.0	
014	2	5.5	5.5	5.5	5.5, R*		
015	3	7.0	7.5	7.5	7.5		
016	3	7.0	7.5	6.5	6.0		
017	2	7.0	7.5	7.5			
018	3	6.0	6.0	6.5, R*			
019	2	5.5	5.5	5.5			
020	6	6.0	6.5	6.0	6.0	6.0	6.0
021	3	6.0	6.5	6.5	5.5	5.5	
022	2	7.5	7.5	7.5			
023	2	6.5	6.5	6.5	6.5		
024	2	7.0	7.5	7.0	7.0		
Mean ± SD		**6.52 ± 0.62**	**6.75 ± 0.68**	**6.46 ± 0.82**	**6.33 ± 0.88**	**6.08 ± 0.82**	**5.93 ± 0.98**

At last follow-up (for all 24 patients): 6.42 ± 0.84.
**R, relapse during the study.*

registered (13 of them of moderate and 28 of mild severity). Thirteen of the patients experienced side effects of any kind. Eleven suffered from headache, six had transient low-grade fever, and three had backache. All these events resolved 1–7 days following the infusions. The full list of adverse events in each patient and each treatment is shown in **Table 2**. Interestingly, at those time points where patients were treated only intravenously with MSCs, there were no side effects at all (0). All the observed adverse events occurred in association with either intrathecal or combined IT + IV treatment.

The definition of the severity of adverse events was according to FDA recommendations; a severe adverse event was any event leading to hospitalization. The single severe event in our study was a case with neck rigidity and back pain, who was hospitalized with suspected meningitis, which was ruled out. The patient was discharged 2 days later.

Clinical Effects

Twenty-two of the 24 patients were either stable or improved in the EDSS score at the last follow-up visit. Ten patients had a lower than baseline EDSS score at last follow-up (nine were among those who received more than two treatments and one in the subgroup of two treatments or less, $p = 0.04$, chi-square test) (**Table 3**). The mean EDSS score reduced from 6.75 ± 0.68 at baseline to 6.42 ± 0.84 at the last visit ($p = 0.028$, Wilcoxon ranked sign test), during a mean follow-up period of 29.24 ± 12.76 months (range: 12–59.5) (**Figure 2** and **Table 3**). The mean change in EDSS in the year prior to inclusion was +0.27 ± 0.25 and −0.35 ± 0.63 ($p = 0.002$, Wilcoxon sign ranked test) at the end of follow-up (last visit) (**Figure 2** and **Table 3**). The numbers of patients who were stable, improved, or deteriorated in EDSS, each year, are shown in **Figure 3**.

Although the aim of our study—in terms of clinical effects—was to follow-up changes in disability in patients with progressive disease, we noticed that 14 of the patients had activity expressed by superimposed relapses during the year prior to inclusion to the study (total numbers of relapses 16). During the period of MSC treatments, only three relapses were noted in three patients ($p = 0.002$, Wilcoxon signed rank test, compared with the year prior to treatment) (**Table 3**).

Immunological Effects

Effect of Mesenchymal Stem Cell Treatment on the Proportions of Various Immune Subpopulations

Immunological follow-up showed a statistically significant upregulation of the CD4+CD25^{high}+FoxP3+ cells (3-fold at month 1 and 4-fold at 3 months), a population representing the majority of T-regulatory cells (T-regs). At 6 months, these

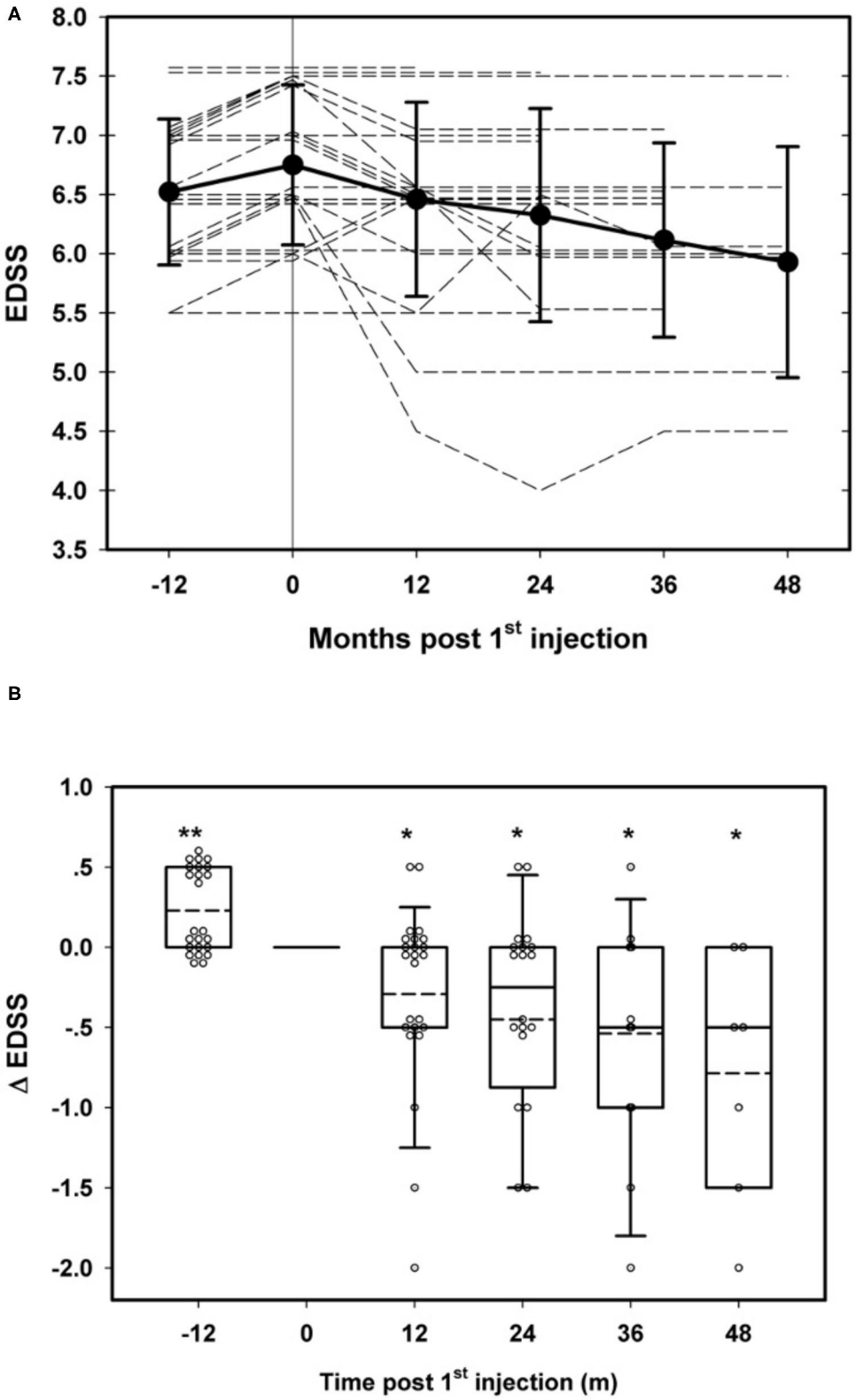

FIGURE 2 | Long-term clinical effects of repeated transplantation of mesenchymal stem cells (MSCs) in multiple sclerosis (MS). **(A)** Changes in EDSS in individual patients before and after MSC transplantation. **(B)** Rate of EDSS change before and after MSC transplantations. *$p < 0.05$, **$p < 0.01$, at the respected time points vs baseline values (Wilcoxon signed rank test).

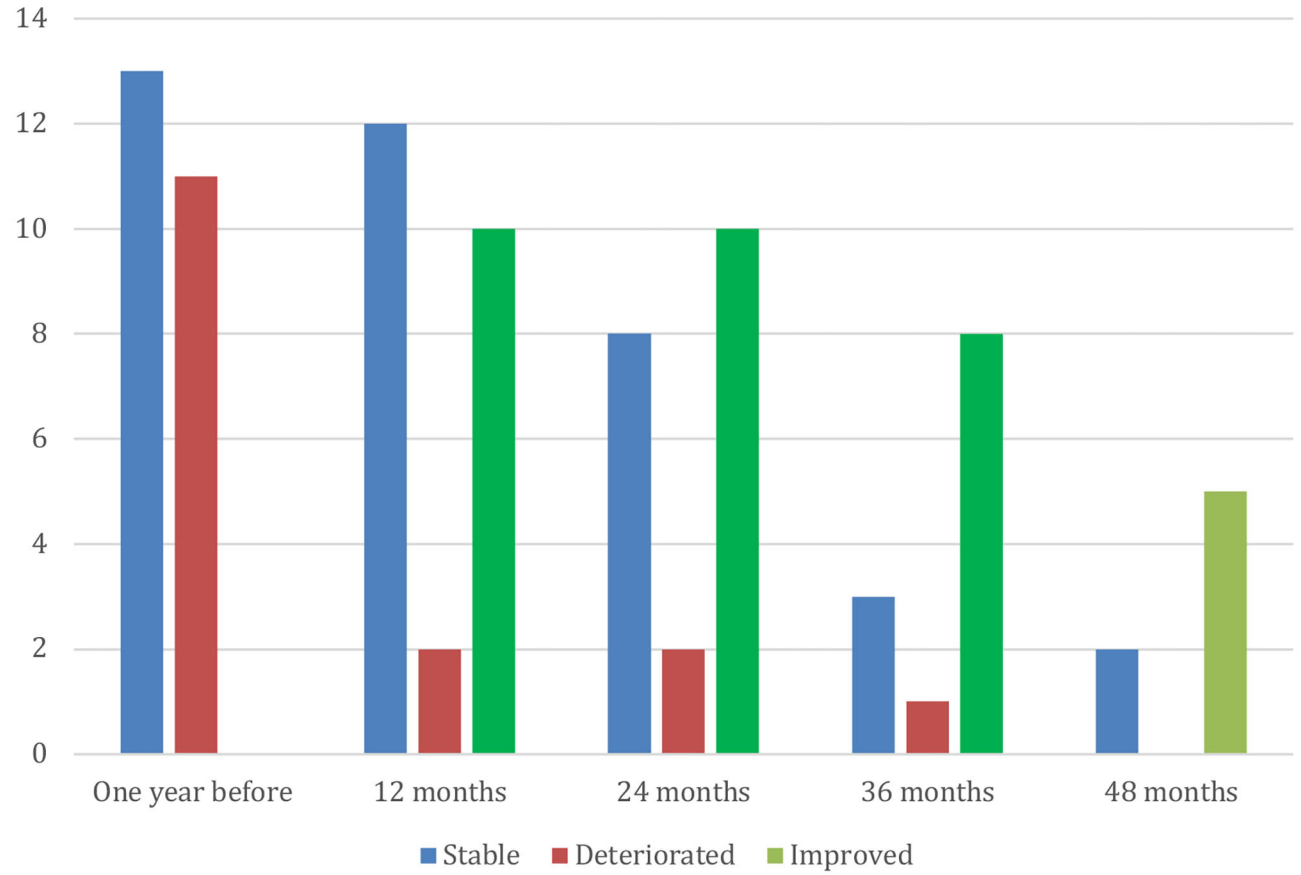

FIGURE 3 | Number of patients with improvement or deterioration in EDSS compared with baseline.

proportions returned to baseline values ($p = 0.002$ at 4 h vs. baseline, $p = 0.0034$ at 24 h, $p = 0.002$ at 1 month, $p = 0.0007$ at 3 months, non-significant at 6 months, Wilcoxon signed rank test) ($n = 8$) (**Figure 4A**).

Other changes included a transient reduction in the proportion of $Lin^{-}CD11c^{+}CD86^{+}$ cells, representing antigen-presenting cell populations (mostly dendritic cells but also macrophages) (from 2.97 to 1.2% at 1 month), following MSC transplantation, indicating a possible downregulatory effect on the antigen presentation process and a mild reduction in the proportion of CD3+CD69+ cells, which was more significant at month 3 (**Figures 4B,C**).

Effect of Mesenchymal Stem Cell Treatment on the Proliferation Ability of Lymphocytes

Following *ex vivo* stimulation of peripheral blood lymphocytes (obtained from the patients at various time points following the first MSC transplantation) with the phytohemaglutinin (PHA), there was a 71% decrease in the proliferative cell response at 24 h, 72% decrease at 1 month, 65% at 3 months, and 28% at 6 months ($p = 0.001$ at 4 h vs. baseline, $p = 0.0002$ at 24 h, $p = 0.0009$ at 1 month, $p = 0.003$ at 3 months, not statistically significant at 6 months) (Wilcoxon signed rank test) ($n = 8$) (**Figure 4**).

DISCUSSION

In this open trial, repeated intrathecal and intravenous administration of MSCs in 24 patients with progressive MS, not responding to the conventional immunomodulatory treatments was shown safe at the short/intermediate term. During the observation period of up to 4 years, there were indications of clinical benefits (i.e., stabilization or improvement in EDSS score), especially in patients treated with more than two injections. Although this was predominantly long-term safety study significant clinical benefits of the MSC treatments, were detected. At the end of the follow-up period, 22 out of the 24 patients treated with MSC had a stabilized had a stabilized or improved EDSS and were defined as "long-term responders". During the 6 months following the first treatment course, immunomodulatory effects of the treatment were also detected, as indicated by an increase in the proportion of the CD4+CD25+FoxP3+ cells (mostly representing the T-regs population) (peaking at 1 day and lasting up to 1–3 months post-transplantation), a transient downregulation of the proliferation ability of the lymphocytes (lasting for up to 3 months) and a moderate downregulation of the CD3+CD69+ and Lin-CD11c+CD86+ cells, representing mainly the activated

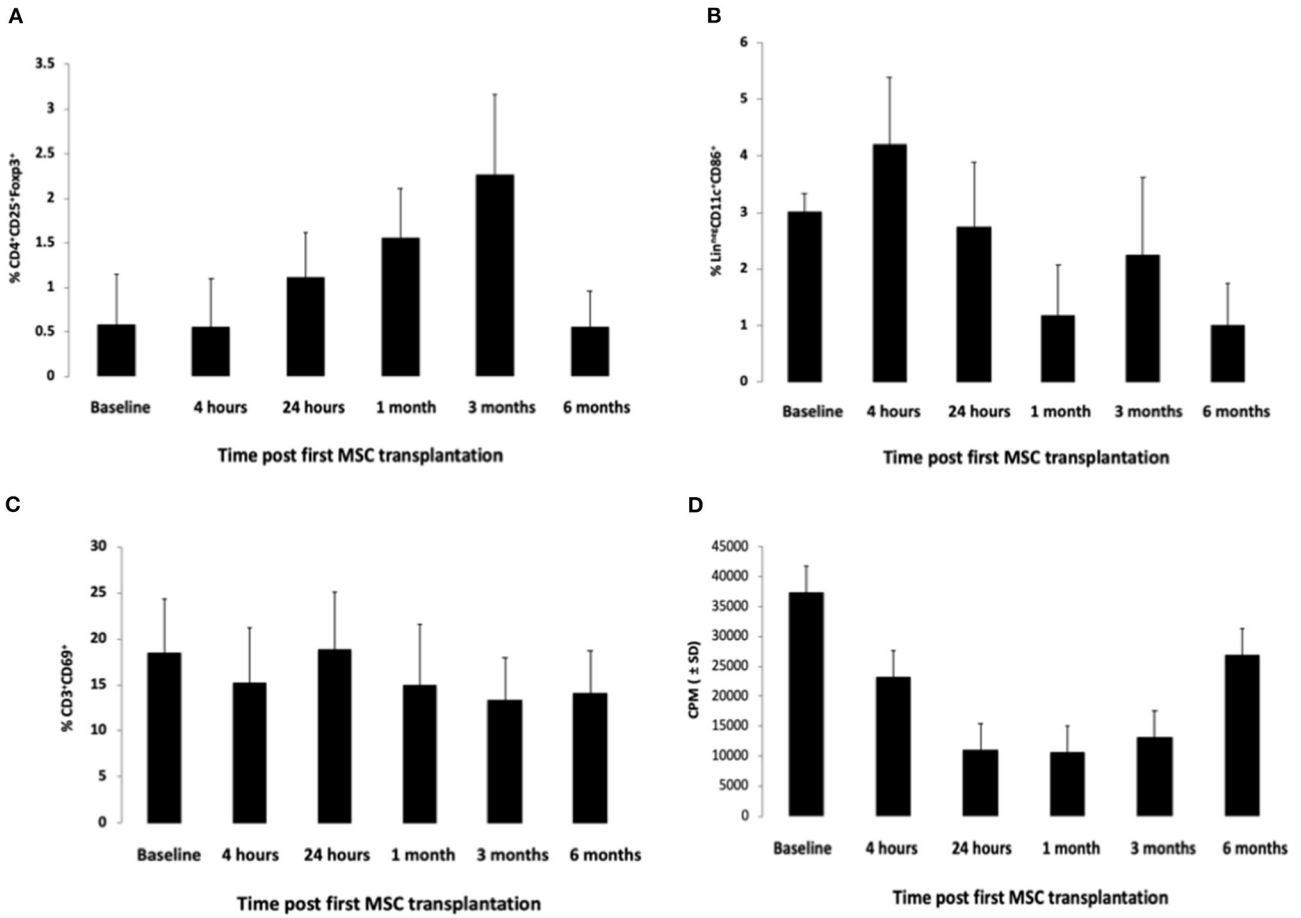

FIGURE 4 | Immunological changes following MSC transplantation at various time points during the 6 months following the first MSC transplantation. **(A)** Changes in CD3-gated, CD4+CD25^{high}+Foxp3+ cells (mostly T-regs), at various time points following the first MSC transplantation. $P = 0.002$ at 4 h vs. baseline, $p = 0.0034$ at 24 h, $p = 0.002$ at 1 month, $p = 0.0007$ at 3 months, nonsignificant at 6 months (Wilcoxon signed rank test) ($n = 8$). **(B)** Changes in Lin-CD11c+CD86+ cells (representing antigen-presenting cell populations, mostly dendritic cells, but also macrophages), at various time points following the first MSC transplantation. $P = 0.0002$ at 1 month vs. baseline, others not statistically significant (Wilcoxon signed rank test) ($n = 8$). **(C)** Changes in CD3+CD69+ cells (mostly activated lymphocytes), at various time points following the first MSC transplantation. $P = 0.029$ at 3 months vs. baseline, others not statistically significant (Wilcoxon signed rank test) ($n = 8$). **(D)** Changes in the *ex vivo* proliferative ability of lymphocytes upon stimulation with phytohemagglutinin (PHA), at various time points following the first MSC transplantation. $P = 0.001$ at 4 h vs. baseline, $p = 0.0002$ at 24 h, $p = 0.0009$ at 1 month, $p = 0.003$ at 3 months, not statistically significant at 6 months (Wilcoxon signed rank test) ($n = 8$).

lymphocytes and antigen-presenting cell populations (mostly dendritic cells and macrophages). Immunological analysis was performed only during the first cycle of treatment, since the subsequent treatments were not given at the same time points in each patient, and therefore, cumulative immunological effects of the repeated treatments could greatly vary among the patients and could complicate the interpretation of the findings.

Despite the development of highly efficient and more targeted immunotherapies for MS, two major unmet needs still exist: (1) the need for treatment to suppress compartmentalized and meningeal inflammation in the central nervous system (CNS), which seems to drive tissue injury and progression of disability (22–24). These compartmentalized inflammatory and degenerative activities seem to be less responsive to the majority of immunomodulatory drugs, accounting for the relatively poor efficacy of the majority of registered MS therapies in progressive MS, with minor exceptions (25, 26).

(2) The need for a treatment that may substantially promote regeneration–remyelination. Generally, the CNS loses its capacity for efficient regeneration and remyelination over time. This is especially pronounced in chronic neuroinflammatory and neurodegenerative diseases such as MS, possibly due to an insufficiency of growth factors or defective mobilization of intrinsic CNS stem cells/oligodendrocyte progenitors (27–29).

Based on their described properties (4, 30–32), stem cells may represent a "logical" treatment approach to achieve those unmet needs and possibly induce neuroprotection and enhance endogenous remyelination (as indicated by animal studies). Moreover, stem cells are strong immunomodulators (6, 29, 33–35) that may potentially downregulate the localized

and compartmentalized inflammation upon their migration to the CNS (22, 24). Several studies have shown that embryonic, neuronal, and other adult stem cells can induce beneficial clinicopathological effects in animal models of neurological diseases, including MS (3, 7–9, 36–39). MSCs are the most commonly used type of stem cells for such cell-based therapies, as they have the following practical advantages for clinical use over other types of stem cells: (1) They can be easily cultured and expanded in large quantities. (2) They can be obtained from the patient, thus, eliminating the need for a donor, the risk of rejection, or the need for chemotherapy. (3) They seem to be safe and carry low risks of malignant transformation. During the last decade, MSC treatments have been applied to various neurological diseases in small or pilot open-label trials (10–18, 40), with promising indications.

The putative mechanism of action of MSC in neurological diseases is controversial. Some investigators claim that the most prominent effects are mediated through peripheral immunomodulation (6, 29, 34, 35). Our group has long advocated that neuroprotective and neurotrophic mechanisms play the most crucial role, as supported by our findings in animal models and pilot trials (4, 13, 17) and that the intrathecal way of administration, which brings a higher proportion of the injected cells into close proximity with damaged areas of the CNS, is preferable to the intravenous injection. Indeed, the findings of our recent double-blind randomized trial in MS (19) showed that the intrathecal injection of MSC was superior to the intravenous at several parameters.

Concerning the (rather short lasting) immunological changes that were shown in the current study, they seem—most probably—to be caused by the intravenous administration of the MSCs, since most of the intravenously administered MSCs have been shown to reside in the periphery and not the CNS (41). Although it is difficult to estimate the clinical relevance of the observed immunological changes, they may have a possible impact on the autoimmune responses of lymphocytes that target myelin antigens and, therefore, be beneficial for MS. Moreover, if the MSCs indeed (via the intrathecal route) migrate to the areas of CNS lesions, they could theoretically downregulate locally the compartmentalized inflammation, potentially acting as "Trojan horses."

On the other hand, downregulation of either antigen-presenting cells or the activation cascade of immune cells and upregulation of regulatory cells may introduce potential risks, such as increased risk for carcinogenesis. Although such risks theoretically exist, they do not seem to be substantial, since these immunomodulatory effects that were induced by the MSCs were transient and rather short lasting, in our study.

In any case, peripheral immunomodulation alone does not seem to sufficiently explain the wide range of clinical beneficial effects induced by MSC transplantation, which were observed in our previous and the current trial (13, 17, 19, 31).

The strengths of our trial include the inclusion of patients with progressive MS, in which conventional immunotherapies were shown ineffective, the long follow-up (up to 4 years), the treatment protocol of repeated (up to eight) administrations of stem cells, and the robust clinical benefits observed in disability progression. The main limitation of our study is obviously related to the small number of patients and the open-label design. Additional limitations of this trial are related to the inclusion of a non-homogenous patients' population (with different types of progressive MS and disease duration) and the lack of uniformity in the treatment protocol (number of injections and intervals between them), for the reasons that are explained in the *Methods* section.

Another possible problem in the interpretation of our findings could be related to the fact that half of our patients had a deterioration in the EDSS score during the year prior to inclusion. Part of the beneficial effects, therefore, could be theoretically related to a "regression to the mean" phenomenon. However, such regression, although may have affected the clinical changes at some degree (especially in the first months of the study), cannot—to our view—explain the findings of the benefits during the subsequent cycles of treatment and the long-lasting clinical improvements.

In conclusion, in our present, open trial, we showed that repeated intrathecal administrations of MSCs in patients with progressive MS was safe at the short/intermediate term and induced clinical benefits (especially in patients treated with more than two injections) that lasted for up to 4 years and included stabilization of the progression of MS and improvements of neurological disability, paralleled by short-term immunomodulatory effects. The data presented here may help in the design of larger trials that could further evaluate the clinical potential of repeated injections of MSCs in MS and other neurological and neuroimmunological diseases.

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/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hadassah Ethics committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DK, PP, and IK participated in the writing of the manuscript. PP was the clinical PI of the trial. All authors participated in the organization and performance of the trial.

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Early High Efficacy Treatment in Multiple Sclerosis Is the Best Predictor of Future Disease Activity Over 1 and 2 Years in a Norwegian Population-Based Registry

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Background: Moderate and high efficacy disease modifying therapies (DMTs) have a profound effect on disease activity. The current treatment guidelines only recommend high efficacy DMTs for patients with highly active MS. The objective was to examine the impact of initial treatment choice in achieving no evidence of disease activity (NEDA) at year 1 and 2.

Methods: Using a real-world population-based registry with limited selection bias from the southeast of Norway, we determined how many patients achieved NEDA on moderate and high efficacy DMTs.

Results: 68.0% of patients who started a high efficacy DMT as the first drug achieved NEDA at year 1 and 52.4% at year 2 as compared to 36.0 and 19.4% of patients who started a moderate efficacy DMT as a first drug. The odds ratio (OR) of achieving NEDA on high efficacy drugs compared to moderate efficacy drugs as a first drug at year 1 was 3.9 (95% CI 2.4–6.1, $p < 0.001$). The OR for high efficacy DMT as the second drug was 2.5 (95% CI 1.7–3.9, $p < 0.001$), and was not significant for the third drug. Patients with a medium or high risk of disease activity were significantly more likely to achieve NEDA on a high efficacy therapy as a first drug compared to moderate efficacy therapy as a first drug.

Conclusions: Achieving NEDA at year 1 and 2 is significantly more likely in patients on high-efficacy disease modifying therapies than on moderate efficacy therapies, and the first choice of treatment is the most important. The immunomodulatory treatment guidelines should be updated to ensure early, high efficacy therapy for the majority of patients diagnosed with MS.

Keywords: multiple sclerosis, disease modifying therapies, no evidence of disease activity, disease activity, treatment decision

INTRODUCTION

Multiple sclerosis is a chronic neuroinflammatory disease with onset in mostly young people, and it is the commonest cause of serious physical disability in adults of working age (1). The condition may have a profound impact on quality of life and employment (2). Interferon as a treatment for multiple sclerosis (MS) was first approved in 1996 (3). In 2006, natalizumab was approved as the first high efficacy disease modifying therapy (DMTs) (4), and in the following years more DMTs followed suit. The therapies are divided into moderate efficacy DMTs, with a well-defined safety profile, and high efficacy DMTs, which are more effective but carries higher risk of serious side effects (5). The current European and American treatment guidelines only advise the use of high efficacy drugs for highly active disease (6, 7). Time to EDSS 6 over the past two decades has increased (8). Although DMTs are not the only reason for this development (9), they likely play an important role (10). There are few head to head randomized clinical trials (RCT), so the importance of real-world evidence has been elevated (11).

The concept of "No Evidence of Disease Activity" (NEDA) has been identified as an ambitious tool for measuring efficacy of DMTs (12). NEDA at 1 year is achieved if there is no history of a clinical relapse, no new activity on magnetic resonance imaging (MRI) and no sign of clinical disease progression measured by expanded disability status scale (EDSS) in the past year (13). Although NEDA is by no means a perfect tool (14), limited disease activity in the first few years of diagnosis is widely regarded as a good prognostic sign (15).

The aim of this study was to determine how many patients in a Norwegian population-based real-world study achieved NEDA at 1 and 2 years and examine the impact of initial treatment choice in achieving NEDA.

MATERIALS AND METHODS

Data Collection and Study Population

The BOT-MS (Buskerud, Oslo and Telemark) is a database comprising the complete population of MS patients in the two counties Buskerud and Telemark, and the majority of the patients in the Norwegian capital Oslo ($n = 3{,}951$). The data were recorded prospectively until 31.12.2017, but retrieved retrospectively by three neurologists specialized in MS between January and December 2018. Detailed information on the database and data collection has previously been published (9).

For this cohort study, we included all patients who had been treated with moderate efficacy DMTs (interferons, glatiramer acetate, teriflunomidem, and dimethyl-fumarate) and/or high efficacy DMTs (natalizumab, fingolimod, or alemtuzumab) for at least 12 months. All patients had access to all disease modifying drugs as all were available and reimbursed since market access in Europe (**Table 1**). The definition of moderate and high efficacy DMTs was chosen because at the time of market access, fingolimod was considered high efficacy and dimethyl-fumarate was considered moderate efficacy treatment (16). Consequently, that is how they were utilized in the follow-up period. We only included patients started on the treatment in 2006 or after, as 2006 was the first year our population had access to the first high efficacy drug, natalizumab. Only patients with yearly (±2 months) EDSS and MRI were included. Patients with missing or incomplete information and patients with incomplete information precluding determination of NEDA were excluded. For NEDA-status at year 1 we did not include those that discontinued due to side effects or wish for pregnancy, but we included the patients who discontinued the drug before the full 12 months due to lack of efficacy. For NEDA-status in year 2, we included any patient who had been on the drug for at least 24 months, including treatment interruption due to lack of efficacy, but not interruption due to side-effects and wish for pregnancy. The population was divided into three subgroups dependent on previous treatments: first drug, second drug or third drug. When looking at drugs previously used, we included all drugs the patient had taken for at least 3 months. Alemtuzumab was considered effective from the first treatment.

TABLE 1 | Disease modifying therapies and year of European Medical Agency (EMA) approval (http://www.emsp.org/about-ms/ms-treatments/#).

Disease modifying therapy		Year of EMA authorization
Moderate efficacy	Interferons	1995
	Glatiramer acetate	2000
	Teriflunomide	2013
	Dimethyl-fumarate	2014
High efficacy	Natalizumab	2006
	Fingolimod	2011
	Alemtuzumab	2013

We considered any new or enlarging lesions or new gadolinium enhancing lesions on follow-up brain MRI to represent MRI change. For EDSS, we considered any increase in EDSS on at least two consecutive occasions to represent a worsening of EDSS. Only EDSS documented 3 months or more after a relapse were included. Relapses documented in the patients' hospital records were counted as a relapse, regardless of steroid treatment. If we had a negative finding in one of the three components of NEDA, we considered the patient as NEDA fail even though we did not have one or two of the other components (EDSS, MRI and/or relapse).

We created a predictive variable for the future risk of disease activity based on our previous findings in this population (9) and known prognostic risk factors. Age (17–19), sex (17, 18, 20, 21), symptoms at onset (17, 22, 23), involvement of more than one Kurtzke functional system at onset (24), number of relapses within the first 2 years of onset (17, 21, 24–26), findings on a first MRI (20) and EDSS (27) were used, see **Table 2**. We graded the risk of disease activity based on seven categories of characteristics at the time of diagnosis that are believed to have an impact on future disease activity. Symptom at onset was defined by Kurtzke's functional system (28) and multiple symptoms at onset was defined as symptoms from two or more functional system. We divided the population into low risk for disease activity (0–3 points), medium risk (4–7), and high risk (8–14p). Based on seven categories, the population was divided into three risk

TABLE 2 | The risk of disease activity at the time of diagnosis was calculated according to these seven factors.

	2 points	1 point	0 points
Age at diagnosis	>35	18–35	
Gender		Male	Female
Symptom at onset	Motor, brainstem, cerebellar		ON, sensory, other
Multiple symptoms at onset	Yes		No
Multiple relapses before diagnosis	Yes		No
MRI findings at diagnosis	>10 lesions	5–10	<5
EDSS at diagnosis	3.0 or more	2 and 2.5	1.5 or less

The population was then divided into low risk (1–3 points), medium risk (4–7 points), and high risk (8–14 points). ON, optic neuritis; MRI, magnetic resonance imaging; EDSS, expanded disability status scale.

groups: low risk for disease activity (1–3 points), medium risk (4–7), and high risk (8–14p). We did not include spinal cord lesions or gadolinium enhancing lesions as this is not done routinely in the clinical practice.

The first generation drugs are referred to as injectables (interferon, glatiramer acetate) and were used as a reference category in calculations of odds ratio. Age was dichotomous in the age category with "old" (≥40 years at time of drug initiation) and "young" (<40 years). To investigate the impact of possible changes in prescription practice, and to correct for missing patients, we split the groups into those patients initiated before 2013, and those initiated after 2013. The year 2013 was chosen as this was when teriflunomide, the first oral moderate efficacy drug, became available.

Statistics

We used IBM SPSS Statistics 25.0 (IBM Corp., Armonk, NY, USA) for data analysis. Differences in continuous variables between two groups were assessed by independent sample *t*-test. Between groups, differences in continuous variables were tested with Student *t*-test for normally distributed data and Mann-Whitney *U*-test for skewed data. The chi-square-test for contingency tables was used to detect associations between categorical variables. Binary logistic regression analysis was used to investigate the association between the treatments and NEDA, and to adjust for possible confounding effects of sex, age at start of medication, time from onset to start of medication and risk group (low, medium and high risk). The results from the regression analysis are presented as odds ratio, adjusted and unadjusted, with 95% confidence intervals (CI). All *p*-values were two-sided and a 5% significance level was used.

Ethics

This study was approved by the Regional Ethics Committee in Norway (REK 2015/670). One of the conditions for approval was that strict privacy concerns were respected, and that data was not made publicly available. Specific requests regarding data sharing should be directed to the corresponding author.

RESULTS

We included 694 patients with a total of 1,146 drug initiations; demographics are shown in **Table 3** and drug swaps are illustrated in **Figure 1**. Of the patients who started a high efficacy DMT as the first drug, 68.0% achieved NEDA at year 1 and 52.4% achieved NEDA at year 2. Conversely, 36.0% of patients who started a moderate efficacy DMT as a first drug achieved NEDA in year 1 and 19.4% in year 2 (**Table 4**). The superior effect of high efficacy vs. moderate efficacy DMT on NEDA was highly significant ($p < 0.001$) at both year 1 and 2.

The odds ratio of achieving NEDA on a high efficacy DMT as first drug at year 1 was 3.9 (95% CI 2.4–6.1, $p < 0.001$) and at year 2 was 4.6 (96% CI 2.8–7.6, $p < 0.001$) compared to moderate efficacy DMTs (**Table 5**). The odds ratio did not change meaningfully after adjusting for sex, age at start of medication and time from onset to start of medication. The difference in the proportion of patients achieving NEDA on high efficacy drugs and the odds ratio of achieving NEDA were lower for the second drug, but still highly significant ($p < 0.001$). There was no significant difference for the third drug (**Tables 4**, **5**).

We also looked at moderate and high efficacy drug initiations before and after 2013 (data not shown), and the findings remained largely unchanged. One exception is that the odds ratio adjusted for initiation before and after 2013 for the second drug increased from 2.5 (95% CI 1.66–3.9, $p < 0.001$) to 3.1 (95% CI 2.0–4.9, $p < 0.001$).

Age did not have a notable impact on the proportion achieving NEDA on the first drug. The proportion of older patients achieving NEDA on a moderate efficacy drug as the second drug was lower than younger patients (37.8 vs. 50.5%), but this was not significant ($p = 0.08$). As a third drug, however, there was a significant difference between moderate and high efficacy drugs in the younger population (72.6 vs. 62.5%, $p = 0.004$), but there was no significant difference in the older age group (**Supplementary Tables 1**, **2**).

Table 6 shows the demographic observations of those who achieved NEDA vs. those who did not achieve NEDA on moderate and high efficacy drugs in the three subgroups. Patients who achieved NEDA on moderate efficacy DMTs were in general slightly older, had longer time from onset to diagnosis and from onset to initiation of treatment. In contrast, this finding tended

TABLE 3 | Demographics of study population.

	First drug			Second drug			Third drug		
	All	**Moderate efficacy**	**High efficacy**	**All**	**Moderate efficacy**	**High efficacy**	**All**	**Moderate efficacy**	**High efficacy**
Number of drug initiations	594	491	103	381	191	190	171	56	115
Women, %	67.5	68.4	63.1	69.8*	74.3	65.3	70.8	64.3	73.9
Older than 40 years at start, %	47.6	48.9	41.7	54.6	57.1	52.1	59.6*	48.2	65.2
Mean age at initiation, years (SD)	38.6 (10.4)	38.8 (10.1)	37.8 (11.9)	40.0 (9.9)	40.8 (9.5)	39.2 (10.2)	40.8 (10.0)	39.8 (11.1)	41.3 (9.4)
EDSS at start, median (IQR)	2.0 (1.0–2.5)	2.0 (1.0–2.5)	1.5 (1.0–2.4)	2.0 (1.5–3.0)	2.0 (1.5–2.5)	2.0 (1.4–2.0)	2.0 (1.5–3.5)	1.5 (1.0–2.0)	2.0 (1.5–2.3)
Mean age at onset, years (SD)	33.8 (9.9)	33.8 (9.6)	33.5 (11.3)	31.8 (9.2)	32.6 (9.0)	30.9 (9.2)	30.3 (9.0)	29.9 (8.7)	30.6 (9.2)
Years from onset to diagnosis, mean (SD)	3.3 (5.3)	3.3 (5.3)	3.2 (4.9)	3.3 (4.9)	3.5 (4.9)	3.2 (4.9)	3.3 (4.1)	2.7 (3.7)	3.5 (4.2)
Years from onset to drug initiation, mean (SD)	4.9 (6.5)	5.0 (6.6)	4.4 (6.1)	8.2 (6.9)	8.2 (6.6)	8.2 (7.2)	10.7 (7.6)	9.8 (8.4)	11.2 (7.1)
Years from diagnosis to drug initiation, mean (SD)	1.6 (3.9)	1.7 (4.0)	1.1 (3.1)	4.9 (4.9)	4.7 (4.5)	5.1 (5.3)	7.3 (5.4)	6.7 (6.0)	7.5 (5.1)
>10 MRI lesions at diagnosis, %	43.6**	40.3	59.2	40.7*	39.8	41.6	40.4	30.4	45.2
Multiple symptoms at onset, %	30.3	27.7	42.7	30.1	39.8	29.4	32.6	39.1	29.6
≥2 relapses before diagnosis, %	71.9*	70.6	78.4	77.3	75.1	79.4	72.8	66.0	75.9
Sensory symptoms at onset, %	40.7*	41.1	38.6	40.3	35.3	45.2	43.1	37.0	46.0
Motor symptoms at onset, %	11.8	11.8	11.9	11.2	12.3	10.1	14.4	13.0	15.0
EDSS at diagnosis, median (IQR)	2.0 (1.0–2.0)	2.0 (1.5–2.5)	2.0 (1.0–2.5)	2.0 (1.3–2.0)	2.0 (1.3–3.5)	2.0 (1.0–3.0)	2.0 (1.1–2.0)	1.5 (1.0–2.3)	2.0 (1.5–3.3)
Low risk (0–3 points), %	12.5**	14.3	3.9	10.8	12.6	8.9	14.6	21.4	11.3
Medium risk (4–7 points), %	52.2	54.0	43.7	54.9	53.4	56.3	52.6	48.2	54.8
High risk (8–14 points), %	35.4	31.8	52.4	34.3	56.3	34.7	32.7	30.4	33.9

SD, standard deviation; IQR, interquartile range; EDSS, expanded disability status scale.
**p < 0.05, **p < 0.001.*

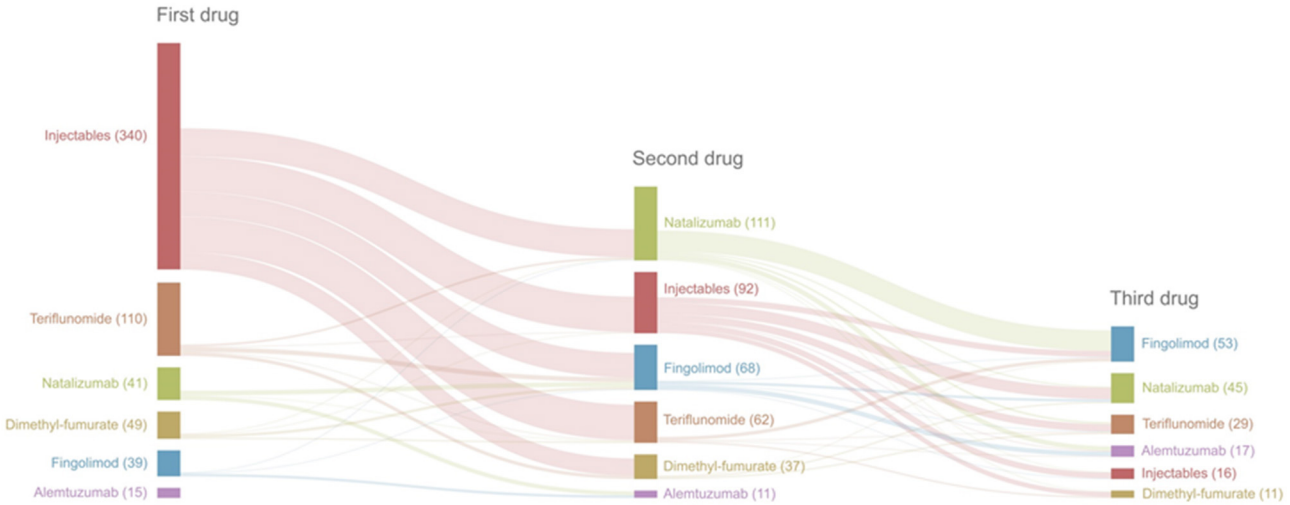

FIGURE 1 | Flow chart of drug swaps.

TABLE 4 | NEDA year 1 and NEDA year 2.

		Year 1				Year 2				
		Achieved NEDA			Total	Achieved NEDA			Total	Missing year 2
		n=	%	*p*		*n*=	%	*p*		
First drug	Moderate efficacy	177	36.0	<0.001	491	83	19.4	<0.001	428	7
	High efficacy	70	68.0		103	43	52.4		82	3
Second drug	Moderate efficacy	86	45.0	<0.001	191	38	23.5	<0.001	162	5
	High efficacy	127	66.8		190	85	52.1		163	5
Third drug	Moderate efficacy	24	42.9	0.18	56	13	27.1	0.25	48	1
	High efficacy	62	53.9		115	36	36.7		98	2

Moderate efficacy: Injectables (interferon and glatiramer acetate), teriflunomide and dimethyl-fumarate. High efficacy: Fingolimod, natalizumab, and alemtuzumab.

to be reversed in patients on high efficacy therapies who achieved NEDA. Patients with a medium or high risk of disease activity (87.6% of patients on a first drug, 89.2% of patients on a second drug and 85.3% of patients on a third drug) were significantly more likely to achieve NEDA on a high efficacy therapy as a first drug. There was no significant difference in patients on moderate efficacy therapy, or any second or third drug, regardless of potency.

The numbers of patients on the individual drugs achieving NEDA are presented in **Table 7**. Natalizumab and fingolimod are the only DMTs that are significantly more likely than the injectables to achieve NEDA at year 1 and 2 as a first drug, though the numbers of alemtuzumab were small and 100% of patients on alemtuzumab as a second drug achieved NEDA. All the DMTs were superior to the injectables as a second drug. The adjusted odds ratio of each individual drug vs. the injectables are presented in **Table 7** and **Figure 2**. Natalizumab as a first drug has an odds ratio of 7.4 (95% CI 3.5–15.4, $p < 0.001$) for reaching NEDA, which is superior to all the other drugs (see **Figure 2**), though the confidence interval is large. Teriflunomide and dimethyl-fumarate as a first drug did not have significantly better odds ratios at year 1 or 2 than the injectables. As a second drug, all the DMTs were superior to injectables at year 1 and 2. The odds ratio of achieving NEDA on a third drug was less convincing. Adjusting for sex, age at start of medication, time from onset to start of medication and risk groups did not meaningfully alter the results.

Unsurprisingly, patients on moderate efficacy therapy as a first drug were more likely to discontinue treatment than patients on a high efficacy therapy as a first drug (65.2 vs. 29.2%, $p < 0.001$). This was also the case in patients on a second drug (55.4 vs. 42.7%, $p = 0.02$) but not in patients on a third drug (42.9 vs. 29.5%, $p = 0.10$). **Table 8** shows the number of patients who discontinued therapy on moderate and high efficacy therapies and causes of discontinuation. Patients on moderate efficacy therapy as a first drug were more likely to discontinue due to side effects than patients on high efficacy therapy as a first drug (45 vs. 14%, $p = 0.002$). This was also the case for the second drug (40

TABLE 5 | Odds ratio (OR) analyzed by binary logistics for NEDA year 1 and 2 in high efficacy DMT vs. moderate efficacy DMT, stratified by risk and adjusted for age at initiation of medication, time from onset to initiation of drug and sex.

	Year 1				Year 2			
	OR	95% CI for OR		*p*-value	OR	95% CI for OR		*p*-value
		Lower	Upper			Lower	Upper	
First drug	3.9	2.4	6.1	<0.001	4.6	2.8	7.6	<0.001
Low risk ($n = 74$)	2.3	0.3	18.4	0.44	2.1	0.1	30.5	0.59
Medium risk ($n = 310$)	2.4	1.3	4.6	0.008	3.0	1.4	6.5	0.005
High risk ($n = 210$)	6.2	3.0	13.0	<0.001	5.9	2.8	12.5	<0.001
Second drug	2.5	1.7	3.9	<0.001	3.5	2.1	5.6	<0.001
Low risk ($n = 41$)	8.8	1.4	56.6	0.02	12.1	1.6	94.4	0.02
Medium risk ($n = 209$)	2.8	1.6	4.9	<0.001	3.9	2.0	7.7	<0.001
High risk ($n = 131$)	1.9	0.9	4.0	0.07	2.0	0.9	4.5	0.08
Third drug	1.5	0.8	2.9	0.25	1.5	0.7	3.4	0.30
Low risk ($n = 25$)	3.3	0.4	28.7	0.28	3.4*	0.3	39.3	0.33
Medium risk ($n = 90$)	0.9	0.3	2.1	0.7	1.1	0.4	3.3	0.36
High risk ($n = 56$)	2.2	0.7	7.7	0.2	1.5	0.4	6.3	0.57

There is minimal difference between unadjusted and adjusted OR.
**Not adjusted for gender due to small numbers.*

vs. 14%, $p < 0.001$). The number of patients who discontinued a third drug were too small to draw a conclusion ($n = 4$ and $n = 5$).

DISCUSSION

In this Norwegian population-based, real-world study we found that patients who start high efficacy therapies are significantly more likely to achieve NEDA at year 1 and 2 than patients starting moderate efficacy therapy. However, the odds ratio of achieving NEDA is reduced for each attempted drug.

Patients started on a high efficacy drug as a first DMT had an odds ratio of achieving NEDA of 3.9 compared to the moderate efficacy drugs, adjusting for sex, age and time from onset to diagnosis. The odds ratio was reduced to 2.5 as a second drug, and the odds ratio of 1.5 was not significant for the third drug. Age did not have a notable impact on the proportion of patients achieving NEDA on the first and second drug, but older patients were less likely to achieve NEDA on the third drug. Our findings illustrate the importance of choosing the most effective drug at the time of diagnosis. These findings were especially strong in the 90% of patients who were classified as having a medium to high risk of disease activity.

NEDA is by no means a perfect tool as it is overly reliant on MRI (29), it does not take into account subtle deterioration in fine motor skills and cognitive changes, and there is no consensus regarding the definitions of the different components (13). Failure to achieve NEDA is not necessarily a good predictor of long-term disability (14). However, neuronal injury occurs early in the disease, and limited disease activity within the first few years of diagnosis is widely regarded as a good prognostic sign (15).

Our findings are in accordance with studies supporting high efficacy therapy at the time of diagnosis compared to an escalation approach (30, 31). The escalation approach may be inadequate to prevent unfavorable outcomes in a real-world population (32), and this is important as the disease activity in the first couple of years influence the disease course (33, 34). The risk of progression at 10 years is highly dependent on EDSS score at 5 years, and it progresses more rapidly from EDSS 4 onwards compared to EDSS 2 and onwards (35). In the absence of a cure, an increasing body of evidence supports early initiation of high efficacy disease modifying treatment in MS to halt disease activity and reduce disability progression (36, 37).

However, many neurologists still utilize a stepwise approach in initiating disease modifying therapy, starting with the safer, but less effective therapies, and only escalate once there is sign of disease activity (38). This is reflected in national guidelines, regulatory bodies and insurance policies (1, 30, 39). In addition, some argue that there is no need for high efficacy treatment in patients with positive prognostic factors and a suspected "mild" disease (5). In our cohort, patients who achieved NEDA on moderate efficacy drugs tended to be older and have longer time from onset and diagnosis to start of drug initiation. This most likely reflects the disease rather than the drug efficacy. Patients with delayed drug initiation after onset and diagnosis have more likely been followed with a watchful wait approach (40). These patients have fewer relapses and less MRI activity, and thus less disease activity and less incentive to initiate immunomodulatory treatment early. However, the concept of mild or benign MS is controversial (37, 41). One study found only nine of 1,049 patients with disease duration of >15 years and EDSS <4 were truly benign (42). Ellenberger et al. found one in four patients with benign MS at 15 years were unemployed, and only one in three remained benign after 30 years (43). Smestad et al. found that although only one third of MS patients in an Oslo cohort had mild disability based on EDSS, half of them were cognitively impaired (44).

TABLE 6 | Demographics by NEDA and no NEDA.

	First drug						Second drug						Third drug					
	Moderate efficacy			High efficacy			Moderate efficacy			High efficacy			Moderate efficacy			High efficacy		
	NEDA	No NEDA	*p*	NEDA	No NEDA	*p*	NEDA	No NEDA	*p*	NEDA	No NEDA	*p*	NEDA	No NEDA	*p*	NEDA	No NEDA	*p*
	n = 177	*n* = 314		*n* = 70	*n* = 33		*n* = 86	*n* = 105		*n* = 127	*n* = 63		*n* = 24	*n* = 32		*n* = 62	*n* = 53	
Women, %	63.8	71	0.10	62.9	63.6	0.94	79.1	70.5	0.18	3.8	68.3	0.54	66.7	62.5	0.75	67.7	81.1	0.10
Older than 40 years at initiation of drug, %	52	47.1	0.30	42.9	39.4	0.74	64.0	51.4	0.08	50.4	55.6	0.50	**66.7**	**34.4**	**0.02**	59.7	71.7	0.18
Age at start, years mean (SD)	**40.2 (9.6)**	**38.0 (10.3)**	**0.02**	38.1 (12.7)	37.3 (9.9)	0.77	41.9 (8.5)	39.8 (10.1)	0.13	39.2 (10.7)	39.2 (9.4)	1.00	**45.2 (9.1)**	**35.7 (10.8)**	**0.001**	40.6 (9.7)	42.1 (9.1)	0.42
EDSS at initiation, median (IQR)	1.8 (1.0, 2.5)	2.0 (1.0, 2.5)	0.87	2.5 (1.5, 3.4)	2.0 (1.5, 2.8)	0.43	2.0 (1.0, 2.5)	2.0 (1.0, 3.0)	0.28	2.5 (2.0, 3.5)	2.5 (1.6, 4.0)	0.64	**2.0 (1.5, 2.5)**	**1.5 (0.8, 2.0)**	**0.01**	2.0 (1.5, 3.5)	2.8 (2.0, 3.5)	0.10
Age at onset, years mean (SD)	34.4 (9.6)	33.5 (9.6)	0.35	33.6 (12.2)	33.2 (9.4)	0.86	32.6 (8.5)	32.7 (9.5)	0.93	31.7 (9.3)	29.4 (9.0)	0.11	32.6 (9.1)	27.8 (7.9)	0.04	31.4 (9.2)	29.7 (9.2)	0.32
Years from onset to diagnosis, mean (SD)	**4.1 (5.8)**	**2.8 (5.0)**	**0.01**	3.3 (5.4)	3.2 (3.7)	0.93	3.8 (5.5)	3.3 (4.4)	0.44	2.7 (4.8)	4.1 (5.1)	0.07	3.1 (4.1)	2.4 (3.3)	0.51	3.1 (3.8)	4.1 (4.6)	0.21
Years from onset to drug initiation, mean (SD)	**5.8 (7.1)**	**4.5 (6.3)**	**0.03**	4.4 (6.1)	4.2 (6.0)	0.65	**9.3 (7.6)**	**7.3 (5.6)**	**0.05**	**7.3 (6.7)**	**10.0 (7.9)**	**0.02**	**12.5 (9.5)**	**7.8 (6.9)**	**0.03**	10.1 (6.0)	12.4 (8.0)	0.08
Years from diagnosis to drug initiation, mean (SD)	1.7 (3.8)	1.7 (4.1)	0.87	1.2 (2.8)	1.0 (3.6)	0.91	**5.4 (5.2)**	**4.1 (3.8)**	**0.05**	4.8 (5.1)	5.8 (5.7)	0.24	**9.4 (6.9)**	**4.7 (4.4)**	**0.003**	6.8 (4.3)	8.3 (5.9)	0.10
Multiple symptoms at onset, %	27.5	27.9	0.93	47.5	32.1	0.17	35.1	27.4	0.28	29.1	30.2	0.89	47.6	32	0.28	30.9	27.9	0.75
>10 MRI lesions at diagnosis, %	**41.9**	**58.1**	**<0.001**	**75.4**	**24.6**	**<0.001**	**55.3**	**44.7**	**<0.001**	65.8	34.2	0.41	41.2	58.8	0.19	57.7	42.3	0.61
≥2 relapses before diagnosis, %	72.2	68.7	0.57	79.1	76.7	0.79	71.4	78.5	0.28	78.8	80.6	0.77	54.5	75	0.13	74.1	78	0.64
Sensory symptoms at onset, %	36.3	43.7	0.11	37.1	41.9	0.65	33.7	36.5	0.69	45.6	44.4	0.88	37.5	36.7	0.95	**35**	**58.5**	**0.01**
Motor symptoms at onset, %	11.7	11.9	0.95	14.3	6.5	0.26	12.0	12.5	0.93	7.2	15.9	0.06	16.7	10	0.47	20	9.4	0.12
EDSS at diagnosis, median (SD)	1.5 (1.0, 2.0)	2.0 (1.0, 2.5)	0.40	1.8 (1.0, 3.5)	2.0 (0.0, 2.3)	0.35	**2.0 (1.0, 2.0)**	**2.0 (1.5, 2.5)**	**0.04**	2.0 (1.5, 3.0)	2.0 (1.0, 2.5)	0.54	1.5 (1.0, 2.3)	1.0 (1.0, 2.5)	0.52	2.0 (1.5, 2.4)	2.0 (1.0, 2.0)	0.63
Low risk of disease activity, %	27.1	72.9	0.09	50.0	50.0	0.43	50.0	50.0	0.60	**64.7**	**35.3**	**0.05**	25.0	75.0	0.16	46.2	53.8	0.55
Medium risk of disease activity, %	37.0	63.0	0.64	**57.8**	**42.2**	**0.05**	40.0	59.8	0.15	64.5	35.5	0.43	55.6	44.4	0.06	52.4	47.6	0.72
High risk of disease activity, %	38.5	61.5	0.45	**77.8**	**22.2**	**0.03**	50.8	49.2	0.25	65.2	34.8	0.72	35.3	64.7	0.45	59.0	41.0	0.44

SD, standard deviation; IQR, interquartile range; EDSS, expanded disability status scale. Significant findings are highlighted in bold.

TABLE 7 | NEDA and odds ratio of reaching NEDA on individual drugs at year 1 and 2.

		Year 1						Year 2						
		NEDA					Odds ratio	NEDA					Odds ratio	Missing year 2
		n=	%	*p*	Total	OR	(95% CI), *p*	*n*=	%	*p*	Total	OR	(95% CI), *p*	
First drug	Injectables	118	34.7		340	1.0		61	20.3		301	1.0		3
	Teriflunomide	39	35.5	0.89	110	0.9	(0.6–1.6), $p = 0.94$	11	12.0	0.07	92	0.5	(0.3–1.0), $p = 0.07$	2
	Dimethyl-fumarate	20	48.8	0.08	41	2.0	(1.0–3.8), $p = 0.05$	11	31.4	0.13	35	2.0	(0.9–4.3), $p = 0.09$	2
	Natalizumab	39	79.6	<0.001	49	6.9	(3.2–14.4), $p < 0.001$	27	64.3	<0.001	42	6.5	(3.2–13.1), $p < 0.001$	2
	Fingolimod	21	53.8	0.02	39	2.0	(1.0–4.0), $p = 0.04$	12	40.0	0.01	30	2.4	(1.1–5.3), $p = 0.03$	1
	Alemtuzumab	10	66.7	0.01	15	4.4	(1.5–13.5), $p = 0.009$	4	40.0	0.13	10	2.8	(0.8–10.5), $p = 0.12$	0
	Total	**247**	**41.6**		**594**			**126**	**24.7**		**510**			**10**
Second drug	Injectables	30	32.6		92	1.0		11	13.6		81	1.0		0
	Teriflunomide	34	54.8	0.006	62	2.7	(1.4–5.3), $p = 0.005$	13	28.3	0.04	46	2.5	(1.0–6.3), $p = 0.04$	4
	Dimethyl-fumarate	22	59.5	0.005	37	3.3	(1.5–7.6), $p = 0.004$	14	40.0	0.001	35	4.4	(1.7–11.1), $p = 0.002$	1
	Natalizumab	72	64.9	<0.001	111	4.2	(2.3–7.7), $p < 0.001$	54	51.4	<0.001	105	6.5	(3.1–13.7), $p < 0.001$	0
	Fingolimod	44	64.7	<0.001	68	4.4	(2.2–8.7), $p < 0.001$	26	49.1	<0.001	53	6.2	(2.7–14.6), $p < 0.001$	4
	Alemtuzumab	11	100.0	<0.001	11	*	*	5	100.0	<0.001	5	*	*	1
	Total	**215**	**55.9**		**381**			**123**	**27.8**		**325**			**10**
Third drug	Injectables	4	25.0		16	1.0		1	7.1		14	1.0		0
	Teriflunomide	12	41.4	0.27	29	1.9	(0.5–7.7), $p = 0.38$	7	26.9	0.14	26	4.4	(0.5–40.8), $p = 0.20$	1
	Dimethyl-fumarate	8	72.7	0.01	11	8.5	(1.3–53.7), $p = 0.02$	5	62.5	0.005	8	20.9	(1.7–260.6), $p = 0.02$	0
	Natalizumab	27	60.0	0.02	45	4.4	(1.2–17.1), $p = 0.03$	19	47.9	0.007	40	12.5	(1.4–107.9), $p = 0.02$	1
	Fingolimod	24	45.3	0.15	53	1.9	(0.5–7.0), $p = 0.36$	13	27.7	0.11	47	3.7	(0.4–33.0), $p = 0.24$	1
	Alemtuzumab	11	64.7	0.02	17	4.9	(1.0–24.3), $p = 0.05$	4	36.4	0.07	11	6.9	(0.6–83.6), $p = 0.13$	0
	Total	**86**	**50.3**		**171**			**49**	**33.6**		**146**			**3**

P-values and odds ratio compared to injectables. Odds ratio, analyzed by binary logistics regression, was adjusted for age at start of medication, time from onset to start of drug, sex and risk group.
**100% of patients achieved NEDA. The total number and percentage of drugs as a first, second or third drug are highlighted in bold.*

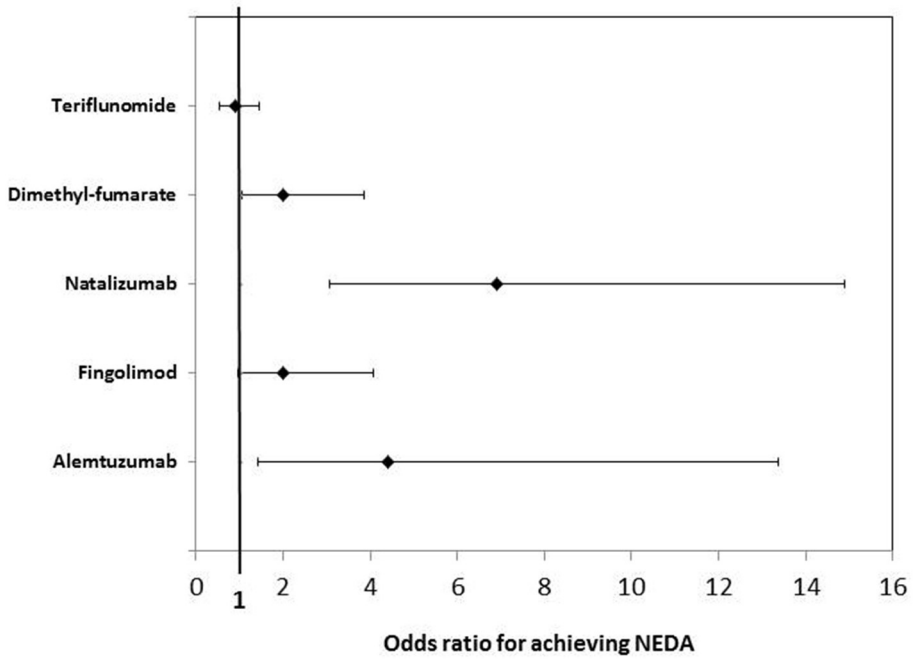

FIGURE 2 | Forest plot of odds ratio for reaching NEDA at year 1 compared to the injectables (interferon and glatiramer acetate).

TABLE 8 | Information on drug discontinuation.

	First drug		Second drug		Third drug	
	Moderate efficacy	**High efficacy**	**Moderate efficacy**	**High efficacy**	**Moderate efficacy**	**High efficacy**
Discontinued, *n*=	276 (65.2%)	28 (29.2%)	93 (55.4%)	79 (42.7%)	21 (42.9%)	33 (29.5%)
Months on drug before disontinuation (SD)	32.1 (23.9)	33.7 (21.1)	29.0 (17.0)	48.3 (27.4)	28.4 (17.0)	41.5 (24.6)
Causes for discontinuation, %						
Lack of efficacy	31.0	17.9	36.6	10.1	52.4	18.2
Side effects or fear of side effects	44.9	14.3	39.8	13.9	19.0	15.2
Pregnancy	7.2	7.1	5.4	12.7	9.5	6.1
Lack of compliance	5.4	3.6	9.7	6.3	4.8	9.1
Converted to progressive disease	2.9	10.7	4.3	12.7	4.8	12.1
NAB positive	7.6	0	3.2	1.3	4.8	3.0
JCV positive	0	46.4	0	43.0	0	33.3
Unknown	0.4	0	1.1	0	4.8	3.0

SD, standard deviation; NAB, neutralizing antibodies; JCV, John Cunningham virus.

One argument for not initiating high efficacy treatment early is the safety profile (15). However, natalizumab has few side effects beyond the risk of progressive multifocal leukoencephalopathy (PML), and this risk has been mitigated with intensified follow-up regimes, monitoring of the JC-virus index and possibly extended interval dosing (45). The three hospitals included in this study utilize natalizumab frequently. We check JC-virus index biannually and discontinue the drug in cases of elevated titres. Due to risk stratification, none of the hospitals has experienced PML, despite a combined population of more than 2,500 patients, or a quarter of the national MS population. In addition, patients treated with alemtuzumab are monitored closely for 5 years after treatment initiation, and there have been no deadly outcomes from alemtuzumab treatment. Also moderate efficacy drugs are certainly not without side effects that can significantly affect quality of life (46). Our population was significantly more likely to discontinue moderate efficacy therapies due to side effects than high efficacy therapies. The injectables have poorer acceptability profiles than other DMTs, and the high efficacy drugs have lower dropout rates than moderate efficacy drugs (47). Although side effects from moderate efficacy therapies are rarely life threatening, there are several reported cases of PML in Tecfidera

treated patients (48). In the end, higher disability at a younger age seems a more significant risk than most of the adverse effects associated with established high efficacy DMTs.

The European (ECTRIMS/EAN) guidelines of 2018 suggest the choice of treatment depends on patient characteristics, disease severity, safety profile and drug accessibility (6). They advise escalating treatment if there is disease activity despite injection therapy. The American Academy of Neurology guidelines notes that patients with a highly active disease should be treated with high efficacy DMT (7). Neither the European guidelines, nor the American guidelines recommend a specific treatment strategy. Two large randomized clinical trials (TREAT-MS, NCT035300328 and DELIVER-MS, NCT03535298) examining escalation vs. early high efficacy therapy are currently underway and will provide valuable information on the short-term differences between these two treatment strategies. However, the differences in long-term disability will require decades of follow-up time, and the available evidence favors early high efficacy therapy. In our opinion, international guidelines should consider updating their recommendations according to current knowledge.

The strength of this study is the well-defined study population. The ratio of neurologists per capita in 2017 was 9.5/100,000 (data from The Norwegian Doctors' Union), and almost all Norwegian MS patients are followed by neurologists at public hospitals. There are few neurologists in private practice. All Norwegian MS neurologists had complete access to all therapies available in Europe at the time of approval, and all these drugs are reimbursed. Real-world studies, such as this, are not restricted by stringent inclusion criteria but instead assess the entire heterogeneous population and can therefore be generalized beyond their study frames (49). BOT-MS is a population-based registry, and a major strength as a real-world study is that we have limited selection bias and know who is missing and why.

Real-world data is also subject to missing data, which is a source of potential information bias. Many of the patients started on the injectables might not have been followed as strictly as those started on the newer drugs. Thus, patients with enough information on the composites of NEDA to be included were likely to have more disease activity. This means there may be an underrepresentation of NEDA patients in this group. This possible information bias was partly counteracted by only including patients started on treatment as of 2006, the year the first highly potent disease modifying drug, natalizumab, was made available to our patients. From this point onwards, there was a more stringent follow-up process of all MS patients. In addition, the odds ratio for teriflunomide was the same as injectables. Our findings also remained largely unchanged before and after 2013, which marks the introduction of teriflunomide and dimethyl-fumarate.

We have created a risk score to categorize patients as having low, medium and high risk of disease activity. Our choices in creating this score were based on available literature (17–26), though we acknowledge that others may categorize the risk differently. In fact, the MS community's ability of predicting individual disease development is limited (37). Our score is based on easily accessible data, though ideally it should have included information on smoking (50), vitamin D (51), and spinal cord lesions, gadolinium enhancing lesions (52) and atrophy (53) on MRI, to name a few. This score has not yet been validated, and we would like to validate it in a new population. We could have used propensity score analyses to control for confounding, but propensity score matching does not yield different estimates compared to conventional multivariate methods (54) and is often used inappropriately in MS research (55).

We acknowledge that treatment allocation bias may play a role in this study. The cohort exposed to high efficacy drugs as a first drug were younger, with lower disease duration and more MRI lesions and relapses at presentation. It is likely that this would lead to a greater response to immunotherapy (15). We do not believe this weakens our study, but rather strengthens our findings and our conclusion that more people should be offered high efficacy therapies. Of the 199 people with medium or high risk of disease activity diagnosed in 2013 or after, 64% were started on moderate efficacy therapy as a first drug. These patients should have received high efficacy therapy from the start (37).

We have chosen to categorize fingolimod as a high efficacy therapy since that is how it was portrayed when it first arrived on the market (56). International, national and local guidelines (6) consequently recommended it as a choice for treatment escalation in highly active MS during the span of this study, and treatment choices were subsequently decided based on this premise. However, many studies conclude that fingolimod has a similar efficacy profile to the moderate efficacy therapy dimethyl-fumarate (57, 58), though not all (16, 47). We have shown NEDA-data on each individual drug in this study in addition to the two efficacy groups. Regardless, the allocation of fingolimod as a moderate efficacy therapy would only strengthen our conclusion that achieving NEDA is significantly more likely in patients on high-efficacy disease modifying therapies.

Another potential bias is observation bias. All patients treated with natalizumab are seen monthly, and the patients treated with teriflunomide are seen frequently in the first year after initiation. These patients were thus more inclined to mention relapses to their treating MS team, as opposed to the remaining MS patients who are seen less often (59). Despite this, natalizumab patients did better than other patients. Another weakness is the retrospective data retrieval, subjecting the study to investigator bias. This was ameliorated by only having three neurologists specialized in MS to include in the database based on a mutually accepted manual. Finally, we did not have enough observations to make a confident statement on the odds ratio of reaching NEDA on alemtuzumab, and we have not included cladribine, another high efficacy DMT, which was approved after the inclusion period.

CONCLUSION

Achieving NEDA is significantly more likely in patients on high-efficacy disease modifying therapies than on moderate efficacy therapies, and the first choice of treatment is the most important. Moderate efficacy therapies should be used with caution in most MS patients, unless the clinician is confident the patient

has a less active form of MS. There is a need for updating immunomodulatory treatment guidelines ensuring early, high efficacy therapy for the majority of patients diagnosed with MS.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because one of the conditions for ethics approval was that strict privacy concerns were respected, and that data was not made publicly available. Requests to access the datasets should be directed to cecsim@vestreviken.no.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Regional Ethics Committee in Norway (REK 2015/670). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

CS, HF, LB, PB-H, SM, and EC: design and conceptualization. CS, HF, and LB: data acquisition. CS, HF, LB, CB, PB-H, SM, and EC: data analysis, data interpretation, and writing original draft. 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.2021.693017/full#supplementary-material

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REVIEW
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Influence of Pregnancy in Multiple Sclerosis and Impact of Disease-Modifying Therapies

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Purpose of this Review: This article is a systematic review on the influence pregnancy has on multiple sclerosis and the resulting impact of disease-modifying therapies.

Findings: Multiple sclerosis predominantly affects young women with a clinical onset most often during the child-bearing age. The impact of multiple sclerosis and disease-modifying therapies on fertility, pregnancy, fetal outcome, and breastfeeding is a pivotal topic when it comes to clinical practice. The introduction of disease-modifying therapies has changed not only the natural history of the disease but also the perspective of pregnancy in women with multiple sclerosis. Family planning requires careful consideration, especially because many disease-modifying drugs are contraindicated during pregnancy. In this article, we review current evidence collected from published literature and drug-specific pregnancy registers on the use of disease-modifying therapies. Additionally, we discuss safety profiles for each drug and correlate them to both risk for the exposed fetus and risk for the mothers interrupting treatments when seeking pregnancy.

Keywords: multiple sclerosis, pregnancy, delivery, breastfeeding, newborn, disease modifying therapy, postpartum

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INTRODUCTION

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system with a chronic course, mainly affecting young women, the majority of whom are of childbearing age.

Several factors have been suggested to explain the progressive increase in MS incidence in adult women in the last 30 years, including interactions between genes and environment, lifestyle modifications (contraception, diet, obesity, smoking, sunlight exposure, and vitamin D deficiency), older age at the birth of the first child, younger age at the menarche, or fewer pregnancies during a woman's lifetime (1–4).

Until the end of the 1990s, women affected by MS were frequently falsely discouraged to undertake pregnancy. Only later, the management of pregnancy in women with MS, from planning to conception and postpartum period, has been radically reviewed. The study published by Confraveux et al. (5) was pivotal in reshaping the idea of pregnancy in women with MS. Moreover, the progressive introduction of disease-modifying therapies (DMTs) has completely transformed the natural history of MS, consequently improving the perspective of pregnancy in affected women.

Several concerns afflict women who intend to plan a pregnancy, namely, the impact of the disease on fertility, the risk of transmitting MS to the progeny, the possible adverse effects of drugs

on the fetus, the influence of pregnancy on MS course, the impact of the disease on the mother's ability to care for her baby, and finally, the socioeconomic burden of the disease on the family (6).

All these reasons pose an extra challenge in guiding MS women in their fertile age in making choices about pregnancy (7).

Our article is a systemic review on the influence that pregnancy has on MS and the resulting impact of DMTs. For this purpose, we performed a complete revision of literature data through MEDLINE, PubMed, and Cochrane Database, in the period from June 1982 to March 2021. MS, pregnancy, delivery, breastfeeding, newborn, disease-modifying therapy, and postpartum have been the main keywords we used to identify the most relevant studies on the topic.

FERTILITY IN MS

The effect MS may have on fertility is still debated. Sexual dysfunction is a common and frequent complaint in women with MS, eventually affecting their quality of life (8, 9). Reduced libido, difficulty in achieving orgasm, and dyspareunia are often reported, as well as bladder and bowel symptoms, which may affect sexual activity, interfering with social and intimate interactions in both sexes (10, 11). Furthermore, changes in sexual hormones have been reported in women with MS. High levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) have been described, associated with low estrogen levels in the initial part of the follicular phase of the menstrual cycle, hyperprolactinemia, and hyperandrogenism (12), this latter suggesting a relationship with the slightly higher incidence of oligo/amenorrhea (13). Despite these noticeable evidences, no statistically significant association between sexual dysfunction and blood hormone abnormalities has been reported (14).

There are epidemiological studies reporting that women with MS have less children compared to the general population (15). Several factors have been suggested to interfere with parenthood, including health issues, drug-induced decreased fertility, symptoms such as fatigue, sexual or bladder dysfunction, and personal decision influenced by the disease to avoid pregnancy (16–18).

Although MS does not seem to impact on fertility, a history of infertility may be reported in women with MS, which is not necessarily linked by a cause–effect relation with the disease. In case of infertility, assisted reproductive technology (ART) becomes another relevant matter. There is evidence of an unfavorable effect of gonadotropin-releasing hormone (GnRH) agonists on disease activity, because of their effect in stimulating proliferation of leukocytes, as well as production of cytokines, chemokines, endothelial growth factor, and estrogen. On the contrary, use of GnRH antagonists would appear to be safer, although data still needs confirmation (19–22). Nevertheless, a recent study from pooled data of Boston, France, Germany, and Argentina cohorts reported an increased risk of relapses in the short-term period (3 months) after ART, both with GnRH agonists and antagonists. Furthermore, the authors suggest that continuing some MS DMTs might decrease this risk of relapse in women undergoing ART (23).

There are only few studies in literature focusing on male fertility in MS and are principally focused on sexual dysfunction, although according to one study found, males with MS present a reduced semen quality, associated with hypogonadotropic hypogonadism (24, 25).

IMPACT OF PREGNANCY ON MS DISEASE ACTIVITY

For what concerns the short-term impact of pregnancy on the course of the disease, several evidences point toward a positive decrease in the annualized relapse rate during pregnancy, in particular during the last trimester, which is however followed by a postpartum rebound mainly in the first 3 months after delivery (5, 26, 27). The PRIMS (Pregnancy in Multiple Sclerosis) study confirmed a decrease in annualized relapse rate (ARR) during the third trimester of pregnancy (0.2 compared to 0.7 in the year before pregnancy), as well as an increase in ARR during the first 3 months postpartum (1.2 compared to 0.7 in the year before pregnancy) so that the ARR in the post-pregnancy year was similar to that in antepartum (26). A recent study conducted in a large population-based cohort from Southern and Northern California medical centers, including 466 patients, confirmed in the entire cohort a significant lower ARR during pregnancy compared with the 2 years before conception. However, unlike previous reports, the relapse rate did not increase in the first 3 months postpartum. Moreover, the majority of women who were relapse free in the postpartum period were not taking any DMTs, and, even more surprisingly, they had only a suboptimally controlled disease at the time of conception (28).

Changes in frequency of the relapse risk both during gestation and in the postpartum period seem to be linked to fluctuations in estrogenic levels. Indeed, estrogens have a dose effect that is biphasic, since they boost the immune system at low levels, as in childbearing age, while they are immunosuppressive, hence protective, at higher levels, such as in pregnancy. The most acknowledged theory to explain the protective effect pregnancy exerts on disease activity is that, during pregnancy, estrogens with other sex hormones induce a switch in the T helper (Th) cell profile from Th1 (pro-inflammatory cytokines) to Th2 (anti-inflammatory cytokines). After delivery, the immune system gradually returns to its pre-gestation profile, which translates to disease rebound (29–33).

Early postpartum relapses notably have a poor prognostic value for what concerns MS disability progression (34). The main risk factors identified include a higher ARR in the 2 years before conception, the number of relapses during gestation, higher EDSS score at fecundation, and lastly, no history of DMT use 2 years before conception (5, 26, 35–37). Similarly, active pre-pregnancy MRI is a strong and sensitive predictor of early

Abbreviations: AAR, annualized relapse rate; ALZ, alemtuzumab; MS, multiple sclerosis; BBB, blood–brain barrier; DMF, dimethyl fumarate; DMT, disease-modifying therapy; EMA, European Medicine Agency; FDA, Food and Drug Administration; GA, glatiramer acetate; IFN β, interferon beta; NAT, natalizumab; PPMS, primary progressive multiple sclerosis; OCR, ocrelizumab; RRMS, relapsing–remitting multiple sclerosis.

postpartum relapse, which is independent of clinical evidence of disease activity prior to conception and delivery (38). These clinical and MRI findings could offer neurologists a valuable strategy to minimize postpartum relapse risk in female MS patients planning pregnancy. Some studies report that women taking DMTs for a minimum of 8 weeks during pregnancy carried a decreased risk of postpartum relapses compared to patients not taking any DMT during gestation or in the trimester before conception (39). All these observations would suggest taking DMT, if safe, until conception.

There are no systematic studies on MRI activity during pregnancy, because of the risk of exposing the fetus to the thermal effect of radiofrequency as well as of intravenous contrast agent use (40, 41).

In case of relapse during pregnancy, patients should avoid corticosteroids in the first trimester, due to risk of fetal malformations such as cleft palate (42). Still, short courses of high-dose methylprednisolone are the ideal first-line treatment choice, as they are relatively safe during the second and third trimesters. However, they should be exclusively used in case of particularly severe relapses. For disabling steroid-refractory relapses, plasma exchange may be recommended, with very low associated risks (e.g., thromboembolic events).

IMPACT OF PREGNANCY ON MS DISABILITY PROGRESSION

Another important aspect to evaluate is the impact pregnancy has on the long-term disability accumulation. Some studies showed that pregnancies have no influence on the time needed to reach a certain disability level (43–45), which instead could be predicted by a previous progressive disease course and older age at disease onset (46). On the contrary, a slower progression of disability was reported in women who conceived after disease onset, compared to the nulliparous women (47, 48). However, a bias of this latter finding could be the initial severity of the disease, which could lead the women to choose or not to become pregnant.

A recent study, including a large sample of 501 women, confirmed that pregnancy occurring after disease onset was associated with a slower disability progression only when pregnancy was analyzed as a baseline variable; conversely, this protective effect disappeared when pregnancy was considered as a time-dependent variable. The value of this study is primarily grounded on the elevated number of subjects and on the consistency of statistical analysis, which is characterized by a time-dependent approach to avoid any time-dependent bias and a propensity score to avoid selection biases (49).

Furthermore, the total number of pregnancies in an MS patient's lifetime did not appear to have a negative influence on the long-term course of the disease (26, 45, 46, 48, 50).

IMPACT OF MS ON PREGNANCY

Several studies support the evidence that MS does not impact pregnancy outcomes, which are not significantly different from the general population (51–53). A higher incidence of small for gestational age newborns, an increased predisposition to experience urinary tract infections and constipation, and more frequent interventions to induce labor, particularly in women with higher disability levels, have been reported (17, 52–55). Previous studies reported an increased rate of planned caesarean section or forceps assistance during vaginal births in MS mothers (54, 56) as well as an increased need for vacuum assistance (56, 57). On the contrary, in a large study conducted by British Columbia, mothers with MS were not more likely to receive assisted vaginal delivery or cesarean section (52).

With regard to epidural or general anesthesia, studies underline that both procedures are completely safe and they do not affect the risk of postpartum relapses (26, 58). All these observations remark that choices made during delivery have to remain with the obstetrician.

BREASTFEEDING

The role of breastfeeding remains controversial. Studies on the risk of MS relapse in the period after delivery reported that breastfeeding may reduce the postpartum relapse rate (28, 36, 59–61). In this regard, Langer-Gould et al., emphasizing the protective role of exclusive breastfeeding, suggested that MS women should be encouraged to breastfeed (28). The favorable effect of breastfeeding could be mediated by immunological mechanisms related to lactational amenorrhea (62). On the other hand, studies reported that exclusive breastfeeding had no influence on postpartum relapse rate (26, 63). These controversial results for a possible protective role of exclusive breastfeeding might depend on the selection bias in some studies, such as a limited number of cases, or the lack of correction for confounding variables (e.g., number of relapses in the year before pregnancy, treatment comparison, disease duration). To our knowledge, only two of the aforementioned studies presented with the following characteristics: a large number of subjects, a time-dependent approach, and inclusion of a propensity score. Those two studies, however, reached different conclusions (60, 63). The Italian study counting 302 pregnancies (46% of which treated with DMTs, in particular interferon or glatiramer acetate) and with a postpartum follow-up period of 1 year concluded that the only factor predicting postpartum relapses was the relapse rate before and during pregnancy and not breastfeeding (63). On the other hand, the German study on 201 pregnancies (76% treated with interferons or glatiramer acetate, 11% treated with natalizumab) and with a postpartum follow-up period of 1 year concluded that exclusive breastfeeding was associated with a lower risk of postpartum relapses, while the main factor predicting disease activity after delivery was the number of relapses during pregnancy (60). The authors concluded that exclusive breastfeeding may act like a modestly effective immunosuppressant for a limited time. Differences in the number of patients undergoing treatment and in the relapse rate before pregnancy (calculated on 2 years before conception in the German study, while only on 1 year before conception in the Italian one) could account for these different conclusions. Furthermore, the benign effect of breastfeeding in reducing postpartum relapses was more evident in women with a benign disease course who chose to breastfeed compared to women with a higher disease activity who stopped breastfeeding to restart

TABLE 1 | Impact on fertility induced by disease-modifying therapies in multiple sclerosis.

Drug	Effect on fertility
Interferon beta	No effect on fertility.
Glatiramer acetate	No effect on fertility.
Dimethyl fumarate	In animal studies reduction of estrogen, but no effect on fertility.
Fingolimod	No effect on fertility.
Siponimod	No effect on fertility.
Teriflunomide	In male animal studies reduction of sperm count, but no effect on fertility.
Cladribine	In male animal studies reduction of germ cells and sperm count, but no effect on fertility.
Natalizumab	In animal studies reduction in fertility. No data in humans.
Alemtuzumab	In animal studies reduction in corpora lutea and implantation in uterus. No data in humans.
Ocrelizumab	No effect on fertility.

DMTs (63, 64). In this regard, it is important to underline that the necessity to restart maternal treatment with DMTs becomes an essential factor in the decision-making process for breastfeeding.

In a recent systematic review and meta-analysis of 24 studies including 2,974 women, a significant reduction of relapse rate postpartum (more than 43% reduction) was confirmed in women who were breastfeeding compared to those not breastfeeding. The studies included in this meta-analysis did not distinguish exclusive from not-exclusive breastfeeding. Therefore, conclusions about the favorable or unfavorable effect of partial breastfeeding could not be deduced (65).

All this evidence underlines how clinicians should discuss the possibility of breastfeeding with the patient, pondering both her wish and her disease activity before and during gestation (7, 66).

In the following therapy section, we argue about current recommendations for each DMT in relation to breastfeeding.

IMPACT OF DISEASE-MODIFYING THERAPIES

The introduction of DMTs in MS inevitably leads to several concerns. Clinicians and patients referring to MS centers need to discuss and balance the potential hazards of exposing the fetus to possibly teratogenic drugs vs. the maternal risk of relapses and MS progression if therapies are stopped. Fortunately, current evidence based on real-life experience in DMT use, as well as the large number of available therapies, has made easier both management and counseling of women in child-bearing age. The mechanism of action and adverse effect profiles of each DMT are classified and continuous reviewed by the Food and Drug Administration (FDA) and European Medicine Agency (EMA), which evaluate drugs according to their risk weighted against potential benefit.

The impact of DMTs on fertility is summarized in **Table 1**.

Population-based studies showed that among MS patients who became pregnant, more than 40% were not taking DMTs in the 12 months before conception, suggesting that many women prefer to avoid any risk of drug-induced adverse outcomes for their fetus. Furthermore, women with little or no disability, rare relapses, and low lesion burden load on MRI or who required low effective therapies to control disease activity in the past were the most likely to interrupt treatment during and after pregnancy (28).

Neurologists should discuss with their patients about the benefit/risk profile of DMTs before, during, and after pregnancy at or soon after MS diagnosis, and then discussions should be regularly repeated afterward. The choice of optimal time for a woman with MS to become pregnant should be evaluated individually, according to her disease activity, her response to drugs, and the availability of resources to manage the motherhood. As such, family planning should be a crucial step for women of reproductive age with MS, and they should undergo regular counseling on the use of effective contraception in order to plan pregnancies.

Reliable contraception is recommended for patients taking DMT, but it is tailored on each drug. A systematic review was performed to estimate the safety of contraceptive use in MS patients (67). The four studies selected by the authors of the review concluded that the use of combined oral contraceptives (type not specified) did not worsen the clinical–neuroradiological course of the disease (defined by disability level, disease severity or progression, relapse, or number of new brain lesions on MRI after 96 weeks of follow-up) (67–71). The US Medical Eligibility Criteria for contraceptive use in MS women reported that most contraceptive methods are safe—the only exception being use of contraceptives in MS patients with prolonged immobility due to concerns on venous thromboembolism risk (72).

Self-Injectable DMTS

EMA and FDA recommendations for the management of self-injectable DMTs in pregnancy and breastfeeding are summarized in **Table 2**.

Interferons Beta and Glatiramer Acetate

Interferons β (IFNβs) were the first DMTs to be approved in MS. Their mechanism of action is pleiotropic. They induce the shift in T cell balance toward the anti-inflammatory profile of Th-2 cells, as well as inhibition of T-cell migration blocking metalloproteases and adhesion molecules (73).

Glatiramer acetate (GA) is a mixture of four synthetic polypeptides (l-glutamic acid, l-lysine, l-alanine, and l-tyrosine), like the myelin basic protein. Although its precise mechanism of action is still unknown, GA has been reported to induce a shift from Th1 to Th2 responses, with an increase in T-regulatory cells and downregulation of both Th1 and Th17 cells (73).

Fertility

Studies on the impact of IFNβs and GA on the fertility are rare. Clinical trials on IFNβ-1b report a similar rate of pregnancies in both treatment and placebo groups (74, 75). In addition, IFNβ or GA showed no alterations on sperm count (76, 77).

TABLE 2 | EMA and FDA recommendations for the management of self-injectable DTMs in pregnancy and breastfeeding.

Treatment	EMA	FDA	Milk secretion	Clinical practice
Interferons beta	**Pregnancy**: initiation of treatment contraindicated during pregnancy. Update of EMA in 2019 allows to consider continuing IFNβ-1a until conception and during pregnancy as clinically needed. **Breastfeeding**: no harmful effects on breastfed infants are anticipated; can be used during breastfeeding.	**Pregnancy**: Should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus. **Breastfeeding:** administer with caution to a nursing mother.	Not known.	Continue until pregnancy confirmed. In selected patients with highly active disease, may be administered throughout pregnancy after careful evaluation of the risk–benefit ratio.
Glatiramer acetate	**Pregnancy**: pregnancy contraindication removed from the EU label in 2017. **Breastfeeding:** decide on the balance between infant breastfeeding versus maternal therapy.	**Pregnancy:** Only use during pregnancy if clearly needed. **Breastfeeding:** consider benefits of breastfeeding against possible risks to the fetus.	Not known.	Continue until pregnancy confirmed. Continued use in pregnancy now supported in some cases.

EMA, European Medicines Agency; FDA, U.S. Food and Drug Administration.

Pregnancy and Fetal Development

Several evidences suggest that IFNβs and GA do not increase the risk for spontaneous abortions, preterm birth, or major congenital malformations. Indeed, registry-based and post-marketing studies on a large series of pregnant women exposed to IFNβ do not report any increased risk of either spontaneous abortions or congenital malformations in newborns compared to the general population (78–82).

Some studies reported a lower body weight in newborns (39, 78, 83, 84), which was not confirmed by others (85, 86).

European prescribing information has been updated in 2019; it now allows considering continuing IFNβ-1a at dose of 44 mcg (Merck-Serono®) until conception, during pregnancy as clinically needed, and while breastfeeding[1].

For what concerns GA, animal studies did not report evidence of teratogenicity, fetal development, or malformations. One study on a small number of patients reports a reduced birth length of 2.3 cm in newborns exposed to GA during the first trimester of pregnancy (36). On the contrary, post-marketing surveillance in a large sample of pregnancies confirms the safety of the treatment, also when exposure occurred in the first trimester (86–90).

Recent data collected by Teva Pharmaceuticals as part of a global pharmacovigilance database provided important evidence on the safety of branded GA during gestation, highlighting the lack of teratogenic effects (91). For these reasons, GA is not contraindicated during pregnancy, if the maternal benefit outweighs the risk to the fetus. Reports on GA exposure during the entire gestation are rare; however, no increased risk of an adverse pregnancy outcome has been disclosed (88, 92, 93). EMA has withdrawn pregnancy contraindication to Copaxone 40 mg/ml (Teva Pharmaceuticals®) in 2017.

No relation has been documented between paternal exposure to IFN beta or GA at the time of fecundation and the risk of adverse outcomes (76, 77).

[1]https://www.ema.europa.eu/en/medicines/human/EPAR/rebif

Breastfeeding

The transfer of IFNβs and GA into breast milk is very unlikely because of their large molecular weight and high polarity (94). Use of IFNβ-1a (Rebif®) is indeed approved by EMA during breastfeeding[1].

Based on these evidences, the discontinuation of IFNβ or GA during pregnancy may be avoided in MS patients with a high level of disability. On the other hand, treatment continuation might lead to a reduced risk of relapses postpartum, even if there is no data about this.

Oral Drugs

EMA and FDA recommendations for management of oral DMTs in pregnancy and breastfeeding are summarized in **Table 3**.

Dimethyl Fumarate

Dimethyl fumarate (DMF) is an oral drug approved for the treatment of relapsing–remitting MS (RRMS). DMF decreases the absolute lymphocyte count, mainly affecting $CD8^+$ T cells but also $CD4^+$ T cells, B lymphocytes, myeloid, and natural killer populations, which all shifted toward an anti-inflammatory state. Furthermore, *in vitro* and animal models demonstrated that DMF promotes neuronal survival within the central nervous system (CNS) by acting on an Nrf2 pathway, with consequent antioxidative, anti-inflammatory, and cytoprotective effects (95).

Fertility

In female rats, high doses of DMF may induce a reduction in estrogen levels, however not affecting fertility (96). In male rats, no evidence of impaired fertility was reported (97).

Pregnancy and Fetal Development

The drug is able to cross the placental barrier. In animal studies, low birth weight, delayed ossification, and a higher risk for spontaneous abortion at very high and toxic doses were registered (97).

Human data are too sparse to draw conclusions. An international registry is currently tracing gestations in women exposed to DMF. A rate of premature fetal death of 9% emerged in a recent report from this database (194 pregnancies with

TABLE 3 | EMA and FDA recommendations for the management of oral drugs in pregnancy and breastfeeding.

Treatment	EMA	FDA	Milk secretion	Clinical practice
Dimethyl fumarate	**Pregnancy:** not recommended during pregnancy and in fertile women not using appropriate contraception. Should be used only if clearly needed and if the potential benefit justifies the potential risk to the fetus. **Breastfeeding**: decide on the balance between infant breastfeeding versus maternal therapy.	**Pregnancy**: Use only if the potential benefit justifies the potential risk to the fetus. **Breastfeeding**: consider benefits of breastfeeding against possible risks to the fetus.	Not known.	Discontinue before conception and maintain effective contraception for an appropriate time before pregnancy. Monitor disease activity with MRI, cease breastfeeding if applicable and resume therapy.
Fingolimod	**Pregnancy:** women should not become pregnant and active contraception is recommended. Since it takes approximately 2 months to eliminate fingolimod from the body, contraception should be continued for 2 months after drug cessation before looking for pregnancy. **Breastfeeding:** contraindicated.	**Pregnancy:** Use effective contraception during treatment and for 2 months after interruption. Use only if the potential benefit justifies the potential risk to the fetus. **Breastfeeding:** consider benefits of breastfeeding against possible risks to the fetus.	Yes, in animals.	Discontinue before conception and maintain effective contraception for an appropriate period of time. Monitor disease activity with MRI, cease breastfeeding if applicable and resume therapy.
Siponimod	**Pregnancy**: contraindicated during pregnancy. Fertile women must have a negative pregnancy test, and they should use effective contraception during treatment and for at least 10 days after discontinuation. ***Breastfeeding***: contraindicated.	**Pregnancy**: contraindicated; women should not become pregnant for at least 10 days after drug cessation. **Breastfeeding**: consider benefits of breastfeeding against possible risks to the fetus.	Yes, in animals.	Discontinue therapy at least 10 days before conception while maintaining effective contraception. Breastfeeding is contraindicated.
Teriflunomide	**Pregnancy:** contraindicated. Use accelerated drug elimination procedure if planning pregnancy or pregnancy occurs on treatment. **Breastfeeding:** contraindicated.	**Pregnancy:** contraindicated: use accelerated drug elimination procedure if planning pregnancy or pregnancy occurs on treatment. **Breastfeeding:** women should not breastfeed while on treatment.	Yes, in animals.	Use effective contraception during treatment and after treatment as long as drug plasma concentration is above 0.02 mg/l. Breastfeeding is contraindicated.
Cladribine	**Pregnancy:** contraindicated. Women should not become pregnant for at least 6 months after the last dose. **Breastfeeding:** contraindicated. Women should not breastfeed for at least 1 week after the last dose.	**Pregnancy:** contraindicated. Women should not become pregnant for at least 6 months after the last dose. **Breastfeeding:** contraindicated. Women should not breastfeed for at least 10 days after the last dose.	Not known.	Women should not become pregnant for at least 6 months after the last dose. Women who become pregnant under therapy should discontinue treatment. Breastfeeding is contraindicated.

EMA, European Medicines Agency; FDA, U.S. Food and Drug Administration.

known outcome), with a rate of birth defects of 4% (98). Therefore, women of childbearing age are recommended to use contraception while being treated with DMF and switching to an alternative DMT should be contemplated depending on the degree of disease activity. In general, it is recommended to stop DMF with the plan to conceive, and DMF received a pregnancy category 2 by EMA (96)[2]. However, due to its very short half-life (1 h) and its almost negligible tissue accumulation, DMF is quickly eliminated, and no washout period is required after drug discontinuation when seeking pregnancy, even though other studies suggest establishing a washout period of 2 weeks (99). DMF should be immediately stopped after discovery of unexpected pregnancy during treatment, and fetal organ screening ultrasound might be considered (100). Lastly, drug agencies have not provided any recommendation regarding paternal exposure to DMF at the time of conception and the consequent risk of adverse outcomes (101).

[2]https://www.accessdata.fda.gov/drugsatfda_docs/label/2016/204063s014lbl.pdf

Breastfeeding

To our knowledge, there are currently no data regarding excretion of DMF or its metabolite in breast milk. Only low amounts of the active metabolite of DMF were found in breast milk, and therefore, no adverse effects in breastfed infants should be expected. However, some authors as well as FDA and EMA recommend avoiding breastfeeding while on therapy (96, 102)[2]. The benefit of breastfeeding for the child and the benefit of receiving therapy for the woman should be taken into account in individual cases.

Fingolimod

Fingolimod (FTY720; Gilenya®) is the first oral drug approved for RRMS treatment. Fingolimod is a sphingosine-1-phosphate (S1P) receptor modulator regulating lymphocyte egression from lymphoid tissues into the circulation. Furthermore, S1P1, S1P2, and S1P3 receptors, being expressed in the endothelial and vascular smooth muscle cells during embryonic development, may regulate vascular development. Finally, the S1P1 signaling

pathway is fundamental in neurogenesis and the subsequent development of the nervous system (103, 104).

Fertility

In animal studies, fingolimod demonstrated no effect on fertility of both male and female rats, even at doses approximately 200 times higher than the recommended dose in humans (105, 106).

Pregnancy and Fetal Development

Fingolimod can cross the placental barrier, and it was found causative of teratogenic effect in rats, such as persistent truncus arteriosus and ventricular septal defect. In humans, it likely increases the risk of malformations. According to regulatory agency recommendations, fingolimod is contraindicated during pregnancy. Recommended washout is 2 months before pregnancy (105, 106).

The clinical development fingolimod program included 89 exposed pregnancies. Pregnancy was considered "exposed to the drug" if fingolimod was ongoing at conception or 6 weeks before. Spontaneous abortion occurred in 24% of pregnancies, slightly exceeding the rate registered in the general population. Abnormal fetal development was reported in 7.6% of cases, being borderline considering normal values expected in the general population (4–8%). Fetal development abnormalities included one case of acrania, one case of unilateral congenital postero-medial bowing of the tibia, and one case of tetralogy of Fallot, and they were all associated with fetal exposure to fingolimod in the first 3 months of pregnancy (107). More recently, an additional 717 pregnancies exposed to fingolimod were collected. In this cohort, the prevalence of major cardiac abnormalities was comparable with that in the general population. The overall percentage of spontaneous (15%) and elective abortion was within the expected range (108).

Contraception is recommended during treatment with fingolimod and in the 2 months after discontinuation (106). In case of accidental exposure to fingolimod after suspension, organ screening ultrasound should be recommended.

In clinical practice, bridging with a depleting agent or natalizumab should be considered. When fingolimod is withdrawn before pregnancy, the risk of disease activity rebound must be taken into account, even if the magnitude of this risk is not known yet, as well as the successful strategies to minimize the risk (109, 110). Natalizumab might be considered for bridging strategies, and an extended dosing regimen is usually proposed in order to guarantee a lower exposure of the fetus to the drug and a lower PML risk for the mother. Natalizumab should be stopped anyway at least at 34 weeks of pregnancy; it is eventually administered in an off-label setting.

Breastfeeding

In animal studies, fingolimod was found to be excreted in rat milk at concentrations 2–3-fold higher than in maternal plasma (101). Excretion in human breast milk is still unknown, but probable (106). For this reason, fingolimod is not compatible with lactation.

Siponimod

Siponimod is a new S1P modulator targeting S1P1 and S1P5 receptors. The molecule is characterized by a molecular weight of 516 DA and a half-life of approximately 30 h, and it is contraindicated in carriers of the CYP2C9_3/_3 genotype (111). FDA has approved siponimod for the treatment of adult patients with RRMS, active secondary progressive MS, and clinically isolated syndrome, whereas it has been indicated by EMA for the treatment of secondary progressive MS with clinical or MRI active disease (112)[3].

Fertility

Animal studies failed to demonstrate any noxious effect on male reproductive organs in rats and monkeys. No alterations in fertility on female rats were demonstrated either (112)[3].

Pregnancy and Fetal Development

Placental passage of siponimod and its metabolites has been demonstrated in animal studies. Siponimod induced embryotoxicity and fetotoxicity in rats and rabbits as well as teratogenicity in rats. Embryonic and fetal deaths, along with skeletal or visceral defects (e.g., urogenital) at exposure levels similar to human dosages (daily dose of 2 mg), have been reported in rats and rabbits (112)[3].

There are a limited number of studies available on the use of siponimod in pregnant patients. Since fetal threat has been demonstrated by animal studies, siponimod is contraindicated during gestation and in fertile women not using effective contraception. Before initiating treatment, fertile women must be educated on the serious fetal risks associated with the drug, and it is recommended to use effective contraceptive measures both during treatment and for at least 10 days after drug suspension[3]. If pregnancy occurs while on treatment, siponimod must be immediately discontinued and medical advice on the risk of possible fetotoxicity should be given. Furthermore, ultrasound investigations should be performed.

When stopping siponimod in order to plan a pregnancy, the risk of disease activity rebound should be carefully considered.

Breastfeeding

No published data is currently available on the excretion of siponimod in human milk, on the effects of the drug on the breastfed infant, or on milk production itself. A study in lactating rats has demonstrated that siponimod and/or its metabolites are excreted in milk; hence, siponimod has been contraindicated during breastfeeding (112)[3].

Teriflunomide

Teriflunomide is an oral immunomodulatory drug taken once daily and approved for RRMS. The drug interferes with *de novo* pyrimidine synthesis by specific inhibition of the mitochondrial enzyme dihydro-orotate dehydrogenase (DHODH), which is highly expressed in proliferating lymphocytes (113).

[3]https://www.ema.europa.eu/en/documents/product-information/mayzent-epar-product-information_en.pdf

Fertility

Studies in animal models have failed to show adverse effects on male or female fertility. Although a small reduction in sperm count has been reported in rats at highest teriflunomide doses, no effects on fertility have been demonstrated (114, 115).

Pregnancy and Fetal Development

Drug-induced embryotoxicity and teratogenicity, with the occurrence of abnormalities of the axial skeleton and the head (e.g., microphthalmia, hydrocephaly), have been reported in animal studies (114, 115).

For this reason, although human data on teratogenicity are lacking, teriflunomide is contraindicated by FDA in the 3 ½ months before pregnancy and during pregnancy (115).

A recent review on the outcomes of 222 pregnancies exposed to teriflunomide in the first trimester based on clinical trials and post-marketing experiences reported a frequency of major birth defects consistent with that in the general and MS population. Similarly, the incidence of spontaneous abortions (21.2% overall) was similar to that reported in the general population (15–20%) (116).

For what concerns male MS patients treated with teriflunomide, pregnancies of their female partners resulted in 12.5% of spontaneous abortions, 4.2% elective abortions, and two cases of fetal anomaly (116).

Teriflunomide is a small molecule with a MW of 270 g/mol, which is rapidly and completely absorbed after oral administration. Consistent with the extensive plasma protein binding of teriflunomide, its elimination half-life is approximately 10–12 days, but to reach total elimination of the drug, 8–24 months may be required (113). Therefore, when planning a pregnancy, a washout strategy may be proposed with either cholestyramine (8 g for three times daily or, if 8 g is not supported, 4 g for three times daily, for 11 days) or activated charcoal (50 g, two times daily for 11 days) in order to accelerate the elimination of the treatment. Fertile women taking teriflunomide must use effective contraception during and after treatment as long as the drug plasmatic concentration is above 0.02 mg/l (115).

Teriflunomide can be identified at low concentrations in semen (117). According to FDA, men wishing to father a child should suspend treatment and undergo accelerated washout (115). On the contrary, EMA states that the risk of male-mediated embryonic or fetal toxicity induced by teriflunomide is negligible (114).

Breastfeeding

Being a small molecule, teriflunomide is likely excreted into breast milk. Therefore, its administration is contraindicated during lactation (114, 115).

Cladribine

Cladribine (2-chlorodeoxyadenosine, 2-CdA) is a synthetic chlorinated analog of deoxyadenosine that interferes with DNA synthesis. It induces a prolonged lymphocyte depletion, which is more selective toward B lymphocytes (118).

The drug is a small molecule with a MW of 285, and it represents an example of oral selective pulse immune reconstitution therapy. Cladribine received approval by FDA for RRMS and active secondary progressive MS and by EMA for highly active relapsing MS (119, 120).

Fertility

Different reproductive toxicology studies carried out in animals (unpublished results, Merck KGaA) failed to demonstrate a role for cladribine in impacting female and male fertility, or in affecting peri–postnatal development abnormalities in the offspring. However, cladribine induced depletion of germ cells, spermatids, and spermatozoa in mice. Studies of ovarian dysfunction induced by cladribine are lacking; it can even induce DNA strand breaks (119, 120).

Pregnancy and Fetal Development

Cladribine showed teratogenicity in mice and rabbits when given intravenously. In humans, the half-life of the drug is short (<24 h), with a rapid elimination after administration (121). However, considering that animal studies demonstrated both teratogenicity at doses higher than those administered to humans, as well as short-term effects on male germ cells, caution should be exerted during and after cladribine dosing.

Data from clinical programs on outcomes from women exposed to cladribine ($n = 44$) and from women whose partners had been exposed to cladribine are very limited. Eighteen of 44 pregnancies exposed to cladribine were carried to term; nine were terminated by spontaneous abortions, three by induced abortions mainly because of ectopic pregnancy and choriocarcinoma. The female partners of nine male patients treated with cladribine had a total of 10 pregnancies, nine of which were carried to term with resulting live births. No congenital malformations were reported (122).

There are no published data from post-marketing clinical studies on the effect of cladribine tablets in pregnancy.

Cladribine is contraindicated in pregnancy (119, 120). The drug crosses the placental barrier. Based on its potential for serious fetal risk, manufacturers recommend adding a barrier method of contraception (even if already on hormonal contraception) during cladribine treatment and for at least 4 weeks after the last dose, to be repeated at every course of treatment. According to current European labeling recommendations, women should not become pregnant for at least 6 months after a course of cladribine; similarly, male patients must adopt effective contraception to prevent pregnancy of their partner during cladribine treatment and for at least 6 months after the last dose (119).

Breastfeeding

Whether cladribine is excreted in human milk is unknown. However, given the potential for serious adverse effects, women should not breastfeed during treatment and for at least a week after the last drug administration (119, 120).

TABLE 4 | EMA and FDA recommendations for the management of injectable monoclonal antibodies in pregnancy and breastfeeding.

Treatment	EMA	FDA	Milk secretion	Clinical practice
Natalizumab	**Pregnancy:** if a woman becomes pregnant, discontinuation of the drug should be considered. Newborns of women exposed to natalizumab during the third trimester of pregnancy should be monitored for potential hematologic abnormalities. **Breastfeeding:** contraindicated. Breastfeeding should be discontinued during treatment.	**Pregnancy:** use during pregnancy only if the potential benefit justifies the potential risk to the fetus. **Breastfeeding:** consider benefits of breastfeeding against possible risks to the fetus.	Yes, in humans.	Continue until pregnancy confirmed. Continue until second trimester of gestation in highly active disease. Resume therapy as soon as possible after delivery.
Alemtuzumab	**Pregnancy:** maintain contraception for 4 months after the last dose. Only use during pregnancy if potential benefit justifies potential risk to the fetus. **Breastfeeding:** avoid breastfeeding during and for 4 months after each treatment course (but balance potential benefit of breastfeeding with potential risks from exposure).	**Pregnancy:** use during pregnancy only if the potential benefit justifies the potential risk to the fetus. Maintain contraception for at least 4 months after last dose. **Breastfeeding:** consider benefits of breastfeeding against possible risks to the fetus.	Yes, in animals.	Discontinue before conception and maintain effective contraception for an appropriate period of time before pregnancy.
Ocrelizumab	**Pregnancy:** fertile women should use contraception while receiving ocrelizumab and for 12 months after the last infusion. **Breastfeeding:** advise women to discontinue breastfeeding during treatment.	**Pregnancy:** there are no adequate data on the developmental risk associated with use of ocrelizumab in pregnant women. Fertile women should use contraception while receiving the drug and for 6 months after the last infusion. **Breastfeeding:** consider benefits of breastfeeding against possible risks to the fetus.	Yes, in animals.	Discontinue before conception and maintain effective contraception for an appropriate period before pregnancy. Monitor disease activity with MRI, cease breastfeeding if applicable, and resume therapy

EMA, European Medicines Agency; FDA, U.S. Food and Drug Administration.

Injectable Monoclonal Antibodies

EMA and FDA recommendations for the management of injectable monoclonal antibodies in pregnancy and breastfeeding are summarized in **Table 4**.

Natalizumab

Natalizumab (NTZ) is a monoclonal antibody approved for the treatment of highly active RRMS. It reduces CNS inflammation by blocking very-late antigen (VLA)-4 on the surface of lymphocytes, thus preventing their transmigration through the blood–brain barrier (BBB) (123).

Fertility

Preclinical studies demonstrated a reduction in female guinea pig fertility at dose levels (30 mg/kg) of NTZ 2.3 times higher than the clinical dose (124). Male guinea pig fertility was unaltered. No studies have analyzed the effects of natalizumab on human fertility (125, 126).

Pregnancy and Fetal Development

NTZ is recognized to have established teratogenic effects in animal models. In humans, adequate and well-controlled studies are lacking.

Being a very large molecule with a MW 150 kDa, NTZ is unable to cross the placenta during the first trimester of pregnancy (123), but it can reach fetal circulation, being carried by active transportation through the placenta, from the second trimester.

In animal studies on guinea pigs and cynomolgus monkeys, NTZ did not show fetotoxicity or teratogenicity (127, 128). However, preclinical studies demonstrated that an exposure to NTZ throughout pregnancy can cause hematological effects in the offspring, which were anyway reversible after drug elimination (128).

In humans, conflicting results for increased abortion rates have been reported, but no specific patterns of malformations suggesting a drug effect emerged (129–131). Effects on spontaneous abortion were also confirmed in an Italian population of 92 pregnancies exposed to the drug, demonstrating that NTZ exposure to up to 12 weeks of gestation was associated with spontaneous abortion (odds ratio [OR] 3.9, 95% confidence interval [CI] 1.9–8.5, $p < 0.001$) if compared to IFNβ exposure or no exposure. The rate of spontaneous abortion (17.4%) was anyway within the limits expected in the general population (132).

On this background, avoiding pregnancy during treatment and a post-treatment washout period of at least 3 months before conception is recommended by regulatory agencies[4]. Nevertheless, maternal risk of disease reactivation might be considered and weighted in respect to fetal risk. A disease

[4]https://www.ema.europa.eu/en/documents/product-information/tysabri-epar-product-information_it.pdf

reactivation or true disease rebound is described in one-third of patients within 2–6 months after NTZ suspension (133–135), and the gestational period seems protective against disease reactivation (136, 137). When approaching the use of NTZ during pregnancy, clinicians always need to consider that MS patients treated with NTZ usually have aggressive MS requiring a very active drug. A recent meta-analysis tried to identify patients that were at a higher risk of post-NTZ suspension disease reactivation. It resulted that younger age, higher number of relapses, gadolinium-enhanced lesions before treatment initiation, and fewer NTZ infusions were associated with increased risk of disease reactivation after NTZ ($p \leq 0.05$) (137). For this reason, an individualized evaluation of the risk-to-benefit ratio must be considered for each patient treated with NTZ when planning pregnancy.

Recent studies suggest that NTZ should be considered as a therapeutic option in pregnant patients with highly active MS. Haghikia et al. report a case series of 13 pregnancies in 12 women with highly active MS who were treated with NTZ in the last trimester of pregnancy. Mild to moderate hematologic alterations were observed in 10 of 13 infants, such as thrombocytopenia and anemia. In the majority of the newborns, these hematological aberrations resolved during the 4 months after birth and no specific treatment were needed, although a subclinical bleeding complication was reported. In a subsample of five mother–child pairs, natalizumab was detected in the umbilical cord blood of the newborns. Pediatricians, at delivery, should be prompt to evaluate potential signs of anemia and thrombocytopenia in newborns exposed to natalizumab during the third trimester of pregnancy (138). Triplett et al. suggested that in order to reduce possible hematological complications of the newborn, NTZ doses could be modified during the third trimester, while prenatal umbilical cord should be sampled, and intravenous immunoglobulins should be administered (139).

Since NTZ suspension is associated with a high risk of disease reactivation, pregnancy could be planned without interrupting the drug and with a strict monitoring of conception.

In practice, a patient-tailored approach is suggested which can be either:

1. *Conservative approach*: discontinue natalizumab prior to conception and maintain contraception for 2–3 additional months after discontinuation;
2. *Semi-active approach:* maintaining natalizumab at least until conception (test beta-HCG before each infusion, 6–8 weeks extended dosing regimen) and restarting treatment early after delivery;
3. *Active approach*: maintaining natalizumab until the 30–34th weeks of pregnancy (6–8-week extended dosing regimen) and early restarting after delivery (8–12 weeks after last infusion);
4. *Bridging approach*: shifting natalizumab to a depleting agent (rituximab or ocrelizumab).

Current consensus UK guidelines recommend an active approach (140). Even if evidence supporting the aforementioned approaches is available, the use of NTZ, as well as depleting agents, during pregnancy remains off label in clinical practice. Therefore, the adoption of any approach must always be shared with the patient and a report of the discussion annotated in clinical records.

Breastfeeding

NTZ is excreted into human breast milk. Although NTZ is not orally bioavailable, the effects of exposure to infants are unknown. One study reported that the transfer of natalizumab into human milk increased over time and with repeated injections, with the highest concentration of 2.83 μg/ml at day 50 and with a relative infant dose of 5.3% (141). For this reason, NTZ should be avoided during lactation, also according to EMA and FDA prescribing information (125, 126, 142). However, the risk/benefit ratio of breastfeeding in case of restarting NTZ after delivery must be discussed, considering that reliable data are lacking (143). If women decide to breastfeed under natalizumab, infants should be monitored for hematological abnormalities.

Alemtuzumab

Alemtuzumab (ALZ) is an anti-CD52 humanized monoclonal antibody that is administered annually, typically used in aggressive or refractory MS. The drug provokes depletion of B and T lymphocytes (144, 145), in regulatory T- and B cells, as well as in secreting cytokines with a less inflammatory profile (146, 147).

Fertility

Animal production studies showed an adverse effect on fertility. In female mice, intravenous infusion of ALZ at doses up to 10 mg/kg/day (4.7 times above the daily dose recommended in humans) for five consecutive days produced a significant reduction of corpora lutea and implantation sites per female mouse. No effects on fertility in male mice were reported. Adequate clinical safety data on fertility in humans (women and men) are lacking (148, 149).

Pregnancy and Fetal Development

Animal studies reported an increased embryonic lethality and a reduced level of B and T circulating lymphocytes in the offspring when pregnant mice were exposed to ALZ during the period of organogenesis (148, 149). Data from the clinical development programs on 264 pregnancies, occurring in 160 out of 972 women treated with 12 or 24 mg ALZ before conception, showed normal live births, without increase in congenital anomalies or birth defects. Furthermore, the incidence of spontaneous abortion was not different from that reported in the general population and in treatment-naive MS patients (150). For what concerns the risk of postpartum relapse, treatment with ALZ induced a significant reduction in the ARR postpartum (0.2) as compared to the rate before treatment (1.7) or that reported in literature, which ranges from 2.0 to 0.5 (5, 35, 151). This data confirms the prolonged clinical effect of ALZ on the risk of disease activity. To our knowledge, there are no published post-marketing studies to date concerning ALZ exposure during pregnancy, except for two ECTRIMS abstracts. On 18 pregnancies exposed to ALZ (last ALZ < 4 months before last menstrual period), 7.7% pregnancies ended in spontaneous abortion, 8.3% babies were affected by malformations or genetic abnormalities, and 8.3% were born

preterm. A favorable disease course without relapses during pregnancy and postpartum was reported in all women with term pregnancies (152).

According to EMA and FDA prescribing information, ALZ is contraindicated in pregnancy (148, 149).

Monoclonal antibodies, such as ALZ, are known to cross the blood–placenta barrier, at least certainly after 20 weeks of gestation. Therefore, their use during pregnancy may potentially affect the fetus. ALZ concentration becomes low or undetectable in plasma approximately 30 days after a course of treatment (153). Despite this, recommendations from manufacturers are to avoid conception and to use effective contraception for 4 months following a course of treatment with ALZ. According to current European and USA labeling recommendations, ALZ should be administered during gestation only if potential maternal benefits justify the possible risk to the fetus. Women have to be informed on the possible drug-induced risks, which include autoimmune thyroid disease (37%), immune thrombocytopenic purpura (1%), Goodpasture syndrome (0.1%), and other autoimmune diseases that may persist for 4 years after a cycle of ALZ. If those autoimmune disorders occur during gestation, they may distress both the mother and the fetus as antibodies cross the placenta (e.g., neonatal thyrotoxicosis) (139, 154). In case of maternal autoimmune thyroid diseases, thyroid hormones have to be monitored monthly during pregnancy. These drug-induced autoimmune diseases, and in particular consequent hypothyroidism, might increase the risk of spontaneous abortions, intrauterine growth retardation, preeclampsia and preterm birth, irregular menstruation, infertility, and delayed mental development of the child (149).

Breastfeeding

Alemtuzumab, being a monoclonal antibody, can be excreted in human milk. Therefore, breastfeeding is discouraged for at least 4 months after the last infusion of the drug in each treatment course.

Ocrelizumab

OCR is a humanized anti-CD20 monoclonal antibody that depletes B cells through antibody-mediated and complement-mediated cellular cytotoxicity. The B-cell depletion is evident within 14 days of infusion, while the B-cell population recovers in 72 weeks (155). The drug is approved by EMA and FDA for treating adult patients with RRMS or early primary progressive MS (PPMS) with MRI findings of inflammatory activity (156, 157).

Fertility

Data from animal studies showed no effects on reproductive organs in male monkeys as well as on estrus cycle in female monkeys that were administered by intravenous OCR at 2 and 10 times the recommended human dose of 600 mg (157).

Pregnancy and Fetal Development

Animal studies reported both teratogenicity and fetotoxicity. In two pre- and postnatal development studies carried out in cynomolgus monkeys, the administration of OCR throughout gestation was correlated with glomerulopathy, lymphoid follicle formation in bone marrow, lymphoplasmacytic renal inflammation, and decreased testicular weight in the offspring. There are five cases of neonatal moribundities caused by opportunistic bacterial infection impacted by B-cell depletion. Animal offspring born from mothers exposed to OCR exhibited depleted B cell populations after delivery. No teratogenic effects were reported in animal studies (156).

Data on the safety profile of OCR before and during pregnancy in women are limited. Preliminary data on pregnancy outcomes were reported from OCR clinical trials and post-marketing sources. Out of 267 pregnancies exposed to OCR (dose range 20–2,000 mg), 62 live births, 86 ongoing pregnancies, 25 elective abortions, 10 spontaneous abortions, 1 stillbirth, 3 ectopic pregnancies, 22 lost to follow-up, and 58 unknown outcomes have been reported. The outcomes of these cases do not suggest an increased risk of adverse pregnancy outcomes. No data on B cell count have been published in newborns and infants exposed to OCR during gestation (158).

A recent cohort study on treatment with anti-CD20 (OCR or rituximab) in women with MS or neuromyelitis optica spectrum disorder reported that pregnancy outcomes after treatments have been administered in the years before pregnancy were similar to those expected in the general population. On the contrary, treatment given during pregnancy could result in more preterm births and congenital malformations.

For what concerns disease activity, anti-CD20 treatment induced a significant decrease in the number of relapses during pregnancy and in the postpartum period (159). Considering that OCR may cross the placental barrier, the manufacturers recommend that fertile women should use adequate contraception while receiving the drug. In Europe, current recommendations suggest planning pregnancy only after 12 months after the last infusion of OCR (compared to the 6-month interval recommended by FDA). OCR should be avoided during pregnancy unless "the potential benefit to the mother outweighs the potential risk to the fetus" (156, 157).

Breastfeeding

Published data in animals have demonstrated excretion of OCR in breast milk, with measurable levels of OCR (approximated 0.2% of serum levels) during the lactation period. Reliable data on human are lacking. Recently, a study with another anti-CD20 (i.e., rituximab) reported that levels of the drug in milk were <240 times the amount detected in maternal serum, suggesting that this minimal excretion was related with the drug's pharmacological property, with monoclonal antibodies being macromolecules, and therefore the breastfeeding would be allowed (160). However, EMA and FDA recommend that women should be advised not to breastfeed during or 6 months after discontinuing the treatment (156, 157).

CONCLUSIONS

This review attempts to summarize current evidence and expert recommendations about specific issues regarding pregnancy planning, pregnancy course, partum and postpartum period,

breastfeeding, and the management of DMT use in MS women. Based on current evidence, MS does not impact the fertility in either sex, or the women's ability to conceive and to carry the fetus to term. The disease does not increase the risk of spontaneous abortion, malformations, and caesarean delivery. Pregnancy appears to be protective against MS disease activity, particularly during the third trimester, but an increased risk of relapse is reported in the first 3 months postpartum. Pregnancies do not impact either the long-term disease course or the accumulation of disability. Results from registers, real-world databases, and pharmacovigilance have increased our awareness on the impact DMTs exert on the pregnancy. Consequently, family planning strategies for patients with MS have changed. Women with MS should be supported and encouraged to have children and to breastfeed, also considering the possible favorable effect of exclusive breastfeeding. Neurologists and patients should tailor together the best therapy for any pregnant woman, considering the chances of conception in relation to DMTs without exposing the fetus to any possible risk and the safety of a benign postpartum period. Specific recommendations regarding whether and when to discontinue DMTs or switch to other therapy are continuously evolving, which is why neurologists are required to be constantly updated with both literature and international guidelines.

AUTHOR CONTRIBUTIONS

IS: paper design and conception. IS, CT, and AG: manuscript writing. AG: figures. IS: manuscript revision and editing. All the authors agreed to be accountable for the content of the work.

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Rituximab in Multiple Sclerosis: Are We Ready for Regulatory Approval?

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Despite the availability of a lot of effective disease-modifying drugs, multiple sclerosis (MS) (in particular the progressive forms) still represents an important unmet medical need, because of issues in terms of effectiveness, duration of response, safety, and patient compliance. An increasing body of evidence from randomized clinical trials and real-world data suggest that rituximab is a highly effective alternative in both relapsing and progressive MS, with a low discontinuation rate, related to a good benefit/risk profile, and a good compliance. To date, the use of rituximab in patients with multiple sclerosis is not in accordance with the authorized product information (off-label use). However, the use of this medicine is widespread in several countries, and in some cases, it is the most commonly used disease-modifying drug for MS subtypes. This use could be officially recognized by national regulatory authorities, according to specific procedures, to ensure equal access for patients to a safe and effective option.

Keywords: multiple sclerosis, rituximab, off-label, regulatory issue, disease-modifying drugs

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INTRODUCTION

Multiple sclerosis (MS) is the most common chronic demyelinating disorder of the central nervous system (CNS), affecting more than 2.8 million people worldwide in 2020, with a global median prevalence of 36 cases per 100,000 people, and an average incidence rate of 2.1 per 100,000 people per year (1, 2). MS primarily affects young adults, with the age of onset between 20 and 40 years, and it could be considered the second-most expensive chronic condition behind congestive heart failure in the US (3). The clinical manifestations and course of MS are heterogeneous, with different degrees of severity, from an initial clinically isolated syndrome (CIS), to a relapsing–remitting form (RRMS) and the progressive development of permanent neurological deficits and disability (known as secondary progressive MS, SPMS). Moreover, some patients have a progressive disease from the onset, known as primary progressive form (PPMS) (4). CIS and RRMS are typically characterized by active white matter demyelinating lesions, with heavy immunological infiltration and activation (5), whereas the progressive forms are mainly characterized by inactive lesions, reduced inflammation and neurodegeneration (6, 7).

The physiopathological mechanisms behind the damage are still incompletely understood (8). T cells appear early in lesion formation, and the disease is considered to be autoimmune, initiated by autoreactive lymphocytes that mount aberrant responses against CNS autoantigens, the precise nature of which, however, have not been routinely identified (9, 10). B cells and their plasma cell

derivatives also produce antibodies, including clonally expanded immunoglobulin G (IgG) oligoclonal bands (OCBs) detectable in the cerebrospinal fluid of most patients with MS (11). However, B cells probably contribute mainly through antibody-independent mechanisms, due to an abnormal cytokine response profile — with a propensity to produce pro-inflammatory cytokines (including IL-6, GM-CSF, TNF, and lymphotoxin-α) — that can induce aberrant Th1 cell and Th17 cell responses and pro-inflammatory myeloid cell responses, which could in turn contribute to the cellular immune cascades involved in first phases of the pathology and in relapses (12–14). Treg cells can be responsible in inducing remission in MS, through the downregulation of immune responses (15), and activated pro-inflammatory cells may be more likely to be killed by other immune cells (16). In later stages of the disease, ongoing inflammation in the CNS might contribute to the propagation of tissue injury, in terms of neuro-axonal degeneration, astrocyte, and oligodendrocyte damage, and to the clinical manifestations of progressive disease (7). The different inflammatory characteristics among progressive forms and RR forms of MS may explain the lack of efficacy of most disease modifying therapies (DMTs), which are typically systemic anti-inflammatory drugs.

Cognitive impairment (impairment in information processing speed, episodic memory, attention, efficiency of information processing, and executive function), which can start in the earliest phases of the disease but is more frequent and more pronounced in chronic progressive MS, worsens over time and affects the patient's daily life activities (17).

Optimal MS management requires coordinated and comprehensive care from health care professionals with expertise in the complexities of MS (18, 19). Untreated relapses and progression of disease restrict participation in usual activities and increase the risk for serious morbidity. The ultimate goal of modern MS therapies is to achieve no evidence of disease activity (NEDA) in which the therapy has halted relapses and disability progression, as well as new and active magnetic resonance imaging (MRI) lesion development. The treatment of MS includes DMTs, which are used to reduce inflammatory disease activity and its long-term clinical consequences; the treatments for the management of MS relapses and symptomatic treatments are used for short-term amelioration of MS symptoms, such as impaired walking capability, spasticity, pain, loss of bladder and bowel control, and neuropsychiatric symptoms (4).

The most established treatment for the acute management of MS relapses is high-dose corticosteroids. In particular, current protocols typically include 3 to 5 days of intravenous methylprednisolone (20). Relapses that do not respond to corticosteroids can be treated with plasma exchange (3–5 courses) or intravenous immunoglobulins.

DMTs effectively reduce the inflammatory activity, relapse rate, and disability progression, although safety concerns, individual immunological changes, and issues with compliance make their long-term use challenging. To date, several DMTs, with different routes and frequencies of administration, mechanisms of action, effectiveness, and safety profiles, have been approved for the treatment of RRMS in EU — including subcutaneous interferon-β (IFNβ)-1a, IFNβ-1b, and pegIFNβ-1a, subcutaneous glatiramer acetate, small-molecule oral agents (cladribine, dimethyl fumarate, fingolimod, ozanimod, teriflunomide), intravenous monoclonal antibodies (mAbs) (alemtuzumab, natalizumab, ocrelizumab), and intravenous mitoxantrone — offering to physicians the possibility of tailoring therapy to individual patient needs (**Table 1**). Effective treatments for the progressive forms of MS are more limited, with only a small number of therapeutic agents available with beneficial effects.

Because of the wide variability in the disease course and in the individual responses to treatment, access to several DMTs, with different routes of administration and dosing schedules, mechanisms of action, efficacy and safety profiles, contraindications, and side effects, is essential to ensure a good long-term control of the disease.

Escalation therapy is appropriate for most patients with non-aggressive RRMS, provided that they are closely monitored to detect suboptimal response or disease progression. Subjects with an intolerable degree of disease activity despite high-efficacy treatments may be treated with alternative immunosuppressive agents, such as mitoxantrone (currently authorized for the treatment of highly active relapsing MS associated with rapidly evolving disability in the absence of other therapeutic alternatives), cyclophosphamide, and azathioprine (35, 36).

In patients with active SPMS, current ECTRIMS/EAN guidelines recommend (weak recommendation) the following: IFNβ-1a or -1b, taking into account the efficacy, safety, and tolerability profiles of these drugs; mitoxantrone, taking into account its efficacy and specifically considering the safety and tolerability of the drug (cardiotoxicity, delayed congestive heart failure, myelosuppression, and acute treatment–related leukemia); and ocrelizumab or cladribine (35). EU approval of siponimod is too recent for its consideration in these guidelines. Another anti-CD20 agent, ofatumumab, that can be self-administered once monthly at home subcutaneously, has been approved in August 2020 by FDA for the treatment of relapsing form of MS, including CIS, RRMS, and active secondary progressive disease, with an expected approval in Europe by the first half of 2021.

For patients with PPMS, ocrelizumab represents, to date, the only authorized treatment.

The Role of Anti-CD20 in MS

The reduction of B-cells demonstrated to be an effective therapeutic approach for the progression of CNS autoimmune diseases (37).

There are three major mAbs targeting CD20+ B-cells, rituximab, ocrelizumab, and ofatumumab. The mechanisms of apoptotic B-cell depletion include antibody-dependent cell-mediated phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC) (28, 31). Recent studies have shown also a depleting action on CD20+ T cells, which are shown to be present in MS patients, suggesting an alternative contributing mechanism (38).

Rituximab is the first anti-CD20 therapy to be used in MS. It is a chimeric antibody, approved since 1997 for hematological

TABLE 1 | Disease-modifying therapies currently licensed for the treatment of MS; 1a) Common first-line treatment; 1b) Common second-line treatments.

A

DMT	Administration route, dosage and posology	Mechanism of action	Efficacy	Main adverse effects/Safety issues	Monitoring requirements	First EMA approval (year)	Indication
INFβ-1b	Subcutaneous injection, 250 mcg every other day	Not fully understood. Autocrine and paracrine actions *via* activation of the IFN receptor on leucocytes (21)	Moderate	Injection site reactions, flu-like symptoms, abnormal LFTs, lymphopenia, leukopenia, depression (and suicidal ideation), thyroid dysfunction, neutralizing antibodies	At baseline and periodically during treatment: full blood count, differential leukocyte count, platelet count, liver function tests, and TFTs.	1995	CIS RMS
INFβ-1a	Intramuscular injection 30 mcg once a week or subcutaneous injection; 22 mcg or 44 mcg three times a week	The same as above	Moderate	The same as above	The same as above	1997	CIS RMS
Peg-INFβ-1a	Subcutaneous injection, 125 mcg once every 2 weeks	The same as above	Moderate	The same as above	The same as above	2014	RRMS
Glatiramer acetate	Subcutaneous injection, 20 mg daily or 40 mg three times per week	Unclear. Immuno-modulatory and neuroprotective effect through various mechanisms. MBP mimetic, thus competes with MBP antigens to bind with MHC II (22).	Moderate	Injection site reactions, post-injection reactions (vasodilatation, rash, dyspnea, chest pain within minutes), mood disturbance, hypersensitivity reaction, cutaneous necrosis	None required	2005	CIS RRMS
Dimethyl fumarate	Oral capsule, 240 mg twice a day	Not fully understood. Activates the Nrf2 pathway to protect against oxidative stress–induced cellular injury and loss in neurons and astrocytes (23)	Moderate/ High	Flushing, gastrointestinal symptoms (abdominal pain, diarrhea, and nausea), pruritus/rash, anaphylactic reactions, lymphopenia, infections (VZ), PML, abnormal LFTs, proteinuria	At baseline and periodically during treatment: full blood count, differential leukocyte count, LFTs, renal function monitoring	2014	RRMS
Teriflunomide	Oral tablets, 14 and 7 mg daily	Inhibits proliferation of activated T and B lymphocytes *via* mitochondrial dihydroorotate dehydrogenase inhibition (24)	Moderate	Hair thinning, gastrointestinal symptoms (nausea, diarrhea), abnormal LFTs, impaired bone marrow function with anemia, leukopenia, neutropenia, thrombocytopenia, infections, peripheral neuropathy, skin AEs, increased blood pressure, respiratory effects (interstitial lung disease), pancreatitis, teratogenicity	At baseline and periodically during treatment: blood pressure, LFTs (fortnightly for 6 months then every 8 weeks), full blood count	2013	RRMS

B							
DMT	**Administration route, dosage and posology**	**Mechanism of action**	**Efficacy**	**Main adverse effects/Safety issues**	**Monitoring requirements**	**First EMA approval (year)**	**Indication**
Fingolimod	Oral capsule, 0.5 mg daily (0.25 mg daily for pediatric patients ≤ 40 kg)	S1P agonist -prevents egress of lymphocytes from lymph nodes (25, 26)	High	Headache, diarrhea, back pain, elevated liver enzymes, bradyarrhythmia, and/or atrio-ventricular block (first dose), hypertension, respiratory effects, lymphopenia, infections (VZ), PML, macular edema, increased risk of malignancies (basal cell carcinoma), hepatic injury, teratogenicity	First-dose observation protocol (6-h monitoring of heart rate and blood pressure). Baseline: full blood count, serum Ig levels, serology (VZV, HIV 1 and 2, hepatitis B and C, syphilis), LFTs, skin examination. Periodically during treatment: full blood count, blood pressure, ECG, skin examination, ocular examination at 3 months	2011	Highly active RRMS* (adults and pediatrics from 10 years)
Natalizumab	Intravenous infusion, 300 mg every 4 weeks	Selective inhibitor of VLA-4 ($\alpha4\beta1$) integrins, preventing leukocyte migration across BBB (27)	Very high	Arthralgia, urticaria, infusion reactions, opportunistic infections (VZ, encephalitis, meningitis, PML), hepatic injury	Baseline and periodically during treatment: full blood count, LFTs, JCV serology and MR, neutralizing antibodies	2006	Highly active RRMS* (adults) Adolescents (12-18 years) with severe and rapidly evolving RRMS* not eligible to fingolimod (648/1996 law)
Alemtuzumab	Intravenous infusion, 12 mg, first course: daily for 5 days; second course: daily for 3 days, 1 year after the first course	Anti-CD52 mAb depleting B cells, T cells, monocytes, macrophages, and dendritic cells (immune reconstitution therapy) (28, 29)	Very high	Infusion reactions, profound lymphopenia, infections (herpes simplex and zoster), secondary autoimmunity (as thyroid disorders, immune thrombocytopenia, purpura, glomerular nephropathies), Hemophagocytic lymphohistiocytosis (HLH), serious cardiovascular disorders	Baseline: full blood count, urine analysis, LFTs, TFTs, serum immunoglobulin levels, serology (VZV, HIV 1 and 2, hepatitis B and C, syphilis), TB elispot, cervical smear (HPV). Follow-up (for 48 months after last course): monthly full blood count, urine analysis and 3-monthly TFTs	2013	Highly active RRMS* (adults)
Cladribine	Oral 10 mg tablets, cumulative dose of 3.5 mg/kg over 2 years, administered as 1.75 mg/kg treatment cycle per year. Tablets given for 4–5 days in months 1 and 2 in year 1 and the cycle is repeated in year 2 (8–10 days of treatment per year)	Deoxyadenosine (purine) analog, adenosine deaminase inhibitor, selective T- and B-cell depletion (immune reconstitution therapy) (28, 30)	High	Severe lymphopenia, infections (VZ), TB/LTB reactivation, increased risk of malignancies, teratogenicity	Baseline: full blood count (before each treatment year), LFTs, TFTs, serum immunoglobulin levels, serology (VZV, HIV 1 and 2, hepatitis B and C, syphilis), TB elispot, pregnancy test, and cervical smear. Follow-up: full blood count 2 and 6 months after start of treatment in each treatment year	2017	Highly active RMS (including RRMS and SPMS)
Ocrelizumab	Intravenous infusion, 600 mg twice a year (initially 300 mg/250 ml IV, followed 2 weeks later by second dose of 300 mg/250 ml IV; subsequent dosing 600 mg/500 ml IV 6 monthly)	Anti-CD20 mAb, B-cell depleter (immune reconstitution therapy) (28, 31)	Very high	Infusion reactions, infections, PML, increased risk of malignancy, possible hypogammaglobinemia with prolonged use	Baseline: full blood count, LFTs, TFTs, serum immunoglobulin levels, serology (VZV, HIV 1 and 2, hepatitis B and C, syphilis), TB elispot, cervical smear. Follow-up: annual serum immunoglobulin levels	2018	RMS PPMS
Mitoxantrone	Intravenous infusion, 12 mg/m^2 every 3 months or 5 mg/m^2 every 3 months	Immune deplete (topoisomerase II inhibitor) (32)	Very high	Leukopenia, hair loss, nausea, vomiting, infections, cardiomyopathy (congestive heart failure), amenorrhea,	Baseline: full blood count, LFTs, TFTs, serum immunoglobulin levels, serology (VZV, HIV 1 and 2,	2015**	Highly active RMS associated with

(Continued)

TABLE 1 | Continued

B							
DMT	**Administration route, dosage and posology**	**Mechanism of action**	**Efficacy**	**Main adverse effects/Safety issues**	**Monitoring requirements**	**First EMA approval (year)**	**Indication**
				myelosuppression, secondary acute myeloid leukemia, myelodysplastic syndrome, infections, renal failure, teratogenicity	hepatitis B and C, syphilis), TB elispot. Follow-up: 3-monthly (predosing) full blood count		rapidly evolving disability (patients not eligible to other therapeutic alternatives)
Siponimod	Oral tablets, 2 mg daily (maintenance dose after 5 days titration)	S1P agonist (33)	Very high	Lymphopenia, infections (including cryptococcal and herpes viral infections), macular edema, bradyarrhythmia atrioventricular conduction delays, hypertension, respiratory effects, liver injury, hypertension, skin malignancies, fetal risk.	First dose monitoring for patients with sinus bradycardia, first- or second-degree atrio-ventricular block or a history of myocardial infarction or heart failure. Baseline: CYP2C9 genotyping; vital signs and ECG; full blood count; serology (VZV, HIV 1 and 2); ocular examination; LFTs. Follow-up: full blood count; ocular examination at 3 months; skin examination; LFTs; neurologic and psychiatric examination	Jan 2020	SPMS
Ozanimod	Oral capsules, 0,92 mg daily (maintenance dose after 7 days titration)	S1P agonist (34)	Very high	Bradycardia, hypertension, LFTs alterations, liver injury, infections (PML risk), increased risk of malignancies (skin malignancies), macular edema, PRES, respiratory effects, fetal risk	First dose monitoring for patients with sinus bradycardia, first or second degree AV block or a history of myocardial or heart failure. Baseline: full blood count, blood pressure, ECG, LFTs, ocular examination. Follow-up: full blood count, LFTs, blood pressure, ocular examination	May 2020	RMS

BBB, blood-brain barrier; CIS, clinically isolated syndrome; INF, interferon; MBP, myelin basic protein; MHC II, class II major histocompatibility complex; MS, multiple sclerosis; NGF, nerve growth factor; Nrf2, nuclear factor erythroid 2-related factor 2; PPMS, primary progressive multiple sclerosis; RMS, relapsing multiple sclerosis; RRMS, relapsing remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; LFTs, liver function tests; PML, progressive multifocal leukoencephalopathy; PRES, posterior reversible encephalopathy syndrome; S1P, sphingosine 1-phosphate; JCV, John Cunningham virus; TB, tuberculosis; TFTs, thyroid function tests; VZ, varicella zoster.

**Highly active disease despite a full and adequate course of treatment with at least 1 disease modifying therapy OR 2+ disabling relapses in previous year and with MRI activity including enlarging T2 lesions.*

***Mitoxantrone has been first authorized in 2000 as antineoplastic.*

and autoimmune disorders. However, it is not approved for use in MS, but is commonly prescribed as off-label treatment.

On the contrary, its humanized surrogate ocrelizumab received EMA and FDA approval for the treatment of patients with relapsing forms of MS or with early PPMS. In two phase III trials, OPERA I and OPERA II, ocrelizumab reduced annual relapse rate (ARR) up to 47% and disability progression by 40% compared with subcutaneous IFNβ-1a (39). Ocrelizumab also induced a reduction in the count of T1 gadolinium (GAD)-enhancing lesions (up to 94%) and the mean number of new or newly expanding lesions on T2-weighted MRI imaging. 47.9% and 47.5% of patients treated with ocrelizumab in OPERA I and OPERA II, respectively, after 96 weeks demonstrated no evidence of relapses, disability progression, and T2- or GAD-enhancing T1 lesions, without new safety concerns (40). In addition to the robust phase III data in RRMS, ocrelizumab had also favorable phase III data in PPMS (41). In the ORATORIO trial, patients receiving ocrelizumab had lower disability progression at 3 and 6 months and showed a reduced volume of T2 hyperintense lesions and a significant improvement in brain volume loss compared with placebo. Long-term follow-up data from the open-label extension of ORATORIO trial showed persistent efficacy in patients treated continuously with ocrelizumab up to 6.5 study years, with no evidence for increasing risk of adverse events (AEs) related to cumulative exposure (42). The only concern was a decrease in serum immunoglobulin concentration below the lower limit of normal, where the clinical significance is not clear (43). The most common AEs associated with the use of ocrelizumab are infections followed by infusion-related reactions (IRRs) (44). One observational study reported a higher risk of AE-related discontinuations for ocrelizumab versus rituximab (rate ratio [RR], 2.66; 95% confidence interval [CI], 1.09-6.47) (45). Current recommendations to reduce the risk of an IRR include pre-medication with intravenous methylprednisolone and an antihistamine and monitoring of patients during and after the infusion (46). Interestingly, a shorter infusion period (2 h *versus* 3.5 h) was not associated with an increased risk of IRRs (47), and EMA has recently authorized the 2-h infusion time for second and subsequent doses. The most commonly reported serious AEs (SAEs) are serious infections, followed by neoplasms. Treatment with B-cell-depleting anti-CD20 frequently results in a decrease in total immunoglobulins (IgG, IgM, IgA), typically associated to the occurrence of recurrent or complicated serious infections (45, 46, 48–51). As of December 2020, 10 cases of progressive multifocal leukoencephalopathy (PML) (nine cases had prior exposure to either natalizumab or fingolimod, and one case had no prior exposure (52), and six other serious opportunistic infections (including systemic Pasteurella infection, multisegmental herpes zoster infection, enterovirus-induced fulminant hepatitis requiring a liver transplant, Candida sepsis, viral meningitis) have been reported (44). However, because of its relatively recent marketing authorization, PML risk in patients treated with ocrelizumab has not yet been well established. Overall, 64 cases of neoplasms have been reported among patients treated with ocrelizumab across all the trials, to which eight cases reported in observational studies and a total of 95 cases of breast cancer reported among women exposed outside of clinical trials have been added (44). A much longer follow-up in large populations treated in a real-world setting is necessary to assess the real correlation between malignancies and ocrelizumab treatment. Finally, cases of neutropenia have been described after ocrelizumab treatment, as well as one case of a drug-induced hypersensitivity syndrome (DRESS).

Clinical Data Supporting the Use of Rituximab in Multiple Sclerosis

Rituximab recognizes a similar epitope of CD20 protein to that ocrelizumab, but with a relatively higher binding affinity (53). As ocrelizumab, rituximab induces cell death through apoptosis, ADCC, antibody-dependent cell-mediated phagocytosis, and CDC. Because of the differences in the Fc regions, rituximab induces more CDC and less ADCC than ocrelizumab, being, accordingly, theoretically more prone to induce infusion-related side effects (53, 54). As other monoclonal antibodies, rituximab does not pass readily across the BBB, and its CSF concentration has been estimated to reach only 0.1% of that in serum after intravenous administration (55); nevertheless, a profound depletion of intrathecal B cells with standard intravenous doses is evident (56).

It was initially approved for CD20+ non-Hodgkin lymphoma and subsequently for CD20+ chronic lymphocytic leukemia, rheumatoid, granulomatosis with polyangiitis and microscopic polyangiitis, and pemphigus vulgaris (48).

Available relevant literature (updated on March 2021) was searched on MEDLINE (PubMed), applying the medical subject headings (MeSH) terms "multiple sclerosis" and "rituximab" and "efficacy" and "safety."

We selected peer-reviewed, full-text, and English language manuscripts, randomized-controlled trials (RCTs), prospective studies, non-randomized clinical trials, retrospective studies, and studies made from registries. We excluded meta-analyses and reviews.

Clinical trials and real-world data supporting the use of rituximab in patients with MS are reported below and summarized in **Supplementary Table 1**.

Clinical Trials

The first trial with rituximab in MS was an open-label phase I study providing an initial assessment of safety, tolerability, and activity of the drug in a small cohort of 26 patients with active RRMS, aged 18 to 55 years and mostly not treatment naïve, followed for 72 weeks (57). Patients received intravenous rituximab 1,000 mg on days 1 and 15, and a second course of treatment on weeks 24 and 26. Rituximab treatment induced a reduction of the mean ARR from 1.27 to 0.25 at week 24 and to 0.18 at week 72. The mean number of GAD-enhancing lesions was also reduced from 1.31 at baseline to 0.73 at week 4 after the first course and further to 0.05 at week 48 and to 0 at week 72. The mean number of new T2 lesions decreased as well, from 0.92 at week 4 to 0 at week 72, with a significant reduction also in the volume of the lesions. Rituximab was globally well tolerated:

84.6% of enrolled patients completed the week 72 visit, and all patients received the four infusions of rituximab, with the exception of one patient, in whom an IRR developed at the third infusion. The majority of enrolled patients (77%) experienced grade 1 to 2 AEs and only six reported grade 3 AEs (including fatigue, tooth fracture, muscle weakness, and headache), whereas no grade 4 events were reported. IRRs, likely due to cytokine release accompanying B cell lysis, were documented in 65.4% of the patients during the study (all mild to moderate in severity) and tended to decrease with subsequent infusions. However, no glucocorticoid premedication was administered before infusions. Infections, reported in 61.5% of patients, were also mild to moderate in severity and none led to withdrawal from the study. No opportunistic infections, including PML, were observed. None of the patients had IgG or IgA levels less than the lower limit of normal at week 72. Of the 25 patients who had normal baseline IgM values, 11 (44%) had a value below the lower limit of normal and presented a higher incidence of overall infections. Noteworthy, anti-rituximab antibodies, detected in 35% of patients at week 72, did not appear to influence either efficacy or safety measures.

In a phase II, double-blind, placebo-controlled, manufacturer-sponsored, 48-week trial (HERMES study) in 104 patients with RRMS (58), a single course of rituximab (1,000 mg on days 1 and 15) induced a drastic and sustained reduction of total GAD-enhancing lesions (relative reduction, 91%; p<0.001) and of total new GAD-enhancing lesions (p<0.001) at all investigated time points, together with a significant reduction of T2 lesions volume at week 24 (p=0.008) and 36 (p=0.004). Patients randomized to placebo had fewer GAD-enhancing lesions at baseline, but this imbalance would represent a bias against rituximab. Treatment with rituximab was also associated with a significant reduction, as compared with placebo, of the proportion of patients with relapses at week 24 (14.5% rituximab *vs.* 34.3% placebo; p=0.02) and week 48 (20.3% vs. 40.0%, p=0.04). ARR was also significantly reduced at week 24 (0.37 vs. 0.84, p=0.04), but not at week 48 (0.37 vs. 0.72, p=0.08). By week 48, CD19+ peripheral B lymphocytes, almost completely depleted from 2 weeks after treatment until 24 weeks, returned to increase. CD3+ T lymphocytes were not appreciably altered by rituximab. IgM, IgG, and IgA were normal in both groups, and IgM levels were below the lower limit of normal in more rituximab-treated patients. Sixteen (24.6%) of 65 rituximab-treated patients developed human antichimeric antibodies, although, as previously, no apparent association with the type or severity of AEs or with efficacy response at week 24, week 36, or week 48 was observed. As reported in the phase I trial, the discontinuation rate was very low, with 92.3% of the enrolled patients completing 24 weeks, and 76.0% completing 48 weeks (84.1% in the rituximab group and 60.0% in the placebo group), confirming the good tolerability of the drug in this setting. Only 6% of patients in the placebo group and 4% of patients in the rituximab group withdrew from the study because of AEs. Considering that, even in this trial, no premedication with glucocorticoids was used, 78.3% of rituximab-treated patients (versus 40.0% of patients in the placebo group) had IRRs after the first administration, mostly mild to moderate, decreased to placebo levels with successive infusions. Importantly, no differences in the rate of SAEs and infections were reported. The most common infections in rituximab group were nasopharyngitis, upper respiratory tract infections, sinusitis, and urinary tract infections, whereas no clinically significant opportunistic infections (including PML) were reported.

Globally, the efficacy results of these two trials in RRMS were encouraging and the safety evaluation was favorable: despite the high frequency of IRRs, they were mostly mild to moderate in severity, not inducing hospitalization or treatment discontinuation, and their number was reduced after subsequent infusions. Infections were also quite common in rituximab-treated patients, but, also in this case, they were mainly mild to moderate. As expected, because of the chimeric nature of rituximab, the frequency of anti-drug antibodies was higher than that reported with ocrelizumab (39, 41). However, few cases of delayed hypersensitivity reactions, associated with anti-drug antibodies forming immune complexes and observed in rituximab use for other indications (59), have been reported in MS (60). Moreover, no significant differences in treatment efficacy between the patients with and without anti-drug antibodies have been reported.

Rituximab has also been evaluated in a phase II/III randomized, double-blind, placebo-controlled, manufacturer-sponsored trial (OLYMPUS study) in patients with PPMS (61). A total of 439 PPMS patients were randomized 2:1 to receive two intravenous infusions (2 weeks apart) of 1,000 mg rituximab (n = 292) or placebo (n = 147) every 24 weeks, through 96 weeks. At week 96, treatment with rituximab compared with placebo was associated with a reduction in the proportion of patients with confirmed disease progression (CDP) — defined as an Expanded Disability Status Score (EDSS) increase of ≥1.0 (baseline EDSS 2.0 — 5.5. points) or ≥ 0.5 (baseline EDSS > 5.5. points) point from baseline values sustained for at least 12 weeks — of 8.3 percentage points (30.2% and 38.5%, respectively; p=0.14). This effect, even if not statistically significant, was quite comparable with those seen in the ocrelizumab PPMS trial ORATORIO, in which the corresponding reduction in the CDP rate compared with placebo was of 6.4 percentage points (p=0.03) (32.9% in the ocrelizumab group *vs* 39.3% in the placebo group; hazard ratio [HR]:0.76, 95% CI: 0.59–0.98) (41). Nevertheless, the prespecified subgroup analyses indicated a statistically significant effect of rituximab on CDP rate in patients younger than 51 years (HR: 0.52; p=0.010), in those with GAD-enhancing lesions at baseline (HR, 0.41; p = 0.007), and in those both younger than 51 years and with baseline GAD-enhancing lesions (HR, 0.33; p = 0.009). These results may help identify patients amenable to the treatment, with important implications for treating progressive forms of MS, for which very few therapeutic alternatives are, to date, available. Of note, ocrelizumab ORATORIO trial, in addition of having a higher sample size (488 patients in the ocrelizumab arm *vs* 292 patients in the rituximab arm) and a different statistical analysis plan, only included patients younger than 55 years (mean age, 44.7 ± 7.9 vs 50.1 ± 9.0 years in rituximab-treated patients), which may have contributed to the more favorable results obtained in this setting, supporting the approval for primary progressive MS.

Moreover, with respect to rituximab-treated patients, patients in the ocrelizumab group were characterized by a shorter mean disease duration, had a higher brain volume at baseline, were slightly less disabled, and a higher percentage of them presented GAD-enhancing lesions at baseline and were treatment naïve at randomization (88.7% *vs* 64.7%) (41).

In addition, the open-label extension phase of ORATORIO trial, evaluating the effects of maintaining or switching to ocrelizumab therapy on measures of disease progression, even if demonstrating the benefit of earlier and continuous treatment with ocrelizumab over the 6.5 years of study follow-up compared with patients switching from placebo, confirmed that progression remains an important unmet need in multiple sclerosis in the long term, despite treatment with the only authorized DMT for PPMS (42).

In the OLYMPUS trial, rituximab treatment was also associated with significantly lower ($p<0.001$) increase in T2 lesion volume and with lower worsening in the Multiple Sclerosis Functional Composite (MSFC) timed 25-foot walk test (therefore in the ambulation) at week 96, whereas brain volume decrease was similar to placebo ($p=0.62$). As previously observed, rituximab induced a rapid and almost complete depletion of peripheral CD19+ B lymphocytes, which recovered at week 122 in 35% of treated patients, with no appreciable effects on CD3 T-cell counts. IgG and IgA levels were below the lower limit of normal in less than 5% of patients in either treatment arm, whereas IgM levels were below the lower limit of normal in 31.7% of rituximab-treated patients vs 5.9% of patients receiving placebo. No evidence of a relationship between lower immunoglobulin levels and an increased incidence of infections or other adverse events has been found. Twenty (7.0%) of 286 patients receiving rituximab developed human antichimeric antibodies, although, also in this case, no apparent association with the type or severity of adverse events or with efficacy responses was observed. Safety profile of rituximab reported in the trial was in line with other published data. IRRs, primarily mild to moderate in severity, were more common with rituximab (67.1% *vs* 23.1%) and decreased with successive infusions. Infections (upper respiratory infections, urinary tract infections, and nasopharyngitis) were globally reported in 65.3% of placebo and 68.2% of rituximab-treated patient, with 4.5% of rituximab *vs* <1% of placebo-treated patients reporting serious infections. AEs leading to treatment discontinuation occurred among 3% of patients who received rituximab, whereas none withdrew due to AEs was reported in the placebo group.

Despite the promising results in PPMS, as well as in RRMS, obtained from RCTs, the clinical development of rituximab was interrupted. However, in the light of the well-established long-term safety profile of rituximab from its wide use in other diseases (62) and of the promising results obtained in MS, researchers were highly motivated to pursue further trials.

A small single-center, investigator-initiated phase II trial, including 52 weeks post-treatment follow-up, evaluated the safety, efficacy, and tolerability of add-on intravenous rituximab at a dose of 375 mg/m^2 weekly × four doses in 32 RRMS patients with breakthrough disease while receiving INFβ or glatiramer acetate (63). Enrolled patients were older, more disabled, and with a longer disease duration compared with the population of phase I and phase II placebo-controlled trial of rituximab in RRMS (57, 58). In this setting, add-on rituximab induced a significant reduction of GAD-enhancing lesions in comparison to pretreatment MRIs ($p<0.0001$). 74% of the three post-treatment MRI scans were free of GAD-enhancing lesions *vs* only 26% of the three pre-treatment MRIs. The median number of GAD-enhancing lesions declined from 1 per month to 0 after treatment. Although the study was not designed or powered to examine relapse rate reduction, a reduction in ARR from 1.27 pre-treatment to 0.23 after treatment has been observed. MSFC improved, mainly due to an improvement in Paced Auditory Serial Addition Test (PASAT) scores (a measure of cognitive function), whereas EDSS remained substantially stable during follow-up. As previously, also in this study, no correlation has been found between the development of human antichimeric antibodies and efficacy response. Add-on rituximab was generally well tolerated, with no SAEs and only few AEs reported. Infusion reactions, the most common AEs observed in the study, were typically mild, but resulted in two study discontinuations. Four uncomplicated urinary tract infections and one upper respiratory tract infection, with an unknown relation to rituximab, were documented.

In a more recent investigator-initiated, open-label, phase II trial (STRIX-MS trial), 75 patients with clinically stable RRMS, treated with first-line injectable IFNβ or glatiramer acetate for at least 6 months, were switched to rituximab (64). After a run-in period of 3 months, patients received two doses of 1,000 mg rituximab, followed by repeated clinical assessments, MRIs, and measurement of neurofilament light chain concentrations in the cerebrospinal fluid (NFL-CSF) for 24 months. In the first year of treatment, only one patient experienced a clinical relapse and was switched to natalizumab, whereas no patients fulfilled the prespecified MRI criteria for treatment failure (i.e., occurrence of one GAD-enhancing lesion or more than one new T2 lesions). During the second year, one patient experienced a clinical relapse and the same patient, together with three others, had a MRI worsening and was re-treated with rituximab. The mean cumulated number of GAD-enhancing lesions at months 3 and 6 and of new or enlarged T2 lesions at month 12 after treatment shift was reduced, as well as the mean CSF-NFL levels. These results support the use of rituximab in MS, given the equal or superior effect in reducing disease activity in RRMS compared to first-line treatments during the first year after switch (Class IV evidence). Regarding clinical and patient reported outcomes, there was a statistically significant improvement in the Symbol Digit Modalities Test (SDMT) ($p<0.001$), although the changes were small in absolute values, whereas neurologic impairment assessed by EDSS did not show any progression or improvement of statistical significance, as well as scores for patient-perceived impact of disease on daily life (Multiple Sclerosis Impact Scale, MSIS-29) and fatigue (Fatigue Scale for Motor and Cognitive functions, FSMC) (65). However, the overall treatment satisfaction, measured by a modified version of the Treatment

Satisfaction Questionnaire for Medicine (TSQM-10), improved significantly, in particular for question 4 of the questionnaire ("How easy or difficult is it to use the medication in its current form?") and question 7 ("How easy or difficult is it to live with the side effects of the medicine?") and was sustained after 2 years. The apparent discrepancy between the improvement of patient treatment satisfaction and the lack of significant improvement in EDSS, MSIS-29, and FSMC might be explained with the overall low disability, fatigue, and therefore, global impact of the disease on daily life characterizing the patient population at the time of the switch, as well as with a more convenient treatment schedule compared to injectable first-line DMTs, with probably less interference with daily activities. Even then, the treatment was generally well tolerated. Globally, 17 non serious AEs related or possibly related to rituximab were reported. The most common side effects were, as expected, mild to moderate infusion reactions. Six SAEs were documented, three of which (two pyelonephritis and one influenza) possibly related to rituximab and three not related (stroke, cholangitis, and suicidal attempt by intoxication) (64).

A double-blind, placebo-controlled, randomized, single-center study evaluated the efficacy and safety of rituximab also as first-line treatment in an induction therapeutic approach (66). Fifty-five patients with RRMS and active disease or with a diagnosis of CIS were randomized 1:1 to receive a single cycle of rituximab (two intravenous injections of 1,000 mg 2 weeks apart) or placebo, followed by subcutaneous glatiramer acetate 20 mg/daily up to a maximum of 144 weeks. At the end of the 3 years of the study, 44% of rituximab-treated patients demonstrated NEDA *vs* 19.23% of patients in the placebo group (p=0.049). The greater probability of demonstrating NEDA in the rituximab group, observed from about 6 months from induction, was not sustained and returned to baseline within the study period. Treatment failure (defined as ≥2 new lesions, relapses, and/or sustained accumulation of disability) was observed in a smaller percentage of rituximab-treated patients (37.04% *vs* 69.23% of placebo group, p=0.019), and time to treatment failure was longer (23.32 months vs 11.29 months, p=0.027). Rituximab-treated patients demonstrated also less MRI activity as compared with placebo-treated patients, with a smaller proportion of participants having new T2 lesions (25.93% rituximab *vs* 61.54% placebo, p=0.009), and a smaller total number of new T2 lesions. No significant group differences were observed for GAD-enhancing lesions and for patient-reported outcomes regarding disability or quality of life. These results suggest that a single cycle of rituximab followed by a moderate efficacy/high safety DMT as glatiramer acetate may provide a superior efficacy than glatiramer acetate alone in RRMS, although this benefit does not seem to be long-lasting. As expected, a greater number of infusion-related reactions, all mild to moderate, was documented in the rituximab group compared with controls, whereas no differences in SAEs between the two study groups were observed.

On 2015, a phase I/II trial (RIVITaLISe) was conducted to evaluate the efficacy of combined intrathecal and intravenous rituximab therapy on SPMS compared to placebo (67). The study was prematurely terminated by investigators based on an interim analysis on CSF biomarkers that showed an incomplete and transient depletion of intrathecal B cells by rituximab. However, the early termination of the study made the acquired clinical and imaging data insufficient to perform reliable analyses of clinical effects of rituximab in SPMS patients.

Recently published results from another phase II/III, open-label, randomized clinical trial, in which 84 patients with SPMS were assigned to receive rituximab (1,000 mg every 6 months; n=37) or glatiramer acetate (40 mg subcutaneous 3 times/week; n = 40) for 12 months, documented an apparent lack of efficacy of both treatments in controlling EDSS progression (68). Indeed, the mean EDSS increased after 12 months from 3.05 ± 1.01 to 4.14 ± 0.91 in the rituximab group ($p < 0.001$), and from 3.22 ± 1.20 to 4.60 ± 0.67 in the glatiramer acetate group ($p < 0.001$). No statistically significant differences in EDSS scores were observed between the two groups, although a trend favoring rituximab emerged. In contrast, both rituximab and glatiramer acetate resulted equally efficacious in reducing ARR after 12 months (from 1.30 ± 0.52 to 0.41 ± 0.64 in the rituximab group [$p<0.001$], and from 1.17 ± 0.38 to 0.22 ± 0.42 in the glatiramer acetate group [$p<0.001$]) and the number of active lesions in brain and cervical spine. However, it has to be considered that the study had a short duration, and that patients randomized to glatiramer acetate had a longer disease (17.39 ± 7.53 years *vs* 11.41 ± 6.45, p=0.001) and were older (mean age 45.72 ± 7.64 years vs 40.92 ± 8.12, p= 0.011) compared with those assigned to rituximab group. Non-serious self-limited AEs were observed in both groups without any differences, whereas no SAEs were reported in the study.

In summary, except for the disappointing results in SPMS, even the investigator-initiated clinical trials substantially confirmed the good safety, tolerability, and efficacy profile of rituximab in RRMS, not only as second-line monotherapy but also as add-on therapy in patients not adequately controlled with first-line DMTs and as first-line monotherapy protocol (single cycle of RTX followed by other DMTs).

Real-World Data and Retrospective Studies

Besides clinical trials, a large number of studies have used real-world data, obtained from the wide off-label use of rituximab, to assess its efficacy and safety in MS patients.

One of the largest real-world study, assessing rituximab safety and efficacy in a heterogeneous real-world MS cohort of 822 patients (557 RRMS, 198 SPMS, 67 PPMS) (69), reported a low ARR during treatment (0.044 for RRMS, 0.038 for SPMS, and 0.015 for PPMS patients) and an overall reduction of the occurrence of contrast-enhancing lesions from 26.2% at baseline to 4.6%. Most of the contrast-enhancing lesions that were detected appeared early after rituximab initiation, which eventually disappeared. The mean annual change in brain parenchymal fraction on rituximab treatment (assessed in 160 patients) was –0.19% (a percent change sensibly lower to those observed in MS patients treated with placebo in other studies) (70). During the observation time, median EDSS remained

unchanged in patients with RRMS, and increased 0.5 and 1.0 for patients with SPMS and PPMS, respectively (p=0.42; p=0.10; 0.25). As previously, rituximab showed an acceptable safety profile: 7.8% of infusions led to IRRs, mostly mild, and 89 AEs grades ≥ 2 (76 infections) occurred in 72 patients. No cases of PML were detected. Treatment compliance, in line with other published data, was very high, with 94.8% of patients continuing rituximab.

Interestingly, no statistically significant differences in B-cell depletion and efficacy were reported between the two-dosing regimen used (500 and 1,000 mg doses given as single infusions every 6 months), whereas a trend for fewer AEs with the lower dose regimen was observed. These data suggest that lower doses of rituximab might be as effective in MS as higher doses with a better safety profile and a substantial cost-saving (given that the cost of rituximab is related to the dose administered).

A recently published prospective study by Disanto et al. (71), including 59 patients (37 RRMS and 22 SPMS) treated with rituximab for at least 1 year before study entry, provided evidence that the de-escalation of rituximab dose from 1,000 to 500 mg/6 months is safe and associated with clinical, radiological, and biomarker-based stability over 12 months. Indeed, no relapses were reported in the 12 months after switching to the lower dose regimen, EDSS scores maintained approximately stable, as well as serum NFL concentration, and only three new T2 lesions in brain/spinal cord (all of which without contrast enhancement and clinically asymptomatic) were detected. Such a result is striking considering that most of the included patients had a severe form of MS and started rituximab mainly because of the suboptimal response on previous DMTs. Overall, three SAEs, only one (a late-onset transient neutropenia) probably related to rituximab, occurred in the 12 months after dose de-escalation. The most common AEs were infections, whereas no IRRs were reported after dose switching. A greater risk of infections was detected in those patients with a mean IgG concentration below the reference range (OR=6.27, 95% CI=1.71–22.9, p=0.005). Importantly, an inverse association between the total dose of rituximab received under the 1,000 mg/6 months regimen (rituximab load) and the IgG concentrations measured after the de-escalation emerged in the study, with a higher rituximab load associated with a lower IgG, and, therefore, with a greater risk of infections.

Another huge multicenter, retrospective Italian-Swiss study, analyzing data from over 350 RR and progressive MS patients treated with rituximab, showed a significant reduction of ARR in the 2 years after the treatment start from 0.86 (95% CI: 0.73–0.99) to 0.09 (95% CI: 0.07–0.13) in RRMS and from 0.34 (95% CI: 0.25–0.45) to 0.06 (95% CI: 0.04–0.10) in SPMS patients (p<0.0001), and a slight not significant decrease in PPMS patients (from 0.12 to 0.07, p = 0.45) — probably related to the lower number of events (72). The proportion of patients with an EDSS progression was 14.6 ± 0.07% in the RRMS group, 24.7 ± 0.11% in the SPMS group, and 41.5 ± 0.17% in the PPMS group, after 3 years of treatment. In the multivariable analysis, the risk of EDSS progression was higher for PPMS (p=0.0005) and SPMS (p=0.013) as compared with RRMS patients. AEs observed during rituximab treatment were within the expected range, including mostly IRRs and infections, both rarely reported to be serious. No major safety concerns (especially those related to neoplasms or PML) arose. Overall, the study adds to the published literature, confirming that rituximab is effective and relatively safe in the treatment of MS.

An interesting propensity score matching analysis performed on data retrospectively collected from three MS centers located in Switzerland and the Netherlands, showed, in contrast to what reported in the phase II/III trial in SPMS (68), a significantly lower EDSS score during a mean follow-up of 3.5 years (mean difference, −0.52; p<0.001) and a significantly delayed time to confirmed disability progression (p=0.03) for patients treated with rituximab compared with matched patients never treated with rituximab, suggesting a potential therapeutic benefit of rituximab also in SPMS (73). No major safety concerns were reported during the treatment period, although complications, mainly related to infections, were documented in five cases (9%).

A single-center retrospective observational study in Finland (74), included a total of 72 rituximab-treated patients with RRMS (n=31), PPMS (n=16), and SPMS (n=25) for whom other MS medications failed to achieve an adequate effect, or for whom no other medication was available. EDSS remained substantially stable in all MS group. In particular, among patients with progressive forms, 45% had stable EDSS during the study, whereas 18% of PPMS and 20% of SPMS patients even had an improvement. Moreover, rituximab treatment significantly reduced ARR in both RRMS and SPMS and the mean number of GAD-enhancing lesions in RRMS patients. Treatment discontinuation was observed in 12 patients because of the patient's disappointment with the drug efficacy (n=10) or a drug-related adverse event (n=2). The study confirmed the good tolerability of rituximab, also in this setting, with no serious IRRs or infections.

A large cross-sectional study by Dunn and collaborators (75), including patients receiving off-label rituximab for MS (both RR and progressive forms), reported the development of anti-rituximab antibodies in 34% of patients (a percentage higher to that observed in clinical trials). The presence of anti-drug antibodies, which decreased after repeated rituximab infusions, was associated with incomplete or unmaintained B-cell depletion, but not with infusion reactions, adverse events, or lack of clinical effect, with a strong suppression of disease activity observed in both antibody-positive and antibody-negative patients.

A retrospective observational study (76), based on data collected within a registry, provided further evidence of the efficacy of rituximab in MS treatment, both in RRMS and PMS in terms of number of new relapses, EDSS worsening, new T2 and GAD+ lesions, and proportion of patients without evidence of disease activity during treatment.

A small retrospective study confirmed the good tolerability and acceptable safety profile of rituximab also after long-term treatment (average duration, 33.2 months) (77). AEs reported during the observation period were mostly mild, with the exception of three severe urinary tract infections requiring hospitalization, and no cases of PML.

Effectiveness and safety of rituximab were further confirmed in a recent Italian single-center retrospective observational analysis of 17 patients with demyelinating CNS diseases (including MS, neuromyelitis optica, and neuromyelitis optica spectrum disorders [NMOSD]) who underwent rituximab treatment (78). About 25% of patients were naïve to DMTs. The mean follow-up was 22.6 ± 22.9 months (range, 12–80 months). After rituximab treatment, 11 (65%) of 17 patients got NEDA status, and no patients had disability progression and new T2 or T1-GAD+ brain and/or spinal lesions. Six AEs were recorded in five patients. One patient with RRMS stopped rituximab and switched to azathioprine due to severe lymphopenia, whereas another patient with PPMS switched to ocrelizumab after its license for PPMS treatment.

In another retrospective study on an Italian real-life cohort of RR and progressive MS patients, the most of which not treatment naïve and switched to off-label rituximab due to persistent disease, AEs or reduced compliance, rituximab (1,000 mg, 6 monthly) significantly reduced the ARR from 0.75 to 0.36 at 12 months ($p<0.001$), with no differences between RR and progressive patients (79). The proportion of patients showing MRI activity was reduced from 88% to 8.3% at follow-up ($p<0.001$), again with no differences between RR and progressive patients. Of the 55 patients who had an EDSS evaluation, 13 (23.2%; 10 PMS, and 3 RRMS) showed a progression at 6 months compared with baseline, whereas only one progressive patient showed a progression at 12 months. The NEDA status at 12 months was observed in about 60% of patients. The reported safety profile in this patient group was substantially consistent to that reported in other studies, with a high frequency of mild-to-moderate IRRs and infections. Interestingly, infectious AEs were less common than non-infectious and 10% of the reported AEs were leukopenia. Globally, 12 patients suspended rituximab during the study due to AEs (n=4), scarce tolerability (n=3), persistent clinical (n=2) or radiological disease activity (n=2), or pregnancy (n=1).

A retrospective cohort university hospital-based study (80), analyzing data from 59 RRMS and 30 PMS patients switched to rituximab mainly due to persistent disease activity on other DMTs, showed a reduction of ARR by approximately 89% (relapse-free in 79% in the RRMS and 90% in the PMS group) and no EDSS score progression in both RRMS and PMS patients. Interestingly, there was a trend of improvement in terms of EDSS in RRMS, whereas in the PMS group, it was substantially unchanged. 92.6% in the RRMS and from 82% in the PMS group were free from any new lesions, and 74% achieved NEDA at 1 year of treatment. The most common AEs (n=64; 71.9%) were mild IRRs, whereas the overall rate of infection was relatively low (15.7%). Two rituximab-treated patients (2.2%) experienced SAEs requiring surgical interventions (pyoderma gangrenosum vaginalis with perianal abscess and fistula; increase in the size of a meningioma). No cases of PML were reported.

In another Spanish retrospective university hospital-based study, including both RRMS and PMS patients, rituximab (administered off-label mainly as second- or third-line treatment) significantly reduced ARR by 88.4% ($p<0.001$) and the number of GAD-enhancing lesions from 2.56 to 0.06 ($p<0.001$) (81). Ninety percent of patients remained free of relapses during the follow-up and the relapses observed in the remaining patients occurred almost all in the first 6 months of treatment. A decrease of 0.3 EDSS points in the first year ($p=0.01$) and no variation in the second year of therapy were detected. Considering only PMS patients, most of them remained stable after rituximab treatment, without significant changes in the EDSS score. NEDA status was reached in 70% of the total sample (74.2% of RRMS patients, and 67% of the PMS patients). Therefore, in this study, rituximab demonstrated to be a feasible therapeutic option for PMS patients as well. The main AEs were IRRs, mostly mild and less frequent than those reported in clinical trials (18.8% vs 60–70%). Regarding non–infusion-related AEs, the most common were non-severe infections, while no opportunistic infections like PML were reported. One case of agranulocytosis 3 months after rituximab infusion and three cases of venous thrombotic events (one deep venous thrombosis in one leg, one deep venous thrombosis with secondary mild pulmonary embolism in a patient taking concomitant oral contraceptives, and a serious massive pulmonary embolism secondary to a deep venous thrombosis in a patient with an EDSS score of 8.5 and lack of mobility) were reported. Rituximab was interrupted in 22 (24.4%) patients, mainly as a consequence of suboptimal responses or disability worsening (especially in PMS patients).

At a general hospital level, Hellgren et al., retrospectively analyzing data from 83 patients with RRMS, PPMS, and SPMS, reported a highly significant reduction of ARR induced by rituximab (500 or 1,000 mg every 6–12 months) from mean 0.38 ± 0.5 before treatment initiation to mean 0.05 ± 0.19 at follow-up ($p<0.00001$), with a global reduction by 87% (82). The percent of patients with new inflammatory lesions decreased from 58% at baseline to 26% during the long-term follow-up, from 36 to 18 ($p<0.0001$) in the RRMS cohort, and from 8 to 2 ($p=0.07$) in PMS. Considering only contrast-enhancing lesions, the percent of subjects with one or more lesions dropped from 47% at baseline to 6% at 1 year after rituximab initiation. Globally, contrast-enhancing lesions decreased from 0.94 to 0.24 ($p<0.00001$). In the RRMS cohort, contrast-enhancing lesions/MRI ratio was reduced from 1.05 to 0.31 ($p=0.00003$), whereas no lesions were seen in the PMS patients after rituximab initiation. The most interesting finding of this study was that most scans showing contrast enhancement were done within 6 months after starting rituximab, whereas a total absence of new lesions was reported in almost all patients during the remaining follow-up period (mean duration ~2 years). Reported AEs were mainly mild. Most frequent non-IR AEs were infections (observed in 22% of treated patients), of which four were classified as moderate, requiring hospitalization, and one as severe (a case of pneumonia with concomitant late-onset neutropenia, the first reported in Swedish MS population related to rituximab).

An interesting retro-prospective study performed in a developing country (India), where rituximab, also thanks to the availability of biosimilars, represents an affordable

therapeutic option for MS with respect to other high-cost approved standard treatments, demonstrated the good safety and efficacy profile of three different dosing regimens (a low-, a medium-, and a high-intensity regimen, chosen depending on the severity of MS) of the DMT in RRMS (n=58) and PMS (n=15 SPMS and n=7 PPMS) patients (83). In the RRMS population, the mean ARR decreased from 0.44 ± 0.498 to 0.051 ± 0.223 ($p<0.05$) at 1 year of follow-up, with no relapses in 97% of treated patients. EDSS improved by 0.5 to 2.0 points in 85% of patients (all RRMS patients, four SPMS and six PPMS), remained stable in 12.5% (9 SPMS and 1 PPMS), and worsened in 2.5% (2 SPMS patients). In all treated patients with GAD-enhancing lesions at baseline, follow-up scans at 1 year did not show any lesions either old or new. Interestingly, in the study, the incidence of IRRs was minimal, probably as a consequence of the very low infusion rate adopted (64 ml/h). As in the other real-world studies, no opportunistic infections, like tuberculosis or PML, were reported.

A retrospective study, involving 29 patients with immune-mediated neurological disorders (MS, neuromyelitis optica, and myasthenia gravis) treated with rituximab for up to 7 years (mean treatment duration of 51.3 ± 12.2 months) confirmed the long-term safety and efficacy of rituximab in this setting (84). A total of 32 AE and 4 SAEs (all infections in both cases) were reported, whereas no cases of PML or tumors were detected over the observation period. Rituximab cycles resulted globally well tolerated, with minimal and manageable IRRs, and an overall benefit in terms of relapse rate reduction and improvement in EDSS was observed. Another recent large retrospective study (85), including 1,000 patients with MS, NMOSDs, and other immunological disorders with a mean follow-up of 31.1 months, reported a low incidence of serious AEs, especially infections, associated with rituximab. The overall rate of infections, resulting in hospitalization, intravenous antibiotics, and extended dosing antibiotics, was nearly identical to that reported in a long-term study of rituximab-treated RA patients (86). No cases of PML were observed. IRRs reported in the study were rarely serious, with no infusion deemed life-threatening or resulting in hospitalization, and the rate of malignancy was similar to those of the general population (87). Interestingly, a dramatic increase in infection risk was reported for patients with increasing levels of ambulatory disability, highlighting the importance of using rituximab in younger, less disabled patients early in the disease.

The good safety and efficacy profile of rituximab in both relapsed and progressive forms of MS have been confirmed by different meta-analysis. A meta-analysis by Hu et al. (88), including 15 studies and a total of 946 patients with RRMS, showed a significant decrease of ARR, of EDSS score, and a low percentage of patients experiencing a relapse after starting rituximab therapy. Although mild-to-moderate AEs (mainly infusion-related events and infections) occurred in 29.6% of the patients, no SAEs were reported.

A more recent meta-analysis, including 20 studies for a total of 2020 RRMS patients, reported even more favorable results, with an overall absolute reduction in ARR of 1.00 (95% confidence interval [CI], 0.83–1.17), an overall relapse-free rate at weeks 24, 48, 72, and 96 of 90.4%, 88.5%, 86.4%, and 86.2%, respectively, and an estimated reduction in EDSS score of 0.62 (95% CI, 0.20–1.04) (89). overall AEs (58%; 95% CI, 12%–104%), injection-related events (31%, 95% CI, 18%–45%), and infections (33%; 95% CI, 20%–46%) were common in patients treated with rituximab, whereas SAEs were rarely reported, confirming an acceptable safety profile of rituximab.

Another meta-analysis, including seven studies for a total of 399 patients with any type of MS treated with rituximab, showed a reduction of mean EDSS score (0.29; 95% CI, 0.16–0.42) and of mean ARR (1.24; 95% CI, 1.04–1.44) after treatment, and a proportion of AEs (mostly infusion-related reactions and infections) of 23% (95% CI, 20%–26%) (90).

Indirect Comparisons

Currently, no head-to-head RCTs comparing rituximab with other DMTs have been completed. **Table 2** provides a list of ongoing clinical trials comparing DMTs, including rituximab.

However, real-world studies have allowed to carry out indirect comparisons (76, 91–95). A propensity score–matched Swedish registry study (95), assessing efficacy of rituximab (n=461) in comparison with interferons/glatiramer acetate (n=922), demonstrated a superiority of rituximab over injectable DMTs in the reduction of ARR and EDSS from baseline to 12 and 24 months. Rituximab was also associated with an 85% reduction in the rate of discontinuation relative to IFN-β/glatiramer acetate (HR, 0.15; 95% CI, 0.11–0.20).

The retrospective cohort study by Granqvist et al. (93), including 494 Swedish patients with newly diagnosed RRMS, found a significantly lower discontinuation rate with rituximab (500 mg or 1,000 mg intravenous every 6 months) compared with all other DMTs included in the analysis (interferons, glatiramer acetate, dimethyl fumarate, fingolimod, and natalizumab). The most common cause of treatment discontinuation was pregnancy for rituximab, disease breakthrough and AEs for injectable DMTs, dimethyl fumarate, and fingolimod, and positive JCV serology for natalizumab. Regarding clinical efficacy and safety, a significantly lower rate of relapses and/or disease activity was found with rituximab together with a lower incidence of AEs compared with injectable DMTs and dimethyl fumarate. Compared with fingolimod and natalizumab, ARR and GAD+ lesions were numerically lower but did not reach statistical significance.

Boremalm et al. (94), in a small cohort of 241 RRMS patients switched from interferon/glatiramer acetate due to breakthrough disease, found no significant difference in ARR between natalizumab and rituximab (HR, 1.0; 95% CI, 0.2–5.6), both before and after adjustment for confounders. Both natalizumab and rituximab demonstrated superiority compared with fingolimod, in terms of clinical efficacy. As previously, the discontinuation rate was significantly lower with rituximab compared with both natalizumab and fingolimod. The comparable efficacy of rituximab and natalizumab was further supported by the aforementioned retrospective study by Scotti et al. (76), reporting a similar disease activity reduction in RRMS patients both in multivariate Cox models and after propensity score-based matching.

Globally, these studies showed a greater drug survival and tolerability of rituximab and an efficacy, in terms of control of

TABLE 2 | Ongoing clinical studies comparing disease modifying therapies, including rituximab (www.clinicaltrial.gov; update January 2021).

ID	Title	Trial design	Age (years)	Number of estimated patients (disease)	Arms and interventions	Primary Outcome measures	Follow-up duration	Start date/ Estimated Study Completion Date
NCT03500328	A Pragmatic Trial to Evaluate the Intermediate-term Effects of Early, Aggressive Versus Escalation Therapy in People With Multiple Sclerosis	Phase NA, randomized, parallel assignment	18-60	900 (RRMS)	*Early aggressive therapy choices*: -natalizumab; -alemtuzumab; -ocrelizumab; -rituximab; -cladribine; -ofatumumab *Traditional therapy choices*: -glatiramer acetate; -intramuscular interferon; -subcutaneous interferon; -pegylated interferon; -dimethyl fumarate; -fingolimod; -siponimod; -ozanimod.	-Time to sustained disability progression; -change in overall burden of MS	63 months	May 2, 2018/ August 1, 2023
NCT04047628	A Multicenter Randomized Controlled Trial of Best Available Therapy Versus Autologous Hematopoietic Stem Cell Transplant for Treatment-Resistant Relapsing Multiple Sclerosis	Phase III, randomized, parallel assignment	18-55	156 (MS with EDSS ≥ 2.0 and ≤ 5.5, excluding PPMS)	-Myeloablative and Immunoablative therapy followed by Autologous Hematopoietic Stem Cell Transplantation - Best Available Therapy (BAT) Natalizumab, alemtuzumab, ocrelizumab, or rituximab			
NCT03535298	Determining the Effectiveness of earLy Intensive Versus Escalation Approaches for the Treatment of Relapsing-Remitting Multiple Sclerosis	Phase IV, randomized, parallel assignment	18-60	800 (RRMS)	-*Early highly effective* arm: ocrelizumab, natalizumab, alemtuzumab, rituximab)- *Escalation arm*: any other approved MS therapy (beta interferon, glatiramer acetate, teriflunomide, fingolimod, dimethyl fumarate) - *No intervention*	Brain volume loss	36 months	January 3, 2019/ September 2023
NCT02746744	RItuximab Versus FUmarate in Newly Diagnosed Multiple Sclerosis. A Randomized Phase 3 Study Comparing Rituximab With Dimethyl Fumarate in Early Relapsing-Remitting Multiple Sclerosis and Clinically Isolated Syndrome.	Phase III, randomized, parallel assignment	18-50	200 (RRMS)	-Rituximab -Dimethyl Fumarate -Sham comparator	Freedom of relapse	2 years	May 2016/August 2021
NCT04121403	Norwegian Study of Oral Cladribine and Rituximab in Multiple Sclerosis (NOR-MS) A Prospective Randomized Open-label Blinded Endpoint (PROBE) Multicenter Non-inferiority Study	Phase III, randomized, parallel assignment, open-label blinded endpoint (PROBE)	18-65	264 (active RMS)	-Rituximab -Cladribine	Number of new or enlarging cerebral MRI T2 lesions	96 weeks	October 16, 2019/ December 2023

(Continued)

TABLE 2 | Continued

ID	Title	Trial design	Age (years)	Number of estimated patients (disease)	Arms and interventions	Primary Outcome measures	Follow-up duration	Start date/ Estimated Study Completion Date
		multicenter non-inferiority study						
NCT03193866	COMparison Between All immunoTherapies for Multiple Sclerosis. An Observational Long-term Prospective Cohort Study of Safety, Efficacy, and Patient's Satisfaction of MS Disease Modulatory Treatments in Relapsing-remitting Multiple Sclerosis	Prospective non-intervention observational prospective cohort	≥ 18	3526 (CIS or RRMS)	-Rituximab -all other frequently used immunomodulating (natalizumab, fingolimod, alemtuzumab, interferon-beta, glatiramer acetate, dimethyl fumarate)	Confirmed disease progression in patients with EDSS ≤2.5 at baseline	3 years	February 1, 2017/ December 31, 2022
NCT04688788	Danish Non-inferiority Study of Ocrelizumab and Rituximab in MS (DanNORMS): A Randomized Study Comparing the Efficacy of Ocrelizumab and Rituximab in Active Multiple Sclerosis	Phase III, randomized, open-label, non-inferiority clinical trial with blinded primary endpoint.	18-65	594 (RRMS, PMS)	-Biosimilar rituximab -ocrelizumab	Percentage of patients without new or enlarging T2 white matter lesions on brain MRI scans	24 months	January 15, 2021/ January 15, 2028
NCT04578639	Ocrelizumab Versus Rituximab Off-Label at the Onset of Relapsing	Phase III, randomized double blinded non-inferiority study	18-60	211 (active RRMS)	-rituximab -ocrelizumab	Proportion without new MRI activity	24 months	November 2, 2020/ February 14, 2025

CIS, clinically isolated syndrome; EDSS, expanded disability status score; MRI, magnetic resonance imaging; MS, multiple sclerosis; NA =; PPMS, primary progressive multiple sclerosis; PMS, progressive multiple sclerosis; RMS, relapsing multiple sclerosis; RRMS, relapsing remitting multiple sclerosis; NA, Not Available.

relapses and MRI activity, comparable to natalizumab and superior to dimethyl fumarate and fingolimod, although considerable variability was observed in the magnitudes of the reported differences.

A recent retrospective US study, which performed a head-to-head comparison between 182 rituximab-treated patients and 1,064 patients who received dimethyl fumarate, fingolimod, or natalizumab over 2 years after treatment initiation (91), demonstrated decreased odds of discontinuation and improved efficacy for rituximab compared with fingolimod and dimethyl fumarate, whereas no significant differences were observed between rituximab and natalizumab. However, when investigation of disease activity was restricted between months 6 and 24, an improved effectiveness of rituximab over natalizumab has been reported. Notably, although rate of discontinuation was similar, rituximab discontinuations were driven by insurance issues related to the off-label use, whereas natalizumab discontinuation was mainly related to safety issues.

A new retrospective study, comprising RRMS and SPMS patients treated with rituximab (n=311) and RRMS patients treated with ocrelizumab (n=161), compared tolerability, safety, and immunosuppressive effects of the two anti-CD20 drugs over the first year of treatment (45). The researchers found that ocrelizumab, but not rituximab, was associated with a decrease in IgG of 0.16 g/L (95% CI, 0.01–0.31) with each infusion (a reduction that may increase susceptibility to infections), whereas IgM decreased to a similar extent with both drugs and IgA levels were not affected. CD19+ B depletion was greater with ocrelizumab. Infections and SAEs were more common in the ocrelizumab group, whereas incidence of IRRs was identical to that of rituximab. No statistically significant differences were observed in the proportion of patients discontinuing treatment within the first year (10% with rituximab and 15% with ocrelizumab, p=0.11). However, although the discontinuation due to lack of effect was low and not significantly different in the two groups, discontinuation due to AEs was more common with ocrelizumab than with rituximab. These findings corroborate the idea of the non-inferiority, in terms of tolerability and safety, of rituximab to ocrelizumab, and substantially confirm that the development of anti-drug antibodies, higher with the more immunogenic rituximab and potentially associated with reduced efficacy and increased risks of IRRs, is of marginal clinical importance.

Another recent study analyzed AEs reported for rituximab and ocrelizumab in the real-world practice setting using the Food and Drug Administration's Adverse Event Reporting System (FAERS) database (96). The database contained 623 reports with rituximab and 7948 reports with ocrelizumab. Patients treated with rituximab were on average older than patients treated with ocrelizumab and progressive forms of MS were more frequently found in the reports associated with ocrelizumab (21.2% *vs* 5.6%). Rituximab was associated with a higher proportion of reported SAEs as compared to ocrelizumab (64.8% *vs* 56.3%, $p < 0.001$). AEs resulting in death were found in 5.7% of rituximab reports versus 2.1% of ocrelizumab reports ($p < 0.001$). The study revealed significant differences in reported AE profiles in the real-world setting between the two anti-CD20 drugs, with frequency of reported infections (especially oral herpes, urinary tract infections, and nasopharyngitis) nearly two times higher with ocrelizumab (21.93% *vs* 11.05% of rituximab), whereas no significant differences were reported for IRRs. However, the risk of bias in spontaneous reporting system, above all under-reporting and the tendency for SAEs to be reported more frequently, should be considered.

Preliminary results from an ongoing phase III trial (ClinicalTrials.gov Identifier: NCT02980042), evaluating tolerability and safety of switching from rituximab to ocrelizumab in adult patients with relapsing forms of MS (97), reported a similar incidence of IRRs between patients continuing rituximab and those switched to ocrelizumab and suggested a correlation between levels of CD19/CD20 B cells and risk of IRR (with a decrease by 74% of the risk when CD19 and/or CD20 were ≤1%).

Perez et al. confirmed the similarity of rituximab originator and biosimilar in 145 MS patients (RR and progressive) (98). Patients in the two groups did not differ in CD19+ lymphocyte counts at each follow-up examination and showed a comparable reduction in relapse rate at 12 months (from 0.50 to 0.02 for originator and from 0.40 to 0.025 for biosimilar), whereas EDSS remained stable in both groups at 6 and 12 months. The proportion of patients with MRI activity on the first scan after starting of rituximab was similar between originator and biosimilar (1% and 0% of patients with GAD-enhanced lesions, respectively, p=0.41; 10% and 12% of patients with new T2 lesions, respectively, p=0.76). On the second MRI after rituximab initiation, only one patient in the entire population (treated with originator) showed a new T2 lesion, whereas no new GAD-enhanced lesions were detected. AEs were also similar, with mild-to-moderate IRRs being the most frequent AEs. No severe or opportunistic infections were reported, and no patients discontinued rituximab after 1 year.

Different studies have also assessed rituximab after switching from another DMT in real-world populations. In a retrospective study by Alcalá et al., rituximab has proven to be an effective and safe therapeutic alternative in a small cohort of RRMS patients after fingolimod withdrawal due to suboptimal response or side effects, with an efficacy profile comparable to that of alemtuzumab (99). The ARR was significantly reduced by rituximab with no statistical differences from what was observed with alemtuzumab. Similarly, the median EDSS was significantly reduced with rituximab, without statistical differences compared with alemtuzumab. No difference was detected as regard to patients reaching NEDA. Rituximab, as well as alemtuzumab, was also safe in the study cohort, with reported AEs consistent with that already described in the literature.

Another small retrospective study, including 12 patients with RRMS — all of which had failed first-line therapy (IFN and glatiramer) and seven of which had also failed second-line therapy (natalizumab/fingolimod) — confirmed rituximab as a safe and effective second- or third-line DMTs, even in patients (n=2) who developed a concomitant autoimmune disease (idiopathic thrombocytopenic purpura) during the course of MS (100). During the follow-up period (mean duration 40 months), no patients switched to rituximab experienced

SAEs or discontinued treatment. No patients had a clinical relapse, MRI activity was not detected and the EDSS scores improved in 11 of 12 patients and remained stable in one patient. Furthermore, an improvement of EQ VAS score, and thus an improvement in patient-perceived health status, has been reported almost in all treated patients.

A French nationwide retrospective multicenter study demonstrated the efficacy of off-label rituximab as rescue therapy in 50 patients with active RRMS despite immunosuppressive DMT (fingolimod, natalizumab, or mitoxantrone) (101). The median total number of previous treatments was 3 (range, 2–6), and the median number of immunosuppressive DMT was 2 (range, 1–3). The ARR was significantly reduced by rescue therapy with rituximab from 0.8 during last immunosuppressive DMT to 0.18 ($p<0.0001$), and almost all rituximab-treated patients showed a stable or decreased EDSS score at the last clinical evaluation ($p<0.0001$). The percentage of patients showing contrast-enhancing lesions was also significantly reduced from 72% to 8% after rituximab initiation ($p<0.0001$). Interestingly, almost 95.5% of the MRI performed after rituximab initiation did not show any inflammatory activity. 70% of included patients reached NEDA status at the last clinical evaluation (median, 1.1 years; range, 0.5–6.4 years). The safety profile of rituximab was in line with other observations, with IRRs and infections as the most common AEs, and no cases of PML reported.

In a cohort of 10 patients with RRMS that stopped natalizumab treatment due to high risk of PML, the switch to rituximab resulted efficacious in preventing disease reactivation or rebound and in maintaining radiological stability (102). Rituximab resulted to be a valid post-natalizumab treatment option, with no new relapses recorded, also in small cohort of 16 MS patients switched from natalizumab because of positive JCV serology (76). In another cohort of RRMS patients from two Italian centers who interrupted natalizumab after at least six infusions and with a follow-up of at least 12 months, no evidence of disease reactivation was observed in those switched to off-label rituximab (103). In contrast, clinical and/or radiological reactivation was observed in patients switched to first-line therapies (IFNβ, glatiramer acetate, teriflunomide, azathioprine), fingolimod, and immunosuppressive agents (cyclophosphamide or mitoxantrone).

In a cohort of 256 stable RRMS patients who switched from natalizumab solely due to JCV antibody positivity (92), rituximab showed a better risk-benefit profile compared with fingolimod (the most studied post-natalizumab therapy). In particular, the rituximab-switched group experienced less relapses, fewer contrast-enhancing lesions, and less drug discontinuations compared with fingolimod-switched patients. Regarding discontinuations, most of them in the fingolimod group were due to disease breakthrough, highlighting the higher effectiveness of rituximab. Furthermore, the hazard ratio (favoring rituximab) for AEs (5.3% in rituximab group *vs* 21.1% in fingolimod group) was 0.25 (95% CI: 5 0.10–0.59), indicating a better tolerability of rituximab despite a higher rate of first-dosing AEs compared with fingolimod (26% *vs* 7%).

More limited data are, to date, available on the switch from natalizumab to ocrelizumab. Seven cases of PML have been reported in patients treated with ocrelizumab after natalizumab, therefore, this switch may not be safe (104). However, in a retrospective analysis on 28 patients switched from natalizumab after a median washout period of 44 days (35–83 days), ocrelizumab has been proven to be safe and effective, with absence of new relapses and no cases of PML, although, as for rituximab, the emergence of PML that could elude MRI detection remains a potential concern (105). In another retrospective analysis on 42 RRMS patients who switched from natalizumab to ocrelizumab due to high risk of PML, despite a disease reactivation in 12% patients in the first 3 months, no further relapses were observed with ocrelizumab, EDSS remained stable in 90% of cases and no carryover PML nor significant AEs occurred (106).

Finally, some case reports confirm rituximab to be a safe and effective treatment in controlling MS reactivation after natalizumab interruption. Recently, the case of a young woman, who interrupted natalizumab treatment due to PML diagnosis, has been described (107). After the interruption of natalizumab, the patient experienced an important clinical worsening (EDSS worsened from 4 to 8) and multiple new lesions in the brain and spinal cord. After fingolimod failed to control this MS reactivation, rituximab was started, inducing a dramatic improvement in patient's clinical conditions (EDSS 5.5, no relapses or MRI activity) and no reactivation of PML occurred.

Special Population

Some real-world studies have also suggested that rituximab may represent an optimal therapeutic choice, even superior to other DMTs, for women with MS planning a pregnancy.

Indeed, the management of MS in pregnant women remains challenging due to the lack of approved DMTs for use in this population, and the risk of rebound after discontinuation of certain DMTs. The risk of rebound after discontinuation has not been reported with ocrelizumab (108). However, little is known about the safety profile of ocrelizumab in pregnancy, and current guidelines recommend contraception for women of childbearing age while receiving ocrelizumab and for 6 months after the last infusion of ocrelizumab, given the unknown fetal risk (109).

A large observational cohort study, including 586 women with MS onset, before childbirth identified through the Swedish MS Registry, showed a relapse rate 1 year post-partum significantly higher in women who suspended natalizumab within 6 months before conception and in women untreated within 1 year before conception compared with women who suspended rituximab in the 6 months before conception (adjusted rate ratio [aRR], 7.65; 95% CI, 2.47–23.6 and 4.69; 95% CI, 1.67–13.2, respectively) (110). Moreover, in the suspended rituximab women, only one maternal relapse occurred during pregnancy and only one of four patients who relapsed in the first quarter after delivery experienced new GAD+ lesions. These results suggest a prolonged protective effect on MS disease activity of rituximab, which can encompass pregnancy and postpartum period, without the high risk of disease reactivation or rebound described with natalizumab withdrawal before pregnancy (111). In line with these data, a German cohort study (112), analyzing 88 pregnancies from 81

women with neuroimmune diseases (including MS and NMOSDs) treated with anti-CD20 mAbs in the year before conception, showed a good control of disease activity during pregnancy and postpartum, with no major safety concerns (with the exception of two congenital abnormalities reported in women exposed to ocrelizumab during pregnancy) and with pregnancy outcomes within the range expected for the general population. An interesting case series about 11 pregnancies in 10 women (7 with MS and 3 with NMOSDs) treated with rituximab within 6 months of conception, seems to confirm these safety and efficacy findings: indeed, all completed pregnancies resulted in term live births of healthy newborns, no maternal relapses occurred before/during pregnancy and only one was observed in the post-partum (113).

An interesting case report documented the high efficacy and safety of rituximab in controlling a severe rebound in a woman with MS who interrupted fingolimod during the first month of pregnancy (114). Eight weeks after withdrawal of fingolimod, the patient developed severe symptoms resulting from multiple new and enlarging lesions and a significant worsening of EDSS (from 3.0 to 7.0). Considering the severity of her conditions and to prevent further relapses, rituximab was started at week 22 of gestation and continued during the rest of the pregnancy and beyond. No new relapses occurred, and by the end of the pregnancy, she partially recovered from disability. No adverse fetal or infant effects were reported as the patient delivered, at 38 weeks of gestation, a healthy boy (APGAR score 9 at 1 min, and 10 at 5 min) with a normal 3-month development.

THE REGULATORY PERSPECTIVE: OFF-LABEL USE

To date, rituximab is authorized for various therapeutic indications, including the following onco-hematologic and auto-immune diseases: non-Hodgkin's lymphoma (NHL), chronic lymphocytic leukemia (CLL), rheumatoid arthritis (RA), granulomatosis with polyangiitis, and microscopic polyangiitis, Pemphigus vulgaris. This anti-CD20+ antibody, with the same mechanism of action as ocrelizumab, should be also considered as a therapeutic option for MS patients, although it does not hold regulatory approval for this indication, given its good and well-known efficacy and safety profile, emerging from clinical trials and the wide real-world use as monotherapy for RR and progressive forms. Therefore, the prescription in patients with multiple sclerosis is a typical off-label use, "not in accordance with the authorized product information" (115). This off-label use is common, not only as an escalation therapy but also as a first-line treatment. For example, rituximab is the most commonly used DMT in Sweden for all MS subtypes, although with considerable regional differences (116). Moreover, differently from ocrelizumab added to the repertoire of MS therapies around 2017 to 2018 and with limited post-marketing use, long-term safety of rituximab is well documented not only in MS but also in other conditions, such as rheumatoid arthritis, where prolonged exposure for 11 years was well tolerated and not associated with increased safety risks, including serious opportunistic infections and PML (86). Finally, it has a more favorable price with respect to ocrelizumab, even considering the availability of different biosimilar versions. For example, if we consider the Italian prices, the estimated expenditure with the available RTX products (calculated for a maximum dosage of 1,000 mg*2 and 2 cycle/year -1 cycle every 6 months) are reduced by more than half compared to ocrelizumab (**Table 3**).

Currently, off-label use is not regulated in Europe, but some member states adopted specific national measures (115, 117). For example, the France *Recommandations Temporaires d'Utilisation* (RTU) (118) and the Italian Law 648/1996 (119, 120) ensure a nationwide access to off-label drugs according to criteria for appropriate use and monitoring defined in the light of clinical evidence (at least phase II trials for 648/96). In both cases, public bodies (patient associations, scientific societies, clinical centers) may submit to the national competent authority the requirement for the approval of an off-label use of a medicinal product.

These laws permit to recognize the therapeutic use of effective and safe medicines beyond the interest of pharmaceutical companies for new extension of indications.

Rituximab received a RTU in 2018 for the treatment of patients with severe Immune Thrombocytopenic Purpura (ITP), refractory to other treatments.

Moreover, Italy allows to reimburse the drug for the following off-label use in accordance with Law 648/1996:

- HCV-related mixed cryoglobulinemia refractory to antiviral therapy, HCV-related mixed cryoglobulinemia with severe systemic manifestations, HCV-negative cryoglobulinemia;
- polyneuropathy associated with anti-MAG antibodies;
- hematologic diseases (acute lymphoblastic leukemia, first-line or savage treatment for CD20-positive B cell non-Hodgkin's lymphomas, first-line or savage treatment within polychemotherapy regimens for chronic lymphocytic leukemia, acute and chronic GVHD steroid-resistant, follicular lymphomas in patients not eligible for chemotherapy treatment, Hodgkin lymphoma, autoimmune hemolytic anemia, thrombotic thrombocytopenic purpura, acquired hemophilia);
- primitive or idiopathic membranous nephropathy;
- neuromyelitis optica.

Thus, the Italian NHS currently cover the use of rituximab in some neuroimmune disorders, but the use in MS is not approved and falls within the Italian Law 94/1998, by which physicians can perform off-label prescriptions (not covered by the NHS) but only in individual and exceptional cases. This represents, to date, a limit for the use in this population, due to the exceptionality and not systematicity that should characterize the prescription.

The Norwegian Institute of Public Health (NIPH) has recently conducted a cost-effectiveness evaluation of rituximab concluding that, with respect to the cladribine, rituximab generates more health in terms of QALYs and leads to a significant cost saving, while ocrelizumab, despite generating more health in terms of QALYs, induces large increases in costs (121). Moreover, the institute addressed the topic from the legal point of view too, considering whether the continued off-label use of rituximab for

TABLE 3 | Estimated expenditure for anti-CD20 in MS in Italy.

Pharmaceutical Product	Dosage form	Packaging	Price/box*	Cost/Cycle (max)§	Cost/Year (max)°
Rituximab					
Mabthera®	500 mg concentrate for solution for infusion	1 vial 50 mL	€ 1.252,41	€ 5.009,64	€ 10.019,28
Rixathon®	500 mg concentrate for solution for infusion	1 vial 50 mL	€ 1.001,93	€ 4.007,72	€ 8.015,44
Truxima®	500 mg concentrate for solution for infusion	1 vial 50 mL	€ 1.110,17	€ 4.440,68	€ 8.881,36
Ocrelizumab					
Ocrevus®	300 mg concentrate for solution for infusion	1 vial 10 mL	€ 5.640,63	€ 11.281,26	€ 22.562,52

*ex factory price (source www.codifa.it).
§RTX max 1.000 mg*2.
°RTX max 2 cycle (1 cycle every 6 months).

MS treatment could represent a legal problem when a similar preparation (ocrelizumab) is available. The discussion started from the assumption of the distinction between the right to market and the right to prescribe a medicine, underlining that the marketing authorization involves the possibility to market a drug in accordance with the terms of the authorization; however, physicians are free to prescribe a medicine, even outside these terms, if the requirements for quality, safety, and efficacy can be satisfied. Therefore, NIPH concluded that rituximab can be prescribe for MS even in the presence of ocrelizumab in the specialist health service (121, 122).

CONCLUSIONS

DMTs demonstrated to reduce the inflammatory activity, relapse rate, and disability progression in patients with MS. However, there are still a lot of issues in terms of individual patients' effectiveness, duration of response, safety, and compliance, which make the disease (in particular the progressive forms) an important unmet medical need.

An increasing body of evidence from RCTs and real-world studies suggest that rituximab is a highly effective DMT in relapsing MS and mildly effective in progressive MS, with low drug discontinuation rate thanks to a good safety profile and compliance. The long experience in this and other conditions, and not least a more favorable cost with respect to alternatives (especially if considering the authorized anti-CD20 ocrelizumab), highly support the use of rituximab in patients with RRMS, SPMS, or PPMS.

Most recent data have also highlighted the possibility of optimizing therapeutic scheme, with a potential further improvement of safety and efficacy and incremental saving, and suggested that rituximab may represent an optimal choice for MS women planning a pregnancy.

Currently, with the exception of the head-to-head comparison with glatiramer acetate in SPMS, no results from direct comparisons with other DMTs, including ocrelizumab, are available, but a lot of trials are ongoing, and results are awaited in the next future. However, this use could be officially recognized by national regulatory authorities, to ensure equal access for patients with MS to a therapeutic option, which demonstrated to be safe and effective not only in clinical trials (even if phase II studies but with appropriate clinical endpoints) but also in an extensive off-label use in different countries.

Finally, a dialog across Member States should began to share common standard criteria for off-label approval of medicines.

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SB and LG wrote the first draft of the manuscript. FD checked and revised the draft manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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CASE REPORT
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Dynamic Changes in AQP4-IgG Level and Immunological Markers During Protein-A Immunoadsorption Therapy for NMOSD: A Case Report and Literature Review

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The changes in the serum levels of aquaporin-4-IgG (AQP4-IgG), immunoglobulins, and inflammatory mediators in neuromyelitis optica spectrum disorder (NMOSD) cases treated with immunoadsorption have been rarely described in detail. Here we report a 29-year-old steroid-resistant NMOSD female with a severe disability (bilateral blindness and paraplegia) who received protein-A immunoadsorption as a rescue treatment. During the total 5 sessions, the circulating level of AQP4-IgG, immunoglobulins, and complement proteins (C3 and C4) showed a rapid and sawtooth-like decrease, and the serum AQP4-IgG titer declined from 1:320 to below the detectable limit at the end of the 3rd procedure. Of all the antibodies, IgG had the biggest removal rate (>96.1%), followed by IgM (>66.7%) and IgA (53%), while complement C3 and C4 also dropped by 73% and 65%, respectively. The reduced pro-inflammatory cytokines (interleukin-8 and tumor necrosis factor-α) and marked increased lymphocyte (T and B cell) counts were also observed. The improvement of symptoms initiated after the last session, with a low AQP4-IgG titer (1:32) persisting thereafter. Accordingly, protein-A immunoadsorption treatment could be one of the potential rescue therapies for steroid-resistant NMOSD patients with a severe disability.

Keywords: protein-A immunoadsorption, rescue therapy, AQP4-IgG, neuromyelitis optica spectrum disorder, case report

INTRODUCTION

Neuromyelitis optica spectrum disorder (NMOSD) is commonly considered an antibody-mediated autoimmune debilitating disease, with only a small proportion of acute NMOSD attacks achieving a complete remission (1, 2). The pathogenic aquaporin-4-IgG (AQP4-IgG) can be detected in most NMOSD patients and tends to be associated with frequent relapses (3, 4). Traditionally, the majority of the sufferers can benefit from pulsed high-dose intravenous methylprednisone (IVMP), accelerating clinical improvement and shortening the acute phase. For those refractory patients with severe attacks who are insufficiently responsive to glucocorticoids, plasma exchange (PE) and immunoadsorption can be alternative rescue or adjunctive therapies. In fact, previous studies have noted that the apheresis

techniques, especially used as the first-line therapy in the early stage, can achieve a better outcome than steroids in treating NMOSD attacks (5, 6). Compared to glucocorticoids, this treatment strategy seems to exert a quicker and more potent effect on controlling the excessive immune response *via* direct removal of antibodies, pro-inflammatory cytokines, and complement proteins from the serum, which might reduce the relapse in a short term due to the subsequent reduced serum pathogenic AQP4-IgG concentration.

Nevertheless, the potential exposure to blood-borne diseases, allergens, and the shortage of plasma limit its wide application. Theoretically, protein-A immunoadsorption can selectively remove immunoglobulins and complement proteins without transfusing foreign blood products, spare albumin, and clotting factors, and have fewer adverse effects, which appear to be superior to PE (7–9). Previous reports have mentioned the changes of the antibody levels in patients with AQP4-IgG seropositive NMOSD during the immunoadsorption (8, 10), without involving the complement proteins, cytokine profiles, and lymphocyte system. Here, we report a case with a severe disability due to NMOSD relapse recovered by protein-A immunoadsorption, with a detailed description of the alteration in serum AQP4-IgG titer, the concentration of inflammatory mediators, and the lymphocyte subsets.

CASE PRESENTATION

A 29-year-old female who was first diagnosed with NMOSD in 2012 could recover from pulsed glucocorticoids during the initial several attacks. However, since 2014, she benefited little from this therapy and began to receive PE as the rescue treatment during the 3 severe relapses (Expanded Disability Status Scale, EDSS score ≥6) in the following 5 years. *Azathioprine (150mg/d for 1 year)* and t*acrolimus (3mg/d for 2.5 years)* were given as the maintenance therapies, respectively, but failed to prevent the clinical attacks. The detailed timeline with relevant data of the past episodes and interventions was summarized in **Figure 1**. Three days before admission, she suffered paraplegia and blindness without any immunosuppressant treatment. No other personal or family history of autoimmune diseases was reported. Drug abuse and psychological disorders were denied, either. Owing to the occurrence of the ongoing severe disability and lack of plasma, protein-A immunoadsorption was tried with consent from the patient.

At nadir, neurological examination revealed paraplegia, with hypermyotonia and tendon hyperreflexia. She also had bilateral blindness without light perception, and her EDSS score was assessed at 8. MRI of the cervical and thoracic spine showed a longitudinally extensive T2-hyperintense lesion, with the central portion of the cord involved (**Figures 2A–C**). A significant enhancement and thickening of the optic nerve sheaths were also observed (**Figure 2D**).

The complete blood cell count, basic metabolic panel, and liver function were within normal limits. A cerebral spinal fluid (CSF) study showed a normal cell count ($0*10^6$/L, reference range: 0-$8*10^6$/L), protein level, and oligoclonal band, with an IgG index of 0.6 (reference range: 0-0.7). AQP4-IgG tested by cell-based assay (CBA) revealed a positive result, with a titer of 1:320 in the serum (**Figure 3A**) and 1:1 in the CSF, while the myelin oligodendrocyte glycoprotein antibody (MOG-IgG), glial fibrillary acidic protein antibody (GFAP-IgG), myelin basic protein antibody (MBP-IgG), AQP1-IgG, and Flottilin1/2-IgG in the serum and CSF, which were also measured by CBA, were undetectable.

The patient consent was then obtained before intravenous catheterization and combined anticoagulation with heparin. The protein-A immunoadsorption was started one week after onset, with the pulsed glucocorticoids (1000mg/d) and concomitant supportive therapies given 3 days ago. The immunoadsorption column (KONPIA, KONCEN, China) can be reused no more than 10 sessions as long as the absorbed antibodies are eluted from the column after each procedure according to the product instruction, while the plasma separator and tubing system are for single use only. During 5 sessions, each treatment filtered approximately 3 liters of plasma every other day. The levels of AQP4-IgG, complement proteins (C3 and C4), and immunoglobulins in the serum were detected at the beginning and the end of every procedure. Cytokine profiles (solid-phase two-site chemiluminescent immunometric assay, IMMULITE 1000 Analyzer, Siemens) and lymphocyte subsets (flow cytometry, BD Biosciences) were only analyzed before and after all the sessions. The results revealed that each treatment could lead to a significant reduction in the AQP4-IgG titer, while a slight rebound was always observed before the start of the next therapy (**Figure 3A**). The serum AQP4-IgG decreased rapidly to below the detectable limit

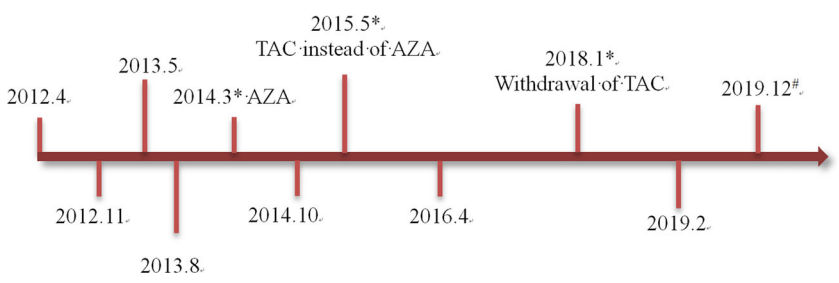

FIGURE 1 | The timeline with relevant data of the past episodes and interventions. *Severe relapse treated with plasma exchange. #This admission. AZA, azathioprine; TAC, tacrolimus.

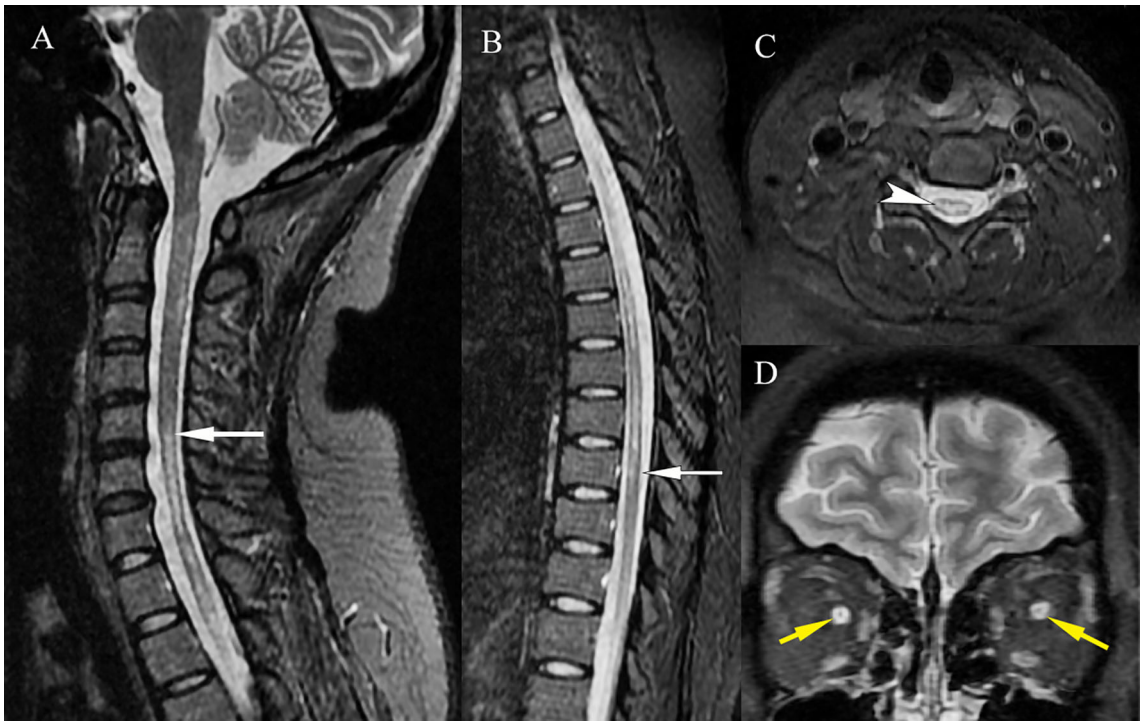

FIGURE 2 | MRI of the NMOSD lesions. MRI of the cervical and thoracic spine showed a longitudinally extensive T2-hyperintense lesion [**(A, B)**, white arrows], which involved the central portion of the cord [**(C)**, white arrowhead]. A significant enhancement and thickening of the bilateral optic nerve sheaths were observed [**(D)**, yellow arrows].

after the 3rd session of treatment and kept a lower titer (1:32) until the patient was discharged 2 weeks later. The IgG, IgA, IgM, C3, and C4 demonstrated almost the same trends (**Figure 3B**). Of all the antibodies, IgG had the biggest reduction rate (>96.1%), followed by IgM (>66.7%), and IgA was least able to be eliminated (53%) (**Table 1**). Interestingly, C3 and C4 components also declined by more than 60% (C3: 73%, C4: 65%). The decline of these immune components tended to become flat after 3 treatment procedures, similar to the change of AQP4-IgG. The analysis of lymphocyte subsets revealed that the natural killer (NK) cells had a remarkable decrease in percent (before *vs* after: 13.85% *vs* 3.54%) and number (before *vs* after: 265 cells/μL *vs* 98 cells/μL), while the number of T cells and B cells rose significantly, with the total lymphocytes (T cells + B cells + NK cells) elevating from 1905 to 2753 cells/μL (**Table 2**). The proportion of the activated T cells (CD3+HLA-DR+) and activated Ts cells (CD3+CD8+HLA-DR+)/Ts decreased from 10.28% and 17.57% to 6.91% and 12.23%, respectively. The percent of regulatory T cells (Treg, CD3+CD4+CD25+CD127 low+) had a mild drop (before *vs* after: 5.08% *vs* 4.6%), with a major decline of the natural Treg cells (CD45RA+CD3+CD4+ CD25+CD127low+) (before *vs* after: 1.69 *vs* 1.16%). There were unapparent differences in the interferon-γ (IFN-γ) producing lymphocytes (PMA/ionomycin-stimulated lymphocyte function assay) before and after treatment. The concentration of pro-inflammatory cytokines including interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) also reduced, with an insignificant change in IL-6 level (electrochemiluminescence method, Roche Diagnostics) (**Table 2**). The patient did not report any discomfort and no infection or thrombosis occurred during the therapy. Notably, her symptoms did not improve with the reduction of AQP4-IgG or other immune components until the end of the 5th session. She got a recovery from bilateral complete blindness to hand move, and the final EDSS score was assessed at 5 one week after the last session at the timing of discharge. *Mycophenolate mofetil (1500mg/d)* instead of *tacrolimus (3mg/d)* was given as the maintenance treatment afterward. She refused a repeated test for immunoglobulins, complement proteins, cytokine profiles, and lymphocyte subsets when discharged. Disability including paraplegia and visual disturbance further ameliorated (visual acuity: OS: 0.6, OD: 0.2), with the EDSS of 3, and no relapse or drug-related adverse event was reported in the next 6-month follow-up.

DISCUSSION

We report a case of AQP4-IgG seropositive steroid-resistant NMOSD with severe relapse who profited from protein-A immunoadsorption. The rapid and sawtooth-like decrease of the complement proteins and antibody levels, especially AQP4-IgG, the declined pro-inflammatory mediators, as well as the subsequent clinical improvement suggest that immunoadsorption could be one of the rescue therapeutic options for severely affected NMOSD patients.

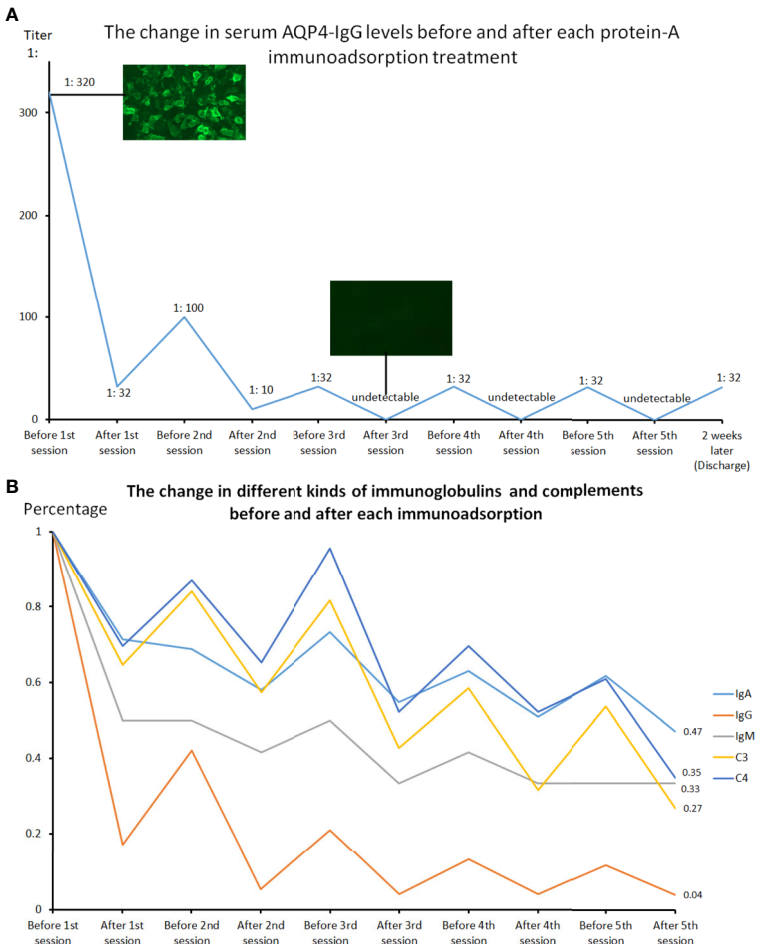

FIGURE 3 | The changes in serum AQP4-IgG levels performed by cell-based assay before and after each protein-A immunoadsorption treatment **(A)** and the changes in serum concentration of immunoglobulins and complement proteins before and after each session **(B)**. Each treatment could lead to a significant decline in the serum AQP4-IgG titer, IgA, IgG, IgM, as well as C3 and C4 levels, with a slight rebound before the next therapy.

Protein-A immunoadsorption selectively eliminates immunoglobulins by filtering plasma through columns containing *Staphylococcus* cell wall–derived protein A. Each protein-A molecule has three potential binding sites for IgG, with a more potent affinity for it than IgM (11). Further, other circulating immune components including complement proteins and inflammatory cytokines can also be removed (12), although the underlying mechanisms remain to be fully elucidated. In line with

TABLE 1 | The changes in the level of serum immunoglobulins and complement proteins (C3 and C4) before and after each session of immunoadsorption.

	IgA, g/L	IgG, g/L	IgM, g/L	C3, g/L	C4, g/L
Before 1st session	1.57	7.6	0.12	0.82	0.23
After 1st session	1.12	1.3	0.06	0.53	0.16
Before 2nd session	1.08	3.2	0.06	0.69	0.2
After 2nd session	0.91	0.4	0.05	0.47	0.15
Before 3rd session	1.15	1.6	0.06	0.67	0.22
After 3rd session	0.86	<0.3	<0.04	0.35	0.12
Before 4th session	0.99	1	0.05	0.48	0.16
After 4th session	0.8	<0.3	0.04	0.26	0.12
Before 5th session	0.97	0.9	0.04	0.44	0.14
After 5th session	0.74	<0.3	<0.04	0.22	0.08
Reduction Rate, %	52.9	>96.1	>66.7	73.2	65.2
Reference Range	0.82-4.53	7.51-15.6	0.46-3.04	0.65-1.39	0.16-0.38

TABLE 2 | The changes of lymphocyte subsets and cytokines before and after treatment.

Lymphocyte subsets and cytokines	Before 1st session	After 5th session	Reference range
Total T cells (CD3+CD19-), %	66.09	74.04	50-84
Total T cell count (CD3+CD19-), cells/μL	1265	2048	955-2860
Total B cells (CD3-CD19+), %	19.58	21.95	5-18
Total B cell count (CD3-CD19+), cells/μL	375	607	90-560
Th cells (CD3+CD4+), %	41.03	43.93	27-51
Th cell count (CD3+CD4+), cells/μL	786	1215	550-1440
*Ts cells (CD3+CD8+), %	19.66	26.29	15-44
Ts cell count (CD3+CD8+), cells/μL	376	727	320-1250
NK cells (CD3-/CD16+CD56+), %	13.85	3.54	7-40
NK cell count (CD3-/CD16+CD56+), cells/μL	265	98	150-1100
T cells +B cells + NK cells, %	99.52	99.53	95-105
T cell +B cell + NK cell count, cells/μL	1905	2753	
Th/Ts	2.09	1.67	0.71-2.78
Th (CD3+CD4+CD28+)/Th, %	93.91	97.77	84.11-100.00
Tc (CD3+CD8+CD28+)/Ts, %	81.42	91.54	48.04-77.14
Activated T cells (CD3+HLA-DR+), %	10.28	6.91	9.04-25.62
Activated Ts cells (CD3+CD8+HLA-DR+)/Ts, %	17.57	12.23	20.73-60.23
Naïve Th cells (CD3+CD4+CD45RA+)/Th, %	35.8	30.34	36.41-57.07
Memory Th cells (CD3+CD4+CD45RO+)/Th, %	64.2	69.66	44.44-68.94
Treg (CD3+CD4+CD25+CD127low+), %	5.08	4.6	3.13-6.49
Natural Treg (CD45RA+CD3+CD4+CD25+CD127low+), %	1.69	1.16	2.07-4.55
Induced Treg (CD45RO+CD3+CD4+CD25+CD127low+), %	3.39	3.44	1.03-2.29
IFN-γ producing CD4+ T cells/Th, %	18.8	18.77	14.54-36.96
IFN-γ producing CD8+ T cells/Ts, %	29.49	25.72	34.93-87.95
IFN-γ producing NK cells/NK cells, %	72.25	65.88	61.2-92.65
IL-1β (pg/ml)	<5	<5	<5
IL-2R (U/ml)	582	378	223-710
IL-6 (pg/ml)	2.25	2	<7.0
IL-8 (pg/ml)	41.7	15.2	<62
IL-10 (pg/ml)	<5	<5	<9.1
TNF-α (pg/ml)	9.9	4.3	<8.1

**Ts cells (CD3+CD8+) included Tc (CD3+CD8+CD28+) and Ts (CD3+CD8+CD28-).*

Th cell, helper T cell; Ts cell, suppressor T cell; Tc cell, cytotoxic T cell; NK cell, natural killer cell; Treg cell, regulatory T cell; IFN , interferon; IL-2R, interleukin-2 receptor; TNF, tumor necrosis factor.

these, we observed a sharp decrease in the serum level of AQP4-IgG and the biggest reduction rate (>96.1%) in (total-)IgG in our case, followed by IgM (>66.7%) and IgA (53%), with a drop of complement C3 and C4 simultaneously. Moreover, 3 sessions could reduce the IgG to below the detectable limit and the decline in immunoglobulin and complement protein levels tended to become flat, implying that 3 or 4 immunoadsorption procedures might be enough in treating antibody-mediated autoimmune diseases. In addition, the immunoglobulin depletion in the serum can cause an osmotic equilibration between extra- and intravascular space (13), probably leading to a reduction of pathogenic antibodies and other immune complexes e.g. complement proteins in the central nervous system (CNS) and finally minimizing the irreversible CNS damage. This redistribution was observed indirectly by a slight rebound of sero-immunoglobulins before every treatment and contributed to the sawtooth-like kinetics of antibody concentrations in the bloodstream (**Figure 3B**, **Table 1**).

Besides, as an IgG1-isotype antibody, AQP4-IgG could trigger the complement cascade and may cause complement-dependent cytotoxicity, which was observed by the vasculo-centric deposition of immunoglobulins and complement components in the acute lesions (14). Theoretically, complement depletion treatment may attenuate the CNS damage through the reduced formation of the membrane attack complex, which is implicated in astrocyte destruction and neuronal injury (15). Also, this therapy resulted in a significantly lower risk of relapse in patients with NMOSD (15). Generally, the complement system can be activated through 3 different pathways: the classical, lectin, and alternative pathways (16). Complement C4 component *via* the classical and/or lectin pathways and C3 *via* the alternative pathway are required in producing inflammatory mediators such as C3a and C4a and proceeding the complement cascade (16). In previous studies, the activation of complement C3 appeared to be positively associated with disease activity and neurological disability in patients with NMOSD (17, 18). Consistent with this, after 5 immunoadsorption procedures, the circulating levels of complement proteins including C3 and C4 in our case had significant removal rates of 73.2% and 65.2%, respectively, with the clinical improvement and a short relapse-free period thereafter. However, when compared to IgG, complement components seemed less vulnerable to be eliminated by immunoadsorption therapy alone (7). Besides, the activation of the complement system could also be possibly suppressed after the clearance of AQP4-IgG and therefore reduced binding to AQP4.

A marked increase of the T cell and B cell counts was observed after the therapy (**Table 2**), with a reduced proportion of the activated T (CD3+HLA-DR+) and Ts cells (CD3+CD8+HLA-DR+), implying a possibly improved immune system, which could be a downstream effect after the clearance of the pro-

inflammatory mediators. In contrast, the percent and number of NK cells had a pronounced drop, which was also noted by previous studies as a potential biomarker candidate for acute-phase NMOSD (19, 20), although the underlying cause has been still unclear. A significantly increased level of Tregs (CD3+CD4+CD25+ CD127low+) after immunoadsorption treatment in a previous study (21) was not found in our case. Also, the concentration of pro-inflammatory cytokines including IL-8 and TNF-α reduced, which may lower the disease activity, while an elevated IL-6 level during the acute phase, noted by previous researches (22, 23), was not observed here, either.

Generally, pulsed high-dose IVMP is widely used as the first-line treatment for acute exacerbations of NMOSD, with considerable benefit in most patients. Glucocorticoids act by inhibiting a series of inflammation processes through multiple mechanisms, including reducing the proinflammatory cytokines and suppressing the T-cell activation. However, steroid resistance is probably the summative effect of polymorphism of the glucocorticoid receptor (24), altered cytokines expression (25), etc., which compromises the anti-inflammatory activity of glucocorticoids, although the exact underlying causes are still not fully understood. Theoretically, protein-A immunoadsorption can rapidly decrease loads of the circulating pathogenic antibodies and pro-inflammatory mediators, possibly exerting a faster and potent anti-inflammatory effect than glucocorticoids or serving as adjuvant therapy to improve the sensitivity to steroids, especially for refractory cases with extensive and serious injuries.

It is noteworthy that although the pathogenic AQP4-IgG can induce a series of inflammatory cascades, causing damage to the CNS, the clinical improvement did not occur instantly with the reduction of the serum AQP4-IgG until the 5th session. This may be associated with delayed depletion of the immune complexes in the CNS and necessary time for neural repair. Moreover, although AQP4-IgG is much more concentrated in plasma than in CSF[73], suggesting the peripheral origin and secondary entry to the CNS, the serum AQP4-IgG in our case persisted (1:32) while the symptoms remitted, implying that the circulating AQP4-IgG alone is insufficient to produce NMOSD lesions (14). It has also been supported by the previous observations that the serum AQP4-IgG might be present for years and will increase in concentration before attacks (3, 26). Nevertheless, it is one of the limitations that the data on IgA, IgG, IgM, C3 or C4, and cytokine levels, as well as the lymphocyte subsets 2 weeks after discharge, were missing due to the patient's refusal.

Besides, the side effects and costs of a particular treatment should also be taken into account. In the previous studies (9, 27) on autoimmune encephalitis, the immunoadsorption therapy was almost well tolerated. The venous catheter-related adverse events should be drawn attention, albeit no thrombosis or patient-reported discomfort occurred in our case. Although this treatment, admittedly, has been still costly from the patient perspective, which is the major limitation for wide application, the potential benefits from the reduced neurologic impairment, accelerated clinical recovery, and the short length of hospital stay may outweigh its risks and costs in patients with severe NMOSD acute attack.

Nonetheless, after all, immunoadsorption is not a panacea for all the NMOSD attacks, for those non-responders after 5 procedures, more sessions seem feasible (28). Moreover, even if this treatment could not contribute to any remission in a short term, the patient could still benefit from the removal of pathogenic immune complexes, which may help provide a temporarily stable immunological seedbed for further neural repair and an improved outcome achieved by the subsequent long-term and slow-acting immunotherapies e.g. *mycophenolate mofetil (1500mg/d)* in our case.

CONCLUSIONS

We demonstrated the changes in the serum level of AQP4-IgG, immunoglobulins, complement proteins (C3 and C4), and cytokine profiles as well as the alterations of lymphocyte subsets in a protein-A immunoadsorption treated case. Immunoadsorption can exert the anti-inflammatory effect *via* rapid clearance of the pathogenic antibodies and other immune components and could be one of the potential rescue therapies for steroid-resistant NMOSD patients with a severe disability.

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.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Tongji hospital of Tongji medical college, Huazhong University of Science and Technology. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

BC analyzed the data and wrote the manuscript. CQ, MC, H-HY, RT, and Y-HC were responsible for collecting the data. B-TB and D-ST cared for the patient, designed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Review: The Nutritional Management of Multiple Sclerosis With Propionate

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Over the last 15 years there has been an accumulation of data supporting the concept of a gut-brain axis whereby dysbiosis of the gut microbiota can impact neurological function. Such dysbiosis has been suggested as a possible environmental exposure triggering multiple sclerosis (MS). Dysbiosis has been consistently shown to result in a reduction in short-chain fatty acid (SCFA) producing bacteria and a reduction in stool and plasma levels of propionate has been shown for MS patients independent of disease stage and in different geographies. A wealth of evidence supports the action of propionate on T-cell activity, resulting in decreased T-helper cell 1 (Th1) and T-helper cell 17 (Th17) numbers/activity and increased regulatory T cell (Treg cell) numbers/activity and an overall anti-inflammatory profile. These different T-cell populations play various roles in the pathophysiology of MS. A recent clinical study in MS patients demonstrated that supplementation of propionate reduces the annual relapse rate and slows disease progression. This review discusses this data and the relevant mechanistic background and discusses whether taming of the overactive immune system in MS is likely to allow easier bacterial and viral infection.

Keywords: short chain fatty acid, propionate, microbiota, immunity, auto-immune, multiple sclerosis

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INTRODUCTION

Multiple sclerosis (MS) is a chronic, progressive autoimmune disease for which there is no current cure. Worldwide, approximately 2.8 million people have MS, making it the most common neurological auto-immune disease (1). The development of MS is considered to result from a combination of genetic and environmental factors including childhood obesity, smoking, low Vitamin D levels and geographical latitude distant from the equator (2–4). More recently, the constitution and activity of the intestinal flora (microbiota) has been suggested as one of the environmental triggers for the development of MS (3). Here we review the data supporting the role the microbiota and short-chain fatty acid (SCFA) metabolites, in particular propionate, play in the pathophysiology of MS. Additionally, we discuss the conflicting goals of immunosuppression and the need to maintain an appropriate immune response to pathogenic bacteria and viruses.

MS AND THE GUT-BRAIN AXIS

Evidence of the gross pathophysiological effect of the gut content that may apply to MS has been demonstrated through fecal transplant studies. Using a murine model of MS [experimental autoimmune encephalomyelitis (EAE)], it was shown that MS score (based on motor deficits) worsened when mice received fecal material from patients with MS compared to mice receiving fecal material from healthy individuals (5). This study was repeated using twin donors where one was healthy and the other with MS. Fecal transplants resulted in a higher frequency of spontaneous EAE with the MS-human donor compared to that from the healthy human donor (6).

A major aspect of microbe–host communication receiving increased attention is the two-way communication between the gut microbiota and the central nervous system (CNS), the so-called gut–brain axis (7, 8). Gut-to-brain communication can occur *via* metabolite effects on the blood-brain-barrier (BBB), entero-endocrine factors, and systemic immune effects of microbe-derived metabolites such as SCFAs, products of tryptophan metabolism, phytoestrogens and bile acid metabolites (9).

A host of publications have described dysbiosis of gut microbiota in patients with MS and other autoimmune diseases compared to healthy controls (5, 6, 10–14). Interestingly, a common finding in these reports is that the alpha diversity (the variance within an individual) and beta diversity (variance between individuals and cohorts) are unchanged with MS. Rather, the dysbiosis is instead manifested as changes in the number of bacteria within the particular family or taxa. **Table 1** summarizes dysbiosis associated with development of MS.

One unifying finding throughout studies reporting dysbiosis in patients with MS is the reduction in the number of SCFA-producing bacteria (5, 6, 10–12), as reviewed elsewhere (15). This is important because it shows that there are common findings on a functional level across studies, despite different microbiota profiles presumably due to background genetics, environmental differences and different nutritional habits. The reduction in SCFA producing bacteria in patients with MS, as discussed later, can have an important physiological impact.

PROPIONATE DEFICIENCY IN MS PATIENTS

Five studies show that patients with MS have low levels of propionate in both feces and plasma (15–19). All 5 studies show that propionate levels in both feces and plasma are lower than those of healthy controls but there is less certainty for the other SCFAs, acetate and butyrate. Zeng et al. reported reduced fecal levels of all SCFAs in Chinese patients with MS compared to healthy controls (15). Interestingly the microbiota profile and level of SCFAs in feces were not affected by dietary and health habits (e.g., vegetarianism, physical activity, smoking, and alcohol intake), indicating that this pattern of dysbiosis may be a result of MS itself.

Park et al. assessed plasma SCFA levels in US patients with chronic MS (secondary progressive disease) and found significant reductions in acetate, propionate and butyrate (18). Duscha et al. measured SCFA levels in German patients with relapsing remitting MS (RRMS) and secondary progressive MS (SPMS) and found decreased propionate in plasma and feces for both MS subtypes, but no differences in butyrate and acetate (16). The findings between the two studies may be discordant with regards to acetate and butyrate levels due to the differences in the MS subtypes studied. However, both studies support a deficiency in propionate and a sub-analysis by Duscha et al. confirms that a propionate deficiency exists in both RRMS and SPMS.

Takewaki et al. studied 12 patients with RRMS and 9 patients with SPMS and showed reduced acetate, propionate and butyrate in the feces of RRMS patients, and a non-statistically significant reduction in SPMS patients (17). **Table 2** presents the outcomes of SCFA measurements in MS studies.

Recently, Trend et al. have demonstrated a small but statistically significant reduction in propionate amongst

TABLE 1 | Comparison of the nature of dysbiosis reported in different studies in patients with MS.

Country	Subjects (n = those with MS)	Bacteria increased in MS	Bacteria decreased in MS	Reference
USA	RRMS n=31	Pseudomonas, Mycoplana, Haemophilus, Blautia, Dorea, Pedobacter, Flavobacterium	Prevotella, Parabacteroides, Adlercreutzia, Collinsella, Lactobacillus, Coprobacillus, Haemophilius	(11)
USA	RRMS n=60	Methanobrevibacter, Akkermansia	Butyricimonas, Prevotella	(12)
USA	RRMS n= 43	Ruminococcus	Fecalibacterium	(10)
USA	RRMS n=7	Akkermansia, Acinetobacter		(5)
Germany	MS twins n=34 (each twin pair had 1 with MS and one healthy)	Akkermansia		(6)
UK	RRMS n=39	Prevotella copri		(13)
Japan	RRMS n=20	Bifidobacteria, Streptococcus, Thermophilus, Eggerthella lenta	Bacteroides, Fecalibacterium, Prevotella, Anaerostipes, Clostridium, Sutterella	(14)
China	RRMS n=34	Streptococcus	Prevotella	(15)
Germany	RRMS and SPMS	Akkermansia, Faecalibacteria	Butyricimonas, Bacteroides, Romboutsia	(16)
Japan	RRMS n=62, SPMS n=15, Atypical MS n= 21, Controls n=55	RRMS: Bifidobacteria, Streptococcus. SPMS: Streptococcus	RRMS: Megamonas SPMS: Roseburia	(17)

Adapted from (15–17).

TABLE 2 | SCFAs in patients with MS.

Author	Acetate		Propionate		Butyrate	
	Feces	Blood	Feces	Blood	Feces	Blood
(16) All types MS	No change from healthy controls	No change from healthy controls	Reduction p=0.0045	Reduction p=0.0006	No change from healthy controls	No change from healthy controls
(15) MS	Reduction p=0.0001	NA	Reduction p=0.0001	NA	Reduction p=0.05	NA
(18) Patients with SPMS	NA	Reduction p=0.001	NA	Reduction p=0.01	NA	Reduction p=0.0001
(17) Patients with RRMS and SPMS	RRMS: Reduction p<0.001	NA	RRMS: Reduction p<0.001	NA	RRMS: Reduction p<0.001	NA
	SPMS: Reduction in levels but without statistical significance		SPMS: Reduction in levels but without statistical significance		SPMS: Reduction in levels but without statistical significance	
(19) Clinically Isolated Syndrome, MS and healthy controls	NA	No difference between MS and healthy control	NA	Reduction reported for CIS/MS group. p=0.0008	NA	No difference between MS and healthy control
(20) RRMS and Clinically Isolated Syndrome	NA	Small but significant reduction in MS	NA	No difference between MS and healthy control	NA	No difference between MS and healthy control

patients with MS, without reductions in butyrate and acetate (19).

Contradictory data has come from the recent study of 58 patients with MS (a mix of RRMS and clinically isolated syndrome) and 50 healthy controls. Here, the serum level of acetate was significantly lower in MS patients but propionate and butyrate levels were similar in patients with MS and healthy controls (20).

Across the studies, there is a clear reduction in propionate levels in feces and plasma in patients with MS, independent of the subtype of MS and across different populations. These studies therefore provide complementary and consistent evidence that patients with MS have a dysbiosis leading to reduced numbers of SCFA producing bacteria which results in reduced levels of propionate across different geographies and disease forms.

MECHANISM OF IMMUNE REGULATION BY PROPIONATE

As well as providing an energy source, SCFAs such as propionate and butyrate exert effects *via* 2 major mechanisms: 1) G-protein coupled receptors (GPRs) of the SCFA receptor family namely Free Fatty Acid Receptor 2 [FFA2 (formally known as GPR43)] and FFA3 (GPR41) (21, 22), and 2) histone deacetylase inhibition (HDACi) (23). A variety of immune cells express FFA2 and FFA3 as well as GPR109a for which butyrate is one of the proposed endogenous ligands. In contrast, T-cells lack the respective GPRs for mediating SCFA effects and therefore any direct modulation of T-cells by SCFAs is likely mediated by histone deacetylase inhibition (24).

An overview of the transporters and receptors for SCFAs and their distribution is presented in **Table 3**.

FFA2 is expressed on myeloid cells and some granulocytes. SCFAs act *via* FFA2 to induce the chemotaxis of neutrophils (27, 28) and neutrophil degranulation (29, 30).

Lipopolysaccharide (LPS) activated neutrophils showed diminished production of nitric oxide and TNF-α when co-cultured with propionate and both histone deacetylase and NF-kappa B activation were inhibited, suggesting their role in propionate-mediated inhibition of inflammation (31).

IMMUNE REGULATION BY SCFAS

The gut microbiota consists of bacteria, fungi and viruses. The mass of these micro-organisms in an adult is typically around 2 kg and could potentially evoke a debilitating and life-threatening immune response if left unchecked. In order to maintain a tolerogenic immune response in the gut, in the face of this considerable microbial load, communication between the commensal microbial population and the body's immune system is essential to maintain immunological homeostasis (8, 9). T-cell maturation in the intestinal tract occurs in the gut associated lymphoid tissue (GALT) and from there cells migrate to the intraepithelial layer or lamina propria. These T-cells are

TABLE 3 | SCFA transporters and receptors and tissue distribution in humans.

	Ligand	Tissue
Transporters		
MCT-1	Butyrate, lactate, pyruvate	Colon, Blood cells (monocytes, granulocytes, lymphocytes)
SMCT-1	Butyrate>propionate>acetate	Intestine (Ileum, proximal colon and distal colon)
G-coupled protein receptors		
FFA3 (GPR41)	Propionate=butyrate>acetate	Adipose tissue Peripheral blood mononuclear cells (PBMCs) Pancreas Spleen Placenta Monocytes, neutrophils, monocyte-derived dendritic cells (DCs)
FFA2 (GPR43)	Acetate=propionate=butyrate	Intestinal epithelium Monocytes, neutrophils, PBMCs, T and B cells Treg cells (colonic>spleen and mesenteric lymph node), colonic myeloid cells
GPR109a	Butyrate	Adipose tissue Colon Monocytes and macrophages

Ref (25, 26).

highly modifiable and can be induced to develop into Treg, Th1, Th2, or Th17 cells. These modifications are regulated by metabolites such as SCFAs but also by interaction with antigen presenting cells [e.g., dendritic cells (DCs)] and intestinal epithelial cells (32). As discussed later, Treg, and Th17 cells are immune cells that play a central role in several auto-immune diseases and normalization of their activity may represent an important target for controlling MS.

Nutritional components and microbial metabolites such as acetate, propionate, butyrate, tryptophan and phytoestrogens, act as immune regulators. Numerous studies demonstrate the action of propionate in regulating T-cell activity *in vitro* (33–35), in animal *in vivo* studies (31, 33, 34, 36), and in human studies (16, 37–39). Supplementation with propionate enhances the activity and numbers of the anti-inflammatory Treg FoxP3$^+$ cells and reduces the activity and numbers of Th17 and to a lesser extent Th1 pro-inflammatory T-cells *via* histone deacetylase inhibition (25, 34, 40–42).

Dendritic cells are important in the activation of T-cells. Human primary DCs express FFA3 and GPR109a but only small amounts of FFA2, thus allowing for regulation by SCFAs. *In vitro* analysis showed that both propionate and butyrate (but not acetate) reduced the DC expression of IL-6, and LPS-induced IL-12 and IL-23 (43). This would have a crucial role in reducing pro-inflammatory Th1 and Th17 populations and allow a shift to anti-inflammatory Treg cells. Additionally, the authors demonstrate SCFA specific effects on gene and protein expression of chemokines. Incubation of colon cultures from colitic mice with 1 mM SCFAs (representing gut levels) led to a reduction in pro-inflammatory chemokines with both propionate and butyrate but not acetate. Propionate reduced the expression of chemokine CC ligands (CCL3, CCL5 and CXCL9, CXCL10, CXCL11) representing an additional indirect effect of propionate towards infiltration of immune cells.

Propionate also has a direct effect on the inflammatory activity of non-immune cells shown by *in vitro* studies. Following LPS stimulation, NF-kappa B activity and TNF-α release were reduced when colon cultures were incubated with propionate or butyrate (44).

MODIFICATION OF THE IMMUNE SYSTEM THROUGH SCFAS CAN IMPROVE AUTO-IMMUNE DISEASE

There is evidence that regulation of T-cells by SCFAs and particularly butyrate has a beneficial effect on Parkinson's Disease (PD). Dysbiosis and reduced levels of butyrate and propionate have been demonstrated in patients with PD (45, 46). Supplemental butyrate reduced the alpha-synuclein deposition in gut nerve cells (enteroendocrine cells) (47) and clinical studies are underway to determine if SCFAs play a role in reversing the pathology of PD (48).

Intestinal inflammation such as in ulcerative colitis has been a target condition for microbiota and SCFA research. Studies to date mostly describe associations between the disease state, the immune inflammatory signature, dysbiosis and reduction in SCFAs (49). Propionate reduced IL-1, IL-6 and iNOS production in an *in vitro* model of ulcerative colitis (44) and administration of propionate during a 3-week period reduced intestinal inflammation in an animal model (34). In an animal model of colitis, colonic SCFA levels were associated positively with Treg activity and inversely with disease state (33), and supplemental propionate has been shown to regulate colonic motility (50), and intestinal inflammation in animal models of Irritable Bowel Disease (51).

In patients with ulcerative colitis, butyrate inhibited the pro-inflammatory transcription factor NF-kappa B in macrophages and improved disease state as measured by the Disease Activity Index (52).

Propionate and other SCFAs have been investigated in relation to a number of other auto-immune disease states. Animal and *in vitro* studies have reported an association of propionate with metabolism, diabetes and hepatic steatosis (53–63), inflammation (35, 64–69) and colitis inflammation (70).

THE EFFECT OF PROPIONATE IN ANIMAL MODELS OF MULTIPLE SCLEROSIS

The EAE animal model is often used for studying certain pathophysiological aspects relevant to MS. With this model, orally supplemented propionate was shown to ameliorate disease progression as measured by clinical scoring based on muscular function (e.g., tail tonicity, partial or total limb paralysis, death) (36, 71, 72). The studies consistently reported an associated increase in Treg cells and a decrease in Th17 cells with propionate supplementation. Of particular interest are the results from Haghikia et al. (36) demonstrating that feeding with propionate led to increased Treg cell frequency associated with the small intestine; transplantation of these cells in the EAE model improved the clinical outcome of the mice showing that gut associated T-cell responses were able to have systemic effects.

HUMAN INTERVENTION STUDIES

In 2020, Duscha et al. confirmed that dysbiosis occurred in patients with MS and that levels of propionate, but not butyrate, were lower in MS patients compared to healthy controls (16). Previous work by the same group had shown propionate to be unique amongst SCFAs in its ability to improve disease score in the EAE animal model (36).

The study went on to investigate daily oral propionate supplementation in patients with MS. Patients showed significantly lower levels of Treg cells and significantly higher levels of Th17 cells at baseline compared to healthy controls representing a pro-inflammatory state for patients with MS. After 14 days of daily 1 g propionate supplementation, a significant reduction in Th17 cells and a significant increase in number and activity of Treg cells was seen.

Long term supplementation with 1 g propionate daily was performed in 97 patients with MS (in which patients had at least 1-year supplementation). Propionate intake was associated with a significant benefit to MS patients as measured by annual relapse rate and expanded disability status scale (EDSS) score.

The study had some design weaknesses including a non-treated control group rather than use of a blinded randomized placebo-controlled design. It is also unclear what medications were used for the treatment group *vs* the control group and whether medications changed during the 3 years of propionate supplementation. Despite these weaknesses, the data point to a beneficial effect of propionate supplementation in patients with MS.

SAFETY OF PROPIONATE FROM AN IMMUNOLOGICAL PERSPECTIVE

Inflammation is a key mechanism in the defense of the body against invading pathogens. Many diets and nutrients are claimed to have anti-inflammatory properties with the presumption that this is always good. However, there could be concern that an anti-inflammatory agent could suppress inflammation needed during infection. At the same time, an uncontrolled inflammatory response is associated with chronic diseases such as diabetes, rheumatoid arthritis, allergies and auto-immune diseases. The question then is whether immune suppression leads to an open door to bacteria and viruses (73).

Interestingly, Kim et al. (74) showed propionate elicits complex immune regulation dependent on the immunological setting. The group demonstrated that *in vitro*, propionate mediated an increase in IL-10 production by T-cells in line with its anti-inflammatory properties, and propionate has been shown by others to be a central regulator of IL-10 production (16, 75). However, they also found that propionate-induced $FoxP3^+$ expression (a marker of T reg cells) was dependent on the strength of T-cell activation. In conditions of high T-cell activation, SCFAs could suppress $FoxP3^+$ cell induction whereas at low T-cell activity SCFAs enhanced $FoxP3^+$ cell induction (from TGFβ1). This suggests that T-cell modulation by propionate and other SCFAs is in accordance with the immunological setting the T-cell is in. The group also suggest that SCFAs could facilitate the differentiation of T-cells to Th1 and Th17 cells given the right immunological conditions. The authors conclude that propionate "aids to promote the right type of T-cells for specific immunological conditions".

The concept of appropriate T-cell modulation is further supported by detailed work by Bhaskaran et al. (76) looking at propionate effects in the light of mucosal infection, where an immune response is desired to fight infection but overt inflammation can lead to tissue damage. This is a common pathophysiological situation where initial inflammation is triggered in response to a pathogen to combat infection, but continued inflammation results in tissue damage. In the case of the study by Bhaskaran et al., propionate (and other SCFAs) again increased T reg cells, but during *Candida albicans* infection propionate also stimulated levels of Th17 cells and IL-17 and promoted clearance of the infection. These results in mice showed propionate improved the immune response against the mucosal fungal infection and at the same time promoted resolution of inflammation. Supporting *in vitro* data showed that a direct effect of propionate on Th17 cells led to reduced disease activity; however, co-culture with spleen and lymph node cells in a Th17 activated medium led to a switch in propionate activity and a promotion of IL-17 production.

Another example is given by the equine herpesvirus which enters the horse through the upper respiratory tract, is spread through infection of leukocytes and T-cells and causes neurological and reproductive disorders. Propionate (and other SCFAs) were shown to have a beneficial impact on the pathogenesis of the virus through several mechanisms including reduction in viral spreading through FFA2- and FFA3-mediated mechanisms (77). Viral spread to endothelial cells from monocytes was inhibited *via* an NF-kappa-B dependent pathway and inhibition of adhesion molecule expression. This mechanism may also be active in suppressing the spread of measles and herpes simplex virus (77).

A neutral effect of propionate was demonstrated with studies of the cholera vaccine. Cholera is an acute diarrheal disease

resulting from bacterial infection. The cholera vaccine uses inactive (killed), whole bacteria that interact with antigen-presenting-cells and lymphocytes in the gut lymphoid tissue. Results from the study suggest that propionate and acetate have no detrimental effect on the response to the vaccine, whereas butyrate may even have beneficial effects (78).

Some contradictory results however point towards SCFAs having a detrimental effect during an infection. A study designed to specifically investigate the role of SCFAs in bacteria-induced inflammation was performed by Correa et al. They investigated the activity of neutrophils against a bacterial skin infection in an animal model. They showed that SCFAs had no effect on leukocyte accumulation but did reduce cytokine production and neutrophil phagocytic capacity suggesting a detrimental effect of SCFAs (79).

The relevance of the findings by Correa et al. can be seen in the light of a similar study by Ciarlo et al. (80) where morbidity and mortality were also measured in mice. Ciarlo et al., in agreement with Correa et al., demonstrated that propionate led to reduced activity of the innate immune system when mouse or human cells were challenged with a range of microbes (*Staph. aureus*, *Strep. pneumoniae*, *E. coli*, *Klebsiella pneumoniae*, *Candida albicans*). In this study, the production of inflammatory cytokines such as IL-6 and IL-12 (but less so for TNF-α) was reduced by propionate in macrophages and monocytes and to a lesser extent in DCs. Despite these effects, 3-week supplementation of infected mice with propionate had no effect on morbidity or mortality. Furthermore, despite the expected increase in Treg FoxP3$^+$ cells following propionate treatment, the immunizing effect of a primary infection to subsequent infections from the same bacteria was not altered. The authors conclude that this was a successful demonstration that anti-inflammatory benefits associated with supplemental propionate did not come at a cost of depleted immune defense to pathogens and therefore supported the use of supplemental propionate.

Thus, the available data support a complex interplay between SCFAs and the immune system, whereby SCFAs in general, and propionate in particular, have a direct effect on T cell activity mediated by histone deacetylase inhibition which can be switched according to immunological context (e.g., chronic inflammation versus an infection).

The aim of this review is to consider the use of propionate in patients with MS in the light of the need for a fully functioning immune system. In the case of MS, an immune system running wild needs to be tamed, but not to the degree that pathogens cannot be controlled. The data collected to date suggest that supplemental propionate can promote a non-inflammatory T-cell profile leading to improved clinical outcomes for MS patients and that this occurs without compromising the immune response to pathogens.

DIETARY MANAGEMENT OF MS WITH PROPIONATE

Although pharmacological treatment of MS has progressed significantly over the last decade or so, the is a continuing need for improved or novel ways of managing the disease. Most patients with MS still experience significant disease progression over time. The emerging importance of gut health and the microbiota in MS etiology offers an opportunity for new adjunctive tools in MS management.

Propionate is classified as a food product in the European Union (81) and the United States (21 CFR 184.1784) and is therefore considered safe for the general public. In the clinical study by Duscha et al. no serious adverse events were reported, and mild gastrointestinal adverse events were reported in less than 5% of participants.

Propionate is included in some food stuffs such as some breads and dairy products for enhancing shelf-life, but quantities are not included in product labels and a consumer would be unable to make informed dietary changes in order to control propionate intake. Therefore, the management of patients can only be through supplementation for which some examples now exist.

Gold et al. (82) reported dietary supplementation with 1g propionate daily as adequate for restoring plasma propionate concentration and immunological parameters in patients with MS to those of healthy individuals. Potential nutritional management of MS patients is described by Duscha et al. (16): 2 x 0.5 g capsules were given daily as an adjunct to disease modifying therapy. Participants under all MS drug regimens studied showed an increase in Treg cell numbers and function (except for where the drug glatiramer acetate was being used). Improved annual relapse rates were noted for all treatment groups (including the non-medicated), although participant numbers were low and results should be considered with caution.

How MS disease modifying treatments might affect the level of propionate or its actions has, to the best of our knowledge, not been investigated. The best indication of treatment effects on propionate levels comes from information on effects on microbiota.

Overall, few studies have been performed assessing the effect of MS-drugs on the microbiota, with inadequate data to draw conclusions. However, results to date suggest some medications may work to aid the levels of propionate. Dimethyl fumarate is an immune modifying treatment in MS with known side effects on gut health. A recent pilot study demonstrated dysbiosis in patients with MS compared with healthy controls and no significant differences were seen from dimethyl fumarate treatment apart from a trend towards increasing propionate (and butyrate) producing Bifidobacteria (83).

Microbiota profiling was performed in glatiramer acetate treated MS, dimethyl fumarate treated MS and healthy subjects (84) showing a tendency of dimethyl fumarate to enhance numbers of some SCFA producing bacteria. In a pilot study, glatiramer acetate treatment reduced the number of propionate-forming bacteria in MS patients whilst addition of Vitamin D somewhat restored these bacteria (10).

Given the safety profile of propionate, the potential clinical benefit in MS and its relatively inexpensive production, it can be considered that propionate is a good candidate agent for the dietary management of MS.

Thus, the current data suggests that patients with MS, either RRMS or SPMS, may have benefits from taking 1g propionate daily as an adjunct to their normal therapy. Studies of MS patients with propionate have been performed with patients on Interferon Beta, teriflunomide, glatiramer acetate, fingolimod, rituximab, and dimethyl fumarate. The numbers of participants studied by treatment regimen are low and the findings should be considered with caution. Adverse events are infrequent but may include gastrointestinal events.

CONCLUSION

The role of propionate in MS is described as a story of dysbiosis, reduction of SCFA producing bacteria, reduced levels of plasma propionate coupled with the impact of propionate on T-cells important in the pathophysiology of autoimmunity. This broad basis supporting the mechanistic action of propionate has been built up over the last 2 decades and supported by studies in the EAE animal model for MS, where disease score is reduced on propionate supplementation. The recent publication of a prospective study showing the benefit of propionate supplementation on MS disease progression suggests this microbial metabolite may have clinical importance in the management of MS and supplementation may be a useful adjunctive tool to current medications.

The potential use of propionate in MS management is grounded in its activity in regulating T-cell profiles and activity. Studies suggest that T-cell modulation is sensitive to the immunological challenge in the body, and this is supported by animal studies showing propionate supplementation is either neutral or beneficial for host immune activity when tackling bacteria and viruses. Studies supporting this claim show outcome data for infections or related inflammatory processes. Whilst the data may show a general beneficial or neutral effect of propionate supplementation on immune activity, the data is of insufficient volume to give a definitive picture of how propionate supplementation can affect the immune system's response to particular pathogens.

However, the nascent data suggest propionate may be useful in the nutritional management of MS (85) and at the same time be neutral or contribute to a normal physiological immune response essential for tackling the pathogenic fungi, bacteria and viruses the body is exposed to.

In conclusion, there is broad mechanistic support for the role of propionate in regulating the immune system *via* modification of T-cell profiles and activity. In the context of auto-immune disease and gut regulation of immunity, propionate and other SCFAs are considered as important mediators of the gut microbiota. In accordance with this, distal outcomes of auto-immune disease such as seen with MS are linked to low levels of propionate due to gut dysbiosis. The use of propionate as a supplemental adjunct to current medical treatment has been strengthened by consistent evidence from animal models (EAE) and a recently published human intervention trial demonstrating long term improvement in disease progression across MS subtypes. Evidence that propionate may also promote T-cell activity in the face of infection further supports that propionate may be a safe nutritional adjunct to MS treatments.

AUTHOR CONTRIBUTIONS

DT drafted the manuscript. RV and PC contributed and commented on the manuscript. All authors contributed to the article and approved the submitted version.

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Alemtuzumab in a Large Real-Life Cohort: Interim Baseline Data of the TREAT-MS Study

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The non-interventional long-Term study foR obsErvAtion of Treatment with alemtuzumab in active relapsing–remitting MS (TREAT-MS) study collects the so far largest real-life cohort regarding utilization, long-term effectiveness, and safety of alemtuzumab, a humanized monoclonal antibody directed against the cell surface glycoprotein CD52, in adult patients with active relapsing–remitting multiple sclerosis (RRMS). An interim analysis of baseline parameters at inclusion of a non-interventional real-world study about alemtuzumab in Germany including previous multiple sclerosis (MS) medication utilization, MS activity, severity, and duration, as well as comorbidities was performed. Of the 883 patients, 71.6% were women. Mean age was 35.7 ± 9.2 years, time since first MS symptoms (=disease duration) is 8.0 ± 6.8 years, and Expanded Disability Status Scale (EDSS) is 2.7 ± 1.8 points (range, 0.0–7.5 points). The number of relapses in the 12 and 24 months prior to inclusion were 1.6 ± 1.2 and 2.2 ± 1.8, respectively. Of the patients, 14.4% were treatment naive, while for the majority, a wide spectrum of MS disease-modifying treatments (DMTs) and treatment sequences were documented. Overall, interferon beta (IFN-beta) was reported most frequently (52.4%), followed by fingolimod (35.2%), natalizumab (34.9%), and glatiramer acetate (28.9%). Patients with longer disease duration and higher EDSS had a higher number of previous DMTs. Compared to the pivotal phase 2/3 studies, RRMS patients starting alemtuzumab treatment had a longer disease duration in real-world conditions. There was variety of different treatment sequences before the final switch to alemtuzumab. In the future, linking these treatment sequences or other baseline characteristics with effectiveness and safety outcomes might be useful to support treatment decisions. Registered at Paul-Ehrlich-Institut under NIS 281.

Keywords: alemtuzumab, non-interventional study, risk-management plan, Germany, real world data, multiple sclerosis, effectiveness, safety

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INTRODUCTION

The treatment landscape for multiple sclerosis (MS) has substantially changed, with the approval of more than 10 new drugs in the last decade. High-efficacy treatments appear to improve the long-term outcomes of MS patients (1) but are often only considered as second- or third-line options due to label restrictions or at the discretion of the treating physician. Two general treatment paradigms

can be applied, either a maintenance-escalation approach, where a medication is given continuously and patients are switched to a higher efficacy drug upon disease activity, or a pulsed immune reconstitution therapy, which involves few treatment pulses with long intermittent treatment-free phases (2). Alemtuzumab (Lemtrada®, Sanofi Genzyme) is given as a pulsed immune reconstitution therapy in usually two treatment phases, which leads to sustained and treatment-free effectiveness (3, 4). Alemtuzumab is a humanized monoclonoal IgG1kappa-type antibody binding to the cell surface protein CD52, which is expressed in large amounts on B and T lymphocytes (5). After binding of alemtuzumab to CD52, circulating lymphocytes are depleted either by complement-induced or antibody-dependent cell-mediated cytotoxicity (6). After depletion, B- and T-lymphocyte repopulation occurs in a defined pattern and has demonstrated beneficial long-term effects (7).

Overall, alemtuzumab appears to reprogram the immune repertoire, which manifests in the special kinetics of immune cell populations, the increased production of antiinflammatory cytokines, and the very long duration of action (8). Three randomized, rater-blinded clinical trials assessing the efficacy of alemtuzumab in MS treatment, using an effective comparator drug, have been performed: CAMMS223 (9), CARE-MS I (10), and CARE-MS II (11). In sum, alemtuzumab significantly reduced clinical and radiological disease activity and slowed down progression of relapsing–remitting MS (RRMS) to secondary progressive MS, also in the long-term and in patients with highly active disease (HAD) (4, 12–15).

In the European Union, in 2013, alemtuzumab has been marketed as a treatment for RRMS with active disease defined by clinical or imaging features. In the USA, in 2014, the drug has been approved for RRMS and progressive–relapsing MS treatment but only for patients who did not have a satisfying response to two or more drugs (16) (i.e., for third-line therapy). In 2019, alemtuzumab has undergone a procedure under Article 20 of Regulation (EC) No 726/2004 resulting from pharmacovigilance data, which led to label change effective January 2020 (17). Alemtuzumab should now only be used to treat RRMS if the disease is highly active despite treatment with at least one disease-modifying therapy or if the disease is worsening rapidly (18). Alemtuzumab must also no longer be used in patients with certain heart, circulation, or bleeding disorders or in patients who have autoimmune disorders other than multiple sclerosis.

Data on the utilization and the treatment outcomes of alemtuzumab in the real-world clinical practice are limited to few reports on small, mostly monocentric cohorts (19, 20) or a retrospective data collection, respectively (21). There is a need for high-quality, comprehensive, and valid real-life evidence data, as these data cover additional aspects of patient care and expand the data available by complementary information (22, 23).

The aim of the non-interventional long-Term study foR obsErvAtion of Treatment with alemtuzumab in active relapsing–remitting MS (TREAT-MS) study is to establish a broader real-world database on the utilization and effectiveness, safety, and other aspects of the drug in everyday clinical practice in Germany (24). The current interim analysis describes the cohort of patients before the alemtuzumab label change with particular focus on the treatment profile, disease characteristics, and comorbidities before alemtuzumab start.

DESIGN AND METHODS

Design

TREAT-MS is a prospective and retrospective, multicenter, open-label, non-interventional long-term study that collects data from neurologists in specialized MS centers (clinics or outpatient departments) in Germany (24). The study was registered in a publicly accessible database at Paul-Ehrlich Institute (regulatory authority) under NIS 281.

Patients

Patients are eligible for documentation if they are newly treated with alemtuzumab or have initiated treatment earlier and are followed up on the long term.

Study Flow and Parameters

Study parameters include the following: demographics, comorbidities, MS anamnesis and characteristics including relapses over time, Expanded Disability Status Scale (EDSS), lesions on MRI, and as patient-related outcomes, Symbol Digit Modality Test (SDMT), Patient-Reported Indices for MS (PRIM US), EuroQol 5D-3L, and Work Productivity and Activity Impairment Questionnaire (WPAI) (25). The Clinical Global Impression-Severity (CGI-S) test expresses the experience-based impression of the treating physician on the severity of the patient's illness in a 7-point scale (26). Analogously, the CGI-S can be determined by the patient to show the evaluation of the patient on his or her clinical condition.

Treatment

Alemtuzumab is administered as two annual courses (on 5 consecutive days at baseline and on 3 consecutive days 12 months later), and patients are followed up for safety as per local labeling. Patients could receive up to two additional courses (12 mg/day $\times$ 3 days) $\geq$12 months after the most recent course or treatment with other DMTs as needed.

Neurologists and MS nurses were guided by the MS documentation system for physician, nurse, and patient (MSDS 3D) Lemtrada-TREAT-MS module through the entire management of treatment, including monitoring of the first and second infusion courses, necessary examinations, and regular laboratory screenings (27, 28).

Statistical analyses were performed in an exploratory manner using descriptive statistical methods. For continuous variables, the number of patients with non-missing and missing data, mean, standard deviation, minimum, 25% quantile, median, 75% quantile, and maximum were calculated. For ordinal and categorical variables, frequencies were calculated. Incomplete data sets were included in the analysis. Imputations were only done for missing dates for days (substituted by the 15) and for months (substituted by June), while years were not substituted. Given the descriptive character of the study, no further imputations were deemed appropriate.

No sensitivity analyses were done.

A treatment pathway is defined as a unique longitudinal sequence of discrete MS treatments [disease-modifying therapies (DMT)] and is differentiated based on introduction of discrete DMTs in patients' MS treatment course. Treatment pathways were visualized in Sankey diagrams, generated through SAS, version 9.4 (SAS Institute Inc., Cary, NC, USA) (29). As another visualization approach, scatterplots were generated based on Multiple Sclerosis Severity Score (MSSS), which relates scores on the EDSS to the distribution of disability in patients with comparable disease durations (30).

The total cohort at the data cutoff date February 10, 2020 comprised 883 patients. Statistical analyses were done with IBM SPSS for Windows, Version 15.0.0.

RESULTS

Setting

Of all physicians who contributed at least one eligible patient for the present analysis, 41 (34.7%) were hospital-based and 77 (65.3%) were resident neurologists. Data from 426 (48.2%) and 457 (51.8%) patients were documented by hospital-based and resident neurologists, respectively.

Baseline Characteristics of Patients

Baseline characteristics of the 883 patients are summarized in **Table 1**. Mean age at baseline was 35.7 ± 9.2 years (range, 16–63 years). The majority (71.6%) were female. Mean time since first MS symptoms (=disease duration) was 8.0 ± 6.8 years and since MS diagnosis was 7.2 ± 6.3 years. The median EDSS was 2.5, with a range from 0.0 to 7.5. While 63.2% of the patients had an EDSS ≤3, 36.8% had a baseline EDSS >3. **Figure 1** displays the distribution of EDSS categories. The mean number of relapses in the 12/24 months prior to inclusion was 1.6 ± 1.2/2.2 ± 1.8. Clinical Global Impression (CGI) assessed by the physician or patients at inclusion assessment was 4.8 ± 2.7 and 3.2 ± 1.7, respectively.

MS Pre-treatment With DMT

About every seventh patient (n = 127; 14.4%) was treatment naive. In contrast, 722 (81.7%) had received any DMT (3.9% unknown). In detail, 21.7, 30.4, 18.5, 9.5, and 2.3% had received one, two, three, four, or five or more pretreatments with MS medications, respectively.

The MS treatment history before the initiation of alemtuzumab is listed by decreasing frequency in **Table 2**. Interferon-beta (IFN-beta) was reported most frequently (52.4%), followed by fingolimod (35.2%), natalizumab (34.9%), and glatiramer acetate (28.9%). With regard to the last MS medication before alemtuzumab initiation, 22.0% received fingolimod, 14.8% natalizumab, and 8.6% IFN-beta therapy.

Characterization of the Disease Status at Baseline

The EDSS, the duration since initial MS symptoms, and the number of MS relapses in the previous year are useful parameters to evaluate the disease status. In order to visualize these

TABLE 1 | Baseline characteristics.

Variable	Total cohort	
	N	Value
Age (years)	883	35.7 ± 9.2
Range		16–63
Sex, Female, %	632	71.6
Male, %	251	28.4
Multiple sclerosis characteristics		
Time (years) since first MS symptoms until inclusion into study	668	8.0 ± 6.8
Time (years) since MS diagnosis until inclusion into study	793	7.2 ± 6.3
RRMS, %	823	95.4
Relapses during last 12 months before inclusion into study, %		
0	127	16.3
1	295	37.8
2	221	28.3
3	87	11.1
Missing	102	
Mean ± SD	781	1.6 ± 1.2
Relapses during last 24 months before inclusion into study, %		
0	89	12.7
1	187	26.6
2	182	25.9
3	128	18.2
Missing	181	
Mean ± SD	702	2.2 ± 1.8
Magnetic resonance imaging		
Contrast medium enhancing lesions present at 1st pretreatment visit, %	397	54.7
Gd+ lesions		
0	226	31.4
1	69	9.6
2	56	7.8
3+	96	13.3
T2 lesions		
0	38	4.8
1	16	2.0
2	10	1.3
3+	174	21.8
EDSS total	798	2.7 ± 1.8
≤3	504	63.2
>3	294	36.8

EDSS, Expanded Disability Status Scale, RRMS, relapsing–remitting multiple sclerosis. Values are percentages or means ± standard deviation (SD).

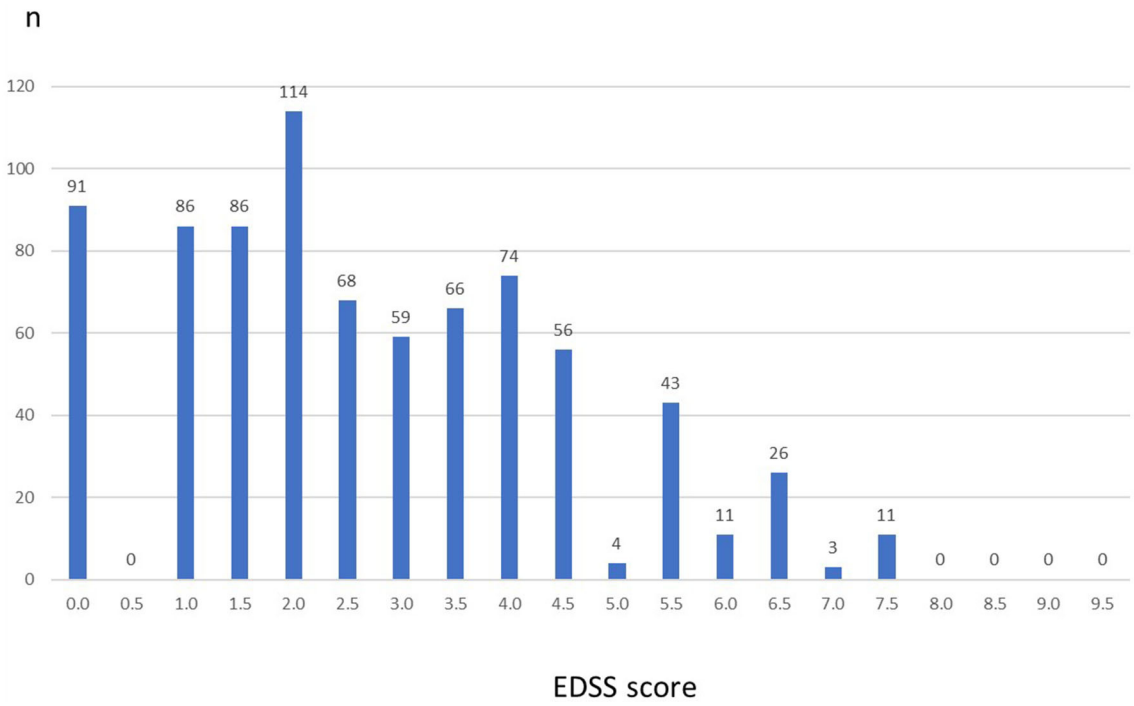

FIGURE 1 | Expanded Disability Status Scale (EDSS) at baseline ($n = 883$). Columns represent number of patients for each EDSS value. There were no patients with values above 7.5.

TABLE 2 | Disease-modifying treatments (DMTs) pretreatment.

DMT pre-treatment	Total ($N = 883$) *n* (%)
Total	727 (82.3)
Interferon-beta	463 (52.4)
Fingolimod	311 (35.2)
Natalizumab	308 (34.9)
Glatiramer acetate	255 (28.9)
Other[a]	131 (14.8)
Dimethyl fumarate	109 (12.3)
Teriflunomide	44 (5.0)
Mitoxantrone	18 (2.0)
Azathioprine	9 (1.0)
Unknown	5 (0.6)
Methotrexate	2 (0.2)
Rituximab	1 (0.1)

Values are n and percentages of total.
[a] *"Other" includes unspecified drugs in 31 patients, daclizumab in 20 patients, immunoglobulins in six patients, and a variety of other drugs in the remaining patients.*

parameters and to relate them to the number of previous MS medications, scatterplots were used combining the parameters. This allows an evaluation of the MS disease status of TREAT-MS alemtuzumab-treated patients at baseline.

The scatterplots (**Figures 2A–D**) show the distribution of EDSS values (y-axis) vs. disease duration (years before inclusion into the study, x-axis) by DMT pretreatment and described in detail in the figure legend.

Each dot in the diagram represents one patient's value in relation to both parameters at baseline. For pretreated patients, the most recent DMT is distinguished by different symbols as indicated in the legend, and the basic therapies glatiramer acetate, dimethyl fumarate (DMF), interferon-beta, and teriflunomide are colored red so that they can be easily distinguished from escalation therapies. The horizontal bars on the right show the distribution (histogram with frequencies in percent) of EDSS values and the vertical bars above the scatterplot the distribution of time intervals across all patients (percentage). In treatment-naive patients, the majority had a short disease duration (in two-thirds of patients <1 year before inclusion) and were predominantly in the lower EDSS categories (with peaks at 0–2 and 3.5) (**Figure 3A**). In patients who previously received one DMT, the EDSS pattern does not differ much. In contrast, the time pattern does, since peaks occur 2 years after diagnosis and after 10+ years. The distribution of the various DMTs appears similar across the different EDSS and the different time periods, respectively. The majority of patients received baseline therapies as indicated by the red color (**Figure 3B**). In patients previously treated with two DMTs, a trend to higher EDSS values is visible. Furthermore, the proportion of patients with long disease duration (10+ years) is nearly at 50%. Fewer patients are on interferon beta

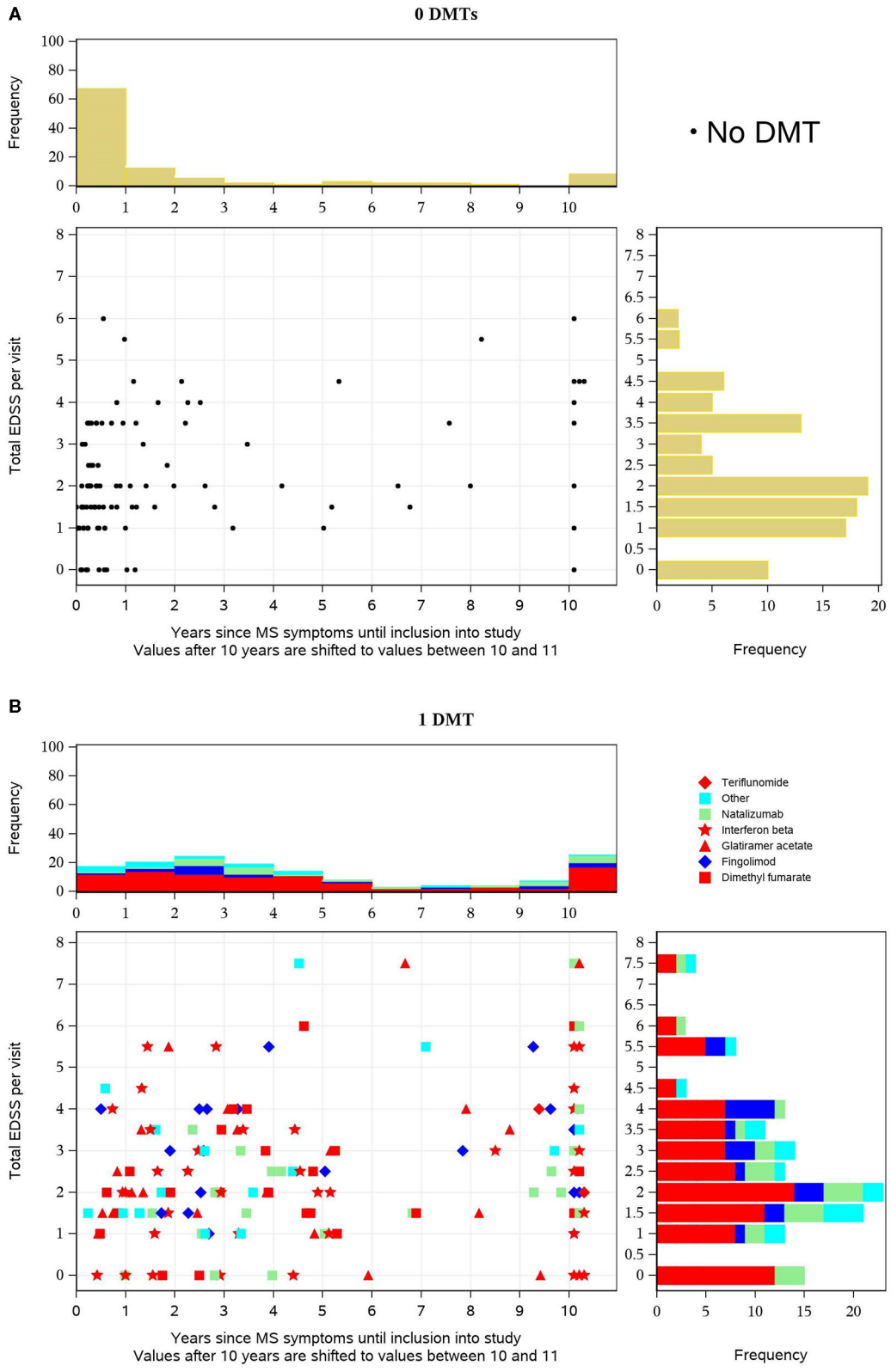

FIGURE 2 | Continued

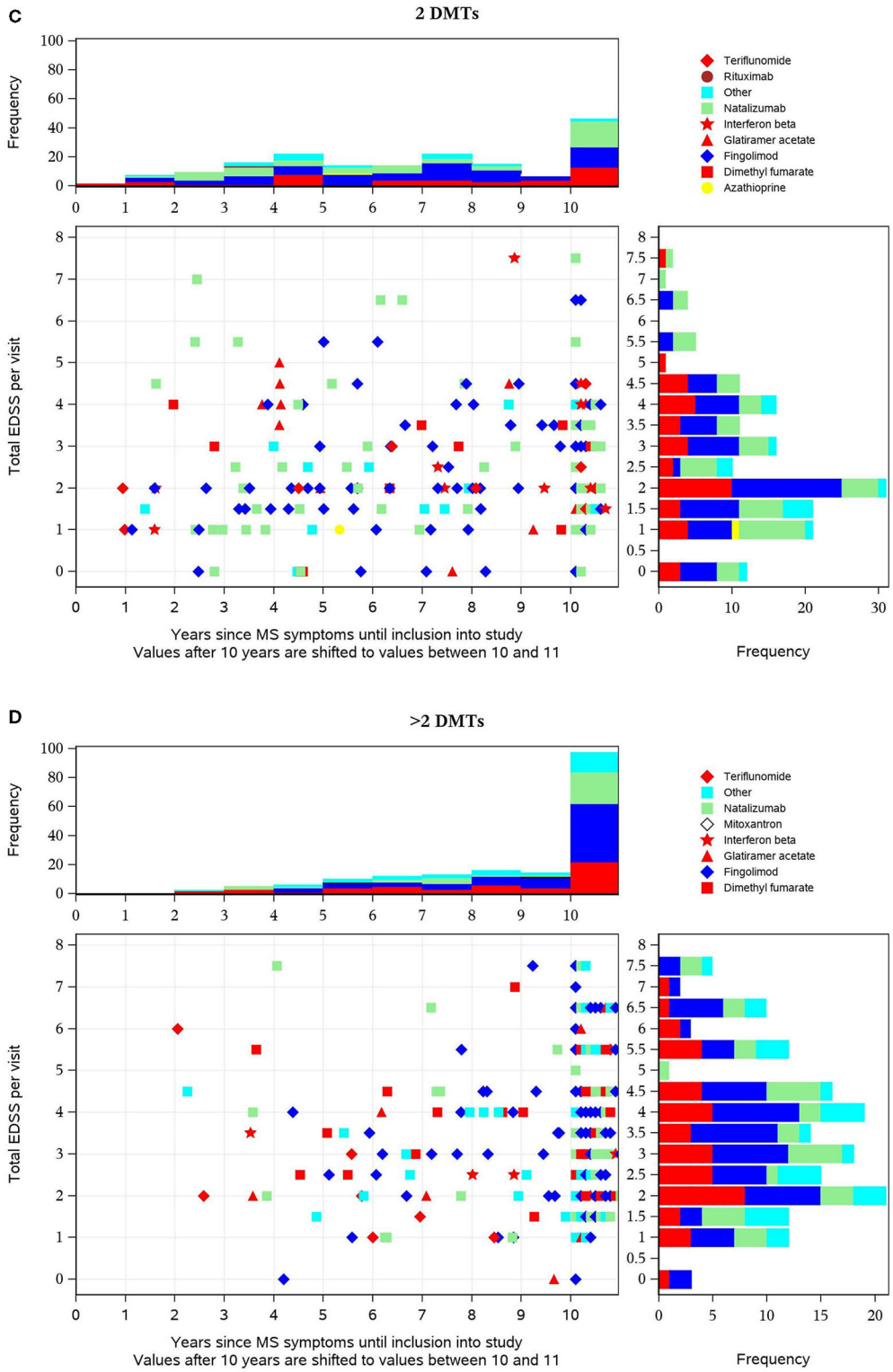

FIGURE 2 | (A–D) Distribution of Expanded Disability Status Scale (EDSS) values (y-axis) vs. disease duration before inclusion into the study (x-axis) by disease-modifying treatment (DMT) pretreatment. Each dot in the diagram represents one patient's value in relation to both parameters at baseline. For pretreated patients, the most recent DMT is distinguished by different symbols as indicated in the legend and the basic therapies glatiramer acetate, dimethyl fumarate (DMF), interferon-beta, and teriflunomide are colored red so that they can be easily distinguished from escalation therapies. The horizontal bars on the right show the distribution (histogram with frequencies in percent) of EDSS values, the vertical bars above the scatterplot the distribution of time intervals across all patients (percentage).

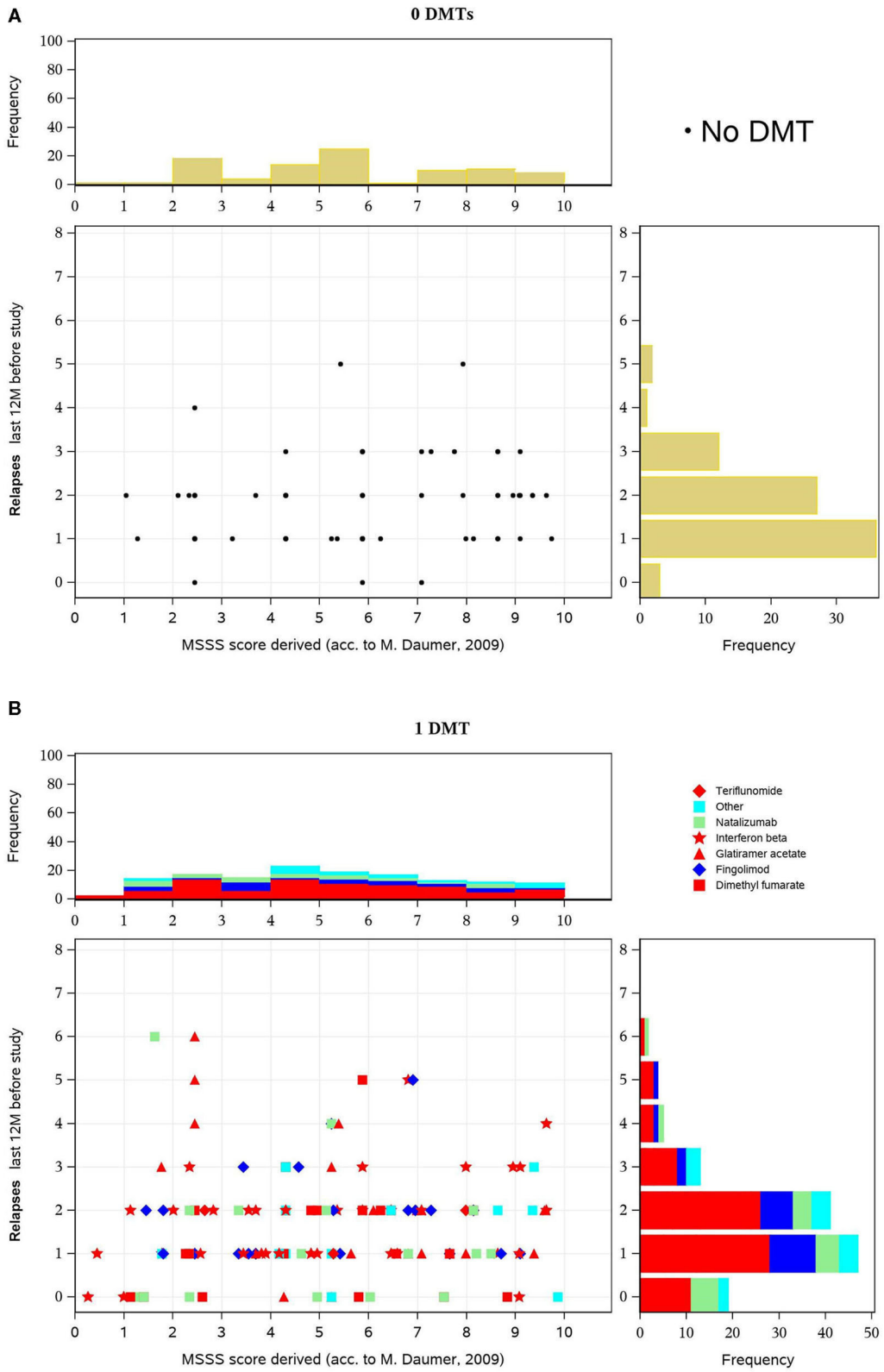

FIGURE 3 | Continued

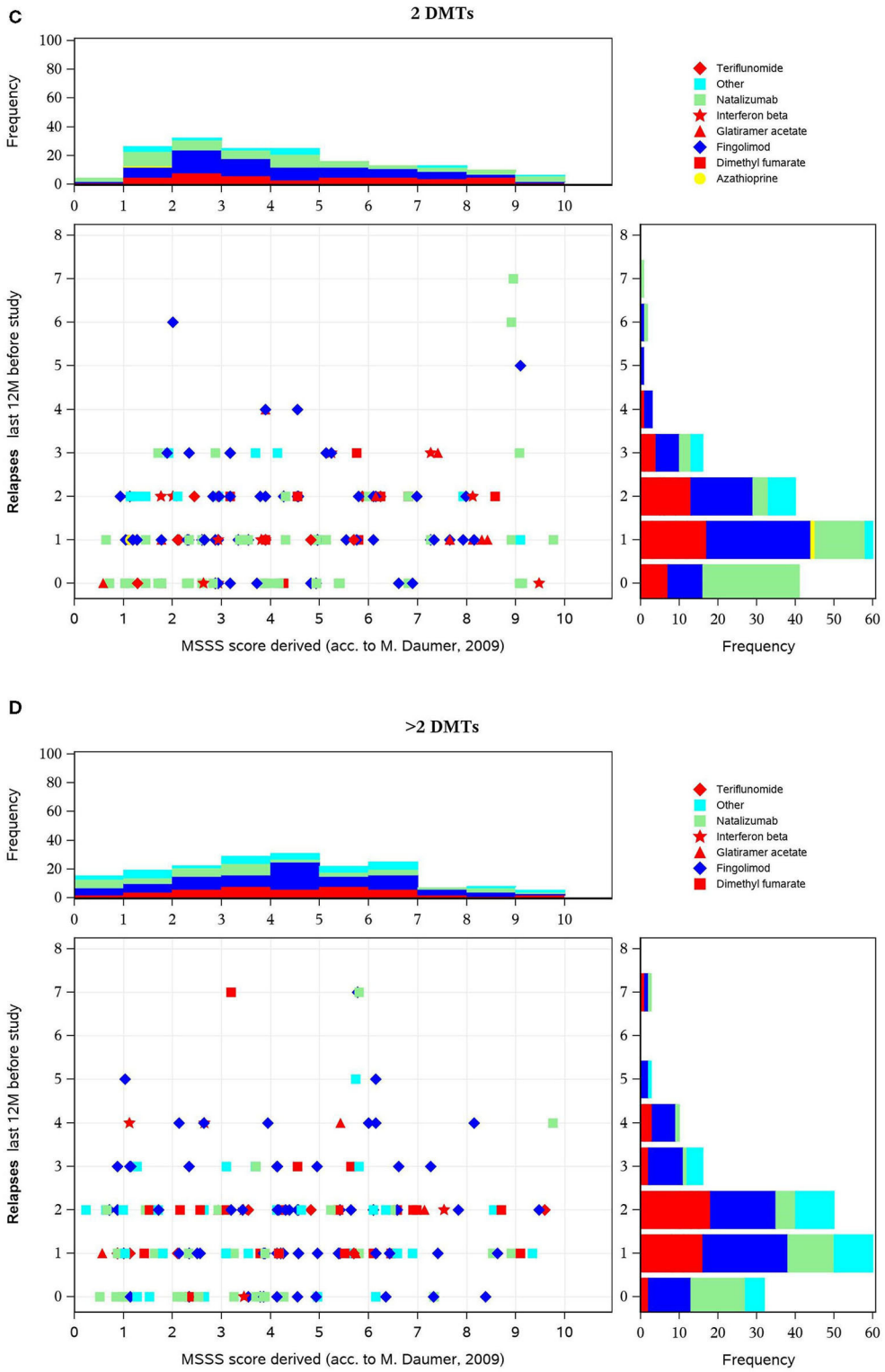

FIGURE 3 | (A–D) Multiple Sclerosis Severity Score (MSSS) (y-axis) vs. number of exacerbations (x-axis) in the 12 months before inclusion, by disease-modifying treatment (DMT) pretreatment. These figures display scatterplots of MSSS (x-axis) vs. number of relapses (y axis) in the 12 months before inclusion, in patients with no, one, two, or more DMT at baseline. In all subgroups, there was a peak of one or two relapses and a similar distribution with a predominance of lower MSSS values. There is no distinct pattern of medication use in the various groups. However, escalation therapy is more often used in patients with a higher number of disease-modifying treatments (DMTs).

TABLE 3 | Most frequent treatment pathways prior to switch to alemtuzumab.

DMT pre-treatment			*n* Patients	% Of total (*N* = 886)
First	**Second**	**Third**		
Interferon-beta			52	5.9
Interferon-beta	Fingolimod		42	4.7
Interferon-beta	Natalizumab		36	4.1
Natalizumab			28	3.2
Dimethyl fumarate			26	2.9
Interferon-beta	Natalizumab	Fingolimod	24	2.7
Fingolimod			23	2.6
Glatiramer acetate			23	2.6
Other			17	1.9
Glatiramer acetate	Fingolimod		13	1.5
Interferon-beta	Interferon-beta	Fingolimod	11	1.2
Interferon-beta	Glatiramer acetate	Fingolimod	10	1.1
Interferon-beta	Glatiramer acetate		10	1.1
Interferon-beta	Dimethyl fumarate		9	1.0
Glatiramer acetate	Natalizumab		9	1.0

In line with three DMTs, patients received the named three drugs in the order first–second–third before switching to alemtuzumab.
Values are n and percentages of total.

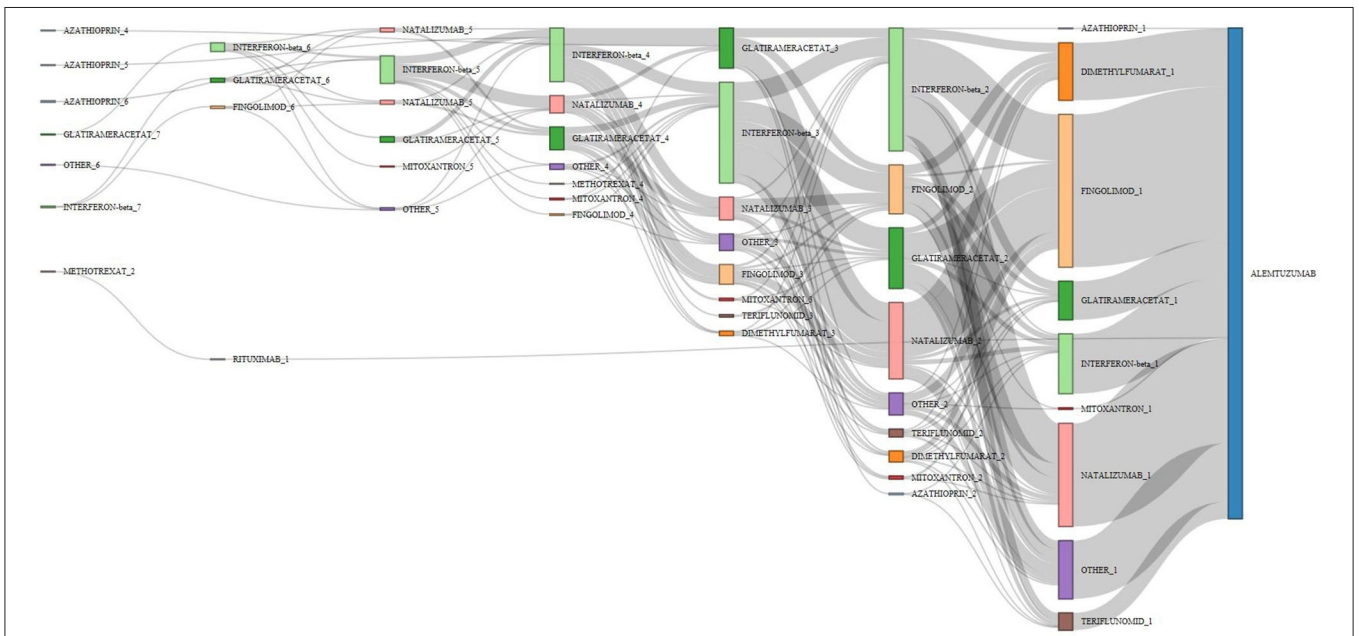

FIGURE 4 | Pathways of disease-modifying treatments (DMTs) before final switch to alemtuzumab. The figure displays the pathways of DMTs before the final switch to alemtuzumab (before or at the inclusion visit of the study). The various MS medications are represented by different colors (alemtuzumab blue, fingolimod light orange, interferon-beta light green, glatiramer acetate green); the height of the stacked vertical bar represents the number of patients treated with the respective MS medication. The width of the lines (ribbons) that connect the individual stacked columns visualizes the number of patients who are transferred to the same (same color) or another medication (different color). It is clearly visible by the wide ribbons that "typical" pathways in this study were from interferon-beta to natalizumab, from interferon-beta to fingolimod, and from natalizumab to fingolimod. Patients were excluded if the exact treatment order could not be determined (29 cases unknown, 127 no pretreatment, 104 last pretreatments not identifiable), leaving 623 patients in the chart. Chronological treatment sequence order starts on the left and ends with alemtuzumab on the right. See also editable **Figure 5**.

and more are on fingolimod, natalizumab, and other DMTs. Overall, the proportion of basic therapies (red) was lower compared to patients with one DMT (**Figure 3C**). In patients pretreated with three or more DMTs, the described changes are even more pronounced: compared to the previously described subgroups, a higher proportion of patients have a higher EDSS

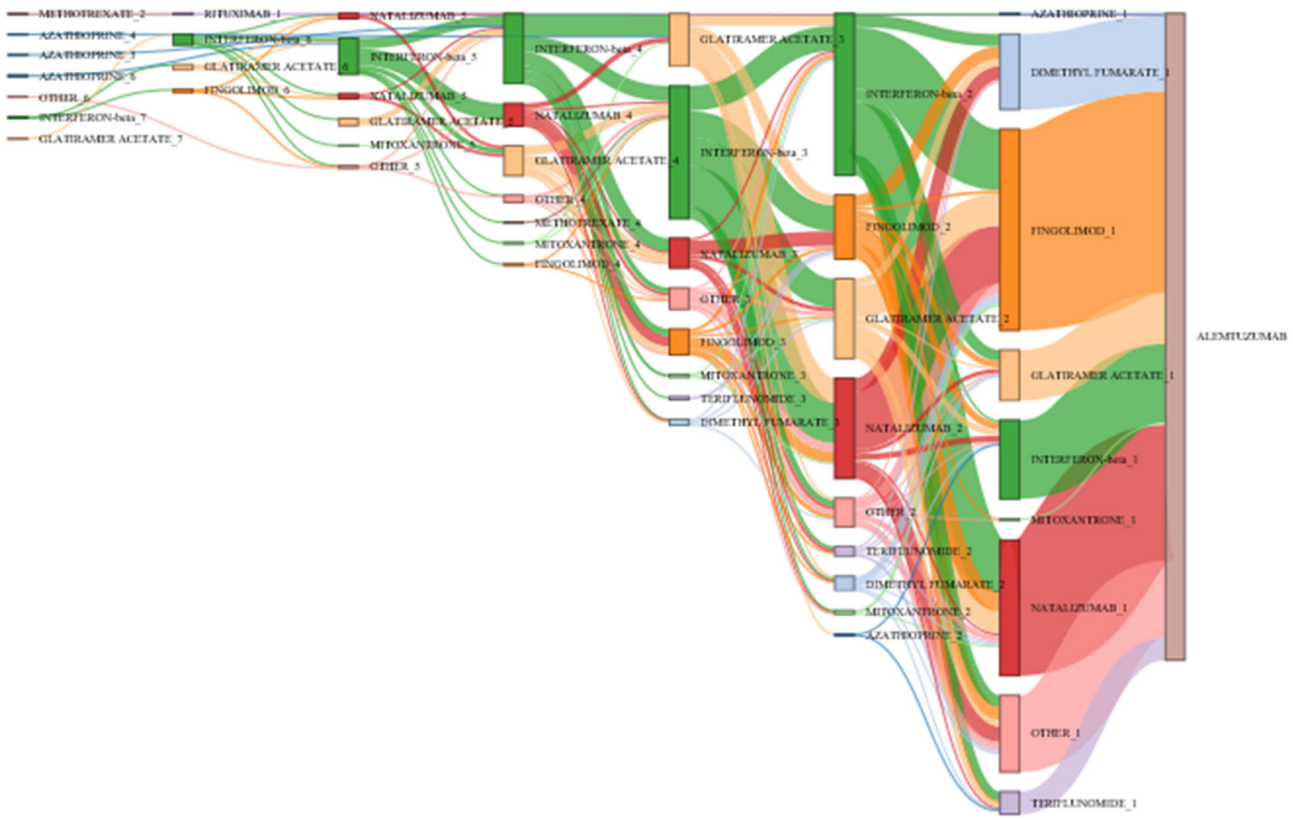

FIGURE 5 | Pathways of disease-modifying treatments (DMTs) before final switch to alemtuzumab. The figure illustrates the various treatment pathways as in **Figure 4**. By moving the cursor over the connecting ribbons, the respective number of patients treated with the previous DMT are indicated. The number following the DMT name indicates the position in the treatment sequence. The bars can be moved vertically to change the view of the Sankey plot.

value, and nearly 90% had a disease duration of 10+ years (**Figure 3D**).

The MSSS combines EDSS and disease duration and is considered to be a powerful method for comparing disease progression using single assessment data (30). The score predicts disease severity over time (31). **Figures 3A–D** show the scatterplot of MSSS vs. number of relapses in the 12 months before inclusion, in patients with no, one, two, or more DMTs at baseline. In all subgroups, there was a peak of one or two relapses and a similar distribution with a predominance of lower MSSS values. There is no distinct pattern of medication use in the various groups. However, escalation therapy is more often used in patients with a higher number of DMTs.

Visualization of Treatment Pathways

Among the pretreated patients, 214 different treatment sequences were documented. **Table 3** shows the 15 most frequent pretreatments and pathways in descending order.

Duration of previous therapy was reported in 55% of patients. Among these, in <5%, duration was <3 months. **Figure 4** visualizes the main treatment pathways, which finally end up in 623 (pretreated) patients displayed in the blue alemtuzumab column on the right.

Figure 5 shows the same plot in HTML format in which the cursor roll-over the connecting ribbon will indicate the respective number of DMTs and the sequence number before alemtuzumab. The nodes can also be shifted vertically to change the view of the Sankey plot.

Concomitant Diseases

Concomitant diseases at baseline were reported in 30.0% of patients (**Table 4**). The System Organ Classes that were most frequently affected were psychiatric disorders (11.6%), metabolism and nutrition disorders (10.0%), and immune system disorders (4.6%). The latter comprised mostly allergies but also one case of autoimmune disorder. As relevant disease (which prevent therapy as specified in the latest update of the Lemtrada® SmPC in January 2020), thyroid diseases were named in 31 cases, nephropathy in 2 cases, and immune thrombocytopenic purpura (ITP) in 1 case (**Table 5**).

There were no patients with history of angina pectoris, myocardial infarction, or stroke at baseline.

DISCUSSION

The present analysis focused on the detailed characterization of MS patients who, irrespective of the type of prior treatment

TABLE 4 | Concomitant disease by system organ class.

SOC	Total ($N = 883$) *n*	%
Any disease	344	39.0
Blood and lymphatic system disorders	12	1.4
Cardiac disorders	13	1.5
Congenital, familial, and genetic disorders	24	2.7
Ear and labyrinth disorders	5	0.6
Endocrine disorders	18	2.0
Eye disorders	29	3.3
Gastrointestinal disorders	25	2.8
General disorders and administration site conditions	18	2.0
Hepatobiliary disorders	7	0.8
Immune system disorders	41	4.6
Infections and infestations	28	3.2
Injury, poisoning and procedural complications	14	1.6
Investigations	13	1.5
Metabolism and nutrition disorders	88	10.0
Musculoskeletal and connective tissue disorders	43	4.9
Neoplasms benign, malignant, and unspecified	14	1.6
Nervous system disorders	114	12.9
Pregnancy, puerperium, and perinatal conditions	1	0.1
Psychiatric disorders	102	11.6
Renal and urinary disorders	30	3.4
Reproductive system and breast disorders	10	1.1
Respiratory, thoracic, and mediastinal disorders	31	3.5
Skin and subcutaneous tissue disorders	28	3.2
Surgical and medical procedures	28	3.2
Vascular disorders	45	5.1

SOC, system organ class (from the Medical Dictionary for Regulatory Activities).

TABLE 5 | Diseases of particular interest.

Disease	*n*	% Of total ($N = 883$)
Immune thrombocytopenic purpura	1	0.1
Nephropathy	2	0.2
Thyroid diseases		
Hypothyroidism	53	6.2
Hyperthyroidism	10	1.2
Hashimoto's thyroiditis	11	1.3
Graves' disease (Basedow)	2	0.2
Other	12	1.4

Values are n and percentages of total.

and the MS duration, are finally treated with alemtuzumab. The data complement the body of evidence from 1,500 patients that received alemtuzumab in the randomized controlled trials [CAMMS223 (9), CARE-MS I (10), and CARE-MS II (11)].

Compared to the initial alemtuzumab registration studies, the treament landscape and armamentarium of drugs have substantially changed, which needs to be considered in the interpretation of results. Compared with the baseline characteristics from the pivotal CARE-MS I and CARE-MS II trials, patients in TREAT-MS at enrollment had a comparable mean duration of disease since first symptoms (CARE-MS I, 2.1 years; CARE-MS II, 4.5 years; TREAT-MS, 3.4 years), a higher percentage with EDSS score >3 (CARE-MS I, 2%; CARE-MS II, 31%; TREAT-MS, 37%), a higher percentage who received treatment with fingolimod (only introduced in 2011: CARE-MS I and II, 0%; TREAT-MS, 35%) or natalizumab (CARE-MS I, 0%; CARE-MS II, 4%; TREAT-MS, 35%) prior to enrollment. They tended to have similar relapse activity in the 2 years before alemtuzumab treatment initiation. Furthermore, in TREAT-MS, the sex and age distribution at baseline was similar to the two registration studies. Generally, patients with more advanced MS are treated with alemtuzumab under clinical practice conditions in Germany. However, every seventh patient was treatment naive prior to alemtuzumab initiation.

In line with the many treatment options for MS patients available today, a great variety of pretreatment patterns were documented. The Sankey diagram visualizes this diversity, over time and across DMTs. Few typical patterns emerged, with switches from IFN-beta to natalizumab or fingolimod and from natalizumab to fingolimod being the most eminent ones.

The relatively high number of patients recruited from centers in all parts of the country and different types of centers (51.8% resident neurologists, 48.2% from various types and sizes of hospitals) is a strength of the study. It describes "typical" alemtuzumab patients as treated under real-life conditions; however, physicians may have assigned patients to the study based on the severity of their disease, on the observation that they did not respond well to other drugs, or the presence of complex comorbidities. These factors might lead to a non-representative study population.

Based on the assessment of the periodic safety update report (PSUSA) for alemtuzumab, in 2020, contraindications were added to the SmPC, in particular relating to cardiovascular disease (including history of stroke, angina pectoris, and myocardial infraction) and concomitant autoimmune diseases besides MS (32). While no patients had the named cardiovascular disease and only few had autoimmune diseases at baseline, the results of the present cohort will be an important contribution to the alemtuzumab safety database.

In conclusion, the present analysis revealed a broad variety of different treatment sequences before the final switch to alemtuzumab. In comparison to the pivotal phase 2 and 3 studies, RRMS patients starting alemtuzumab treatment had a longer disease duration in real-world conditions.

Recently, a dual-center retrospective study from Germany in 170 patients treated with alemtuzumab (PROGRAM^{MS}) described the pretreatment (35 none, 52 basic, 50 natalizumab, 33 fingolimod) and found differences in treatment responses based on the previous use of DMT (33). In the future, linking treatment sequences or other baseline characteristics with effectiveness and safety outcomes might be useful to support treatment decisions (34, 35).

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethikkommission der Universitätsklinik Dresden. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TZ and UE developed the study design. FH, SR, and RW participated in the design of the study and contributed to the interpretation of results and the manuscript. RW initiated the drafting of the report and wrote the manuscript. All authors read and approved the final version of this manuscript.

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REVIEW
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The Role of Distinct Subsets of Macrophages in the Pathogenesis of MS and the Impact of Different Therapeutic Agents on These Populations

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Multiple sclerosis (MS) is a demyelinating inflammatory disorder of the central nervous system (CNS). Besides the vital role of T cells, other immune cells, including B cells, innate immune cells, and macrophages (MΦs), also play a critical role in MS pathogenesis. Tissue-resident MΦs in the brain's parenchyma, known as microglia and monocyte-derived MΦs, enter into the CNS following alterations in CNS homeostasis that induce inflammatory responses in MS. Although the neuroprotective and anti-inflammatory actions of monocyte-derived MΦs and resident MΦs are required to maintain CNS tolerance, they can release inflammatory cytokines and reactivate primed T cells during neuroinflammation. In the CNS of MS patients, elevated myeloid cells and activated MΦs have been found and associated with demyelination and axonal loss. Thus, according to the role of MΦs in neuroinflammation, they have attracted attention as a therapeutic target. Also, due to their different origin, location, and turnover, other strategies may require to target the various myeloid cell populations. Here we review the role of distinct subsets of MΦs in the pathogenesis of MS and different therapeutic agents that target these cells.

Keywords: multiple sclerosis, macrophages, microglia, therapeutic agents, neuroinflammation

INTRODUCTION

Multiple sclerosis (MS) is a demyelinating inflammatory disorder of the central nervous system (CNS). Neurodegeneration (loss of myelin and axons) in MS is caused by an immune response to self-antigens, interrupting signal transmission (1). MS patients exhibit various clinical symptoms related to the site of lesions and associated with the invasion of inflammatory cells across the blood–brain barrier (BBB). In most patients, the disease begins with a single episode, known as a clinically isolated syndrome (CIS), which might be developed in the future or not (2). Patients with at least two relapses are classified as relapsing-remitting multiple sclerosis (RRMS) that makes up >70% of the MS population. Primary progressive multiple sclerosis (PPMS) is another phenotype that occurs in approximately 10%–15% of individuals, and PPMS patients have no remission after the onset of

disease (3, 4). Within 10–20 years after the disease onset, 60%–70% of RRMS patients develop secondary progressive MS (SPMS) symptoms by steady progression with or without periods of remission (5).

Although the cause of MS is unknown, genetic, epigenetic, and environmental factors have been introduced as the possible risk factors of the disease. Individuals with an inherited HLA-DRB1*15:01 allele and its associated haplotypes (*DQB1**06:02, *DQA1**01:02, *DRB1**15:0, *DRB5**01:01) are more likely to develop MS (6). Also, based on genome-wide association studies (GWAS), HLA locus has related with disease susceptibility in 20%–30% of MS patients (7), while some alleles are associated with resistance to MS. Accordingly, studies have found that HLA-*DRB1**01:01, HLA-*DRB1**09, HLA-*DRB1**11, HLA-*DRB1**12, and HLA-*DRB1**16 alleles play a role in protection against MS (8, 9). Besides, other non-HLA genes such as interleukin (IL)-2RA, IL-7RA, CD58, signal transducer and activator of transcription (STAT)3, interferon regulator factor (IRF)8, and tumor necrosis factor receptor superfamily member 1A (TNFRSF1A) are involved in susceptibility to MS (10).

Environmental risk factors such as low vitamin D levels, smoking, obesity, stress, infections, and immunization have been considered as risk factors for MS development (11).

Experimental autoimmune encephalomyelitis (EAE) is an animal model for MS that is used in experimental studies. Many aspects of the MS pathophysiology, such as inflammation, immune surveillance, immune-mediated tissue injury, and roles of immune cells, have been revealed by using EAE models (12). Also, studies have shown that there is a correlation between EAE and MS therapeutic success. For example, licensed drugs such as disease-modifying therapies (DMTs), interferon (IFN)-beta, glatiramer acetate, and the anti-very late antigen (VLA)-4 antibody (natalizumab), have shown therapeutic efficacy in both MS and EAE (13–18). Therefore, EAE as an appropriate model has contributed to our scientific knowledge of neuroinflammation.

Besides the vital role of T cells, other immune cells, including B cells, innate immune cells, and macrophages (MΦs), also play a critical role in MS pathogenesis (19, 20). MΦs are innate immune phagocytes that detect pathogen-associated molecular pattern (PAMP) and damage-associated molecular pattern (DAMP) molecules. These molecules are expressed by pathogens and apoptotic cells, respectively. MΦs also present antigens to T lymphocytes as an antigen-presenting cell (APC) in adaptive immunity. According to *in vitro* features, MΦs are divided into M1 and M2 phenotypes. This nomenclature primarily represents the state of MΦ's activation and is used to facilitate the description of the inflammatory status; otherwise, their phenotype should be seen as plastic manner (21). *In vitro* exposure of monocytes and MΦs to Th1 cytokines, lipopolysaccharide (LPS), and granulocyte-macrophage colony-stimulating factor (GM-CSF) induces their polarization to inflammatory M1 phenotype. These cells produce high levels of pro-inflammatory cytokines, including TNF-α, IL-6, IL-1β, and inducible nitric oxide synthase (iNOS) (22, 23). M1 MΦs are the first line of defense against intracellular pathogens and control tumor growth. Also, M1 MΦs probably play a role in tissue destruction and autoimmune disorders (24). On the contrary, *in vitro* differentiation of monocyte to M2 MΦs is induced in the presence of Th2 cytokines and other immunomodulatory agents, including macrophage colony-stimulating factor (M-CSF), IL-10, transforming growth factor (TGF)-β, and vitamin D3 (25, 26). Recently, M2 MΦs have been classified into four subgroups including M2a, M2b, M2c, and M2d. Generally, the M2 phenotype has anti-inflammatory characteristics and plays a role in the immune response against parasitic infections, allergic reactions, tissue regeneration, and tumor growth (27).

Recent studies have indicated that MΦs possess distinct metabolic characteristics that correlate with their functional state, known as metabolic reprogramming. In the context of metabolic reprogramming, M1 MΦs express iNOS enzyme to produce nitric oxide (NO) from arginine, present enhanced glycolytic metabolism, pentose phosphate pathway (PPP), fatty acid synthesis (FAS), and impaired Krebs [or tricarboxylic acid (TCA)] cycle and mitochondrial oxidative phosphorylation (OXPHOS). On the other hand, M2 MΦs hydrolyze arginine to ornithine and urea by Arg-1 and are characterized by enhanced OXPHOS, FAS, glutamine metabolism, and decreased PPP. It is noteworthy that the different intracellular metabolic pathways regulate the polarization and function of M1 and M2 MΦs (28–30). For example, in the M1 MΦs, NO and NO-derived reactive nitrogen species inactivate the mitochondrial electron transport chain (ETC) and prevent repolarization to the M2 phenotype. On the contrary, ornithine can further participate in downstream pathways of polyamine and proline synthesis, which have a role in cell proliferation and tissue repair in M2 (31, 32). Also, based on studies, glycolysis may promote the immune function of M1 MΦs by increasing the secretion of inflammatory cytokines and enhancing phagocytic activity (33).

Several subsets of MΦs are present in the CNS. The resident MΦs in the parenchyma are known as microglia. Also, non-parenchymal MΦs are located in the choroid plexus, perivascular space, and meninges. These cells have a critical role in the maintenance of CNS homeostasis (34–36). The other types of MΦs in the CNS are the monocyte-derived MΦs entering the CNS following alteration in CNS homeostasis. This phenomenon is a physiologic mechanism to protect the CNS, resolve abnormalities, and restore homeostasis. Besides the neuroprotective and anti-inflammatory actions of monocyte-derived MΦs and resident MΦs, they can promote neuroinflammation by secretion of inflammatory cytokines and reactivation of primed T cells (37). In EAE, activation of microglia/MΦs leads to disease progression (38). Also, in the CNS of MS patients, elevated myeloid cells and activated MΦs have been found and associated with demyelination and axonal loss (39, 40). According to the role of MΦs in neuroinflammation, they have attracted attention as a therapeutic target. Also, due to their dissimilar origin, location, and turnover, different strategies may require to target the

various myeloid cell populations. As shown in multiple studies, direct targeting of myeloid cells has been shown to be effective in some other inflammatory diseases such as psoriasis, Crohn's disease, and ulcerative colitis by targeting IL-12 and/or IL-23 (41, 42). Although MΦs and their function in neuroinflammation have been described in detail in previous studies, their direct/indirect targeting by therapeutic agents has been less discussed. Here, we review the role of distinct subsets of MΦs in the pathogenesis of MS and the impact of different therapeutic agents on these cells.

THE ROLE OF MICROGLIAL CELLS IN MULTIPLE SCLEROSIS PATHOGENESIS

Microglia are known as one type of glial cells and mononuclear phagocytes. These tissue resident cells are located in the brain and spinal cord. The number and location of these cells vary in different species, and human microglia dominate in white matter compared to gray matter (43). Microglia are developed from erythromyeloid progenitors (EMPs) in the yolk sac during primitive hematopoiesis (44), and their differentiation is regulated by some transcription factors such as IRF8, PU-1, and Runx-1 (45). Colony-stimulating factor 1 receptor (CSFR1) signaling is necessary for the survival of microglia, and its ligands, CSF1 and CD34, are produced in normal CNS (46).

Like MΦs, these immune cells recognize infections, toxins, and injuries (47) and have a role in maintaining homeostasis in the adult CNS (48). Microglia use a specific signature called sensome in the homeostatic condition that scans changes in the CNS. So, they are the first cells that respond to damages in the CNS. Sensomes can recognize microorganisms and endogenous ligands. Some of the sensomes are specific integrins, purinergic receptors, and cluster differentiation (CD) markers, including P2ry12, Tumor Microenvironment of Metastasis 119 (TMEM119), Gpr34, CD33, CXCR4, and CX3CR1 (49). Studies in transgenic animals have shown that the interaction between CX3CR1 on microglia and MΦs with fractalkine (CX3CL1) on neurons leads to the communication between immune and neural systems (50–53). Although microglia phenotype is considered resting or quiescent in stable and normal CNS, they have many functions (47, 54). Resting microglia influence surrounding cells through producing some neurotrophic factors such as insulin-like growth factor-1 (IGF-1), brain-derived neurotrophic factor (BDNF), TGF-β, and nerve growth factor (NGF) (55, 56). In addition, microglia participate in myelin debris removal and modulate neural activity and synaptic organization (57, 58). Moreover, they are involved in oligodendrocyte progenitor cell (OPC) maintenance in the CNS (59, 60) and partake in brain development through clearance of neuronal apoptotic bodies (61, 62). Advanced technologies such as single-cell RNA sequencing (scRNA-seq) and genetic fate mapping have improved the distinguishing of microglia subtype, function, and differentiation ways from MΦs (63).

Jordão et al. (64) have used single-cell sequencing and found that in the homeostatic state, the microglia of EAE mice are distinguished into two subtypes, hMG1 and hMG2, and during inflammation, four populations [disease-associated microglia 1–4 (daMG)] have been observed. Furthermore, the gene profile of daMG demonstrates that they have more potential in chemokine production and subsequently disease progression compared to homeostatic parenchymal microglia (hMG) (64).

Microglia morphology in this situation is known as ramified. On the other hand, they have a long cytoplasmic protrusion for monitoring any changes in the CNS (47). This morphology is similar to the morphology of Langerhans cells in the skin (65). Due to their plasticity, microglia alter their phenotype under different conditions and environmental factors (66–68). They activate in response to the unstable state of the CNS (trauma, ischemia, or any threat in the CNS) and change their phenotype (69, 70). Like other innate immune cells, microglia recognize PAMPs and DAMPs through their pathogen recognition receptors (PRRs) (71–73). In this state, the morphology of activated microglia is known as amoeboid, which refers to cell mobility (74, 75). In addition, these cells are highly potent phagocytic cells that phagocytose dead cells and myelin debris (76).

Microglia, similar to MΦs, show inflammatory and anti-inflammatory (alternatively) phenotypes in *in vitro* studies (77), and M2 phenotype microglia have subgroups including M2a, M2b, and M2c (78, 79). However, scRNA-seq and mass cytometry findings show that microglia phenotype and gene expression patterns are associated with age and regional differences (80).

According to previous findings, microglia's role in MS pathogenesis is still unclear (76). Singh et al. (81) have shown that microglial nodules, which are the clusters of activated microglia, are present in the white matter of MS patients in the vicinity of plaques. They participate in response to axon degeneration and stressed oligodendrocytes (81, 82). Microglia and recruited MΦs display a pro-inflammatory phenotype (M1 microglia) in the early MS and EAE disease stages. According to this phenotype, they have many functions, including oxidative injury, antigen-presenting, and T cell stimulating (76, 83). MΦs and dendritic cells have more antigen presentation capacity to T cells than microglia in the early phases of EAE, but during inflammation, microglia express major histocompatibility complex (MHC)-II and costimulatory molecules that can stimulate T cells, so they act like an APC. Despite this ability, new MHC-II gene deletion experiments in microglia indicate that this population has no critical roles in EAE onset and progression (80, 84).

Furthermore, oxidative damage, which is mediated by reactive oxygen species (ROS), induces demyelination (85, 86). Many studies have indicated that innate immune-mediated oxidative injury (by activated microglia and other immune cells) has been proposed as an essential process underlying the progression of MS (87, 88).

Following scRNA-seq, Mendiola et al. (87) have demonstrated that in EAE, microglia are divided into five clusters according to the expression of genes involved in oxidative stress and Ag presentation. For example, cytochrome

b-245 beta chain (Cybb), which encodes the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit, histocompatibility 2, class II antigen, and beta1 (H2-Ab1) gene that participate in MHC-II expression are microglial clusters during oxidative stress and Ag presentation. Also, the ability of different clusters of microglia varies in oxidative damage and antigen presenting. So, the MgV cluster is more involved in the oxidative injury, while the MgIII cluster is enriched for Ag presentation (87).

Activation of microglia also induces the expression of different transcription factors such as nuclear factor (NF)-κB, Janus kinase (JAK)/STAT, c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK)1/2, and p38. Moreover, different cytokines, including IL-6, IL-8, IL-12, IL-23, IL-1β, and TNF, are produced after microglia activation (89, 90). In addition, the induction of chemokines such as CCL2, CCL3, and CCL4 also is induced by activated microglia, which can facilitate leukocyte recruitment in the early phase of EAE (91) (**Figure 1**).

Oxidative processes and pro-inflammatory cytokines result in injury to oligodendrocytes (76). Heppner et al. (92) have shown that microglia paralysis of transgenic mice ameliorates

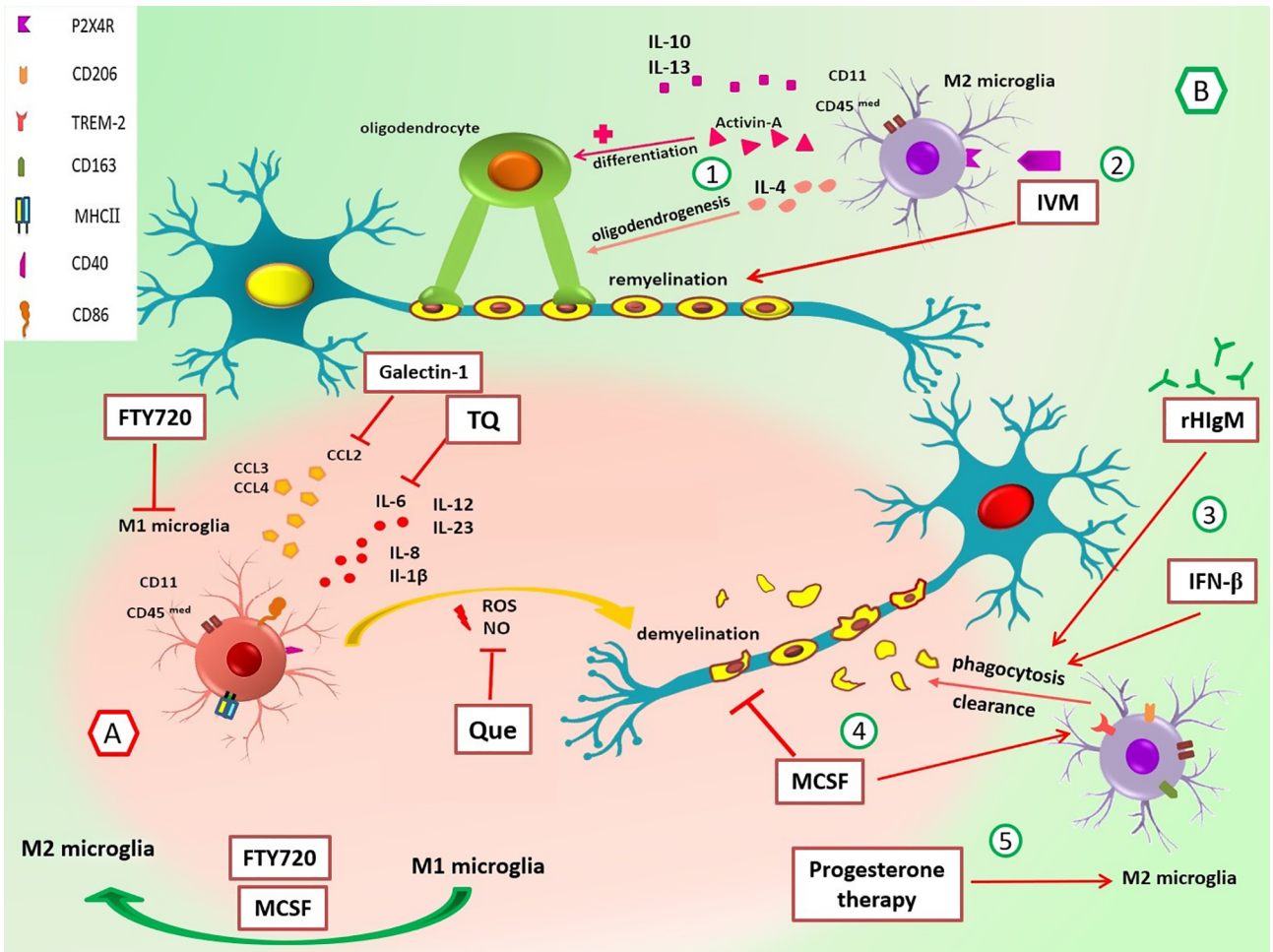

FIGURE 1 | Roles of M1/M2 microglia and the effect of different drugs on these cells in multiple sclerosis. **(A)** In the early stage of EAE and MS, M1 MG have different roles in the promotion of inflammation through cytokine/chemokine release, and ROS and NO production leads to demyelination. Suppression of M1 MG and their functions can be useful in MS control. For example, galectin-1 decreases the production of CCL2, TQ reduces IL-6, Que decreases the release of NO, and FTY-720 suppresses MG activation and switches M1 to M2 phenotype. **(B)** M2 MG have anti-inflammatory functions and promote remyelination *via* cytokines release and phagocytosis of myelin debris. 1) IL-4 promotes oligodendrogenesis, and activin-A helps differentiation of oligodendrocytes. 2) IVM interacts with P2X4R and enhances phagocytosis and remyelination. 3) rHIgM22 and rIFN-β promote phagocytosis and myelin uptake. 4) M-CSF increases the expression of TREM2 mRNA, diminishes demyelination, and improves the organization of myelin sheaths through polarization of M1 MG to M2 MG. 5) Progesterone therapy increases marker expression of M2 phenotype (CD206). CCL, chemokine (C-C motif) ligand; CD40, cluster of differentiation 40; CD86, cluster of differentiation 86; CD163, cluster of differentiation 163; CD206, cluster of differentiation 206; EAE, experimental autoimmune encephalomyelitis; FTY720, fingolimod; IL, interleukin; IVM, ivermectin; M-CSF, macrophage colony-stimulating factor; MG, microglia; MHC-II, major histocompatibility complex class II; NO, nitric oxide; P2X4R, P2X4 receptor; Que, quetiapine; rHIgM22, recombinant human IgM; rIFN-β, recombinant interferon-beta; ROS, reactive oxygen species; TQ, thymoquinone; TREM-2, triggering receptors on myeloid cells-2.

inflammation in the CNS and improves clinical symptoms of the disease. Also, Bhasin et al. (93) have demonstrated that microglia inhibition at the onset of EAE attenuates disease signs and decreases EAE progression.

Autophagy is a conserved homeostatic pathway in eukaryotic cells, which has recently become evident in neurodegenerative disorders (94). There is a consideration that autophagy is associated with the regulation of inflammation in microglia during neuroinflammation (95). Many studies revealed that following autophagy induction in inflammatory microglia, the expression of inflammatory genes is suppressed and anti-inflammatory phenotype is promoted (96–98). In EAE mice, induction of autophagy leads to inflammasome inhibition and attenuation of symptoms (99). Also, Atg5 knockdown in microglia leads to more neuroinflammation in cell culture (98, 100). Moreover, ATG is involved in remyelination and debris cleaning in microglia (101, 102).

The ratio of M1/M2 is an essential factor in the relapse of EAE, and M1 microglia is more than M2 in the early phase of repair. Environmental changes can shift phenotype; however, underlying mechanisms responsible for this switch are unknown (83, 103, 104). M2 microglia play an essential role in the recruitment and differentiation of oligodendrocyte progenitor cells (OPCs) through the clearance of myelin debris. An *in vitro* study has shown that M2 cell medium inhibits OPC apoptosis even in the absence of serum and growth factors. Also, evaluation of myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) reveals that M2 microglia promote oligodendrocyte differentiation (103, 105). M2-produced anti-inflammatory cytokines (IL-4, IL-10, and IL-13) and substances such as activin-A are involved in differentiation of oligodendrocyte during remyelination (103, 104, 106, 107) (**Figure 1**). Also, Miron et al. (103) have indicated that blocking antibodies against M2 cell-derived activin-A diminishes oligodendrocyte differentiation.

Furthermore, anti-inflammatory cytokines such as IL-4 promote oligodendrogenesis; thus, it is helpful for remyelination (108). In contrast, the protective function of TNF as a pro-inflammatory cytokine has been shown in EAE (109). Accordingly, transmembrane TNF (tmTNF) and TNFR2 induce remyelination in EAE, while soluble TNF (solTNF) suppressed phagocytosis of myelin debris and thus inhibited remyelination in the cuprizone demyelination model (110).

MicroRNAs (miRNAs) are a group of small non-protein-coding RNAs, which have a role in biological functions through the regulation of gene expression. Different miRNAs can affect microglia and MΦ functions. Mir-124 is a specific miRNA in the brain and plays a role in CNS development and neurogenesis of adults (111, 112). Mir-124 is highly expressed in microglia compared to other cells and can maintain the resting phenotype of microglia. In experimental studies, no evidence of microglial activation has been shown in EAE mice treated with mir-124. Ponomarev et al. (112) have found that transfection of bone marrow-derived macrophages (BMDMs) with mir-124 induced downregulation of markers such as CD45 and CD11b, suppressed the expression of TNF-α and iNOS, and increased the expression of anti-inflammatory cytokine TGF-β. Moreover, they have indicated that inflammatory responses and EAE symptoms were alleviated in treated mice (112).

Long intergenic noncoding RNA (lincRNA)-Cox2 belongs to long noncoding RNA and can regulate immune functions. LincRNA-Cox2 plays a role in inflammatory responses through binding to the p65 subunit of NF-κB and modulating NLRp3 and Asc expression. Xue et al. (113) showed that knockdown of lincRNA-Cox2 promoted resting microglia (CD11b+ CD45med) and suppressed IL-1β secretion. Also, lincRNA-Cox2 silencing inhibited NLRP3 inflammasome activation and thereby promoted autophagy in BMDMs and microglia. Moreover, knockdown of lincRNA-Cox2 in EAE models decreased inflammatory cells in the white matter and improved EAE symptoms.

Collectively, evidence indicates that activated microglia act as a double-edged sword in MS pathogenesis (38). So, targeting microglia activation and inducing a shift to M2 phenotype would be a promising choice in the future of MS treatment.

MICROGLIA AND MACROPHAGES MARKERS

Resting microglia do not highly express MHC-II and costimulatory molecules, so they cannot prime T cells (114, 115). The expression of surface markers changes following activation of microglia. For example, myeloid marker expression and adenosine A2A receptors are upregulated during their activation, while P2Y12 receptors are downregulated (54, 116, 117). Also, MHC expression and costimulatory molecules such as CD80, CD86, and CD40 have been increased after microglia activation in EAE (106, 118, 119). However, specific deletion of MHC-II in microglia does not promote disease progression, so microglia is not enough to stimulate autoreactive T cells (120). Moreover, studies have shown that microglia are impaired APCs despite their ability to uptake myelin (121, 122).

In the inflammatory state, microglia express p22phox, CD68, CD86, and MHC-II antigens, while in the inactive lesion, they mostly express anti-inflammatory markers including CD206, CD163, and ferritin (76, 123). Efficient myelin debris removal and clearance by phagocytosis is an essential step in effective remyelination, and the surface expression of triggering receptors on myeloid cells-2 (TREM2) plays a key role in phagocytosis (124). Piccio et al. (125) have indicated that the expression of TREM2 on microglia is increased during EAE, and blocking of this receptor with mouse monoclonal antibody is accompanied by cellular infiltration and EAE exacerbation. Also, other molecules such as complement receptor 3 (CR3), signal regulatory protein (SIRP), IFN-β, and transmembrane TNF (tmTNF) participate in this process (106, 110, 126). Discriminating microglia from MΦs is challenging; however, some markers such as CD45 and CD11b have been introduced as differential markers.

According to this classification, CD11b+ CD45med cells are microglia, and CD11b+CD45hi cells are MΦs (127); however, this classification is controversial, and the expression of some markers such as CD45 changes under different conditions (127–130). Furthermore, there are more reliable differential markers, including TMEM119, Sal-like1 (Sall1), sialic acid-binding Ig-type lectin H (Siglec-H), and P2Y12R (76, 131–136).

TMEM119 is a cell-surface protein that is highly expressed on human and mouse microglia. This protein indicates a highly conserved sequence and does not express on MΦs and immature microglia; however, its function is still unknown (131). The purinergic receptor (P2Y12) directs microglia movement toward damage sites (137). The other molecule, Sall1, which is a transcriptional regulator, plays a role in microglia morphology and gene expression (134). Siglec-H is mainly expressed on microglia in mice, but the homology of human Siglec-L2 with Siglec-H is approximately 40% (138, 139).

MONOCYTE-DERIVED MACROPHAGES IN THE CENTRAL NERVOUS SYSTEM

Peripheral blood monocytes are derived from bone marrow hematopoietic stem cells (HSCs) and defined as classical (CD14+CD16-), non-classical (CD14lowCD16+), and intermediate monocytes. However, there are few infiltrating monocytes in the CNS under physiological conditions. Also, substantial accumulation of monocytes, predominantly non-classic CD16+, in both gray and white matter MS lesions is significant, especially during disease relapses (140, 141). During the effector stage of EAE, monocytes rapidly infiltrate surrounding meninges, perivascular space, and choroid plexus through and differentiate into MΦs (142, 143). These MΦs contribute to the progression of the paralytic stage of EAE and demyelination by expressing MHC-II, costimulatory molecules, and producing pro-inflammatory factors (38). Thus, in EAE, MΦ depletion is associated with a lower CNS injury and attenuated signs and symptoms of disease (144, 145).

Expression of cell adhesion molecules such as intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and activated leukocyte cell adhesion molecule (ALCAM) by CNS endothelial cells and their interaction with integrins like leukocyte function-associated antigen [(LFA)-1, αLβ2], VLA-4 (α4β1), and CD6 are essential steps of immune cell migration into the CNS (146). Nerve injury-induced protein (Ninjurin)-1 and junctional adhesion molecule-like (JAML) are other adhesion molecules involved in monocyte-derived MΦ migration (147, 148).

Moreover, CCR2 is a crucial chemokine receptor in the recruitment of Ly6Chigh monocytes to the inflamed CNS, which exacerbates disease progression in the EAE model. So that mice without CCR2 are resistant to EAE induction (149). Besides, CCR4, a chemokine receptor for CCL17 and CCL22, is upregulated in MΦs of CNS lesions, and interestingly, mice lacking CCR4 have also been reported to be resistant to EAE (150).

Both M1 and M2 MΦs are detected in MS lesions, and they may repolarize to apposite phenotype depending on the local environment and stage of disease. According to The study by Vogel et al. in active and chronic active MS lesions, the expression of typical M1 markers is higher than M2 markers (151). Also, in EAE, both M1 and M2 MΦs enhance and regulate the disease's pathogenesis (152, 153).

During MS, M1 MΦs secrete high amounts of pro-inflammatory agents such as IL-6, IL-12, IL-1, TNF-α, IL-23, reactive oxygen species, and nitrogen species and CCL4, CCL5, CCL8, CXCL9, CXCL10, and CXCL2. This condition leads to the recruitment of immune cells, exacerbating neuroinflammation and tissue damage (142). IL-6 is a crucial cytokine in CNS autoimmunity establishment, as IL-6-deficient mice have shown attenuated EAE symptoms. Furthermore, IL-1β has been considered as an inducer of Th17 polarization and EAE progression (154). Recent research on bone marrow chimeric mice has revealed that monocyte-derived MΦs express TRPM2 protein and subsequently produce CXCL2, leading to enhanced neutrophil infiltration and EAE progression (155). Studies on brain autopsy of MS patients have shown that M1 MΦs express CD68 (as a phagocytosis marker), HLA, and CD86, which contribute to antigen-presenting to primed T cells. Also, iNOS has increased in M1 MΦs. iNOS enzyme and nitric oxide production have an important impact on microglia activation, BBB disruption, demyelination, oligodendrocyte injury, axonal degeneration, and axonal conduction impairment (76, 156). According to single-cell oxidative stress transcriptome analysis of CNS innate immunity in EAE, similar to microglia, seven monocyte/MΦ clusters (MpI–VII) have been identified, which have different potentials in ROS production and Ag presentation. Regarding the results, Clusters MpI and MpII had increased Cybb and H2-Ab1 expression, whereas clusters MpIII and MpIV had only high expression of H2-Ab1 and are more potent in Ag presentation (87). So, according to previous studies, the M1 MΦs are generally considered harmful in MS (**Figure 2**).

On the other hand, studies have demonstrated the neuron-protective activities of MΦs in EAE. High levels of M1 MΦ infiltration present in the CNS during exacerbations of disease in mice, but a gradual increase in M2 MΦs is associated with improved neurological impairment (157). The increase in the expression of tissue transglutaminase (TG2) mRNA level in monocytes derived from MS patients indicates anti-inflammatory MΦs and subsequently immunomodulatory cytokines (158). M2 MΦs cause an anti-inflammatory state and tissue repair by secreting IL-4, IL-10, IL-13, and TGF-β cytokines. These cells also drive the recruitment and differentiation of Th2 and regulatory T cells (Treg), which suppress the inflammatory response in EAE mice (159).

Moreover, M2 MΦs express scavenger receptors to clear myelin debris in the damaged spinal cord, promoting CNS repair (160). These populations of MΦs can produce neurotrophic factors, including IGF-1, BDNF, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and IL-1 receptor antagonist that leads to alleviate sympathetic neuron dysfunction (161). Also, they block the iNOS enzyme to decrease inflammation, increase environment

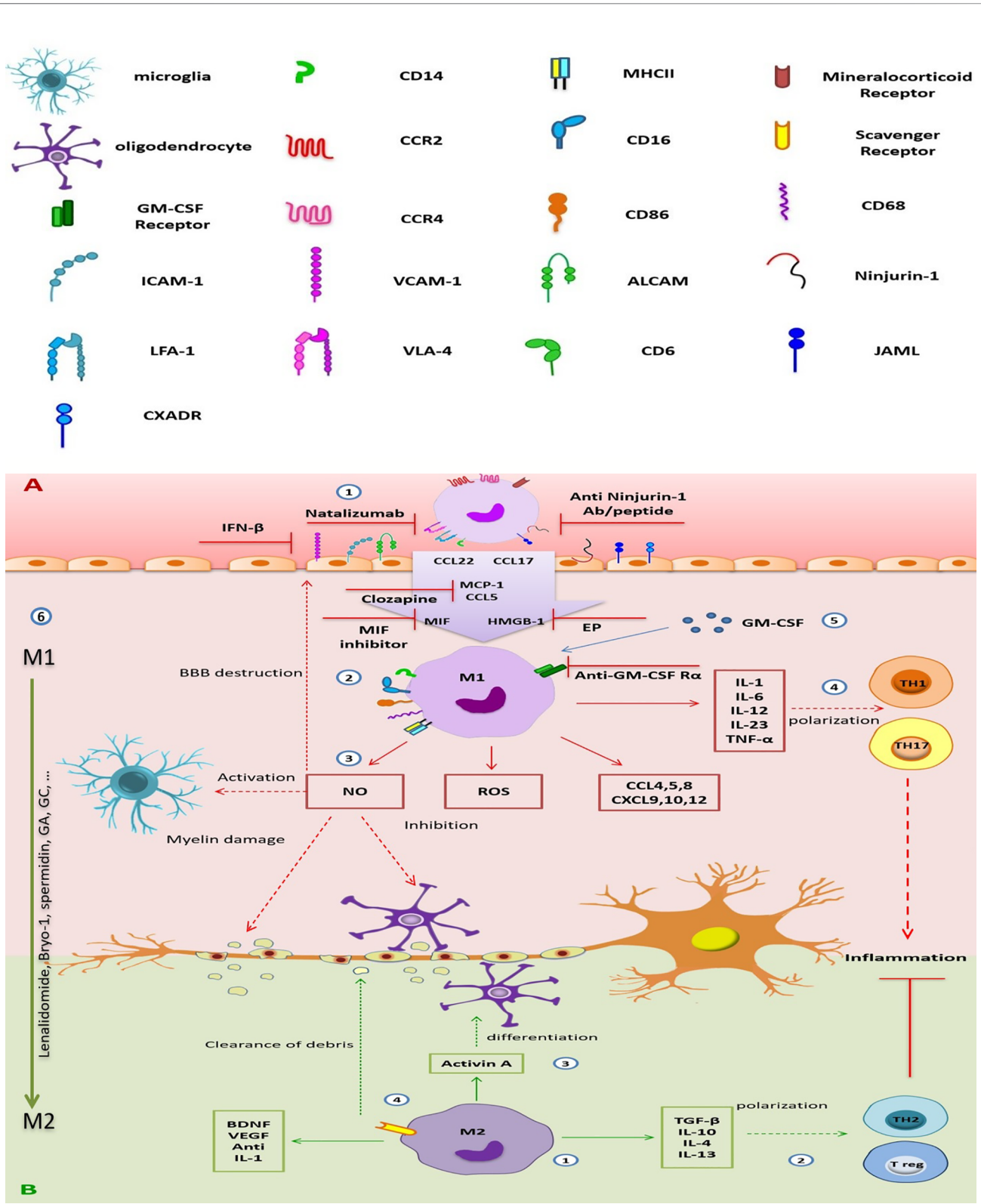

FIGURE 2 | Continued

FIGURE 2 | The destructive/regenerative roles of M1/M2 macrophages in multiple sclerosis and possible treatments. **(A)** 1) Peripheral blood monocytes enter the inflamed CNS following the attachment to adhesion molecules (e.g., the interaction of ICAM with LFA-1, VCAM-1 with VLA-4, ALCAM with CD6, homophilic interaction of ninjurin-1 and JAML with themselves, and also JAML with the other receptor CXADR), the concentration gradient of chemokines [CCL5, CCL17, CCL22, and MCP-1(CCL2)], MIF, and HMGB-1 through damaged BBB, and differentiated into monocyte-d MΦs. Inhibition of adhesion molecules (e.g., ICAM-1 and VCAM-1 by IFN-β, VLA-4 by natalizumab, and ninjurin-1 by anti-ninjurin-1 blockade), receptors (e.g., MR), or chemokines and other stimulators (e.g., MCP-1 by clozapine or HMGB-1 by EP), which is involved in monocyte migration could be a therapeutic approach. 2) M1 MΦs (CD86+, CD68+, MHC-II+) are the dominant subpopulation of monocyte-d MΦs. They enhance CNS inflammation by producing pro-inflammatory cytokines, chemokines, ROS, and NO. 3) NO production leads to increase BBB destruction, microglial activation, myelin damage, and inhibits oligodendrocyte function. 4) Pro-inflammatory cytokines are involved in TH1 and TH17 polarization, enhancing neuroinflammation. 5) GM-SCF is essential for differentiation and function of M1 MΦs, so, GM-CSFR blockade can improve inflammation. 6) Repolarization of inflammatory M1 MΦs into anti-inflammatory M2 phenotype could be a good choice for MS treatment. **(B)** 1) A smaller population of monocyte-d MΦs is M2 MΦ with anti-inflammatory phenotype. It secretes immunomodulatory cytokines, chemokines, and tissue regenerative agents. 2) Anti-inflammatory cytokines induce the polarization of TH2 and Treg cells, which suppress neuroinflammation. 3) Secreted activin A leads to oligodendrocyte differentiation. 4) Expression of scavenger receptor is involved in cleaning the myelin debris. CNS, central nervous system; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; ALCAM, activated leukocyte cell adhesion molecule; LFA-1, leukocyte function-associated antigen-1; VLA-4, very late antigen-4; CD6, cluster of differentiation 6; ninjurin-1, nerve injury-induced protein-1; JAML, junctional adhesion molecule-like; CCL2, chemokine (C-C motif) ligand 2; CCL17, chemokine (C-C motif) ligand 17; CCL22, chemokine (C-C motif) ligand 22; CCL3, chemokine (C-C motif) ligand 3; CCL4, chemokine (C-C motif) ligand 4; CCL5, chemokine (C-C motif) ligand 5; CCL8, chemokine (C-C motif) ligand 8; CXCL9, chemokine (C-X-C motif) ligand 9; CXCL10, chemokine (C-X-C motif) ligand 10; CXCL12, chemokine (C-X-C motif) ligand 12; MCP-1, monocyte chemoattractant protein-1; MIF, macrophage migration inhibitory factor; HMGB-1, high-mobility group box-1; BBB, blood–brain barrier; MΦ, macrophage; MR, mineralocorticoid receptor; CD86, cluster of differentiation 86; CD68, cluster of differentiation 68; MHC-II, major histocompatibility complex class II; ROS, reactive oxygen species; NO, nitric oxide; TH1, T helper type 1; TH17, T helper type 17; TH2, T helper type 2; Treg, regulatory T cell; IL-1, interleukin-1; IL-6, interleukin-6; IL-12, interleukin-12; IL-23, interleukin-23; IL-10, interleukin-10; IL-4, interleukin-4; IL-13, interleukin-13; TNF-α, tumor necrosis factor-alpha; TGF-β, transforming growth factor-beta; GA, glatiramer acetate; EP, ethyl pyruvate; GM-CSFR, granulocyte-macrophage colony-stimulating factor receptor; VEGF, vascular endothelial growth factor.

stability, and protect neural cells against injury (162) (**Figure 2**). In summary, M2 MΦs dominantly play a role in suppressing inflammation and promoting tissue regeneration. However, the dichotomy of MΦ polarization is not accurate. Accordingly, in the active MS lesion, the presence of MΦs with an intermediate phenotype, co-expressed M1 and M2 markers, has been confirmed. So, it seems that MΦ phenotype and function are influenced by environmental conditions (151). In the following, we will discuss the effects of different therapeutic agents on MΦs and microglia in the CNS of MS patients.

Actual Therapeutic Approaches That Affect Macrophages and Microglia Population in Multiple Sclerosis

DMTs are a group of drugs that reduce the early clinical and subclinical disease activity that may contribute to long-term disability. More than 10 Food and Drug Administration (FDA)-approved DMTs target the immune-mediated disease process and differ in routes of administration in addition to their frequencies (163). Generally, T cells and B cells are most frequently discussed as targets of DMTs, but some of the current MS disease-modifying therapies also affect myeloid cells, although these cells are not the main target of the drug (164). The probable effects of DMTs on microglia and monocyte-derived MΦs have been shown in **Table 1**, and some of them are discussed below:

IFN-β is a member of the human type I interferons family that has different roles in the regulation of the immune system, including the decrease of tissue damage and inflammation through downregulation of matrix metalloproteinase 9 (MMP-9), inhibition of effector cell migration by downregulating the adhesion molecule VLA-4, and prevention of T-cell proliferation (196–198). Besides, IFN-β decreases cell migration to the CNS through CCR7 inhibition and reduces pro-inflammatory cytokines such as IL-12 in monocytes (199, 200).

This cytokine is the first FDA-approved drug used in the treatment of RRMS to reduce relapses and severity of MS disease due to its various immunomodulatory properties and several actions on immune cells (201, 202).

Kocur et al. (126) have found that IFN-β-treated microglia accumulate in areas containing myelin debris for phagocytosis. Moreover, adult wild-type and IFN-β−/− mice microglia and BV2 microglia in culture media promote phagocytosis of myelin debris after treatment with recombinant IFN-β (rIFN-β), while IFNAR1 −/− microglia show a bit of a promotion. Therefore, IFN-β and IFNAR1signaling are necessary to stimulate microglial phagocytosis of myelin debris (126) (**Figure 1**). Another study by Floris et al. (169) in IFN-β-treated EAE animals has shown reduced clinical score and improved disease symptoms. Furthermore, they have found that following this treatment, expressions of ICAM-1 and VCAM-1 were reduced in the CNS endothelial cells, leading to the subsequent reduction in monocyte-derived MΦ migration into the inflamed CNS (169) (**Figure 2**).

The other therapeutic agent, glatiramer acetate (GA, Copolymer-1, Copaxone), is a drug that affects MΦs. It is prescribed in RRMS, and its clinical effects have been indicated in both MS and MS models (203). Weber et al. have addressed one of the immunological mechanisms of GA treatment in EAE mice. They have found that GA can develop anti-inflammatory type II monocyte polarization with an increase in the production of IL-10 and TGF-β. It also decreases the secretion of IL-12 and TNF-α and the expression of CD40 and CD80. Furthermore, GA-treated type II monocytes can reverse clinical EAE, accompanied by a reduction in the number of CNS lesions. This GA mechanism has shown the importance of type II monocytes in the future of drug intervention in MS (175) (**Figure 2**).

Fingolimod (FTY720) is an FDA-approved drug for the treatment of RRMS. It is a high-affinity agonist of sphingosine-1-phosphate (S1P) receptor, with an immunosuppressive effect. Qin et al. (177) have reported that fingolimod (FTY720) suppresses microglial activation (fewer Iba-1+ or CD68+ microglia) and attenuates neuroinflammation in a mouse model of white matter (WM) ischemic damage caused by chronic hypoperfusion. It

TABLE 1 | Probable effects of disease-modifying therapies (DMTs) on microglia and/or monocyte-derived macrophages.

DMTs	Definition	Effect on microglia and/or monocyte-derived macrophages
Interferon-β	Cytokine released by host cell in response to viral infection and regulating immune responses (165)	- In MS patients, induces anti-inflammatory phenotype by reducing the production of nitric oxide and in contrast, increasing the expression of BDNF and Ig like transcript-3 in monocyte-derived MΦs (166–168). Inhibits infiltration of monocyte-derived MΦs into the CNS (169). - In EAE, upregulating IL-27 expression in monocyte-derived MΦs leads to Th17 suppression (170) - *In vitro*, promotes phagocytosis capacity of microglia (126).
Glatiramer acetate	Synthetic amino acid polymer (15, 171)	- In MS, induces anti-inflammatory phenotype by inhibiting the production of nitric oxide in both microglia and monocyte-derived MΦs. Enhances the phagocytic activity of microglia and monocyte-derived MΦs (172, 173). Decreases microglial activation (174). - In EAE, promotes anti-inflammatory phenotype in monocyte-derived MΦs by increasing the production of IL-10 and TGF-β and decreasing the secretion of pro-inflammatory cytokines and the expression of adhesion molecules (175). - *In vitro*, increases the production of IL-10 and reduces TNF-α in microglia (176).
Fingolimod	Agonist of sphingosine-1-phosphate (S1P) receptor (177)	- In MS, induces anti-inflammatory phenotype by inhibiting the production of pro-inflammatory cytokines and expression of pro-inflammatory miR-155 in monocyte-derived MΦs. (116/1, 164.167/2) - In EAE, decreases CD40 expression and production of TNF in monocyte-derived MΦs (178). - *In vitro*, switches M1 microglia to M2 phenotype (177).
Natalizumab	Anti-VLA-4 humanized monoclonal antibody (18)	- In MS, reduces microglia activation (179). - In EAE, suppresses the activated microglia and monocyte-derived MΦs (180).
Dimethyl Fumarate	Methyl ester of fumaric acid (181)	- In MS, decreases the expression of pro-inflammatory mir-155 in monocyte-derived MΦs (182). - In EAE, reduces the infiltration of monocyte-derived MΦs in to the CNS (183) - *In vitro*, induces anti-inflammatory phenotype by inhibiting the production of nitric oxide and pro-inflammatory cytokines in microglia (184).
Teriflunomide	A reversible inhibitor of mitochondrial enzyme dihydrooratate dehydrogenase (DHODH) (185)	- In MS, induces anti-inflammatory phenotype by increasing the production of IL-10 and PDL-1 expression (186). - In EAE, inhibits the migration of monocyte-derived MΦs in to the CNS (187, 188). - *In vitro*, induces anti-inflammatory phenotype in microglia by increasing IL-10 production (187, 189).
Rituximab	Chimeric Anti-CD20 monoclonal Ab (190)	- Inhibits monocyte activation by depleting GM-CSF expressing memory B cells (190).
Mitoxantrone	Cytotoxic agent of the anthracenedion family (191)	- *In vitro*, reduces migration capacity of monocytes (192).
Siponimod	Selective sphingosine-1-phosphate receptor modulator (193)	- In EAE, reduces the production of IL-6 and CCL5 in activated microglia (194) - *In vitro*, inhibits IL-6 production in siponimod-treated microglia (193).
Cladribine	Chlorodeoxyadenosine (CdA), is purine nucleoside analog (195)	- *In vitro*, inhibits the proliferation of microglia. Induces apoptosis in microglia (195)

MS, Multiple sclerosis, BDNF, Brain-derived neurotrophic factor; Ig, Immunoglobulin; CNS, Central nervous system; EAE, Experimental autoimmune encephalomyelitis; TH17, T helper type 17; IL-27, Interleukin-27; IL-10, Interleukin-10; TGF-β, Transforming growth factor-beta; TNF-α, Tumor necrosis factor-alpha; miR-155, microRNA-155; CD-40, Cluster of differentiation 40; VLA-4, Very late antigen-4; PD-L1, Programmed death-ligand 1; CD-20, Cluster of differentiation20; Ab, Antibody; GM-CSF, Granulocyte-macrophage colony-stimulating factor; IL-6, Inreleukin-6; CCL5, chemokine (C-C motif) ligand 5.

switches microglial polarization from M1 to M2 phenotype in WM ischemic injury through activating STAT3 (177). Furthermore, other studies have shown that fingolimod influences MΦs, and monocytes induce switching to M2 phenotype in culture and decrease IL-12 production (199) (**Figure 1**).

Another DMT is natalizumab, a humanized monoclonal antibody used in the treatment of RRMS and reduces relapse rate and axonal damage. This Ab binds to α4 subunit of α4β7 integrin, and actually, it can inhibit adhesion molecule VLA-4, which has a role in the pathogenesis of EAE and MS (18). Mindur et al. (180) have shown that natalizumab can suppress the activated microglia and MΦs in the onset of EAE. Also, studies have demonstrated that monocyte-derived MΦs entered into the CNS using VLA-4 so that anti-VLA-4 may decrease MΦ infiltration to the CNS (180) (**Figure 2**). Moreover, Sucksdorff et al. (179) have reported that natalizumab can decrease microglia activation in normal-appearing white matter and at chronic active lesions of MS patients' brains. In another study, Öhrfelt et al. (204) indicated that the CSF-soluble TREM2, a marker of microglial activation, is reduced to baseline levels in MS patients following treatment with natalizumab. But the exact effect of this Ab on microglia is not understood (204).

Promising Therapeutic Approaches That Affect Macrophages and Microglia Population in Multiple Sclerosis

Inhibition of Migration and Infiltration of Immune Cells to the Central Nervous System

Nerve injury-induced protein-1 (ninjurin-1) is a cell surface protein that is found in many tissues such as CNS vascular endothelial cells and leukocytes (remarkably in monocytes), leading to an interaction between these cells in a homophilic manner (205). As Ifergan et al. showed, the expression of ninjurin-1 was upregulated in inflammatory APCs in the CNS of EAE mice and in MS lesions. So, it is associated with the

migration of monocytes across the brain endothelium. Furthermore, this group found that blockade of ninjurin-1 with either the Ab or the peptide resulted in alleviating EAE symptoms and reducing demyelination and immune cell infiltration in mice (147). According to this result, ninjurin-1 targeting may be helpful in MS treatment (**Figure 2**).

The chemokines, including monocyte chemoattractant protein 1 (MCP-1 or CCL2) and CCL5 or RANTES (Regulated on Activation, Normal T cell Expressed and Secreted), are expressed by different cell types in the CNS and secreted by infiltrating blood-derived MΦs following their infiltration into the CNS. These chemokines are associated with acute symptoms of CNS disease in rats and mice (206, 207). Recently, Robichon et al. (208) have treated EAE mice with clozapine, an atypical antipsychotic agent and can cross the BBB (209). They have indicated that clozapine reduces the infiltration of monocytes, neutrophils, and T cells by decreasing the expression of CCL2 and CCL5 in the CNS. This agent also directly upregulates cyclic AMP in immune cells, which leads to alteration of CCL5 and CCL2-mediated signaling pathways and inhibition of migration. As CCL2 and CCL5 are involved in MΦ migration and regulation in EAE, drugs such as clozapine that target CCL2 and CCL5 expression should be considered in future studies (208) (**Figure 2**).

Ethyl pyruvate (EP) is the other compound, a redox analog of dimethyl fumarate (Tecfidera). In a study, Djedović et al. (210) have shown that EP decreases the EAE symptoms at the time of disease peak by inhibiting high-mobility group box 1 protein (HMGB1) in ED1+ and Iba1+ reactive microglia. This effect is induced by reducing the degeneration of axons (210, 211) (**Figure 2**).

Also, mineralocorticoid receptor (MR or NR3C2) has immunoregulatory effects and plays an important role in developing the polarization of myeloid cells toward the inflammatory M1 phenotype (212). Montes-Cobos et al. (213) have deleted the expression of this receptor in myeloid cells in EAE mice (MrlysM Mice) and showed that it is accompanied by reducing neuroinflammation and frequency of inflammatory monocytes and microglia (CD45high CD11bhigh Ly6Chigh) in the CNS. Also, the onset of the disease in MrlysM Mice and control populations was similar, but in the mutant mice, in the chronic phase of the disease, the severity has been significantly reduced. Based on these results, blockade of MR by different drugs has the potential improvement effects in MS disease (213) (**Figure 2**).

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine that is associated with various inflammatory diseases. Its elevation has been identified in the CSF of patients during a relapse of MS (214). Kithcart et al. have observed that administering an MIF inhibitor to C57Bl/6 mice protects them from EAE. Furthermore, they have found little or no infiltration of MΦs in the spinal cord. They also have found that MIF-deficient C57Bl/6 mice have significantly fewer severe clinical signs of disease during both the acute and chronic phases of the disease. Therefore, MIF inhibitors or MIF deletion could be a novel therapeutic option for MS treatment (215) (**Figure 2**).

Targeting the Activation and Function of Microglia and Macrophages

Galectin-1 is a family of endogenous lectins encoded by the Lgals1 gene. Starossom et al. (216) have found that recombinant galectin-1 decreases surface expression of MHC-II, CD86, and iNOS mRNA in microglial cells *in vitro*. Galectin-1 also diminishes the production of TNF and CCL2 levels in IFN-γ-polarized M1 microglial cells. Moreover, induction of EAE in Gal1-deficient (Lgals1-/-) mice has led to an increase in Iba+ MHC-II+ microglial cells and axonal loss and a decrease in axonal outgrowth during autoimmune neuroinflammation. Interestingly, the adoptive transfer of Gal1-secreting astrocytes to these mice has suppressed EAE by inhibiting microglia (216). So, galectin-1 is a critical molecule in the regulation of microglia and can be considered in treating neuroinflammation diseases (**Figure 1**).

Quetiapine (Que) is an atypical antipsychotic drug (APD), and previous studies have indicated that APDs influence activated microglia through the reduction of TNF-α and nitric oxide (NO) production (217–219). Que regulates immune responses in EAE by suppressing the release of pro-inflammatory factors from activated microglia (218, 220).

Wang et al. (221) have used long-term Cuprizone-treated mice (mimics the chronic phase of neuroinflammation disease) and treated them with Que. They have found Que inhibits the activation of microglia/MΦs in corpus callosum lesions. Also, pretreatment with Que inhibits the translocation of NF-κB p65 subunits and Ca2+ elevation by reducing the upregulation of STIM1 and modulation of store-operated Ca2+ entry (SOCE). Since the Ca2+ signaling pathway is significant for microglial activation (221), Que probably influences these cells through the above mechanisms. Thus, Que and other drugs that affect calcium channels and regulate microglial activity could be incorporated into new research (**Figure 1**).

Moreover, thymoquinone (TQ), which is extracted from the *Nigella sativa* plant seed oil, has reduced inflammatory cytokines such as IL-2, IL-4, IL-6, IL-10, and IL-17a in LPS/IFN-γ-activated microglia. In addition, it has downregulated several NF-κB signaling target genes, including IL6, complement factor B (CFB), CXCL3, and CCL5. Furthermore, TQ treatment has increased neuroprotective protein expression in LPS/IFN-γ-activated BV-2 microglial cells (222–225) (**Figure 1**).

Like microglia, some therapeutic agents influence the functional activity of monocyte-derived MΦs. Accordingly, GM-CSF is a cytokine that plays a critical role in neuroinflammation onset, as GM-CSF KOs are resistant to disease induction (226). Ifergan et al. (227) have found that targeting the GM-CSF receptor (expressed on monocytes, DCs, MΦs, and neutrophils) can alleviate chronic EAE. Its blockade has resulted in a significant reduction of the relapse severity in treated mice compared to controls (227). Furthermore, following anti-GM-CSF Rα treatment, the costimulatory molecules such as CD80, CD86, CD40, and MHC II expression and inflammatory cytokines, including IL-1β, IL-6, IL-12p40, IL-23p19, and TNF-α by mDCs and inflammatory monocytes, have reduced. Also, in the presence of anti-GM-CSF Rα, chemotactic agents are

required in inflammatory monocyte migration like CXCR2 (binds to MIF) and CCR6 decrease and ameliorate EAE. Moreover, Lotfi et al. (228) have indicated that GM-CSF blockade in monocytes is accompanied by CXCL11 production and T-cell suppression *in vitro*. Because CNS-infiltrating inflammatory monocytes and mDCs highly express GM-CSF Rα in both EAE and MS, anti-GM-CSF Rα treatment could be a good suggestion for the treatment of MS in the future (229) (**Figure 2**).

Several therapeutic methods have attempted to target the NF-κB pathway as a critical inflammatory signaling pathway in MΦs. The NF-κB family member, c-Rel, is a crucial transcription factor in inflammation and induces pro-inflammatory cytokine production in MΦs. Moreover, c-Rel upregulation has been indicated in the spinal cord-infiltrating MΦs. Accordingly, Deng et al. (230) have found that silencing of c-Rel in CNS-infiltrating MΦs by SiRNA PEG-PLL-PLLeu micelles (cationic micelles based on hybrid polypeptide copolymers [poly (ethylene glycol)-b-poly (L-lysine)-b-poly (L-leucine) (PEG-PLL-PLLeu)] is an effective gene delivery system, which suppresses the clinical signs of EAE and alleviates inflammation in the CNS. Their results showed that these nanoparticles are mainly taken up by F4/80+ cells (CNS-infiltrating inflammatory MΦs and microglia). Furthermore, following downregulation of the c-Rel expression in MΦs, IFN-γ and IL-17A production by MOG-specific T cells were suppressed in EAE mouse spleen. So, C-Rel targeting in MΦs, which dampens Th1 and Th17 responses in EAE, will be helpful for future research on MS treatment (231).

Promote Activation, Migration, and Phagocytosis of Myelin Debris

Although autoantibodies are a hallmark of MS disease, natural IgM antibodies usually have beneficial functions in the body (232). rHIgM22 is a human recombinant type of IgM that has been shown to promote remyelination in cuprizone-mediated animal models of MS (233). Zorina et al. (234) have demonstrated that treatment with rHIgM22 increases myelin uptake in microglial cells compared to the Ctrl IgM treatment. CR3 and IgM Fc domain are required for rHIgM22-mediated phagocytosis (235). Therefore, the addition of anti-CD11b antibody (CR3 consists of two subunits, CD11b and CD18) and Fc5μ antibody results in a negative response to rHIgM22. Moreover, in compstatin (C3 inhibitor)-pretreated BV-2 cells, rHIgM22-mediated myelin uptake has wholly blocked. Thus, it seems that complement opsonization is necessary, whereas multiple receptors may be involved (234, 236) (**Figure 1**). Nevertheless, more research will shed light on rHIgM22 functions and their effectiveness in the treatment of MS.

M-CSF is a major cytokine in changing microglial phenotype into an anti-inflammatory subtype. Also, it has many roles in the survival, proliferation, and differentiation of myeloid cells (237, 238). In a study, Laflamme et al. (239) have found that M-CSF administration in the cuprizone EAE mouse model diminishes demyelination and improves myelin sheath overall organization. In addition, M-CSF augments microgliosis (increasing immunoreactivity for Iba-1 indicates microgliosis) and increases the expression of TREM2 mRNA (239) (**Figure 1**).

Ionotropic P2X receptors (P2XRs) are nucleotide-gated ion channels of the P2R family (240). In EAE and human MS, activated microglia highly express Purinergic P2X4R, which makes these receptors remarkable (241).

Ivermectin (IVM) is a semisynthetic macrocyclic lactone that FDA has approved for parasitic disease treatment. IVM interacts with P2X4R and allosterically modulates ion channels (242, 243). Interestingly Zabala et al. (244) have reported that IVM promotes remyelination in the lysolecithin-induced demyelination model in organotypic cerebellar slices. Also, decreased expression of pro-inflammatory genes *vs*. increased anti-inflammatory gene expression has been found during polarization (244). Furthermore, another study has shown that P2X4R locates intracellularly in late endosomes and lysosome membranes (245). Interaction between IVM and P2X4Rs induces lysosome fusion subsequently and leads to acidic endolysosome generation and altogether promotes phagocytic capacity in anti-inflammatory microglia (244) (**Figure 1**).

Polarization of Microglia and Macrophages to an Anti-Inflammatory Phenotype by Some Therapeutic Agents

In a study, Yu et al. (246) have presented that msh-like homeobox-3 (MSX3) increases M2 polarization and impedes microglia M1 polarization through interfering with MSX3 expression in microglia. In this state, expression of IGF-1, CD206, and FIZZ-1 mRNA levels decreased, but the expression of IL-1β, iNOS, and TNF-α mRNA increased. In contrast, overexpression of MSX3 in microglia has induced a reduction in IL-1β, iNOS, and TNF-α mRNA expression and increased FIZZ-1, CD206, IGF-1, and activin-A mRNA expression. IGF-1 and activin-A are M2-derived factors that promote maturation and survival of oligodendrocyte precursor cells (103, 246–248). Moreover, the overexpression of MSX3 has induced upregulation of peroxisome proliferator-activated receptor (PPAR)γ, JAK3, and STAT6 genes associated with M2 polarization. Interestingly, transplantation of MSX3-overexpressed microglia has improved remyelination and alleviated signs of disease in EAE mice. Also, overexpression of MSX3 in human microglia has shown similar results (246). Based on these results, targeting MSX3 could be assessed as a therapeutic protocol in the future.

In another study, Aryanpour et al. (249) have shown that progesterone therapy increases M2 phenotype-related mRNAs (TREM-2, CD206, Arg-1, and TGF-β) and, in contrast, leads to depletion of M1-microglia markers (iNOS, CD86, MHC-II, and TNF-α) in cuprizone-induced demyelinated mouse model. Moreover, the protein and mRNA expressions of NLRP-3 and IL-18 have been decreased after progesterone therapy. According to a significant decrease in the percentage of demyelination areas after progesterone therapy and its effect on diminishing inflammation (249), future research should consider the potential impacts of this therapy in MS (**Figure 1**).

The monocyte-derived MΦs are highly plastic cells, like microglia, which can repolarize to other phenotypes based on

exposure to a different condition. Lenalidomide, an oral FDA-approved drug, is used for myelodysplastic syndromes and multiple myeloma treatment (250). Also, its immunosuppressive and neuroprotective effects have been indicated in EAE. Weng et al. (251) have found that lenalidomide ameliorates EAE symptoms from the early stage and lasts until the end of experiment. It also reduces demyelination due to MΦ polarization toward M2 phenotype *via* IL10–STAT3–IL10 positive feedback loop. This state leads to IL-10 production and subsequent suppression of pro-inflammatory Th1 and Th17 cell responses. So lenalidomide could be considered as a potential therapeutic drug candidate for attenuating neuronal demyelination in CNS of MS patients (251) (**Figure 2**).

On the other hand, studies have shown that voltage-gated potassium channels 1.3 (Kv1.3) in T cells, microglia, and MΦs are necessary for activation, proliferation, and cytokine production of cells (252, 253). Accordingly, Fan et al. (254) have designed an EAE vaccine composed of a B-cell epitope from a pore reign peptide between extracellular loop S5 and S6 on Kv1.3 channels with a universal synthetic T-cell epitope, Pan HLA DR-binding peptide (PADRE). Following the immunization of rats by the PADRE-Kv1.3 vaccine and subsequent induction of EAE, microglia and MΦ populations have significantly reduced at the first peak day of the disease. Also, they have shifted to the M2 phenotype with the decrease in iNOS expression and increase in Arg-1. Regarding the protective role of this vaccine in preventing or treating EAE through balancing immune responses, this could be a promising option for MS treatment in the future (254) (**Figure 2**).

Bryostatin-1 (bryo-1) is a macrocyclic lactone that can pass through CNS and affect the immune system. This compound favors an anti-inflammatory environment by inducing a type 2 phenotype (255, 256). Kornberg et al. (257) have administered bryo-1 to EAE mice at the first clinical sign of motor weakness, corresponding to tail paralysis and also on 10 days after peak disease, and observed the promotion of anti-inflammatory phenotype in MΦs. So, exploring bryo-1 effects on inflammation in MS might be a promising idea for future research (**Figure 2**).

A natural polyamine, spermidine, is produced from arginine by arginase enzyme (258), and according to the study by Yang et al. (259), administration of spermidine in EAE mice has attenuated disease symptoms and reduced the infiltration of CD4+ T cells and CD11b+ MΦs into the CNS. The amelioration by spermidine has relied on shifting MΦs phenotype from inflammatory (high expression of CD80 and CD86 and secretion of IL-1β, IL-12, IL-6, and TNF-α) to anti-inflammatory M2 phenotype (downregulation of NF-κB, Il6, Il1b, Il12, as well as Nos2 and upregulation of Arg1). Interestingly, this study's results have shown that MΦs of spermidine-treated mice could transfer the protective effect and alleviate disease severity in EAE. This study has introduced spermidine as a possible drug candidate for MS treatment in the future (259) (**Figure 2**).

Moreover, Veremeyko (260) have demonstrated forskolin's (coleonol) effects, a plant-derived traditional oriental medicine, on the experimental model of MS. In forskolin-treated EAE mice, downregulation of MHC-I, CD86, and NOS2 on microglia and MΦs in the CNS has been observed. In contrast, forskolin treatment has induced upregulation of miR-124, Arg1, Mrc1, Ym1, and Fizz1 on CNS microglia and MΦs, leading to polarization anti-inflammatory M2 phenotype. In this state, the changing balance through activating the ERK pathway has decreased neuroinflammation in EAE mice (260).

The other compound, fasudil [1-(5-isoquinolinesulfonyl)-homo-piperazine], with impact on CNS MΦs, is a selective Rho kinase (ROCK) inhibitor that inhibits cell migration, proliferation, and survival and used to treat some neural diseases (261, 262). Following fasudil administration by Liu et al. (263), the disease severity has alleviated in early and late treated EAE mice. Besides, MΦs have shifted from M1 to M2 phenotype (decrease in M1 markers iNOS, TLR-4, and CD40 expression *vs*. increase in M2 markers CD206 and Arg-1). Furthermore, the level of IL-10 as an anti-inflammatory cytokine has increased after fasudil treatment. This study has suggested further research on the possible role of fasudil in MS treatment (263) (**Figure 2**). In addition to the effects of some drugs or natural compounds on MΦs, some cytokines also change CNS MΦ phenotype and have potential therapeutic impacts. For example, IL-33 is one of the crucial cytokines of the immune system that can promote Th2-cell expansion and skews MΦs toward the M2 activation state (264). In a study, Jiang et al. (265) have presented that treatment of EAE mice with IL-33 facilitates the polarization of alternatively activated MΦs and reduces inflammation of the CNS. However, the exact function of IL-33 in the CNS is unclear and needs more investigation in MS therapy (265) (**Figure 2**).

Recent studies have shown that neural stem cell transplantation (NSCT) ameliorates CNS inflammation in animal models by modulating the immune responses (266, 267). Peruzzotti-Jametti et al. (268) have shown that NSCT in EAE mice alleviates disease signs and inflammation by reducing succinate levels in CSF, leading to: 1) a decrease in mononuclear phagocyte (MP) infiltration and 2) secretion of prostaglandin E2 (PGE2), which reprograms type 1 MPs toward an anti-inflammatory phenotype. This study has recommended a new anti-inflammatory mechanism for possible treatment of MS in the future (268) (**Figure 2**).

We know glucocorticoids (GCs) as strong immunosuppressive drugs widely used in treating MS and various inflammatory diseases. GCs can suppress the immune system by many mechanisms like inhibition of cytokine secretion and leukocyte migration, increasing T-cell apoptosis, and shifting MΦ polarization (269). It is documented that MΦ reaches anti-inflammatory phenotype following exposure to GC, accompanied by the limitation of immune responses and resolution of disease symptoms (270). Montes-Cobos et al. (271) have applied GC *via* inorganic–organic hybrid nanoparticles (IOH-NP) with [ZrO]2 +{[betamethasone phosphate (BMP)]0.9[Flavin mononucleotide (FMN)]0.1}2-(BMP-NP). They have found that MΦs are polarized to anti-inflammatory phenotype (decreased percentages of MHC class II+ and CD86+ cells) in EAE treated mice. Thus, MΦ

polarization is crucial for the efficacy of BMP-NP treatment. Based on the potential of BMP-NP as a suitable nanoformulation for GC therapy without toxicity, future investigations should be expanded to examine its potential effects in the treatment of MS and other autoinflammatory diseases (271) (**Figure 2**).

CONCLUSION

The role of MΦs and microglia in neuroinflammation and MS pathogenesis calls our attention to the use of different therapeutic agents that target these cells. Microglia recognize infections, toxins, and injuries and have a role in maintaining homeostasis in the adult CNS. Activation of microglia also induces the expression of different inflammatory transcription factors such as NF-κB, JAK/STAT, JNK, ERK1/2, and p38. Moreover, different cytokines, including IL-6, IL-8, IL-12, IL-23, IL-1b, and TNF, are produced after microglia activation. The production of chemokines such a CCL2, CCL3, and CCL4 is also induced by activated microglia, which can facilitate leukocyte recruitment in the early phase of MS disease. During the effector stage of EAE, monocytes rapidly infiltrate surrounding meninges, perivascular space, and choroid plexus through and differentiate into MΦs. These MΦs contribute to the progression of the paralytic stage of EAE and demyelination by expressing MHC-II, costimulatory molecules, and producing pro-inflammatory factors. In EAE, MΦ depletion is associated with a lower CNS injury and attenuated signs and symptoms of the disease. So, both resident MΦs in the CNS and monocyte-derived MΦs that enter into the CNS following alteration in CNS homeostasis play an essential role in neuroinflammation. Moreover, due to their different origin, location, and turnover, other strategies may target various myeloid cell populations. Although the main targets of some drugs in MS treatment are not MΦs and microglia cells, they influence these cells indirectly. For example, DMTs, such as IFN-β, fingolimod, and GA, can change the activation, migration, and polarization of M1/M2 MΦs and microglia. Also, many therapeutic agents whose impacts on MΦs have been assessed *in vitro* or in animal models. Researchers have recently examined various methods of drug delivery by MΦs or their products to the CNS. For example, Tong et al. (272) have found monocyte-derived MΦs mediate the delivery of superparamagnetic iron oxide nanoparticles (SPIONs, cell-based delivery systems) into the inflamed brain. They have indicated that monocyte-derived MΦs uptake SPIONs with different sizes and carry them into the inflamed brain *in vivo* (272) (**Figure 2**). Also, MΦ-derived exosomes have been investigated as possible drug delivery agents to the CNS (273). Overall, understanding the exact mechanism of therapeutic agents on MΦ population and determining the precise role of MΦs as a drug delivery system in CNS will help their usage in clinical studies.

AUTHOR CONTRIBUTIONS

MR and PK wrote most parts of manuscript and searched for data and collected information. NE helped in finding information and reviewed the article before submission not only for spelling and grammar but also for its intellectual content and contributed to the design and implementation of the manuscript. All authors discussed the information and commented on the manuscript. All authors contributed to the article and approved the submitted version.

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Long-Term Efficacy Outcomes of Natalizumab vs. Fingolimod in Patients With Highly Active Relapsing-Remitting Multiple Sclerosis: Real-World Data From a Multiple Sclerosis Reference Center

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Background: Natalizumab (NTZ) and fingolimod (FTY) are second-line disease modifying treatments (DMTs) approved for Relapsing – Remitting Multiple Sclerosis (RRMS). Few studies are available on a direct comparison between NTZ and FTY, based on post-marketing experience, with conflicting results and reporting relatively short follow-up period.

Aim: We hereby report real-world experience of a MS Center with respect to NTZ vs. FTY comparison in terms of efficacy and safety, referencing long-term follow-up.

Methods: We used retrospective data for all patients that received 2nd-line treatment NTZ (since May 2007) or FTY (since September 2011). Primary endpoints were, among others, annual EDSS score (mean change from baseline), time to disability worsening or improvement, Annualized Relapse Rate (ARR) after 12 and 24 months and upon total treatment duration, time to first relapse and time to radiological progression.

Results: A total of 138 unmatched patients, 84 treated with NTZ and 54 treated with FTY were included. Following Propensity Score (PS) matching, 31 patients in each group were retained. Mean follow-up period for NTZ- and FTY-treated patients was 4.43 ± 0.29 and 3.59 ± 0.32 years ($p = 0.057$), respectively. In the matched analysis, time to disability improvement and time to disability worsening was comparable between groups. A higher proportion of patients remained free of relapse under NTZ, compared to FTY (Log Rank test $p = 0.021$, HR: 0.25, 95% CI: 0.08–0.8), as well as free of MRI activity (Log Rank test $p = 0.006$, HR: 0.26, 95% CI: 0.08–0.6). Treatment discontinuation due to MRI activity was significantly higher for FTY-treated patients compared to NTZ (Log Rank test $p = 0.019$, HR: 0.12, 95% CI: 0.05–0.76).

Conclusion: Our results indicate toward NTZ superiority with respect to relapse and MRI activity outcomes. The fact that NTZ-treated patients may achieve long-standing clinical and radiological remission points toward the need for long follow-up data.

Keywords: relapsing-remitting multiple sclerosis, natalizumab, fingolimod, disease-modifying treatment, annualized relapse rate, highly-active multiple sclerosis

INTRODUCTION

Natalizumab (NTZ) and fingolimod (FTY) are second-line disease-modifying treatments (DMTs), European Medicines Agency (EMA) approved for Relapsing–Remitting Multiple Sclerosis (RRMS) (1, 2), a classification based on the safety profile of these agents. Both treatments were shown to be effective in controlling clinical and MRI activity in patients with RRMS with highly active disease at diagnosis. Although there is no consensus on the definition for highly active RRMS (3), NTZ and FTY are indicated in patients with RRMS for whom at least one DMT has previously proven ineffective and/or exhibit rapidly evolving severe RRMS defined by two or more disabling relapses in 1 year, one or more Gd(+) lesions on brain MRI, or a significant increase in T2 lesion load as compared to a previous recent MRI (1, 2). The use of NTZ has significantly been affected by the occurrence of Progressive multifocal leukoencephalopathy (PML), a rare but severe adverse event linked to anti-JCV (John Cunningham virus) Ab (antibody) seropositivity, prior use of immunosuppressants, and prolonged (>2 years) exposure to NTZ (4). PML risk stratification has further been implemented in clinical practice according to EMA guidelines and based on the anti-JCV Ab index as well as the duration of exposure to NTZ (5). In this respect, NTZ administration is subjected to weighted risk-benefit estimation for the patient, in the clinical practice. The use of FTY is being affected by the risk of opportunistic infections linked to lymphopenia, macular edema, rare cardiologic abnormalities, and adverse events stemming from the drug's mode of action (6).

More recently, several newly-available treatments for Relapsing Multiple Sclerosis (RMS) and highly-active RRMS have been approved by the EMA (7–9). These treatments are either monoclonal antibodies (alemtuzumab, ocrelizumab) targeting immune cell populations via complement- and/or antibody-dependent cytotoxicity (CDC/ADCC) (10, 11), or, as in the case of cladribine, a purine analog that interferes with cell proliferation (12). Alemtuzumab, an anti-CD52 monoclonal antibody, effectively depletes T- and B-cells from the peripheral blood (10), whereas ocrelizumab, an anti-CD20 monoclonal antibody, selectively targets B-cell populations and a small fraction of anti-CD20-bearing T-cells (11). These treatments, although exhibiting a differential depletion profile with respect to the cell populations affected and the duration of their biological effect, are collectively considered as newer highly effective treatments and have drastically contributed a new approach in the management of MS. The principle of pulsed immune reconstitution in the context of early aggressive treatment for MS has been advocated as an attractive alternative to classic escalation treatment schemes and has been linked with long-term disease remission in carefully selected patients (13, 14). However, potential adverse events of these treatments, resulting mainly from the prolonged immune reconstitution kinetics, limit their use and underline the necessity of personalized treatment decisions (15, 16). The use of the traditionally regarded as second-line DMTs, namely, NTZ and FTY, remains central in the management of highly-active RRMS, as dictated by the long-term experience of the medical community with these agents and the overall favorable safety profile, compared to the newly available highly effective agents.

Available studies on a direct comparison between NTZ and FTY, are based on post-marketing experience, with partly conflicting results (17–27), and few meta-analyses (28, 29). More specifically, the majority of the existing literature indicates natalizumab superiority with respect to markers of clinical and radiological activity (17–20, 22). In two studies, natalizumab superiority was not retained following propensity score (PS) matching and correction of the analysis taking into consideration confounding factors stemming from baseline characteristics of the two cohorts, respectively (21, 23). In one study, the effect of NTZ and FTY on disease clinical outcomes was comparable (27). In this respect, treatment choice in clinical practice is mostly empirical, with anti-JCV Ab seropositivity status and route of administration remaining the main determining factors. Moreover, the results of existing studies include a relatively short follow-up period of ∼2 years. We hereby report real-world experience of a multiple sclerosis (MS) Center with respect to NTZ vs. FTY comparison in terms of efficacy and safety, referencing long-term follow-up.

Abbreviations: ARR, Annualized relapse rate; Ab, Antibody; DMTs, Disease Modifying Treatments; EMA, European Medicines Agency; EDSS, Expanded Disability Status Scale; FTY, Fingolimod; Gd+, Gadolinium; HRs, Hazard ratios; IFNs, Interferons; JCV, John Cunningham virus; MRI, Magnetic Resonance Imaging; MSD, Mean Standardized Difference; MS, Multiple sclerosis; NTZ, Natalizumab; PML, Progressive multifocal leukoencephalopathy; PS, Propensity score; RRMS, Relapsing–Remitting Multiple Sclerosis; SPMS, Secondary progressive multiple sclerosis.

MATERIALS AND METHODS

Inclusion/Exclusion Criteria

All patients included in the present study were followed by the Multiple Sclerosis Center of the 2nd Department of Neurology of the Aristotle University of Thessaloniki in AHEPA University General Hospital. We used retrospective data for all patients that received second-line treatment NTZ (since May 2007) or FTY (since September 2011) and who either discontinued treatment

or were currently under treatment (as for August 2020). All patients started NTZ or FTY due to failure of first-line agents [interferons (IFNs) and/or glatiramer acetate] or at treatment-naïve state due to highly active MS at diagnosis, according to EMA label. All patients upon NTZ or FTY treatment initiation were older than 18 years. Treatment with immunosuppressants in the previous year and progressive MS were exclusion criteria. A minimum NTZ or FTY treatment duration of 12 months was necessary for inclusion. Moreover, patients with lost-to-follow-up status during NTZ/FTY treatment were not included. NTZ/FTY treatment initiation was retrospectively regarded as the baseline.

Data Collection

All demographic, clinical, and MRI data were recorded in paper and electronically in the MS database of the Center (iMED until May 2020 and MDS since June 2020). An Expanded Disability Status Scale (EDSS) score was reported at baseline and every 3 months for all patients included in the study as well as clinical evaluation regarding the type of the disease with respect to possible progression onset. As a relapse, a new or worsening neurologic symptom with at least 24-h duration confirmed by neurological examination following the exclusion of fever and/or infection was considered. A relapse occurring within 3 months of NTZ or FTY onset was not taken into account for annualized relapse rate (ARR) estimation. Brain and cervical Magnetic Resonance Imaging (MRI) data, as well as thoracic MRI, where available, were collected before NTZ/FTY initiation and annually thereafter. Brain and cervical MRI data were available for all patients at all time points. MRI studies were conducted in different facilities, as in routine clinical practice, but were all evaluated by the treating Neurologists of the Center, with at least 5-year experience in treating patients with MS. Where electronic files of MRI scans were available, a record was retained in the Center's MRI database. JCV Ab status evaluation was conducted by STRATIFY JCV™ (Unilabs, Copenhagen, Denmark) for patients before second-line treatment initiation, whereas for NTZ treated patients the EMA guide in JCV Ab status monitoring and PML risk stratification was followed. For all patients discontinuing NTZ or FTY, the exact reason for discontinuation was recorded [e.g., PML concern, EDSS increase and/or secondary progressive multiple sclerosis (SPMS) disease course, treatment inefficacy, adverse event, pregnancy planning, and patient's will].

Patient Consent and Ethical Declaration

The study was conducted in accordance with the Helsinki Declaration. All participants provided written informed consent. The study received the approval of the Bioethics Committee of the School of Medicine of the Aristotle University of Thessaloniki (Approval Nr. 5321/23-2-2021).

Outcomes

Primary endpoints were as follows:

- Annual EDSS score
- Time to disability worsening, defined as 1 point of EDSS increase (0.5 points if baseline EDSS $\geq$ 5.5 and 1.5 points if baseline EDSS = 0.0), confirmed after 6 months;
- Time to disability improvement (defined as an EDSS score decrease of $\geq$1 point, or $\geq$1.5 points in case baseline EDSS was 0, confirmed after 6 months);
- Annualized relapse rate ARR after 12 and 24 months
- Annualized relapse rate (ARR) during total treatment duration
- Time to first relapse
- Time to treatment discontinuation due to breakthrough disease (clinical activity)
- Nr of new/enlarging T2 lesions with respect to previous brain and cervical scan on annual MRI
- Nr of T1 gadolinium (Gd+) lesions on annual brain and cervical MRI scan
- Time to radiological progression/MRI activity (defined as the presence of $\geq$1 new/enlargingT2 lesion with respect to previous brain MRI and/or the presence of $\geq$1 gadolinium Gd+ lesion) annual brain and cervical MRI scan
- Time to treatment discontinuation due to MRI activity.

Statistical Analysis

For continuous variables, normality was assessed by a Kolmogorov-Smirnoff test prior to the variables' comparison between the two cohorts. We compared continuous variables by the use of non-parametric Mann–Whitney test and dichotomous and/or categorical variables by the use of Chi-square. For the analysis of unmatched cohorts with respect to mean EDSS, ARR, and MRI activity, and in order to minimize potentially significant imbalances at baseline, we investigated mean parameter change vs. baseline by the use of paired samples *T*-tests. Values were presented as mean ± standard error of the mean. Moreover, for the unmatched cohorts with respect to mean EDSS, ARR, and MRI activity, mixed models for repeated measures were used according to which gender, age (years), MS duration (years), ARR in the precedent year, degree of brain MRI activity at baseline (number of new/enlarging T2 and Gd+ lesions), and baseline EDSS scores were used as covariates. Furthermore, in order to compare the two cohorts following minimization of imbalance at baseline, we used propensity score (PS) 1:1 exact matching method, without replacement, with a caliper of 0.1. Covariates used for PS estimation were as follows: gender, age (years), MS duration (years), ARR in the precedent year, degree of brain MRI activity at baseline (number of new/enlarging T2 and Gd+ lesions), and baseline EDSS score. Anti-JCV Ab status was not included in the PS calculation because not all patients starting NTZ since 2007 performed the test. We assessed the degree of imbalance between matched and unmatched cohorts by calculating measurements of effect size estimation, namely, Mean Standardized Difference (MSD/Cohen's *d*) for continuous variables and Cramer's *V* for dichotomous/categorical variables. A logistic regression model with the parameters used for PS estimation as independent variables were used in order to explore potential variables associated with NTZ or FTY treatment before and after PS matching. We compared survival time endpoints using Kaplan–Meier curves (log rank test) for matched and unmatched cohorts. Moreover, we estimated hazard ratios (HRs)

and relative 95% CI using proportional hazards model adjusted (a) by all covariates used for PS calculation and (b) by PS for unmatched cohorts and adjusted by all covariates used for PS calculation for matched cohorts. Also, for unmatched cohorts, a Cox Regression analysis was conducted following inverse probability weighting, adjusted by all covariates used for PS calculation. The analysis was conducted by the use of SPSS IBM v. 25. A significance level of 0.05 was taken into account. For the comparison of baseline characteristics, as well as for the comparison of the mean parameter change vs. baseline by the use of serial paired-samples *T*-test for EDSS and MRI parameters, the *p*-value Bonferrroni's correction for multiple comparisons was applied.

RESULTS

Study Population

The study included a total of 138 unmatched patients: 84 treated with NTZ and 54 treated with FTY. Mean Standardized Difference for PS between the two groups before matching was 1.21 (mean ± SD for NTZ: 0.72 ± 0.18, FTY: 0.45 ± 0.25, $p < 0.001$), and it was reduced to 0.09 following matching (mean ± SD for NTZ: 0.61 ± 0.21 vs. FTY: 0.59 ± 0.22, $p = 0.783$) (**Figure 1**). Following matching, 31 patients in each group were retained. The reduction in the size of the cohorts after PS matching is primarily attributed to the imbalance of the unmatched cohorts, especially with respect to the ARR in the year before NTZ/FTY onset as well as the EDSS score at baseline (NTZ/FTY onset) (**Table 1**; **Figure 2**). Matched cohorts exhibited comparable demographic and clinical characteristics (**Table 1**). Baseline variables exhibited a degree of imbalance based on standardized differences before matching (for absolute values min: 5.8; max 75.14; range 69.34; mean ± standard error of mean: 23.53 ± 6.96), whereas the degree of imbalance was reduced (<20%) with the exception of the number of first-line DMTs received pre- (20.71) and the EDSS score (26.95), following matching (for absolute values min: 0.1; max 26.95; range 26.85; mean ± standard error of mean: 13.02 ± 2.21) (**Table 1**; **Figure 2**). The logistic regression model used for PS estimation indicated that the ARR pre- (OR: 4; 95% C.I. 1.93–8.32, $p < 0.001$) and the EDSS at baseline (OR: 1.96; 95% C.I. 1.39–2.75, $p < 0.001$) were factors associated with NTZ or FTY allocation before PS matching, whereas no factors were associated with NTZ or FTY allocation following PS matching. In the NTZ group, all patients were followed for at least 1 year and 68 patients for 2 years. In the FTY cohort, 54 patients were followed for 1 year and 37 patients for 2 years. Overall, the mean follow-up period for NTZ-treated patients was 4.43 ± 0.29 years, whereas for FTY-treated patients it was 3.59 ± 0.32 years ($p = 0.057$). In the matched groups, the mean follow-up period for NTZ-treated patients was 4.28 ± 0.45 years, whereas, for FTY-treated patients, it was 3.53 ± 0.43 years ($p = 0.231$). A baseline brain MRI scan was performed within 3 months before NTZ/FTY onset. With the exception of one patient in the NTZ group and three patients in the FTY group, overall patients underwent brain MRI scans annually. In total, 17 (20.2%) of NTZ-treated and 34 (63%) of FTY-treated patients were not tested for anti-JCV Ab throughout the treatment duration. Anti-JCV Ab testing was performed in few patients under FTY for reasons of PML risk assessment in the clinical practice, although essentially FTY treatment is linked with minimal PML risk, and no PML risk stratification guideline for FTY-treated patients is available. For NTZ-treated patients, the percentage of patients that were not tested for ant-JCV Ab status is attributed to patients that received NTZ during the early period of the treatment availability (2007–2011). Moreover, due to the same reason, 64 (76.2%) patients that received NTZ were not tested for anti-JCV Ab at baseline. However, the majority of NTZ-treated patients were tested for anti-JCV Ab during the treatment duration.

Treatment Withdrawal and Safety

Anti-JCV Ab was detected during the treatment period in 35 of 84 (41.67%) patients treated with NTZ and in 14 of 54 (25.93%) patients treated with FTY. In total, 31 of 84 (36.9%) patients under NTZ and 6 of 54 (11.11%) patients under FTY were negative throughout the treatment duration. For 33 (39.29%) patients under NTZ, anti-JCV Ab seropositivity was the main reason for treatment withdrawal. For two patients under NTZ that tested positive for anti-JCV Ab, treatment discontinuation was not suggested due to low index value. One patient positive for anti-JCV Ab developed PML. Treatment discontinuation had been suggested for this patient. Overall, treatment discontinuation occurred earlier on average for 29 FTY-treated patients compared to 70 patients under NTZ, however, the difference in the mean treatment duration did not reach statistical significance (treatment duration in months: 38.17 ± 4.38 vs. 49.8 ± 3.75, $p = 0.094$). Reasons for treatment discontinuation were mainly PML concern in 29 patients (34.52%), SPMS course and/or EDSS increase in 15 patients (17.86%), patient's will in 12 cases (14.29%), inefficacy in 10 cases (11.9%), pregnancy planning in 2 cases (2.4%) and insurance issues in 1 (1.2%) case for the NTZ-treated group. One patient developed PML (1.2%). For the FTY-treated group, reasons for treatment discontinuation were inefficacy in 13 (24.07%) cases, lymphopenia in 12 (22.22%) cases, SPMS course and/or EDSS increase in 3 (5.56%) patients, and pregnancy planning for 1 (1.85%) case. Two patients (2.38%) in the NTZ-treated group experienced adverse events with respect to infections, namely, recurrent urinary tract infections and herpes zoster, respectively. In the first case, the adverse events were managed via symptomatic treatment and did not consist reason for discontinuation. In the second case herpes zoster was a secondary reason for discontinuation, together with anti-JCV seropositivity status and PML concern. Nine patients (16.67%) in the FTY-treated group experienced adverse events with respect to infections, namely, recurrent urinary tract infections. Lymphopenia of grade that did not require treatment discontinuation was evident in all patients under FTY, with the exception of the 12 patients for whom lymphopenia dictated treatment discontinuation due to safety concerns. Apart from infections and lymphopenia, no other adverse event was present in the FTY-treated cohort. Mean time (in years) of treatment withdrawal due to relapse and/or MRI activity did not differ

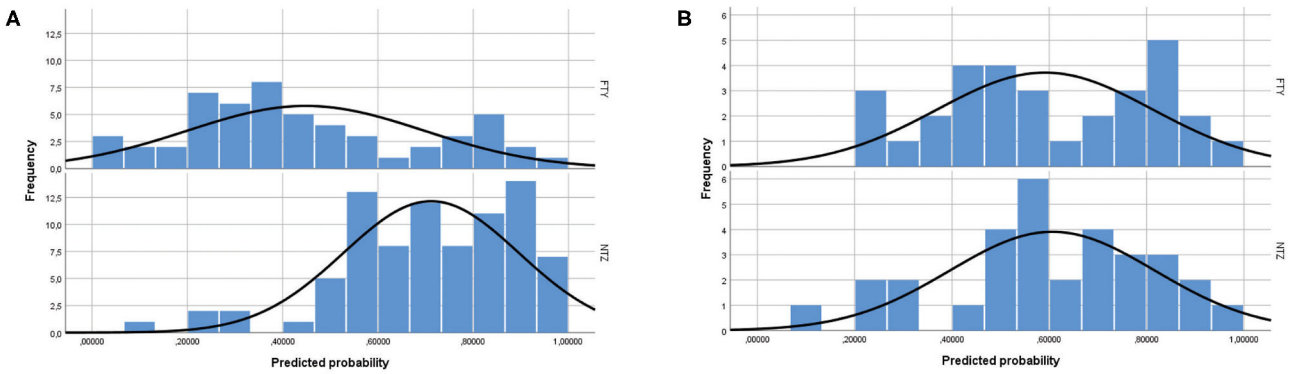

FIGURE 1 | Propensity score distribution in natalizumab and fingolimod patients **(A)** before and **(B)** after propensity score matching. NTZ, natalizumab; FTY, fingolimod.

TABLE 1 | Baseline clinical and demographic characteristics of patients under natalizumab and fingolimod before and after propensity score matching.

Baseline characteristics	Before matching				After matching			
	NTZ (*N* = 84)	FTY (*N* = 54)	*MSD/V*	*p**	NTZ (*N* = 31)	FTY (*N* = 31)	*MSD/V*	*p**
Gender (male/female)	23/61	12/42	5.8	n.s.	5/26	8/23	11.9	n.s.
Age	36.11 ± 1.07	34.07 ± 1.25	−21.24	n.s.	36.23 ± 1.4	35.06 ± 1.52	−14.38	n.s.
Disease duration (years)	9.76 ± 0.62	9.28 ± 0.95	−7.76	n.s.	10.9 ± 1.05	10.91 ± 1.32	0.15	n.s.
First-line DMT treatment duration (years)	5.01 ± 0.41	4.64 ± 0.54	−9.73	n.s.	5 ± 0.71	5.55 ± 0.73	13.59	n.s.
Number of first-line DMT treatments	1.24 ± 0.06	1.19 ± 0.09	−8.99	n.s.	1.13 ± 0.1	1.26 ± 0.12	20.71	n.s.
Type of first-line DMT treatments (IFNs/GA/both)	52/3/23	25/6/9	20.3	n.s.	18/1/6	14/3/7	17.8	n.s.
DMT-free period pre-(months)	5.74 ± 1.52	8.56 ± 3.12	15.74	n.s.	6.14 ± 2.71	8.87 ± 4.14	14.11	n.s.
ARR 1 year pre-	1.58 ± 0.06	1.15 ± 0.09	−71.01	**<0.001**	1.32 ± 0.1	1.42 ± 0.12	15.95	n.s.
Patients with active MRI Scan, N (%)	49 (58.33)	36 (66.66)	8.4	n.s.	18 (58.06)	21 (67.74)	0.1	n.s.
Number of New/enlarged T2 lesions (brain & cervical MRI)	1 ± 0.24	1.69 ± 0.37	28.23	n.s.	1.06 ± 0.37	1.26 ± 0.44	8.58	n.s.
Number of Gd+ lesions (brain & cervical MRI)	1.93 ± 0.35	1.63 ± 0.35	−10.04	n.s.	1.65 ± 0.51	2 ± 0.55	12.05	n.s.
EDSS score	3.81 ± 0.15	2.73 ± 0.17	−75.14	**<0.001**	3.58 ± 0.24	3.21 ± 0.26	−26.95	n.s.

NTZ, natalizumab; FTY, fingolimod; MSD/V, Mean Standardized Difference or Cramer's V; DMT, disease-modifying treatment; IFN, interferon; GA, glatiramer acetate; ARR, annualized relapse rate; MRI, magnetic resonance imaging; EDSS, Expanded Disability Status Scale. Numbers represent mean ± standard error of mean; p, following Bonferroni's correction for multiple comparisons; n.s., non-significant. Comparisons with a p value <0.001 are indicated in bold.*

between NTZ- (2.92 ± 0.51) and FTY- (3.05 ± 0.59, $p = 0.878$) treated patients.

Unmatched Cohorts

Baseline Characteristics

In the unmatched cohort analysis, patients under NTZ exhibited an increased mean EDSS score compared to FTY-treated patients at baseline (NTZ vs. FTY: 3.81 ± 0.15 vs. 2.73 ± 0.17, $p < 0.001$). Moreover, patients under NTZ exhibited a higher mean ARR the year before treatment onset relative to the patients under FTY (NTZ vs. FTY: 1.58 ± 0.06 vs. 1.15 ± 0.09, $p < 0.001$). Patients under FTY exhibited a comparable mean number of new/enlarging T2 lesions on brain MRI at baseline, to NTZ-treated patients (NTZ vs. FTY: 0.68 ± 0.18 vs. 1.22 ± 0.26, p

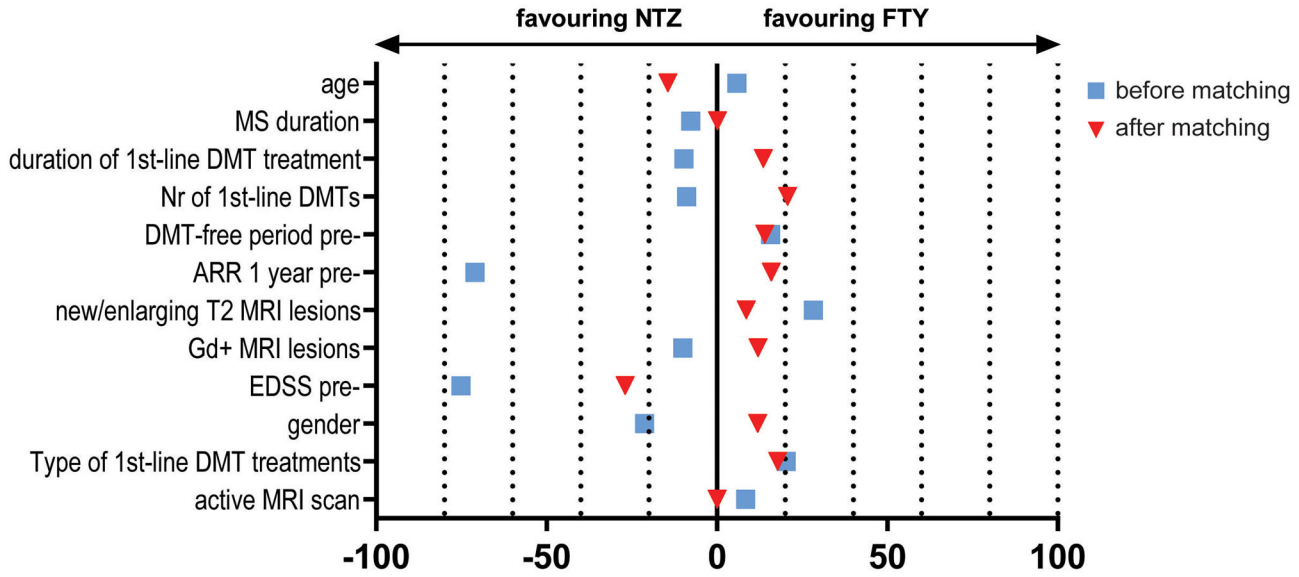

FIGURE 2 | Effect size estimation e.g., Mean Standardized Difference/Cohen's d for continuous variables and Cramer's V for dichotomous/categorical variables, for baseline variables before (blue squares) and after (red triangles) propensity score matching. NTZ, natalizumab; FTY, fingolimod; MRI, Magnetic Resonance Imaging; DMTs, disease-modifying treatments; EDSS, Expanded Disability Status Scale; pre-, before NTZ/FTY; Gd, gadolinium; ARR, annualized relapse rate; Nr, number; MS, Multiple Sclerosis.

= n.s.). Moreover, no difference was observed between NTZ- and FTY-treated patients with respect to the mean number of gadolinium-enhancing lesions at baseline for brain (NTZ vs. FTY: 1.62 ± 0.33 vs. 1.37 ± 0.33, p = n.s.) and cervical (NTZ vs. FTY: 0.31 ± 0.1 vs. 0.26 ± 0.08, p = n.s.) MRIs. Similarly, no significant difference was observed with respect to new/enlarging T2 lesions between NTZ- and FTY-treated patients at baseline for brain (NTZ vs. FTY: 0.68 ± 0.18 vs. 1.22 ± 0.26, p = n.s.) and cervical (NTZ vs. FTY: 0.32 ± 0.11 vs. 0.46 ± 0.15, p = n.s) MRI.

Disability, ARR, and MRI Activity: Analysis at Point Estimates

In order to minimize the impact of different baseline cohort activities, the unmatched cohort analysis was conducted by investigating change vs. baseline for each treatment group, with respect to EDSS, ARR, and MRI activity parameters. In the first year of treatment, patients under NTZ and under FTY did not exhibit alterations with respect to mean EDSS score, compared to baseline (for NTZ: 3.81 ± 0.15 vs. 3.76 ± 0.16, p = n.s.; for FTY: 2.73 ± 0.17 vs. 2.77 ± 0.18, p = n.s). Also, in the second year of treatment patients under NTZ (N = 68) and under FTY (N = 37) did not exhibit alterations with respect to mean EDSS score compared to baseline (for NTZ: 3.61 ± 0.2 vs. 3.68 ± 0.16, p = n.s.; for FTY: 2.74 ± 0.21 vs. 2.68 ± 0.2, p = n.s.) (**Figure 3A**).

For the NTZ-treated patients, there was a significant mean ARR reduction compared to baseline, referencing year 0–1, year 1–2 (N = 68), years 0–2 (N = 68), as well as overall NTZ-treatment duration (mean ARR for year 0–1 vs. baseline: 0.02 ± 0.02 vs. 1.58 ± 0.06, $p < 0.001$; for year 1–2 vs. baseline: 0.04 ± 0.03 vs. 1.58 ± 0.06, $p < 0.001$; for years 0–2 vs. baseline: 0.04 ± 0.02 vs. 1.57 ± 0.06, $p < 0.001$ and for overall NTZ treatment duration vs. baseline: 0.06 ± 0.02 vs. 1.58 ± 0.06, $p < 0.001$). Also for the FTY-treated patients, there was a significant mean ARR reduction compared to baseline, referencing year 0–1, year 1–2 (N = 37), years 0–2 (N = 37), as well as overall FTY-treatment duration (mean ARR for year 0–1 vs. baseline: 0.13 ± 0.05 vs. 1.15 ± 0.09, $p < 0.001$; for year 1–2 vs. baseline: 1.16 ± 0.11 vs. 0.19 ± 0.07, $p < 0.001$; for years 0–2 vs. baseline: 0.14 ± 0.04 vs. 1.16 ± 0.11, $p < 0.001$ and for overall FTY treatment duration vs. baseline: 0.14 ± 0.04 vs. 1.15 ± 0.09, $p < 0.001$) (**Figure 4A**).

Patients under NTZ exhibited significant mean reduction from baseline with respect to the number of new/enlarging T2 lesions on brain MRI at annual point estimates from year 1 to year 4 (at year 1 vs. baseline: 0.11 ± 0.06 vs. 0.68 ± 0.18, p = 0.007; at year 2 vs. baseline: 0.06 ± 0.04 vs. 0.78 ± 0.22, p = 0.014) (**Figure 5A**). Patients under FTY exhibited a significant mean reduction from baseline with respect to the number of new/enlarging T2 lesions on brain MRI at point estimate year 2 (year 2 vs. baseline: 0.15 ± 0.07 vs. 1.09 ± 0.31, p = 0.021) (**Figure 5A**). A similar effect, overall more significant for NTZ, was observed with respect to the number of gadolinium-enhancing lesions on brain MRIs for NTZ- and FTY- treated patients (**Figure 5B**) as well as with respect to the number of new/enlarging T2 lesions and the number of gadolinium-enhancing lesions on first-year cervical MRI (**Supplementary Figures 1A,B**).

Disability, Relapse, and MRI Activity: Mixed Models for Repeated Measures

For NTZ-treated patients, with respect to EDSS, a mixed model for repeated measure was overall statistically non-significant (−2 Restricted Log Likelihood = 1,484.016,

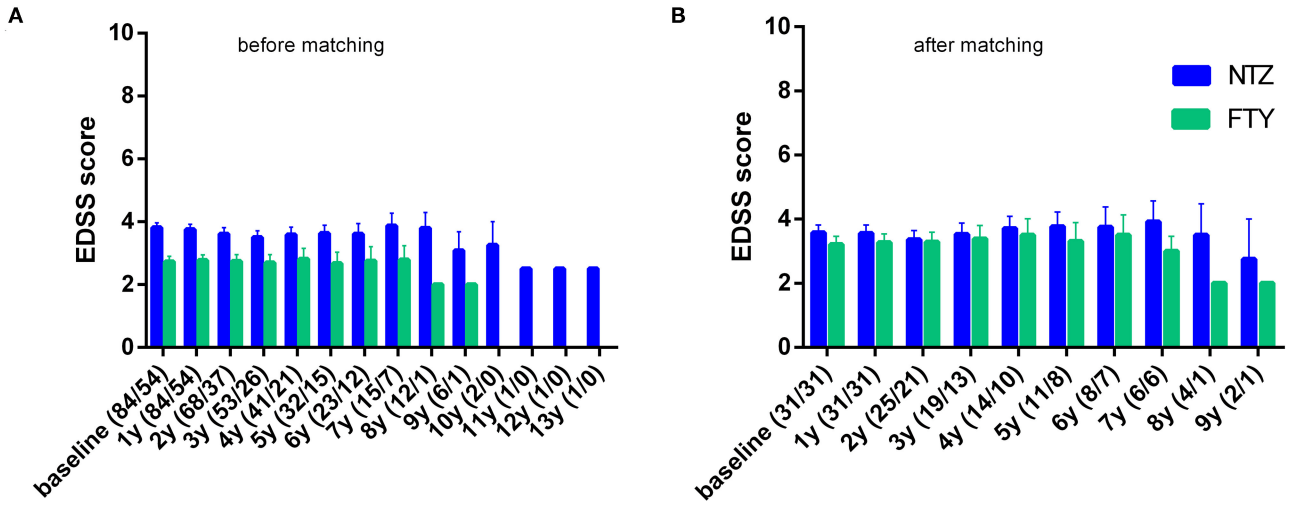

FIGURE 3 | Mean annual expanded disability status scale of natalizumab and fingolimod cohorts at baseline and during the whole follow-up **(A)** before and **(B)** after matching. NTZ, natalizumab; FTY, fingolimod. Analysis was conducted at point estimation by exploring mean change from baseline for each treatment group.

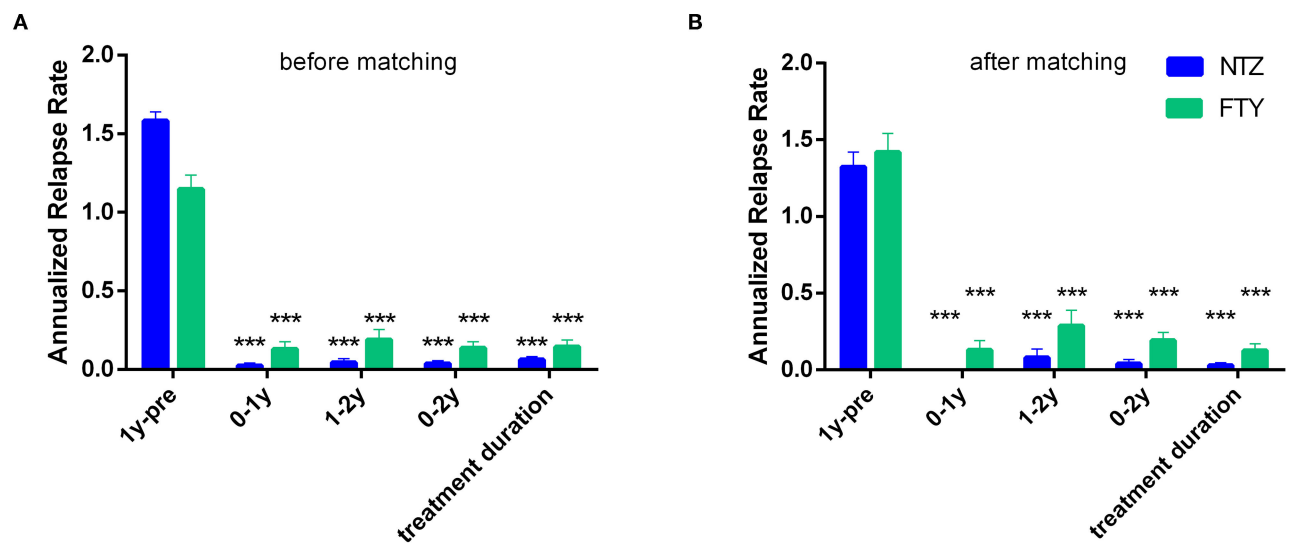

FIGURE 4 | Mean annualized relapse rate of natalizumab and fingolimod cohorts in the year before natalizumab/fingolimod treatment onset (1y-pre; baseline), referencing the first year of treatment (0–1y), referencing the second year of treatment (1–2y), referencing the first and second year of treatment (0–2y), as well as referencing the overall follow-up period under natalizumab/fingolimod treatment (treatment duration). **(A)** before and **(B)** after matching. Statistical significance indicates mean change from baseline (1y pre-). NTZ: natalizumab; FTY: fingolimod; ***$p < 0.001$.

p = n.s.). With respect to ARR, a mixed model for the repeated measure was overall statistically significant (−2 Restricted Log Likelihood = 31.775, $p < 0.001$) with the difference in ARR pre- and post-NTZ treatment initiation being reduced ∼1.54 times ($p < 0.001$), whereas it did not differ between the first and the second year of the follow-up. For MRI activity parameters mixed models for the repeated measure were overall statistically non-significant for NTZ-treated patients.

For FTY-treated patients, with respect to EDSS, a mixed model for the repeated measure was overall statistically non-significant (−2 Restricted Log Likelihood = 623.547, p = n.s.). With respect to ARR, a mixed model for the repeated measure was overall statistically significant (−2 Restricted Log Likelihood = 184.455, $p < 0.001$) with the difference in ARR pre- and post-FTY treatment initiation being reduced ∼0.96 times ($p < 0.001$), whereas it did not differ between the first and the second year of the follow-up. For MRI activity parameters mixed models for

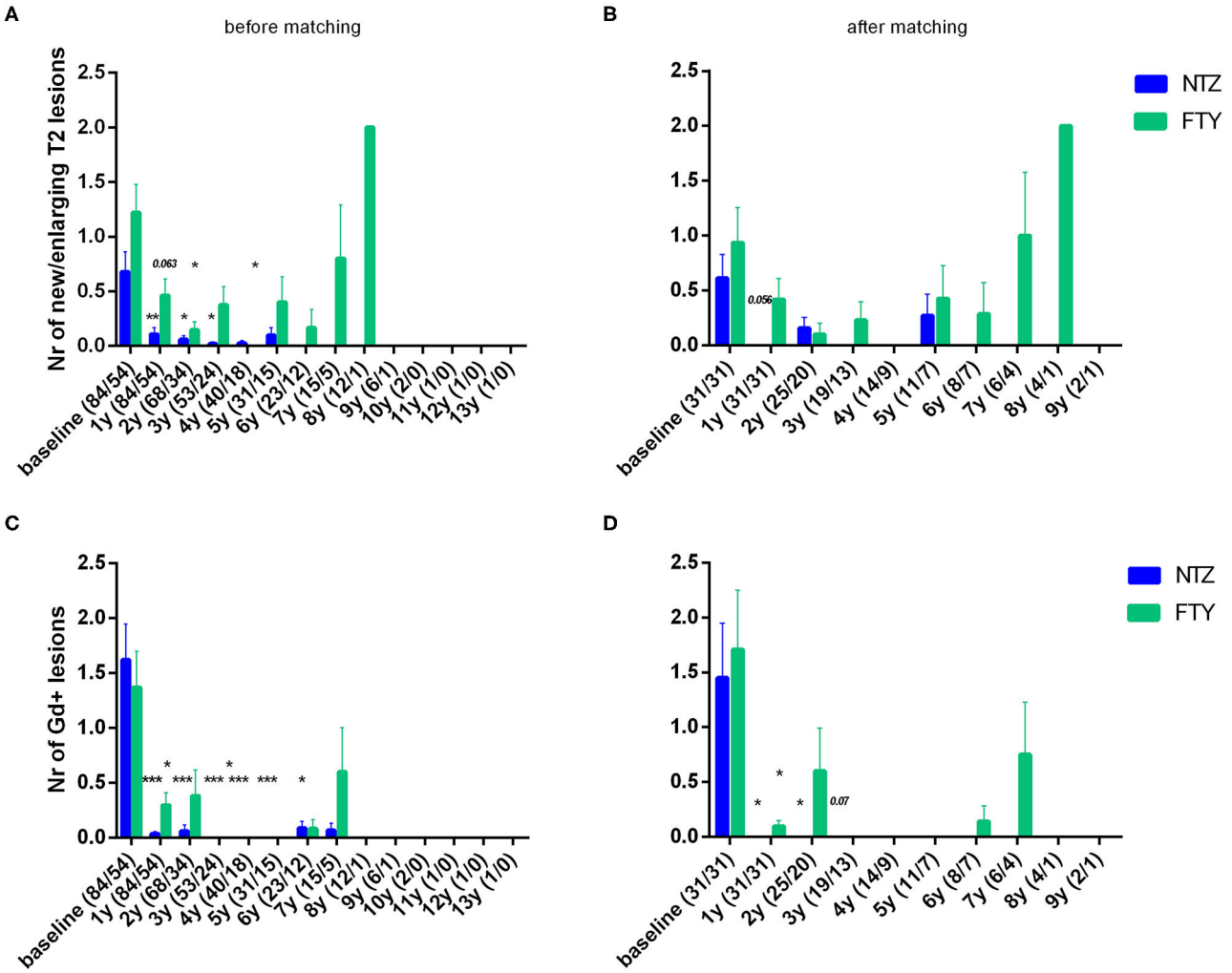

FIGURE 5 | Mean number of new/enlarging T2 lesions **(A,B)** and gadolinium-enhancing lesions **(C,D)** on annual brain MRI of natalizumab and fingolimod cohorts at baseline and during the whole follow-up **(A,C)** before and **(B,D)** after matching. Statistical significance indicates mean change from baseline. NTZ, natalizumab; FTY, fingolimod; Nr, Number; Gd, gadolinium; $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.001$.

the repeated measure were overall statistically non-significant for FTY-treated patients.

Disability, Relapse, and MRI Activity–Survival Time Endpoints

Before matching, NTZ was superior with respect to time to EDSS reduction (% of patients with disability improvement) (HR: 4.76, 95% CI: 1.23–8.67, Log Rank test $p = 0.02$) (**Figure 6**), time to relapse (% of patients free of relapse) (HR: 0.42, 95% CI: 0.18–0.86, Log Rank test $p = 0.021$) (**Figure 7**), time to MRI activity (% of patients free of MRI activity) (HR: 0.38, 95% CI: 0.15–0.54, Log Rank test $p < 0.001$), and time to treatment discontinuation due to MRI activity(HR: 0.09, 95% CI: 0.04–0.3, Log Rank test $p < 0.001$) (**Figure 8**), whereas a tendency toward NTZ superiority was shown for time to treatment discontinuation due to clinical activity (HR: 0.47, 95% CI: 0.17–1.13, Log Rank test $p = 0.065$) (**Figure 7**), without reaching statistical significance.

Sensitivity analyses were conducted by comparing the two unmatched groups (NTZ and FTY) following adjustment either for PS (first sensitivity analysis) or for all covariates that were used for PS calculation (second sensitivity analysis) and are shown in **Table 2**. Overall, sensitivity analyses were in agreement with the main analysis for all survival endpoints with few exceptions: following adjustment for covariates and for PS in the comparison between unmatched groups, NTZ was superior with respect to time to relapse (adjusted for covariates HR: 4.29, 95% CI: 1.76–10.47, $p = 0.001$; adjusted for PS HR: 4.08, 95% CI: 1.7–9.8, $p = 0.002$), time to MRI activity (adjusted for covariates HR: 3.47, 95% CI: 1.68–7.17, $p = 0.001$; adjusted for PS HR: 3.05, 95% CI: 1.5–6.21, $p = 0.002$). and time to treatment discontinuation due to MRI activity (adjusted for covariates HR: 14.38, 95% CI: 2.95–70.1, $p = 0.001$; adjusted for PS HR: 13.86, 95% CI: 2.87–67, $p =$

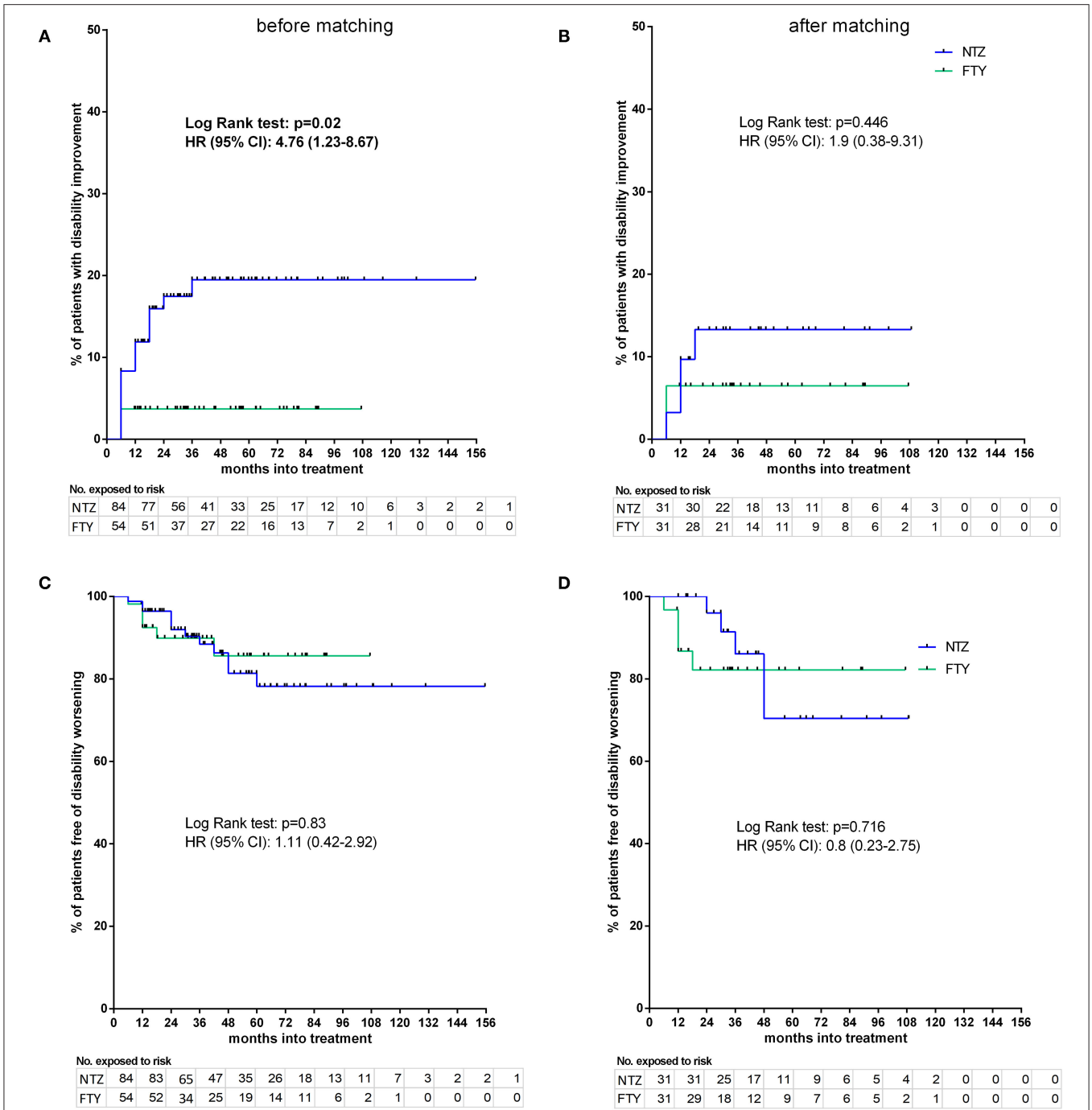

FIGURE 6 | Kaplan–Meier survival estimates for the time to disability improvement **(A,B)** and time to disability worsening **(C,D)** before **(A,C)** and after **(B,D)** matching. HR, hazard ratio; CI, confidence interval; No., number; NTZ, natalizumab; FTY, fingolimod.

0.001) (as in the main analysis), whereas a similar tendency was shown for time to EDSS reduction following the only adjustment for covariates (HR: 0.22, 95% CI: 0.05–1.09, $p = 0.064$) and for time to treatment discontinuation due to clinical activity following adjustment for covariates and for PS, but the difference did not reach statistical significance (adjusted for covariates HR: 2.71, 95% CI: 0.96–7.65, $p = 0.06$; adjusted for PS HR: 2.54, 95% CI: 0.91–7.1, $p = 0.075$).

In the weighted analysis for the unmatched cohorts, the overall test for proportional hazards showed NTZ superiority compared to FTY with respect to time to relapse (Wald F = 3.8, $p = 0.002$), time to discontinuation due to clinical activity (Wald F = 2.69,

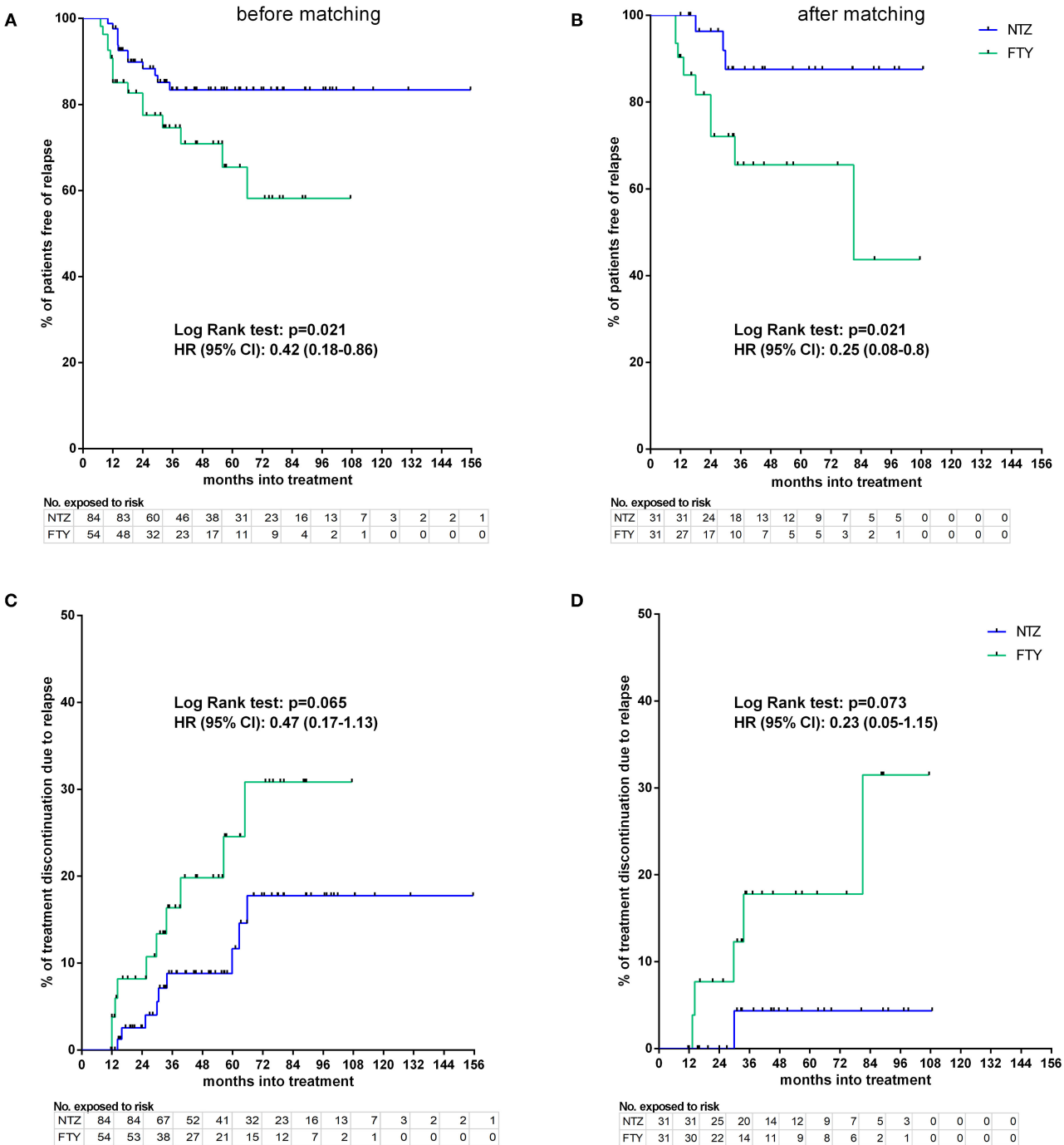

FIGURE 7 | Kaplan–Meier survival estimates for the time to relapse under treatment **(A,B)** and time to treatment discontinuation due to relapse **(C,D)** before **(A,C)** and after **(B,D)** matching. HR, hazard ratio; CI, confidence interval; No., number; NTZ, natalizumab; FTY, fingolimod.

$p = 0.017$), and time to discontinuation due to MRI activity (Wald F = 3.86, $p = 0.001$), whereas the two treatments were comparable with respect to time to disability improvement (Wald F = 1.52, $p = 0.175$), time to disability worsening (Wald F = 0.71, $p = 0.642$) and time to MRI activity (Wald F = 1.66, $p = 0.137$).

Matched Cohorts

Disability

Following PS matching, the mean change from baseline EDSS did not differ from the NTZ- and the FTY-treated patients in annual follow-up time points (**Figure 3**). Moreover, the mean

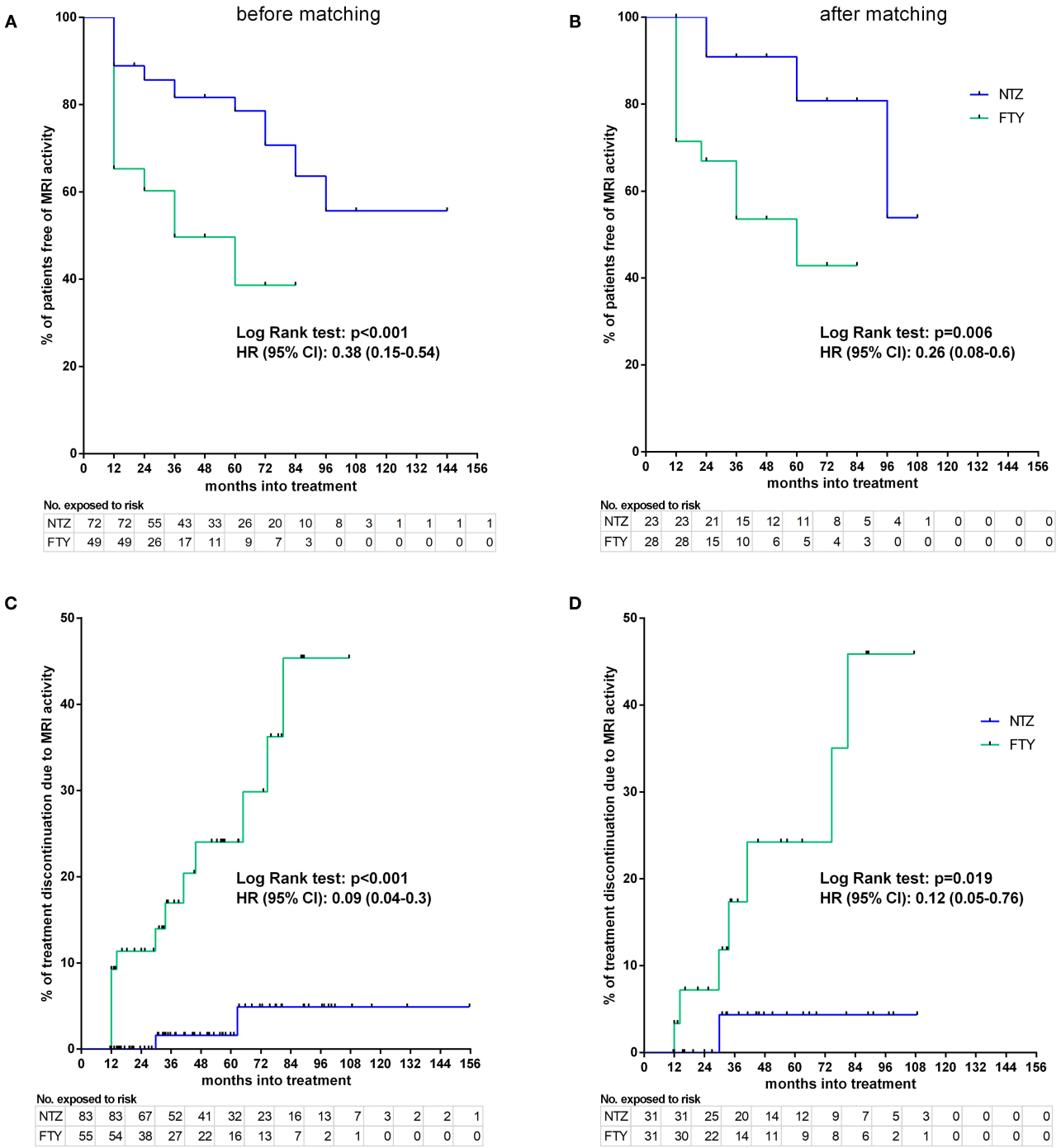

FIGURE 8 | Kaplan–Meier survival estimates for the time to MRI activity **(A,B)** and time to treatment discontinuation due to MRI activity **(C,D)** before **(A,C)** and after **(B,D)** matching. HR, hazard ratio; CI, confidence interval; No., number; NTZ, natalizumab; FTY, fingolimod.

EDSS did not differ between the two groups in annual follow-up time points. Time to disability improvement was not different between NTZ and FTY treated patients (**Figure 6**). In adjusted analysis, a tendency for the severity of the activity at baseline MRI (defined as the number of new/newly enlarged and Gd+ lesions on the brain and cervical MRIs) to predict disability improvement was observed (HR: 1.14, range: 0.98–1.34; $p = 0.097$), but the difference did not reach statistical significance. Time to disability worsening was not different between matched NTZ- and FTY-treated patients (**Figure 6**).

TABLE 2 | Hazard ratios and relative 95% confidence intervals using proportional hazards model adjusted (a) by all covariates used for propensity score calculation and (b) by propensity score for unmatched cohorts and adjusted by all covariates used for propensity score calculation for matched cohorts.

Outcome	Model	Unmatched			Matched		
		HR	CI 95%	*p*	HR	CI 95%	*p*
Time to EDSS reduction	Adjusted for PS	0.34	0.07–1.63	0.179	–	–	–
	Adjusted for covariates	**0.22**	**0.05–1.09**	**0.064**	0.46	0.08–2.66	0.389
Time to EDSS increase	Adjusted for PS	1.29	0.41–4.09	0.666	–	–	–
	Adjusted for covariates	1.43	0.46–4.44	0.542	1.42	0.37–5.37	0.609
Time to relapse	Adjusted for PS	**4.08**	**1.7–9.8**	**0.002**	–	–	–
	Adjusted for covariates	**4.29**	**1.76–10.47**	**0.001**	**5.29**	**1.32–21.29**	**0.019**
Time to treatment discontinuation due to clinical activity	Adjusted for PS	**2.54**	**0.91–7.1**	**0.075**	–	–	–
	Adjusted for covariates	**2.71**	**0.96–7.65**	**0.060**	**8.78**	**0.84–92.02**	**0.070**
Time to MRI activity	Adjusted for PS	**3.05**	**1.5–6.21**	**0.002**	–	–	–
	Adjusted for covariates	**3.47**	**1.68–7.17**	**0.001**	**4.38**	**1.73–16.31**	**0.028**
Time to treatment discontinuation due to MRI activity	Adjusted for PS	**13.86**	**2.87–67**	**0.001**	–	–	–
	Adjusted for covariates	**14.38**	**2.95–70.1**	**0.001**	**8.48**	**0.94-76.98**	**0.057**

HR, hazard ratio; CI, confidence interval; EDSS, Expanded Disability Status Scale; MRI, magnetic resonance imaging. Comparisons with a p value <0.01 are indicated in bold.

Relapse Activity

In the matched cohorts, both treatments resulted in profound mean ARR reduction at point estimates, compared to baseline (**Figure 4**). Moreover, with respect to the direct NTZ/FTY comparison, the mean ARR was significantly lower in the NTZ group compared to FTY at year 0–1 (NTZ vs. FTY, 0 vs. 0.13 $\pm$ 0.06, $p = 0.04$), at year 0–2 (NTZ vs. FTY, 0.04 $\pm$ 0.03 vs. 0.19 $\pm$ 0.05, $p = 0.15$) and with reference to the overall treatment duration (NTZ vs. FTY, 0.03 $\pm$ 0.02 vs. 0.13 $\pm$ 0.04, $p = 0.03$), whereas a tendency toward NTZ superiority was also evident at year 1–2 (NTZ vs. FTY, 0.08 $\pm$ 0.06 vs. 0.29 $\pm$ 0.1, $p = 0.07$) without reaching statistical significance (**Figure 4**). A significantly higher proportion of patients remained free of relapse in the NTZ group, compared to FTY (HR: 0.25, 95% CI: 0.08–0.8, Log Rank test $p = 0.021$) (**Figure 7**). With respect to treatment discontinuation due to clinical activity, a tendency toward NTZ superiority was evident, compared to FTY (HR: 0.23, 95% CI: 0.05–1.15, Log Rank test $p = 0.073$) (**Figure 7**), without reaching statistical significance. These differences were observed also following sensitivity analysis adjusted for covariates between the matched groups (**Table 2**).

MRI Activity

In the matched cohorts, both treatments resulted in a reduced mean number of new/enlarging T2 and Gd+ lesion reduction at point estimates compared to baseline in the brain (**Figure 5**) and cervical MRI (**Supplementary Figure 1**). Moreover, with respect to the direct NTZ/FTY comparison, the number of brain new/newly enlarged T2 lesions was lower for NTZ-treated patients at year 1 (NTZ vs. FTY 0 vs. 0.42 $\pm$ 0.19, $p = 0.021$) and the number of Gd+ lesions was lower for NTZ-treated patients at year 2 (NTZ vs. FTY 0 vs. 0.6 $\pm$ 0.39, $p = 0.048$), whereas a similar tendency was observed for NTZ-treated patients at year 1 (NTZ vs. FTY 0 vs. 0.09 $\pm$ 0.05, $p = 0.078$) (**Figure 5**), without reaching statistical significance. With respect to cervical MRI, the number of brain new/newly enlarged T2 lesions, and the number of Gd+ lesions did not differ between NTZ- and FTY-treated patients at annual follow-up time points (**Supplementary Figure 1**). Also, in the comparison between matched groups, the proportion of patients free of MRI activity was significantly higher for NTZ-treated patients compared to FTY (HR: 0.26, 95% CI: 0.08–0.6, Log Rank test $p = 0.006$) (**Figure 8**). Similarly, treatment discontinuation due to MRI activity was significantly higher for FTY-treated patients compared to NTZ (HR: 0.12, 95% CI: 0.05–0.76, Log Rank test $p = 0.019$) (**Figure 8**). In adjusted analysis, the results were similar to the main analysis with respect to time to MRI activity and the time of treatment discontinuation due to MRI activity (**Table 2**).

DISCUSSION

Early switch from first- to second-line DMTs in patients with highly active RRMS has been advocated as a strategy associated with favorable disease outcomes (30). Moreover, remaining free of relapse following the switch has been linked with improved persistence to the DMT (31), a factor also contributing to favorable overall disease prognosis. Natalizumab and FTY are highly effective DMTs in reducing relapse and radiological activity (1, 2). Although their use in RRMS is subjected to limitations due to safety issues, both treatments are considered to exhibit a more favorable safety profile compared to the newly available highly effective treatments indicated for highly-active RRMS and Relapsing Multiple Sclerosis (RMS), such as cladribine, alemtuzumab, and ocrelizumab, respectively (11, 13). In this respect, NTZ and FTY remain central in the management of highly active RRMS, and the availability of real-world, long-term safety and efficacy data is, therefore, crucial. The recent publication of 10-year real-world data regarding the safety and efficacy of natalizumab partly addresses this need. However, long-term comparative

studies on the safety and efficacy of NTZ vs. FTY are expected to facilitate treatment decision upon switch from first- to second-line DMTs, especially when a newer highly effective treatment is not primarily considered, and to better characterize baseline patients' characteristics linked to optimal treatment response.

Due to the fact that direct comparative randomized prospective studies of NTZ vs. FTY are not available, treatment allocation is primarily based on empirical knowledge and real-world experience. Few post-marketing studies have retrospectively addressed issues with respect to NTZ vs. FTY comparative safety and efficacy, but the follow-up period is short at ∼2 years (17–26) with the exception of one study with a total follow-up up to 4 years (27). However, also in this study, following PS matching, the mean follow-up time was ∼1.8 years (27). In our study, the mean follow-up time for NTZ and for FTY was ∼4.5 and 3.5 years, respectively, in unmatched and matched groups. The shorter follow-up period for FTY-treated patients is likely attributed to the earlier market availability of NTZ, as few patients in the NTZ-treated group had an especially long period of follow-up (15 patients: 7 years, 12 patients: 8 years, 6 patients: 9 years). Similarly, for the FTY-treated group, a long follow-up period was as follows: 15 patients: 5 years, 12 patients: 6 years, 7 patients: 7 years. The maximum follow-up period was 13 years for one patient under NTZ and 9 years for one patient under FTY.

Before PS matching, NTZ-treated patients exhibited higher mean ARR in the year before NTZ onset and higher mean EDSS score, compared to FTY-treated patients. This is in accordance with previous studies (17, 18, 20, 27). The higher mean ARR before treatment initiation may in fact indicate two factors that contribute toward NTZ or FTY treatment choice: (a) NTZ may be initiated preferentially, compared to FTY, in patients with more highly active disease due to the drug's documented capability toward rapid control of disease activity, compared to FTY [as indicated by a REVEAL study, in spite of its early discontinuation due to non-efficacy/non-safety issues (32, 33) and the recently published long-term follow-up safety and effectiveness study on NTZ (34)], and (b) FTY is more readily initiated to patients with relatively less highly active disease due to the more appealing route of administration and relatively low PML concern compared to NTZ. As previously proposed, higher mean EDSS at NTZ onset may indicate disability accumulation due to increased disease activity over the previous year. This observation is further confirmed by the analysis of unmatched groups in our study, according to which NTZ was superior to FTY with respect to disability improvement. In the analysis following PS matching, according to which baseline EDSS and ARR in the year before treatment onset did not differ between the two groups, NTZ was also superior, but the difference did not reach statistical significance. These results indicate that the superiority of NTZ with respect to disability improvement in the unmatched analysis is primarily attributed to patients with especially highly active disease and increased disability accumulation before NTZ onset, a group of patients for whom the sustained and/or reduced degree of disability is of special importance due to the higher burden over the quality of life.

In the matched analysis, NTZ was superior to FTY with respect to relapses (time to first relapse under treatment), as well as with respect to the time to MRI activity under treatment and treatment discontinuation due to MRI activity. Our results are in accordance with previous studies (17, 18, 20, 27) and are further supported by the sensitivity analyses performed in the unmatched and matched groups. The main reason for treatment discontinuation in the NTZ-treated group was PML concern, as in other studies. This fact, together with the lack of EMA guidelines for PML risk stratification in JCV seropositive NTZ-treated patients for treatment administration longer than 6 years renders post-marketing NTZ administration data with reference to longer follow-up especially rare. In our study, few JCV Ab seronegative patients insisted on continuing NTZ treatment following thorough information by the treating neurologist. These patients achieved long-standing clinical and radiological remission under NTZ. One seropositive patient developed PML shortly after NTZ discontinuation was suggested and PML was diagnosed at a pre-symptomatic phase on a routine MRI (35). In the FTY-treated group, treatment inefficacy and lymphopenia were the main reasons for treatment discontinuation. For both treatments, the time of discontinuation due to relapse and/or MRI activity was ∼3 years. Also, in the FTY-treated group, patients with relatively long follow-up time achieved sustained remission of disease activity. These observations underline the need for longer post-marketing data on NTZ and FTY administration. More importantly, the need for NTZ-related PML stratification guidelines for longer follow-up appears of special importance, as evidence suggests that several patients may benefit from long-term NTZ administration.

Our study is subjected to limitations, such as its retrospective design, the lack of a central MRI facility, and the fact that it is a one-center study. However, the latter accounts for a more universal approach in treatment decisions and overall disease management. Moreover, although ARR in the year before NTZ/FTY onset, EDSS score at baseline (NTZ/FTY onset), and MRI measures of disease activity have been included as baseline characteristics, a treatment-naive status was not included as a binary variable in the baseline characteristics of the PS model. It should be noted, however, that the number of first-line DMTs has been included as a baseline characteristic in the PS model. In this respect, patients that did not receive first-line DMTs were represented as cases with a value of zero first-line DMTs prior to NTZ/FTY onset. Moreover, a profound reduction in the cohort sizes was evident following matching due to the fact that the two cohorts exhibited significant imbalance with respect to baseline characteristics, especially the ARR 1-year pre-NTZ/FTY treatment initiation and the EDSS. Following matching, the remaining cohorts were balanced, however, this improvement was at the expense of sample size. This is an inherent limitation of the real-world study setting. For reasons of transparency, we therefore present a comparison of unmatched and matched cohorts, with additional sensitivity and weighted analyses for the unmatched cohorts, as well as analysis of ARR, EDSS, and MRI parameters in a mean-change-from-baseline setting.

To conclude, our study provides real-world experience data on NTZ vs. FTY efficacy outcomes referencing a long follow-up period. Our results indicate NTZ superiority, compared to FTY, with respect to relapse and MRI activity outcomes, whereas the two treatments are comparable with respect to disability outcomes, in the analysis of the matched groups. These results are in accordance with previous studies. Moreover, the results of the present study also further support existing observations that NTZ evidently is empirically preferred for patients with more highly active RRMS with increased disability accumulation before treatment onset. It should be noted, however, that, in the frame of the present study, patients under NTZ were included who saw NTZ treatment initiation since 2007, as soon as NTZ became available, and who, due to the lack of alternative treatment plan, exhibited disability worsening before NTZ onset. The fact that these patients may achieve long-standing clinical and radiological remission upon prolonged treatment administration points toward the need for long follow-up data and universally accepted, evidence-based pharmacovigilance guidelines.

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/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Bioethics' Committee of the School of Medicine of the Aristotle University of Thessaloniki (Approval Nr. 5321/23-2-2021). The patients/participants provided their written informed consent to participate in this study. The study was conducted in accordance to the Helsinki Declaration. All participants provided written informed consent.

AUTHOR CONTRIBUTIONS

MB performed the conception and design of the study, acquisition of data, analysis and interpretation, and critical revision of the manuscript for important intellectual content and gave final approval of the version to be submitted. CB, VG, S-AS, SK, TA, IN, PI, TK, IK, DP, and NG carried out the acquisition of data, analysis and interpretation, and critical revision of the manuscript for important intellectual content and gave final approval for the version to be submitted. 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.2021.699844/full#supplementary-material

Supplementary Figure 1 | Mean number of new/enlarging T2 lesions **(A,B)** and gadolinium-enhancing lesions **(C,D)** on annual cervical MRI of natalizumab and fingolimod cohorts at baseline and during the whole follow-up **(A,C)** before and **(B,D)** after matching. Statistical significance indicates mean change from baseline. NTZ, natalizumab; FTY, fingolimod; Nr, Number; Gd, gadolinium; $*p < 0.05$.

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Intestinal Permeability and Circulating CD161+CCR6+CD8+T Cells in Patients With Relapsing–Remitting Multiple Sclerosis Treated With Dimethylfumarate

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Background: The changes of the gut-brain axis have been recently recognized as important components in multiple sclerosis (MS) pathogenesis.

Objectives: To evaluate the effects of DMF on intestinal barrier permeability and mucosal immune responses.

Methods: We investigated intestinal permeability (IP) and circulating CD161+CCR6+CD8+T cells in 25 patients with MS, who met eligibility criteria for dimethyl-fumarate (DMF) treatment. These data, together with clinical/MRI parameters, were studied at three time-points: baseline (before therapy), after one (T1) and 9 months (T2) of treatment.

Results: At baseline 16 patients (64%) showed altered IP, while 14 cases (56%) showed active MRI. During DMF therapy we found the expected decrease of disease activity at MRI compared to T0 (6/25 at T1, $p = 0.035$ and 3/25 at T2, $p < 0.00$), and a reduction in the percentage of CD161+CCR6+CD8+ T cells (16/23 at T2; $p < 0.001$). The effects of DMF on gut barrier alterations was variable, without a clear longitudinal pattern, while we found significant relationships between IP changes and drop of MRI activity ($p = 0.04$) and circulating CD161+CCr6+CD8+ T cells ($p = 0.023$).

Conclusions: The gut barrier is frequently altered in MS, and the CD161+ CCR6+CD8+ T cell-subset shows dynamics which correlate with disease course and therapy.

Keywords: multiple sclerosis, intestinal permeability, CD161+CCR6+CD8+T cells, mucosal immunity, dimethyl-fumarate

INTRODUCTION

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS), with inflammation, demyelination, and neurodegeneration. The pathogenic process is immune-mediated, and the etiology is probably multifactorial, with interaction of heritable and non-heritable factors (1).

Among the other factors, microbiota and gut function are increasingly recognized as relevant in this immune-mediated disorder (2). Several studies have recently shown that the microbiota, as a part of the intestine–brain axis, plays a role in the etiopathogenesis of MS (3, 4). However, a crucial component of this axis, the intestinal barrier, has received much less attention. The question of whether or not intestinal permeability (IP) is affected during the disease course is at least as important as the changes in the microbiota balance (5, 6).

IP changes may underlie gastro-intestinal or even far-from-gut autoimmune disorders. In fact, increased gut permeability allows the passage of macromolecules, toxins, and bacterial species that may trigger immune-mediated diseases in different systems, even distant from the gastrointestinal tract, such as the CNS (7, 8). On the other hand, CNS inflammation can increase gut permeability and alter mucosal structure in the small intestine (9).

In a previous work, we investigated the gut permeability in relapsing–remitting MS (RRMS) patients and healthy donors, finding that alteration of IP represents a relatively frequent event in patients with MS (10). This study and a previous one, showing that $CD161^{high}CD8+$ T cells, encompassing the mucosal associated invariant T (MAIT) cell subset, play a role in MS pathogenesis (11), prompted us to focus on the gut triggers that may lower the threshold for disease development in susceptible individuals.

Dimethylfumarate (DMF) has both neuroprotective and anti-inflammatory effects, and it is currently used as an oral, first-line, disease-modifying therapy (DMT) in MS. Some of the mechanisms responsible for its efficacy have been clarified, while others remain unexplored. Gastrointestinal tract irritation is one of the most frequent side effects of DMF (12). On the other hand, studies on experimental models of inflammatory bowel diseases showed that DMF might beneficially affect IP (13).

In this study, we investigated IP changes, the circulating CD161+CD8+ T-cell subset, and clinical/neuroradiological data in a cohort of RRMS patients before and after 9 months of DMF therapy, with a longitudinal design aimed at analyzing data at three time points: baseline (before therapy) and after 1 (T1) and 9 months (T2) of treatment.

METHODS

Subjects and Procedures

Twenty-five patients, candidate to DMF therapy according to the approved indications, were enrolled and completed the follow-up. The other inclusion criteria were as follows: age between 18 and 60 years; a treatment-naïve status or being free from "first-line" DMT for at least 3 months; EDSS up to 5.5.

The exclusion criteria were the following: any serious internal medicine disease; any condition that may possibly interfere with the IP test, such as gastrointestinal disorders, renal function, and bladder dysfunction; pregnancy and breast-feeding. The study was conducted after approval of the local Ethics Committee, and a signed informed consent was obtained from each patient.

Each participant underwent the following procedures at baseline (T0) and after 1 (T1) and 9 months (T2) of DMF therapy, and in case of relapse: clinical evaluation, including the recording of gastrointestinal side effects after DMF start; data-sheet safety laboratory tests; urine sampling for IP test; blood sample for CD161+CD8+ T subset analysis; magnetic resonance imaging (MRI) of brain and spinal cord with gadolinium (Gd) to monitor the disease activity.

MRI Protocol

All subjects underwent gadolinium (Gd)-enhanced MRI (brain and spinal cord). MRI was performed in all the patients with a 1.5-T magnet (Philips Gyroscan NT 1.5), with sequences Flair, T2- and T1-weighted after Gd. The presence of at least one Gd-enhancing lesion or of at least one new/enlarging T2-hyperintense lesion was considered indicative of disease activity at MRI.

Intestinal Permeability Analysis

To evaluate IP, we used a solution composed of 5 g of lactulose and 2 g of mannitol in 50 ml of deionized water. All patients followed a lactulose-, mannitol-, lactose-free diet for 72 h before the test, as reported in a form delivered to the patient at the time of enrollment. After the assumption of the solution, the patients collected their own urine for the following 6 h, during which they have been encouraged to drink tap water. A pre-test urine sample was collected at the beginning and subtracted from the ending total. We calculated the total volume, and we stored 10 aliquots of 5 ml and 5 aliquots of 10 ml at -20°C until analysis. Lactulose and mannitol concentrations in urine samples were analyzed using a modified Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) method (14).

The HPLC analysis was performed using an Agilent Liquid Chromatography System series 1100 (Agilent Technologies, USA). Chromatographic separation was performed using a column (Luna® Omega 3 µm SUGAR 100 Å, LC Column 100

TABLE 1 | Demographic, clinical, and neuroradiological characteristics of patients at baseline.

Females/Males (*n*)	17/8
Age, years [mean (sd), range]	40.36 (12.41), 19–59
Disease duration, years [mean (sd), range]	7.28 (7.76), 1–32
EDSS [mean (sd), range]	1.64 (1.08), 0–5
Patients with relapse	4/25 (16%)
Patients with active MRI*	14 (56%)
DTM naive/free from DMT**	15/10

**The presence of at least one Gd-enhancing lesion or of at least one new/enlarging T2-hyperintense lesion.*

***Free from DMT for at least three months.*

× 2.1 mm, Ea Phenomenex, CA, USA) equipped with a security guard precolumn (Phenomenex, Torrance, CA, USA) containing the same packing material. The mobile phase consisted of a solution of HPLC-grade water (eluent A) and 100% HPLC-grade acetonitrile (eluent B); elution was performed at flow rate of 300 μl/min. The oven temperature was set at 40°C. The injection volume was 10 μl, and the total analysis time was 13 min. The mass spectrometry method was performed on a 3200 triple quadrupole system (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo Ion Spray source. The detector was set in the negative ion mode. The Q1 and Q3 quadrupoles were tuned for the unit mass resolution. The transitions of the precursor ions to the product ions were monitored with a dwell time of 200 ms for each analyte. The instrument was set in the multiple reaction monitoring mode. Mass spectrometer parameters were optimized to maximize sensitivity for all analytes. Data were acquired and processed with Analyst 1.5.1 software. Therefore, we calculated the fractional excretion of lactulose as the following ratio, lactulose: lactulose $(mg)^{excreted}$/lactulose $(mg)^{assumed}$. We used the same method to evaluate excretion of mannitol. Our results have been reported as ratio of the lactulose fractional excretion to the mannitol fractional excretion (L/M ratio). Therefore, we were able to quantify the IP status: Lactulose:Mannitol *ratio* > 0.03 corresponded to an altered permeability, which had to be associated with a urinary mannitol concentration <900 mg/L (15).

Flow Cytometry

Peripheral blood samples were collected into sodium heparin Vacutainer tubes (BD Biosciences, San Jose, CA) at baseline and at month 9 after starting DMF treatment. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation using standard procedures (Ficoll-Paque Plus, GE Healthcare). Fresh PBMC (*ex vivo*) from MS patients were labeled with antibodies directed to cell surface proteins along with a dead-cell discrimination reagent for 20 min at room temperature (RT) in the dark. The following antibodies were used: CD8 FITC (Biolegend), CCR6 Alexa Fluor 647 (Biolegend), CD3 BV605 (Becton Dickinson), CD4 BV785 (Becton Dickinson), CD161APC/Fire 750 (Biolegend), and Live/Dead Fixable Aqua Dead cell stain (Invitrogen) to define the frequency of $CD8^+$ T cells ($CD161^{hi}$, $CCR6^+$). All antibodies were titrated to determine optimal concentrations. Stained cells were acquired on a CytoFLEX flow cytometer (Beckman Coulter), equipped with three lasers and able to

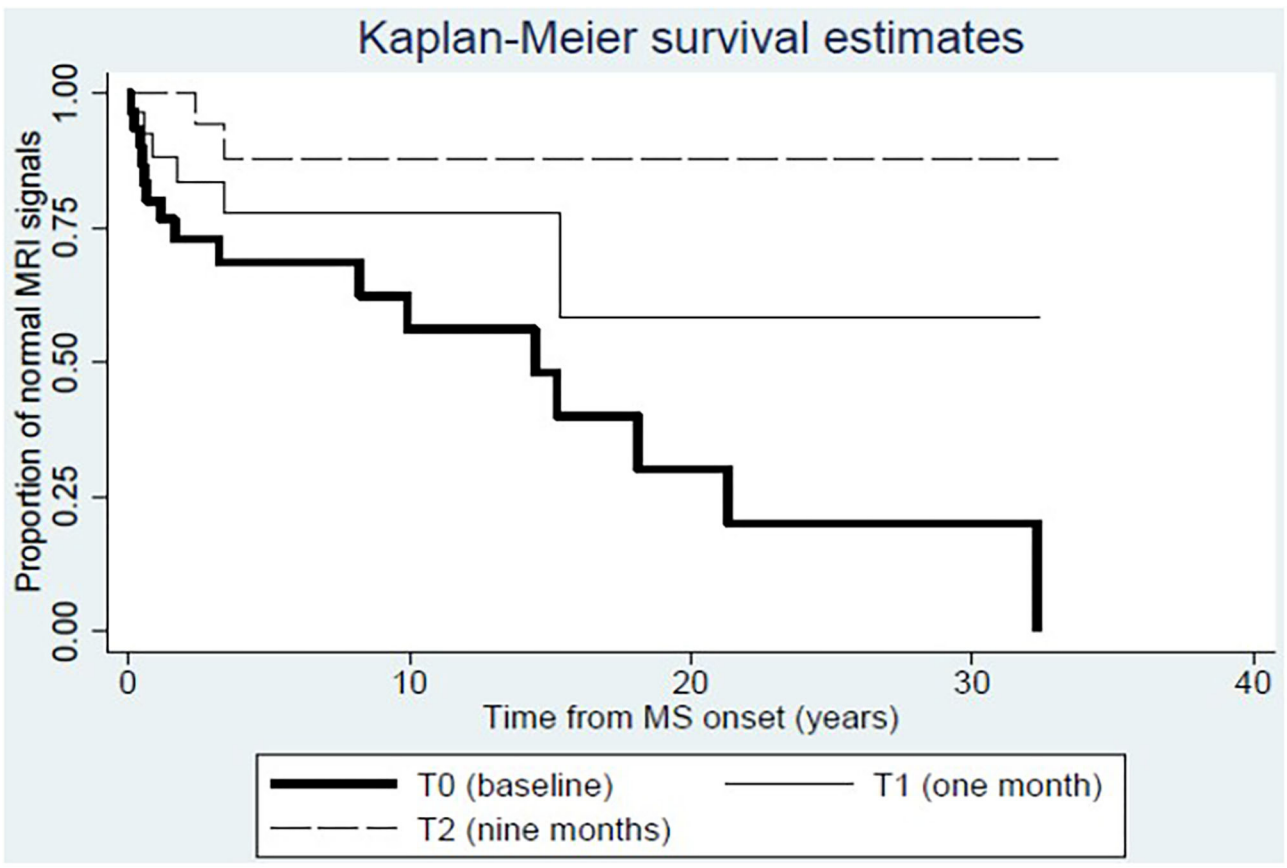

FIGURE 1 | Dynamics of disease activity at MRI before and after dimethylfumarate therapy. Kaplan–Meier estimates show that the proportion of patients with normal MRI signal increases at T1 and T2 compared to T0.

measure up to 15 parameters simultaneously on each cell. For each sample, ∼300,000 lymphocytes were selected based on scatter parameters, and the analysis was conducted after the exclusion of dead cells and coincident events. The data was compensated and analyzed using FlowJo v10.6.1 (TreeStar, Ashland, OR).

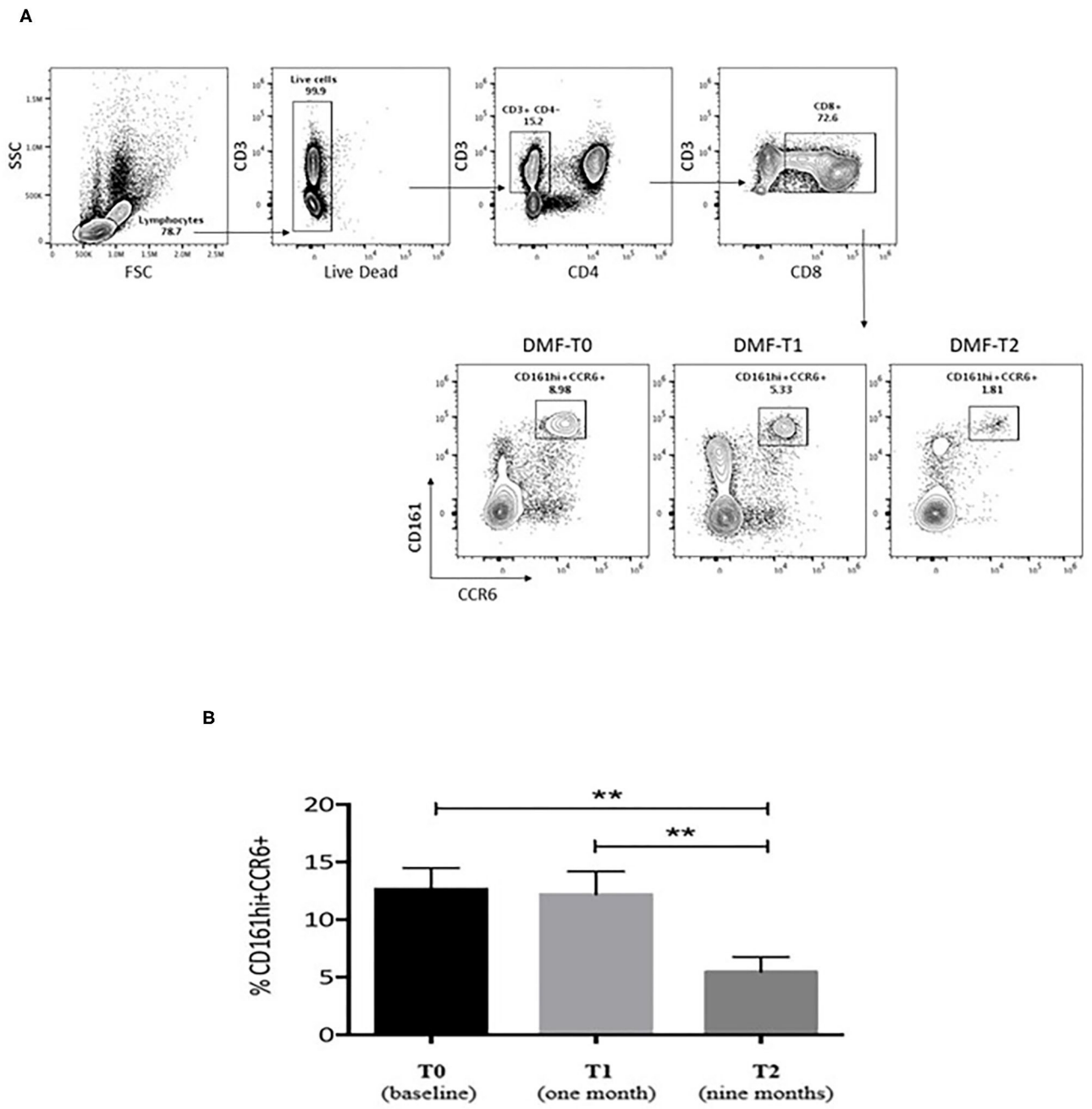

FIGURE 2 | (A) Representative plots showing the frequencies of subpopulations of CD8 T cells (CD161hi CCR6+) in an MS patient during dimethylfumarate (DMF) treatment. Freshly isolated PBMCs were stained with CD3, CD8, CD4, CD161, and CCR6. For this study, we followed 23 patients undergoing treatment with DMF at different time points (3 and 9 months) after initiation of therapy to evaluate the *in vivo* impact of DMF on CD8 T cells in MS. Numbers indicate percent of cells in each quadrant. **(B)** Cumulative data for the frequencies of CD8+CD161hiCCR6+ T cells from the PBMCs of untreated MS patients at baseline (black bar, $n = 23$), T1 (1 month), and T2 (9 months of therapy; gray bars, $n = 23$). The T-cell subset dropped significantly at T2 compared to T0 and T1. Statistical comparisons were performed by ANOVA and Dunnett's multiple comparisons test. Significant values: $^{**}p < 0.001$.

Statistical Analysis

All variables were inspected for normal distribution. Between-group comparisons for continuous variables included parametric Student's *t*-test and ordinary one-way ANOVA, as well as non-parametric Kruskal–Wallis and Dunn multiple comparison tests (GraphPad Prism, v6.2). Between-group comparisons for categorical variables were performed by Pearson's chi-squared test. Statistical significance was inferred for *p*-values below 0.05.

Logistic regression models were fitted to describe the interplay between IP changes and the dynamics of CD161+CCR6+CD8+ T cells and MRI activity across the follow-up period. The same interplay was also explored by Kaplan–Meier and Cox proportional-hazard analysis based on time from MS onset. Multivariate and survival analyses were performed with the Stata software (version 16).

RESULTS

The demographic, clinical, and neuroradiological characteristics of 25 patients at baseline are summarized in **Table 1**. Sixteen patients (64%) showed an altered IP, while 14 cases (56%) showed active MRI (4 of them were also in clinical relapses). Moreover, we investigated the frequencies of CD161+ CCR6+ CD8+ T lymphocytes in PBMCs obtained from 23 MS patients.

During DMF therapy, two significant changes emerged. At first, we could confirm the decrease of disease activity as evaluated by MRI (6/25 at T1, $p = 0.035$ for the comparison between T1 and T0; 3/25 at T2, $p < 0.001$ for the comparison between T2 and T0); consistent with this result, the Kaplan–Meier analysis showed that the proportion of patients with normal MRI signal is higher at T1 and T2 compared to T0 (**Figure 1**). Then, we showed that the frequency of circulating CD161+CCR6+CD8+ T cells in MS patients is reduced after 9 months of DMF treatment. In **Figure 2A**, representative plots depict the progressive drop of frequencies of subpopulations of CD8 T cells (CD161hi CCR6+) in an MS patient during DMF treatment, while **Figure 2B** shows the cumulative data with the significant drop of the T-cell subset at T2 ($p < 0.001$).

We found that the decrease in disease activity evaluated radiologically was 12 times higher in subjects showing reduced frequencies of CD161+CCR6+CD8+ T cells in the peripheral blood, and 15 times higher in cases with IP changes at T1 (**Table 2**). Furthermore, a logistic regression model showed a relationship between the drop of CD161+CD8+ T cells in the peripheral blood and IP changes at T2, considering as covariates both EDSS and MRI activity ($p = 0.023$). Consistent with this result, the Cox analysis showed that the decline of the T-cell subset was more evident in patients with persistent IP changes (Hazard Ratio, HR = 4.19; $p = 0.03$; **Figure 3**).

Concerning the possible effects of DMF treatment on gut barrier alterations, no significant difference emerged: some cases improved, while others worsened during follow-up, without a clear longitudinal pattern (**Supplementary Table**); the proportion of patients having IP changes at T1 and T2 was 16/25 (64%) and 15/25 (60%), respectively (figures that were quite comparable to the baseline data). A minority of patients (3/25) had mild gastro-intestinal side effects during treatment with DMF; other mild side effects were within the known safety profile of the drug (not shown). One patient presented a relapse during the follow-up, and no significant change occurred in patients' EDSS at T2 compared to baseline.

TABLE 2 | Probability of MRI activity reduction at T2 in cases with parallel drop of CD161+CCR6+CD8+ T cells in blood, and in cases with IP changes at T1.

Predictors	OR	*P*-value
Logistic regression model for MRI activity drop at Time 2		
Drop of T cells at Time 2		
No	1	
Yes	12.63	0.071
IP change at T1		
No	1	
Yes	15.42	0.040

Other covariates include EDSS and gastro-intestinal symptoms at baseline

DISCUSSION

This work, together with our pilot studies on IP and mucosal immunity in RRMS (10, 11), provides evidences that the gut barrier is frequently altered in these patients and that the CD161+CD8+ T-cell subset shows dynamics compatible with disease course and therapy. No other studies on IP changes in MS have been reported so far, since the other works are mainly focused on microbiota changes (3). However, we deem IP alterations and dysbiosis as two faces of the same coin (16), and further studies correlating IP and gut microbiota changes in MS will certainly be informative on disease etiopathogenesis.

The CD161+CD8+ T-cell subset encompasses the MAIT cells, which were the object of several investigations after our first study on MS in 2011 (11), all largely confirming the involvement of MAIT cells in MS pathogenesis. Among the evidences repeatedly reported were an IL18-driven activation and consequent CNS infiltration of MAIT cells in the diseased brain, and an increased type-17 differentiation and oligoclonality of circulating MAIT cells in MS patients compared to controls (17–21). The IL18-driven activation, and the consequent CNS infiltration of CD8+ MAIT cells in MS, may cause reduced frequency in blood, helping to reconcile, at least in part, the conflicting results on the frequency of circulating MAIT cells in MS. A recent work showed indeed that MAIT cell subtype, smoking habit, and disease onset (primary progressive vs. relapsing–remitting) affect the number of circulating MAIT cells (22). Smokers with primary progressive MS showed low frequency of circulating MAIT cells, suggesting a tendency to reside in the inflamed organ, in apparent contrast to what was observed in most studies on patient with RRMS.

Concerning the effects of DMF on the variables under study, we found the expected decline of disease activity, which was in keeping with the initial pivotal trials (23–25). The parallel drop in the fraction of circulating CD161+CD8+ T cells is in accord

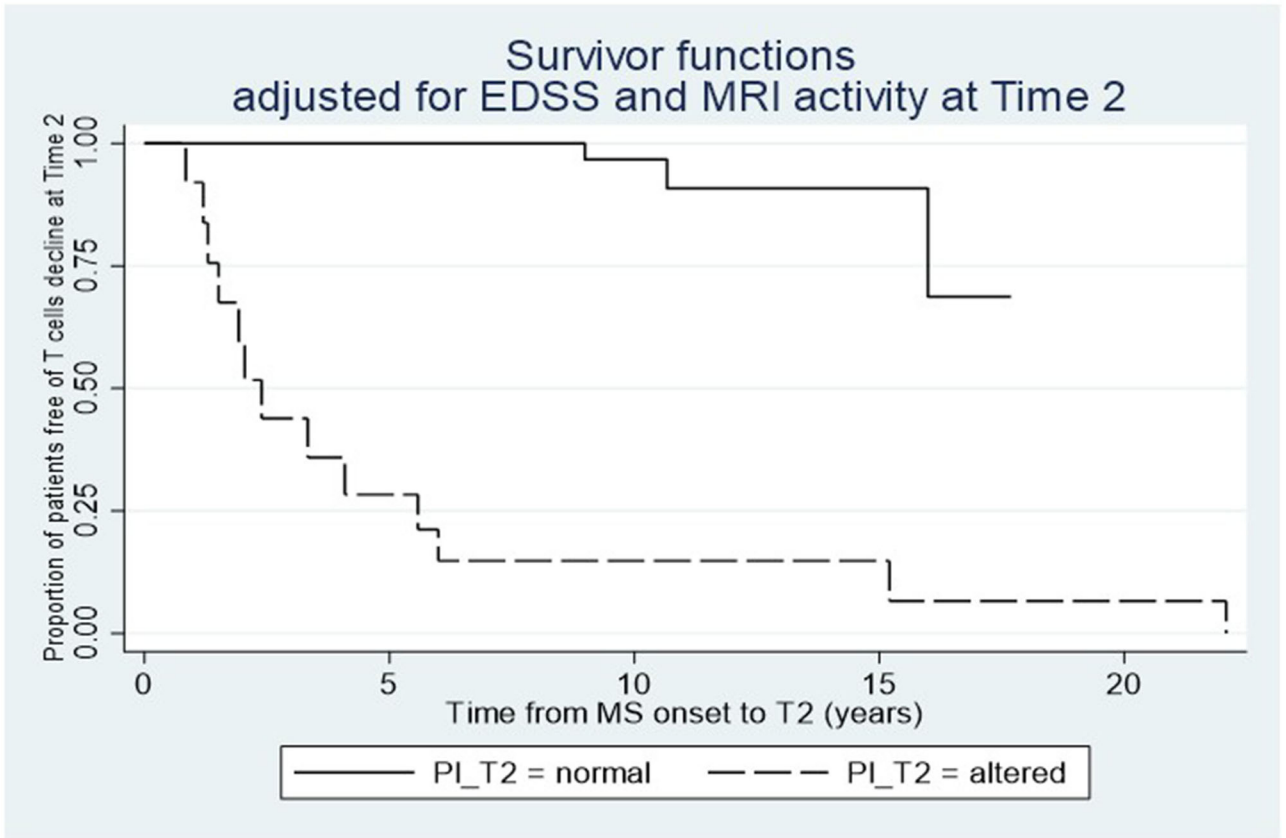

FIGURE 3 | Relationship between the dynamics of circulating CD161+CD8+ T cells after dimethylfumarate therapy and the IP changes at T2. Cox proportional-hazard estimates show that the T-cell subset drop is higher in patients with IP alterations compared to those with normal IP at T2.

with two previous works on the effects of DMF therapy in MS patients (22, 26). The action of DMF on all the proinflammatory T-cell subsets, including the CD161+ IL17-producing T cells, is mediated by a dose-dependent induction of apoptosis and decrease of proliferation (27). Other works, showing a decrease of proinflammatory MAIT cells after hematopoietic stem cell transplant or alemtuzumab for treatment-refractory forms (28, 29), support results obtained after DMF treatment, and indirectly confirm the pathogenic role of MAIT cells in MS.

No clear DMF effects were evident on IP changes, and the gastrointestinal side effects in our group of patients were relatively rare and apparently unrelated to IP changes. The meaning of this finding requires further studies (such as those based on novel multi-sugar assay for site-specific gastrointestinal permeability analysis) and suggests that the alterations of the gut barrier in MS are complex: the decreased disease activity at MRI and the reduction of the percentage of circulating MAIT cells during treatment with DMF seem to occur more frequently in patients with IP changes. These relationships raise the possibility that the gut barrier alteration may represent a predictor of pathophysiological transitions, besides its possible role in disease pathogenesis. Our study adds evidences to the potential role of mucosal immunity in MS pathogenesis, and yet suggests questions that remain unanswered. Among those are whether IP changes somehow drive demyelinating process [as seen in experimental models of MS; (9)] or simply contribute to the organ-specific immune dysfunction. Also, it is unclear through which mechanisms MAIT cells [or subsets of them; (22)] become activated and pathogenic at the CNS level in apparently sterile conditions. Answering these questions may provide new fruitful lines of attack against neuroinflammation, such as IP enhancers or stabilizers, already under scrutiny in gastro-intestinal conditions, as well as compounds coming from reworking the increasingly growing data coming from microbiota studies in experimental and human autoimmune diseases.

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 Azienda Ospedaliero-Universitaria Sant'Andrea,

Università Sapienza. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MSa, GR, and LB conceived the study. MB, GB, and MSi coordinated the project. FG, MC, and LL performed data analysis. CF carried out statistical analysis. All authors contributed to the project, writing of the manuscript, and approved its final 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.2021.683398/full#supplementary-material

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The Dimethyl Fumarate Experience: A Handy Drug With Broad Clinical Utility

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Objectives: The aim of this study was to characterize multiple sclerosis (MS) patients exposed to dimethyl fumarate (DMF) and to evaluate the predictors of therapeutic response. In addition, the study offers a picture of how DMF use has changed over the past few years in naive or switcher patients.

Methods: In this observational monocentric study, we examined the prescription flow of DMF in MS patients categorized as naive or switchers (for safety/tolerability, ineffectiveness, and de-escalation strategy) from 2015 to 2019. Clinical and magnetic resonance imaging data of DMF-treated patients were analyzed, and NEDA-3 status at 24 months was evaluated by the three assessment components (absence of clinical relapses, no Expanded Disability Status Scale progression, no radiological activity). Determinants of therapeutic response were also evaluated using regression analysis.

Results: The sample included 595 MS patients exposed to DMF categorized as naive (158; 26.5%) and switchers for reasons of safety/tolerability (198; 33.3%), inefficacy (175; 29.4%), and de-escalation strategy (64; 10.8%). A 15% increase in DMF use in naive and horizontal shift groups was observed in the last 3 years of observation, whereas there was a drop, with prescription passed from ∼20% to <5%, as an exit strategy from second-line therapies. NEDA-3 status was calculated for 340 patients after 24 months of DMF treatment and achieved in 188 (55.3%) of these. Analyzing the predictors of DMF response, we observed that lower annualized relapse rate (ARR) in 2 years pretreatment [hazard ratio (HR) = 0.49, $p = 0.001$] and being naive patients (HR = 1.38, $p = 0.035$) were associated with achievement of NEDA-3. Analogously, ARR in 2 years pretreatment affected the NEDA-3 achievement at 24 months in patients of the de-escalation group (HR = 0.07, $p = 0.041$), also indicating an effect related to the DMF initiation within 3 months (HR = 1.24, $p = 0.029$).

Conclusion: Our findings confirm DMF as a handy drug with broad clinical utility, with greater benefits for naive patients and horizontal switchers. Additionally, an increase in the flow of DMF prescriptions in these two groups of patients was also observed in our cohort.

Keywords: multiple sclerosis, dimethyl fumarate, real world study, efficacy, NEDA 3

INTRODUCTION

In the last decade, many changes have marked the therapeutic scenario of multiple sclerosis (MS), with the introduction of new disease-modifying therapies with different mechanisms of action, efficacy, and safety profile, resulting in improved choice and steps toward a personalized therapy (1). Dimethyl fumarate (DMF) has been approved as a first-line oral agent for the treatment of relapsing MS, based on the phase III clinical trials data (2, 3). Since its entry into clinical practice setting, postmarketing studies and several real-world experiences have highlighted the multifaceted utility of DMF and added knowledge to identify the best candidate patients. Some studies have shown improved clinical and radiological outcomes, mostly in patients with moderate disease activity before treatment, with better effects in naive patients compared with switchers (4, 5). Moreover, a number of MS-related factors appear to be predictors of response to DMF treatment, such as a shorter disease duration that has been associated with higher rate of NEDA-3 (No Evidence of Disease Activity) (6, 7). This point is in line with the assumption about the influence of the disease-modifying therapies on MS that indicates that "treating early is better than late, but late is better than never," and this is fundamental to define the best choice and window of therapeutic opportunity (8). With the growing experience in the clinical setting, the use of DMF has changed, and the drug is increasingly considered as an option in naive patients and switchers, also in consideration of the data of comparative studies (9, 10), and it has also been evaluated as a possible exit strategy from second-line therapies (11, 12).

Based on these considerations, the present study aimed to (i) define demographic and clinical features of MS patients undergoing DMF therapy categorized as naive or switchers (for safety/tolerability, ineffectiveness, de-escalation strategy), also describing how DMF prescription flow has changed in these four patient categories over the past 5 years, and (ii) evaluate the efficacy data in the different DMF patient groups, also evaluating the predictors of therapeutic response.

METHODS

Study Design and Data Acquisition

This is an observational monocentric study that included MS patients diagnosed with the revised McDonald criteria (13), who started DMF therapy between January 2015 and December 2019. The patients' demographic characteristics (sex and age) and clinical data [age at DMF initiation, disease duration, and disability level, evaluated using the Expanded Disability Status Scale (EDSS)] (14) were collected. The last follow-up of the year 2020 was considered for each patient. Previous disease-modifying therapies, date of last therapy withdrawal and reason of switching to DMF as well as the number of relapses, and annualized relapse rate (ARR) 2 years before DMF start were also recorded. Thus, patients were classified as naive or switchers due to three different reasons (safety/tolerability, ineffectiveness, de-escalation strategy). Additional information about the duration of DMF treatment, the number of relapses, the ARR during the DMF exposure, and magnetic resonance imaging (MRI) outcomes, such as presence of new or enlarging T2 lesions or gadolinium-enhancing T1 lesions at MRI assessments carried out annually after DMF initiation and compared with the rebaseline MRI performed after 6 months, were recorded. The timing of the rebaseline MRI was defined at 6 months, on the pharmacodynamics of the DMF, as recommended, to avoid considering disease activity that may occur in the weeks and months following the initiation of therapy as disease activity unresponsive to treatment. Next, for patients exposed to DMF for 24 months, NEDA-3 status was evaluated by the three assessment components (absence of clinical relapses, no EDSS progression, absence of radiological activity on MRI performed at 24 months of DMF compared to the rebaseline MRI), and determinants of NEDA-3 status were explored (6). Finally, all side effects reported by the patients were registered, as well as the DMF discontinuation causes and subsequent therapeutic choices. Informed consent was obtained from all participants after the local ethics committee approval.

Statistical Analysis

SPSS for Mac version 20.0 (SPSS Inc., Chicago, IL, USA) was used to perform the statistical analysis. Descriptive statistics are presented using mean, SDs, and frequencies (absolute and relative). First, the percentage of naive patients and switchers initiated to DMF therapy was assessed for the years 2015–2019. Thereafter, demographic (sex, age) and clinical differences (disease duration, EDSS score, age at DMF initiation, and DMF duration) among patients exposed to DMF categorized as naive or switchers (for safety/tolerability; ineffectiveness; de-escalation strategy) were evaluated using independent-samples t-tests for quantitative variables and χ^2-tests for qualitative variables. Mann–Whitney U tests were used to compare the ARR calculated 2 years before DMF therapy and at 24 months following DMF therapy for the four groups of patients, naive and switchers, also categorized in relation to the last disease-modifying therapy. Therefore, the achievement of NEDA-3 status at 24 months was calculated as a percentage of patients with no clinical relapses, EDSS progression, and radiological activity, and the predictors of NEDA-3 status were investigated using binary regression analysis. For all assays, statistical significance was set at $p < 0.05$.

RESULTS

The sample included 595 MS patients exposed to DMF categorized as naive (158; 26.5%) and switchers for reasons of safety/tolerability (198; 33.3%), inefficacy (175; 29.4%), and de-escalation strategy (64; 10.8%). Of the patient group, the mean DMF exposure was 28.7 (SD = ±18) months, while the median was 27 months (±32 months of IQR).

Table 1 shows the demographic and clinical differences of naive MS patients versus switchers examined by χ^2 and independent-samples t-tests, showing lower age, MS duration, and EDSS score ($p < 0.005$) for naive patients. The percentage of naive MS patients who initiated DMF use is detailed in **Figure 1**; in particular, in the last 2 years of the observation period (2015–2019), there was a 15% increase in the use of

TABLE 1 | Demographic and clinical features of MS patients exposed to dimethylfumarate categorized as naive or based on the type of therapeutic shift (horizontal for safety; horizontal for DMDs ineffectiveness; de-escalation).

	MS Patients exposed to Dymetilfumarate (595)				
	Naïve (158; 26.5%)	**Switchers (437; 73.5%)**	**Horizontal shift for safety (198; 33.3%)**	**Horizontal shift for DMDs inefficacy (175; 29.4%)**	**De-escalation Shift (64; 10.8%)**
Male Gender	47 (29.7%)*	112 (70.9%)	46 (23.2%)	50 (28.1%)	16 (25%)
Age at DMF initiation (years)	35.9 ± 10.6**	40.3 ± 9.8	40.6 ± 9.3	39.9 ± 10.7	41.2 ± 8.5
MS duration at DMF initiation (years)§	2.9 ± 4.7**	10.3 ± 7.5	10.1± 7.2	9.3 ± 7.5	13.9 ± 7.8
EDSS score at DMF initiation	1.7 ± 1.1**	2.4 ± 1.6	2.2± 1.5	2.2 ± 1.5	3.3 ± 2.1
DMF exposition (months)	25.9 ± 18.3*	29.8 ± 17.7	29.2 ± 18.3	30.1 ± 18.2	30.8 ± 14.5

**p-value 0.05.*
***p <0.005.*
§MS duration at DMF initiation (years) is defined with respect to the first MS symptom presentation.
Chi-square and independent-samples t-tests were used to compare demographic and clinical features of naïve MS patients vs. MS patients previously treated with DMDs (switchers). Data for each group of switchers are also shown.

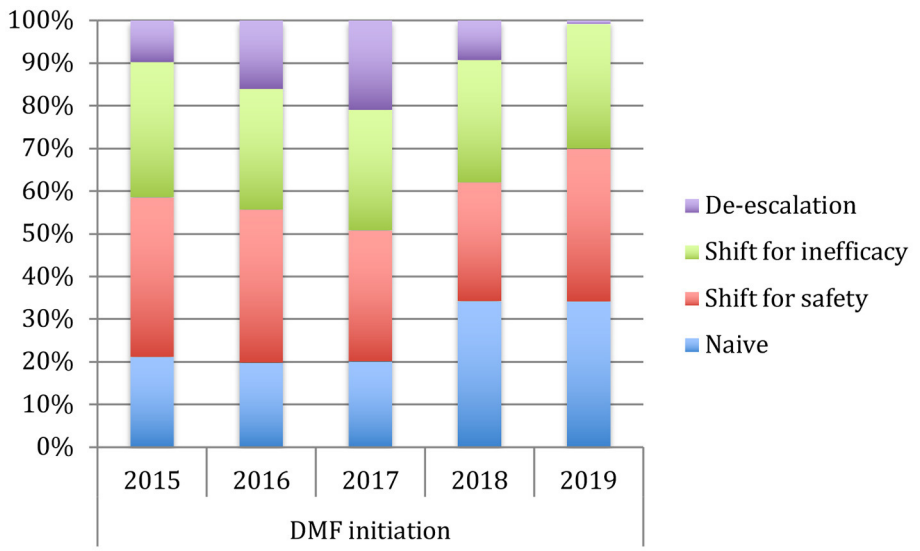

FIGURE 1 | Use of dymetilfumarate in the last five years (2015–2019). The graph indicates the percentage of patients naive or who have undergone a therapeutic shift from 2015 to 2019.

DMF in naive subjects, prescribed in ∼20% of patients initiated on DMF between 2015 and 2017 and then in ∼35% during 2018–2019. Similarly, an increase in DMF use in horizontal shift was observed in the last 3 years of observation, whereas there was a significant drop in DMF use as an exit strategy, with prescription in ∼20% of patients during 2017, in ∼10% during 2018, and then in <5% during 2019. MS treatments before DMF initiation are detailed in **Table 2**. In particular, among the 437 switchers patients, a shift for safety/tolerability was reported by 198 patients [144 (72.7%) after interferon β, 35 (17.7%) after glatiramer acetate, 19 (9.6%) after teriflunomide], whereas a shift for inefficacy was reported by 175 subjects [111 (63.4%) after interferon β, 46 (26.3%) after glatiramer acetate, 18 (10.3%) after teriflunomide]. DMF as exit strategy from second-line therapies was used by 64 patients; of these, 56 (87.5%) shifted from natalizumab for JC virus antibody seropositivity and 8 (12.5%) from fingolimod, with mean time from second-line treatment to DMF initiation of 121 ± 87 days. Of de-escalating patients, four had a relapse in the wash out period, while five within the first year.

Table 3 shows the comparisons between ARR 24 months before and after DMF therapy, analyzed for 340 MS patients (naive or switchers), indicating a significant ARR reduction in the naive group (ARR pre-DMF 0.30 ± 0.34 vs. ARR post-DMF 0.19 ± 0.36, $p = 0.014$), switchers for inefficacy (ARR pre-DMF 0.67 ± 0.68 vs. ARR post-DMF 0.11 ± 0.18, $p = 0.001$), and

TABLE 2 | DMDs treatment before dymetilfumarate initiation in relation to the type of therapeutic shift performed.

	MS Patients exposed to therapeutic shift (437)	
	Horizontal shift for safety (198)	**Horizontal shift for DMDs inefficacy (175)**
INF β	144 (72.7%)	111 (63.4%)
Glatiramer Acetate	35 (17.7%)	46 (26.3%)
Teriflunomide	19 (9.6%)	18 (10.3%)
	De-escalation shift (64)	
Fingolimod	8 (12.5%)	
Natalizumab	56 (87.5%)	

TABLE 3 | ARR before and after 24 months of DMF (340 MS patients).

	ARR 2 years pre DMF	ARR on DMF	*p*-value
MS patients–Naive (77)			
	0.30 ± 0.34	**0.19 ± 0.36**	**0.014**
MS patients–Horizontal shift for safety (114)			
IFN (82)	0.47 ± 0.70	0.12 ± 0.25	<0.001
GA (17)	0.47 ± 0.59	0.08 ± 0.15	0.043
TFU (15)	0.50 ± 0.52	0.15 ± 0.26	0.057
TOT	**0.47 ± 0.65**	**0.12 ± 0.24**	**<0.001**
MS patients–Horizontal shift for DMDs inefficacy (106)			
IFN (71)	0.67 ± 0.66	0.10 ± 0.17	<0.001
GA (23)	0.52 ± 0.39	0.15 ± 0.22	<0.001
TFU (12)	1.0 ± 1.15	0.08 ± 0.17	0.001
TOT	**0.67 ± 0.68**	**0.11 ± 0.18**	**<0.001**
MS patients–De-escalation shift (46)			
NTZ (42)	0.13 ± 0.33	0.19 ± 0.26	ns
FTY (4)	0.25 ± 0.35	0.26 ± 0.37	ns
TOT	**0.17 ± 0.32**	**0.13 ± 0.26**	**ns**

Mann–Whitney tests were used to compare the ARR calculated 2 years before DMF and the ARR at 24 months of DMF for the four groups of patients (naive and switching to DMF). The significant results were shown in bold.

switchers for safety/tolerability (ARR pre-DMF 0.47 ± 0.65 vs. ARR post-DMF 0.12 ± 0.24, $p = 0.001$). No difference in ARR before and after 24 months of DMF therapy was found in the de-escalation group, which continued DMF treatment. However, nine de-escalating patients (five after natalizumab and three after fingolimod) discontinued DMF within the first year, whereas 10 patients (six after natalizumab and four after fingolimod) discontinued DMF between the first and second year, mainly due to ineffectiveness (72.2%).

Finally, NEDA-3 status was calculated for 340 patients after 24 months of DMF treatment and achieved in 188 (55.3%) of these. In detail, relapse-free status was observed in 229 patients (67.5%), no disability progression in 299 (87.9%), and MRI NEDA-3 status in 283 patients (83.2%) (**Figure 2**). Analyzing the predictors of response to DMF, we observed that lower ARR in the 2 years pretreatment [hazard ratio (HR) 0.49, $p = 0.001$)] and being naive patients (HR = 1.38, $p = 0.035$) were associated with achievement of NEDA-3 (**Table 4**). Analogously, ARR in the 2 years pretreatment affected the NEDA-3 achievement at 24 months in the patients of de-escalation group (HR = 0.07, $p = 0.041$), also indicating an effect related to the DMF initiation within 3 months (HR = 1.24, $p = 0.029$; **Table 5**).

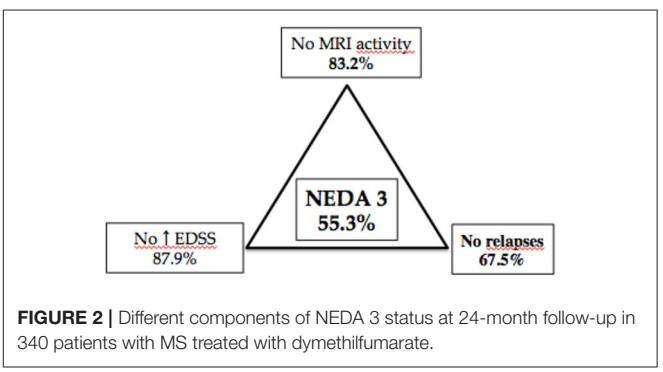

FIGURE 2 | Different components of NEDA 3 status at 24-month follow-up in 340 patients with MS treated with dymethilfumarate.

The overall discontinuation rate was of 17.9% (107/595 patients); of these, 60 of 595 patients (10%) discontinued DMF due to ineffectiveness, 34 (5.7%) of whom within the first

TABLE 4 | NEDA 3. Predictors of therapeutic response in patients exposed to 24 months of dymetilfumarate.

		NEDA 3				
				95% C.I. for EXP (B)		
		B	Exp (B)	Lower	Upper	*p*
Variables	Age at DMF initiation	0.008	1.010	0.982	1.035	0.542
	ARR 2 year pre DMF	−0.588	0.491	0.415	0.744	**0.001**
	MRI activity 2yr pre DMF	0.562	1.121	0.947	3.246	0.074
	Naive	0.662	1.389	0.275	0.967	**0.035**

Multiple regression analysis was used to examine which demographic and clinical variables, included in the model as independent variables, influence the achievement of NEDA 3 status at 24 month of DMF exposure.
The significant results were shown in bold.

TABLE 5 | NEDA 3. Predictors of DMF efficacy after de-escalation switching.

	NEDA 3				
			95% C.I. for EXP (B)		
	B	Exp (B)	Lower	Upper	*p*
ARR 2 year pre DMF	−2.225	0.070	0.13	0.915	0.041
MRI activity 2 year pre DMF	−1.092	0.257	0.063	1.776	0.335
DMF start within 3 months	1.244	1.151	1.139	10.571	0.029

Regression analysis was used to examine clinical variables, included in the model as independent variables, influence the achievement of NEDA 3 status in MS patients switching from Natalizumab.

year of treatment [nine de-escalating from second-line Disease Modifying Treatments (DMTs)]. Analogously, of 21 patients (3.5%) who discontinued DMF between the first and second year, 10 were after de-escalation strategy.

Finally, 47 of 595 patients (7.9%) in our cohort discontinued DMF for safety/tolerability reasons, mainly during the first year of treatment (72% of cases). Of these, gastrointestinal (GI) symptoms were reported as the primary side effect, accounting for 4.6% of drug suspension, followed by flushing (3.1%) and laboratory testing abnormalities (hypertransaminasemia for 0.1% and prolonged lymphopenia for 0.1%). A shift to oral teriflunomide was reported in 18 (38.3%) of these patients, to glatiramer acetate (Copaxone) in 15 (31.9%), and to interferon in 11 (23.4%), whereas 3 patients (6.4%) did not undertake other immunotherapies.

DISCUSSION

Previous studies have evaluated the effectiveness of DMF with analysis of the real-world data. Our data can be differentiated from previous studies in several respects, including data source, cohort (this is a large real-world monocentric study), method of analysis (differentiated assessment of clinical outcomes for patients categorized into four groups), and the evaluation of the results with the examination of predictors of MS outcomes.

In line with other studies that have shown a good efficacy profile of DMF both in naive and horizontal switchers (4, 5, 7), we report a reduction of ARR in these two patient groups after 24 months of treatment. In keeping with this point, an increase in the prescription flow of DMF in naive and switchers for ineffectiveness or safety was observed during the observational period in our cohort, based on DMF persuasive efficacy–risk profile as well as patient preference for oral administration. Furthermore, our data offer new evidence in clinical setting on horizontal therapeutic switching choice, on which a growing literature is trying to discuss the utility of the use of drugs of the same line with different mechanisms of action, as well as the best time and patient candidates for this choice (15, 16). On the contrary, a reduction in DMF use as exit strategy from second-line therapies was reported in our cohort. Bearing in mind that patients de-escalating from second-line therapies to DMF did so mostly for safety reasons, in particular for JC virus positivity during natalizumab treatment, in line with published data (11, 12, 17), we observed that DMF did not eliminate the risk of MS reactivation, with discontinuation of DMF during the first year for 8.9% of our patients previously treated with natalizumab. Interestingly, for patients who persist in DMF treatment, no differences in pre- and post-ARR at 24 months were observed. Moreover, the regression analysis showed that the latency in months in the initiation of DMF therapy is an important determinant of the achievement of NEDA-3 at 24 months, reinforcing the concept of the need to rapidly finalize the therapeutic choice (18), in particular in de-escalation switching.

The reduction of DMF use as an exit strategy observed in our cohort may be attributable to a better selection of patients to be initiated on natalizumab therapy based on JC virus serostatus (19), as well as to the use of new strategies in the clinical setting that allow to continue the treatment while limiting the risk of progressive multifocal leukoencephalopathy (i.e., natalizumab

extending dose protocol) (20, 21). However, it is conceivable that the reduction of DMF use as de-escalating strategy is also attributable to the recent availability of more efficacious agents with rapid effects (21), as well as to the growing awareness that the timing of full effectiveness of DMF does not prevent from disease rebound (11).

Analyzing the predictors of NEDA-3 status, clinical activity in the 2 years preceding DMF and being naive patients emerged as significant determinants confirming, as previously demonstrated by Lanzillo et al., the utility of this oral agent from the earliest stages of the disease (7). Analogously, other studies showed that not only naive patients strongly benefit from DMF, but also patients switched from injectable DMTs due to tolerability and efficacy issues (4, 5). Moreover, our results showed a higher NEDA3 proportion than that reported in the Northern Italy Multicenter Study (5), and this likely is attributable to the differences in patient characteristics and selection. Similarly, we found a higher NEDA3 status of those described in the integrated analysis of the phase III DEFINE and CONFIRM studies (22). Finally, other studies of real-world setting found a higher baseline EDSS, a larger number of T1Gd+ lesions, and a switch because of inefficacy (vs. adverse events) as the principal risk factors for losing NEDA-3 status (23).

Overall, safety data confirmed a favorable profile for DMF, with 7.9% patients dropped out due to safety or tolerability issues, the most frequent being GI tolerability (4.6%). Grade III lymphopenia, which other studies reported as an infrequent event ranging between 3 and 15% of patients (4), was a rare cause of DMF discontinuation in our study (0.01%). Moreover, few recent studies explored the recovery of lymphocyte count after DMF discontinuation that could be very slow in some cases with potential consequences on treatment choice (24, 25). The evaluation of these and other safety aspects is of central importance to better understand adherence, treatment persistence, and the usefulness of other therapeutic decisions.

Another important safety issue linked to the increasingly widespread use of DMF in young women is related to pregnancy, on which preliminary data would have shown safe outcomes (26). Further data, to support these early evidences, are, however, needed.

The present study has several limitations mainly due to its retrospective nature. However, compared to other studies on this topic, the monocentric nature of our study allowed limiting the variability in radiological and clinical data collection. Furthermore, the study focused exclusively on evaluating DMF efficacy outcomes, considering the composite evaluation of clinical relapses, EDSS progression, and neuroradiological activity (NEDA-3) as disease outcome. Safety aspects of DMF and the possible predictors of safety outcomes have not been deliberately explored.

CONCLUSION

Our findings confirm DMF as a handy drug with broad clinical utility. DMF use has progressively increased in clinical practice, showing greater benefits for naive patients and horizontal switchers. Further studies are needed to better investigate the predictors of efficacy, as well as the predictive biomarkers, for the best identification of patients to be initiated on DMF treatment, in the modern perspective of an effective, early, and personalized therapy (1).

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 University of Cagliari. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LL and ECo conceptualized the study and wrote, reviewed, and edited the manuscript. ECa, MF, JF, GF, GC, SP, and MB were responsible for resources and data curation. All authors contributed to the article and approved the submitted version.

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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.

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Silybin Alleviates Experimental Autoimmune Encephalomyelitis by Suppressing Dendritic Cell Activation and Th17 Cell Differentiation

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Silybin, a peculiar flavonoid compound derived from the fruit and seeds of *Silybum marianum*, exhibits strong anti-inflammatory activities. In the present study, we found that silybin effectively alleviated experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), via inhibition of dendritic cell (DC) activation and Th17 cell differentiation. Silybin treatment greatly ameliorated the disease severity and significantly declined inflammation and demyelination of the central nervous system (CNS) of EAE mice. Consistent with the disease development, silybin-treated bone marrow-derived DCs (BM-DCs) exhibited reduced costimulatory molecules (e.g., CD80 and CD86) and MHC II expression. These results demonstrated the distinguished bioactivity of silybin for suppressing DC activation, inhibiting pathogenic Th17 inflammatory cell responses, and, eventually, alleviating EAE severity. Taken together, our results show that silybin has high potential for the development of a novel therapeutic agent for the treatment of autoimmune diseases such as MS.

Keywords: EAE, multiple sclerosis, silybin, dendritic cell, T cell

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INTRODUCTION

Multiple sclerosis (MS) is an inflammatory and neurodegenerative demyelinating disease of the central nervous system (CNS). The pathogenesis of MS is multifactorial, involving genetic and environmental elements interacting in complicated ways. The history of MS therapy is a wonderful instance of a successful investigation translated into treatments and enhanced clinical results (1). Experimental autoimmune encephalomyelitis (EAE), as a classic animal model of MS, is widely applied for drug development and discovery. Although the definite pathogenesis has not been illuminated clearly, increasing evidences endorse that MS is an autoimmune disease with irreversible white matter (WM) damage (2, 3). At the early phase of EAE, myelin-specified $CD4^+$ T cells, as well as dendritic cells (DCs), B cells, and macrophages, are triggered in the periphery and penetrated the CNS. Among diverse $CD4^+$ T-cell subsets, interleukin-17 (IL-17)-positive Th17 cells, which secrete IL-17A, are regarded as the primary effector cells in provoking an inflammatory response in MS/EAE (4). In addition, antigen-presenting cells (APCs) also exert an essential function in MS/EAE by activating naïve T cells, among which DCs are experts at regulating rest T-cell polarization with antigen peptides present (5).

Current clinical treatments for MS possess either insufficient performance or uncertain safety problems. Numerous researches have been devoted to expanding novel therapeutic drugs target Th17 cells without affecting other cells. In the past few years, a lot of immune-modulatory monomers derived from medicinal plants exhibit a tremendous capacity for treating MS/EAE. These small molecule natural compounds present excellent fortune for identifying effective and safe medicine candidates. Silymarin is a peculiar flavonoid compound derived from the fruit and seeds of *Silybum marianum*. It contains a group of flavonolignans, such as silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, silydianin, and taxifolin (6). Silybin, also known as silibinin, is the major biologically active constituent of the silymarin complex (about 70–80%) and is a mixture of silybin A and silybin B. Pharmacological research demonstrated that silybin possesses potent antioxidant, anticarcinogenic, and anti-inflammatory activities (7–9). Moreover, Lee et al. also proved that silybin has excellent therapeutic effects on EAE by inhibiting the polarization of Th1. However, the activity of silybin on Th17 and DC development is still unclear. To tackle these issues, here, we employed the MOG_{35-55}-induced autoimmune animal model to explore the therapeutic activities and the underlying mechanism of silybin. The efficacy of silybin made it a potential therapeutic drug for alleviating EAE as well as other autoimmune diseases.

MATERIALS AND METHODS

Experimental Autoimmune Encephalomyelitis Induction and Drug Treatment

Female C57BL/6 mice (8 weeks old) were purchased from the Air Force Medical University (Xi'an, China). All the animal experiments were performed following the Guidelines for Care and Use of Laboratory Animals of Xian Yang Central Hospital and authorized by the Animal Ethics Committee of Xian Yang Central Hospital. The EAE installation procedure was as outlined previously (10). Briefly, mice were subcutaneously immunized at two sites on the back with 200 mg of myelin oligodendrocyte glycoprotein$_{35-55}$ (MOG_{35-55}; Genscript, Piscataway, NJ, USA) in 200 μl of emulsion comprising 50% complete Freund's adjuvant with 5 mg/ml of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Lawrence, KS, USA). Pertussis toxin (PT) (200 ng/mouse; Sigma-Aldrich, St. Louis, MO, USA) was administrated intraperitoneally (i.p.) to the mice on day 0 and 2 days post-immunization (p.i.). Clinical scores were record daily in a blind manner, according to a 0–5 scale as described previously (11). Accumulative scores of each mouse were calculated by adding scores of the mouse from day 10 to day 30 p.i. Silybin was prepared in dimethyl sulfoxide (DMSO) for stock. Five percent of DMSO was dispersed in phosphate-buffered saline (PBS) designated as the vehicle. Vehicle or silybin (5, 10, and 20 mg/kg) was given by i.p. each day and starting from day 0 p.i., or day 10 p.i. (disease onset, 10 mg/kg), or day 19 (disease peak, 10 mg/kg).

Histopathology

For immunohistochemistry staining, lumbar spinal cords (SCs) were harvested following EAE mice after PBS perfusion and fixed in 4% paraformaldehyde (PFA) for 1 day 25°C. Samples were plated in paraffin for slide stain with hematoxylin and eosin (H & E) and luxol fast blue (LFB). The slides were sectioned coronally at 5 μm. Sections were evaluated and scored in a blind manner for inflammation and demyelination by following previous methods (12). For inflammation, 0 means none; 1, a few inflammatory cells; 2, organization of perivascular infiltrates; and 3, increasing severity of perivascular cuffing with extension into the adjacent tissue. For demyelination, 0 means none; 1, rare foci; 2, a few demyelination areas; and 3, large (confluent) areas of demyelination.

For immunohistochemistry, SC tissues were fixed with 4% PFA for 24 h and then cryo-protected by 30% sucrose solvent for 72 h. Samples were plated in optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura Finetek, Tokyo, Japan) for frozen sections and then cut coronally in 8-μm sections. Transverse sections of SC were stained with myelin basic protein (MBP). The slides were incubated with primary antibody diluted in blocking buffer overnight at 4°C. The primary antibody used was rabbit anti-MBP (Abcam, Cambridge, UK; ab40390; 1:1,000). Secondary detection was performed with Alexa Fluor-488 secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA; 111-545-144; 1:750) for 1 h. ProLong Diamond Antifade Mountant with DAPI buffer (Thermo Fisher Scientific, Waltham, MA, USA; P36962) was used to mount the slides. Results were visualized by fluorescence microscopy (Nikon DS-Ri2, Nikon, Tokyo, Japan). For the myelinated region calculations, 10 areas in the WM of the lumbar SC were chosen and analyzed by Image-Pro Plus software.

Mononuclear Cell Preparation

To collect the mononuclear cells (MNCs) from the periphery for flow cytometry analysis, the mice were treated with silybin or vehicle at day 0 and sacrificed at day 21 p.i. Splenocytes were mechanically dissociated through a 70-μm cell strainer (Corning, New York, NY, USA) and reacted with red blood cell (RBC) lysis buffer (BioLegend, San Diego, CA, USA) for 1 min. The cells were then washed with cold PBS before stimulation. The spleen cells were seeded at 1.0×10^6 cells/ml and cultured in triplicates in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum (FBS) in 24-well plates and pulsed with 25 μg/ml of MOG_{35-55} for 3 days.

To harvest CNS infiltrated MNCs, EAE mice were perfused with 20 ml of PBS to remove blood and collect the penetrated MNCs from CNS. The SCs and brains of each group were dissociated by Neural Tissue Dissociation Kits (Miltenyi Biotec, Bergisch Gladbach, Germany) to digest into cell suspensions and then filtered with a 40-μm cell strainer. MNCs were isolated in a 70%/30% Percoll medium with a 2,000-rpm centrifuge for 20 min. After removal of the myelin debris in the upper layer, the MNCs were harvested from the middle interface to be used in the following experiments (13). The infiltrated MNCs were seeded at 1.0×10^6 cells/ml and were cultured in triplicates in RPMI 1640

supplemented with 10% FBS in 24-well plates and pulsed with 10 μg/ml of MOG_{35-55} for 1 day.

T-Cell Proliferation

For *ex vivo* proliferation, splenocytes were isolated 21 days p.i. from vehicle- and silybin-treated mice. To test the proliferation efficiency, the cells were treated with or without stimuli [25 μg/ml of MOG_{35-55} or 5 μg/ml of concanavalin A (Con A)]. Cell proliferation was determined by the BrdU-incorporation test using BrdU Cell Proliferation ELISA Kit (Abcam, cat no. ab126556).

T-Cell Differentiation

Spleen cells were separated from 8-week-old C57BL/6 naïve mice, and single-cell suspensions were obtained following a previously described method (2). Naïve CD4 microbead (Miltenyi Biotec) was applied to purified $CD4^+$ T cells. Subsequently, under 72 h of differentiation medium, cells were differentiated and analyzed on BD FACSAria (BD Biosciences, San Jose, CA, USA). In short, the cells were cultivated with anti-CD3ε (1 μg/ml) and anti-CD28 (1 μg/ml) under their differentiating medium. IL-12 (5 ng/ml) and anti-IL-4 (10 μg/ml) were added to prompt polarization into Th1 cells. Anti-IFN-γ (10 μg/ml), IL-2 (10 ng/ml), and IL-4 (10 ng/ml) were added to prompt polarization into Th2 cells. IL-6 (20 ng/ml), TGF-β1 (2 ng/ml), IL-1β (10 ng/ml), anti-IL-4 (10 μg/ml), and anti-IFN-γ (10 μg/ml) were added in Th17 polarization medium. TGF-β1 (2 ng/ml) and IL-2 (10 ng/ml) were added to induce polarization into Treg cells.

Dendritic Cell Culture and Activation

To obtain the bone marrow-derived DCs (BM-DCs), femurs and tibias were isolated from the naïve C57BL/6 mice (8 weeks), and the cells were flushed out of the bone marrow with a 30-gauge needle. After flow-through by 100-μm cell strainer, the obtained cells were cultivated in RPMI 1640 medium. Cells were cultured for 10 days; and medium is changed every 4 days supplemented with granulocyte/macrophage colony-stimulating factor (GM-CSF; 10 ng/ml) to obtain mature BM-DCs. DCs are cultured in a medium without any cytokine stimulation after 10 days and activated with 100 ng/ml of lipopolysaccharides (LPSs) for the subsequent experiments. Silybin (50 μM) was added to the medium simultaneously (5).

For DC co-cultured with T cells, DCs were cultivated overnight at a density of 1×10^4 cells/ml in 96-well U-bottomed plates in RPMI added with 10% FBS, 2 mM of L-glutamine, 100 ng/ml of LPS with or without silybin (50 μM), and 10 μg/ml of MOG_{35-55} peptide. After 24 h, the DCs were washed, and then aliquots of 1×10^5 cells/ml of naïve T cells were co-cultured with activated DC cells for 3 days and then used for flow cytometry analysis (14).

Real-Time Quantitative PCR

According to the manufacturer's instructions, RNA was isolated from the RNAprep Pure Tissue Kit (Tiangen Biotech, Beijing, China). Reverse transcription was conducted using the Prime Script™ RT Master Mix Kit (TaKaRa Biotechnology, Dalian, China); detection was performed by the LightCycler® 96 system (Roche Diagnostics, Basel, Switzerland); and ChamQ™ SYBR® qPCR Master Mix (Vazyme Biotech, Nanjing, China) was applied for the experiment. Mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control compared with genes of interest. Nucleotide sequences of the primers are listed in **Supplementary Table 1**.

Enzyme-Linked Immunosorbent Assay

Spleen cells from mice were isolated and cultivated in RPMI 1640, added with 10% fetal bovine serum (Corning), and activated with 25 μg/ml of MOG_{35-55} for 72 h. Supernatants were harvested and determined for IFN-γ, IL-17A, GM-CSF, IL-1β, IL-5, IL-6, and IL-10 by ELISA Kits (R&D Systems, Minneapolis, MN, USA).

Flow Cytometry

For cell surface staining, fluorochrome-conjugated Abs to CD4/CD8 (BD Biosciences, San Jose, CA, USA) or isotype Abs were incubated with cells for 0.5 h on ice. For intracellular cell staining, CNS-penetrated MNCs or splenocytes were stimulated with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml), ionomycin (500 ng/ml) (Sigma-Aldrich), and GolgiPlug (BD Biosciences) for 5 h. The staining process was carried out following our earlier method (15). Details of all flow cytometry antibodies used are listed in **Supplementary Table 2**. Results were evaluated by FlowJo 10.4 (Tree Star, Ashland, OR, USA).

Statistical Analysis

Statistical analyses were carried out by GraphPad Prism 8 (GraphPad, La Jolla, CA, USA). Results are provided as mean ± SD. All data were analyzed by the Mann–Whitney test and ANOVA with Tukey's multiple comparisons test. Statistical details are given in the figure legends. Differences with *p*-values of <0.05 were considered significant.

RESULTS

Silybin Effectively Alleviates Clinical Experimental Autoimmune Encephalomyelitis

To evaluate the anti-inflammatory properties of silybin, we determined its therapeutic ability in EAE. To test various doses, we noticed that silybin at 10 and 20 mg/kg/day is optimal for alleviating EAE development ($p < 0.05$; **Figures 1A,B**). In order to reduce the unmet effects, a lower dosage of 10 mg/kg/day was used for a later *in vivo* test. To dissect the ability of silybin in EAE, treatment began on day 10 p.i., when pathogenic T cells had begun migrating to the CNS (16). The silybin-treated EAE group showed decreased disease development than did the vehicle-treated group (**Figures 1C,D**). These data indicated that silybin might also prevent pathological inflammatory cells from aggravating the disease status. In addition, silybin treatment begins from the peak period, which was also tested in this study. The results showed that silybin failed to relieve the severity of the disease's progression (**Figures 1E,F**).

To examine the influence of silybin on CNS pathology of EAE, SCs from silybin-treated and control groups

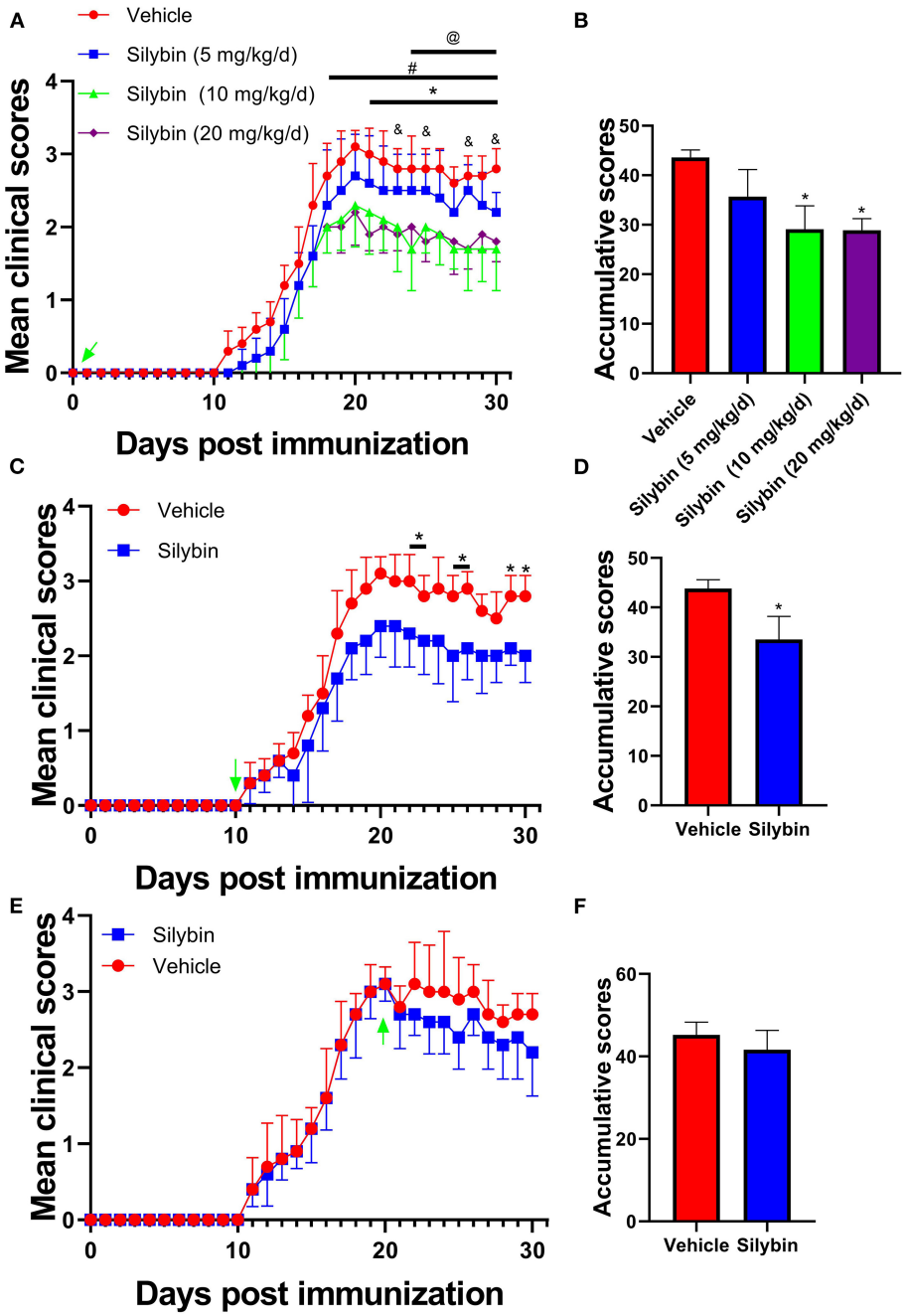

FIGURE 1 | Silybin alleviated the induction of experimental autoimmune encephalomyelitis (EAE). **(A)** Clinical scores were recorded daily for i.p. injection of silybin (5, 10, 20 and mg/kg/day) to EAE mice from the time of disease induction (0 day p.i.). *, comparison between 10 mg/kg/day and vehicle; #, comparison between 20 mg/kg/day and vehicle; @, comparison between 10 and 5 mg/kg/day; &, comparison between 20 and 5 mg/kg/day. **(C)** Clinical scores of mice treated by daily i.p. injection of silybin (10 mg/kg/day) or vehicle alone, beginning at the time of disease onset (10 day p.i.). **(E)** Clinical scores of mice treated by daily i.p. injection of silybin (10 mg/kg/day) or vehicle alone, starting at the time of peak stage of the disease (20 day p.i.) and scored daily following a 0–5 scale. **(B,D,F)** Accumulative score of EAE (sum of daily clinical scores from disease onset). Data are shown as mean values ± SD ($n = 5$ each group). Two-way ANOVA or nonparametric Mann–Whitney test was used. $^{*}p < 0.05$, one representative of three independent experiments is shown.

(treated with silybin or vehicle starting from day 0 p.i. and sacrificed at day 21 p.i.) were analyzed for inflammation and demyelination. Silybin-treated EAE mice had decreased inflammation and demyelination than did controls (**Figures 2A–D**). Furthermore, MBP staining demonstrated that the demyelination lesion was greatly reduced in silybin-treated mice (**Figures 2E,F**). Higher MBP expression in the WM of the silybin-treated group indicates that silybin may prevent

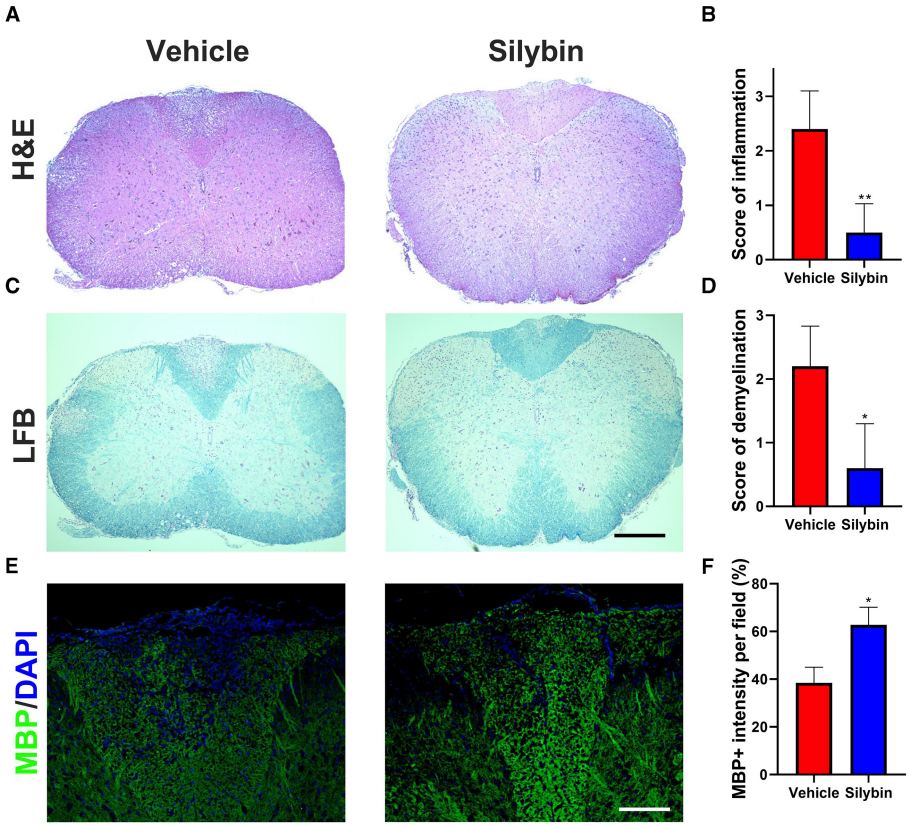

FIGURE 2 | Silybin treatment ameliorated the inflammatory cell infiltration and demyelination. **(A,C)** Mice were treated with silybin (10 mg/kg/day) from day 0 and sacrificed on day 21 p.i. ($n = 5$ each group), and spinal cords (SCs) were harvested for H&E and luxol fast blue (LFB) staining. The white matter of the lumbar SC was analyzed to assess inflammation and demyelination. **(B,D)** Mean scores of inflammation in H&E-stained and demyelination in LFB-stained spinal cord sections. **(E)** Sections were assayed for myelinated area by myelin basic protein (MBP) staining. **(F)** Quantitative analysis of MBP expression. MBP intensity was measured in the lesion areas in the SCs using Image-Pro. Data represent mean ± SD ($n = 8$ each group). Scale bar = 500 μm **(A,C)** or 100 μm **(E)**. $^{*}p < 0.05$ and $^{**}p < 0.01$. Student's *t*-test. One representative of three independent experiments is shown.

demyelination via suppressing inflammatory cell activation or migration.

Silybin Administration Effectively Reduces the Peripheral Immune Response

In the following, the immunomodulatory activities of silybin were assessed. Spleen cells were collected at 21 days p.i., stimulated with MOG_{35-55} (25 μg/ml) for 3 days *ex vivo*, and determined by flow cytometry. In comparison with the vehicle-treated group, $CD4^+$ cells in the silybin treatment group were lower. However, no significant difference was observed between these two groups (**Figures 3A,D**). Furthermore, $CD4^+$IFN-γ^+ (Th1), $CD4^+IL17^+$ (Th17), and $CD4^+$GM-CSF^+ cells (**Figures 3B,C,E**) were examined in the spleen of the silybin-treated group. The gating strategies are shown in **Supplementary Figure 1**. Supernatants of spleen cells were determined by ELISA to test the activities of silybin on MOG-stimulated cytokine production. The pro-inflammatory molecules of IFN-γ, IL-1β, and IL-6 were greatly decreased in the silybin-treated mice. Compared with that in the vehicle-treated group, the IL-17A concentration in the silybin-treated group was significantly reduced, and GM-CSF also showed a similar trend (**Figure 3F**). Therefore, we asked whether silybin affected the maturation of DC. We will verify this speculation in subsequent experiments. In addition, the IL-5 and IL-10 representing the Th2 and an anti-inflammatory cytokine were also reduced after silybin administration. Taken together, these data suggested that silybin weakened the disease progression of EAE by comprehensively inhibiting immune cell activation, especially Th1 and Th17 differentiation *in vivo*.

Silybin Treatment Suppresses Central Nervous System Inflammatory Infiltration

To determine the treatment outcome of silybin on CNS pathology, MNCs were separated from CNS and measured by flow cytometry. The whole number of MNCs was 220.6 ± 45.17 × 10^4 cells/mouse in the vehicle-treated group compared with 102.8 ± 22.95 × 10^4 in the silybin-treated group ($p < 0.001$, **Figure 4A**). Fewer numbers of penetrating $CD4^+$ T cells, Th1, Th17, and GM-CSF^+CD4^+ T cells were observed in the CNS of silybin-treated mice compared with

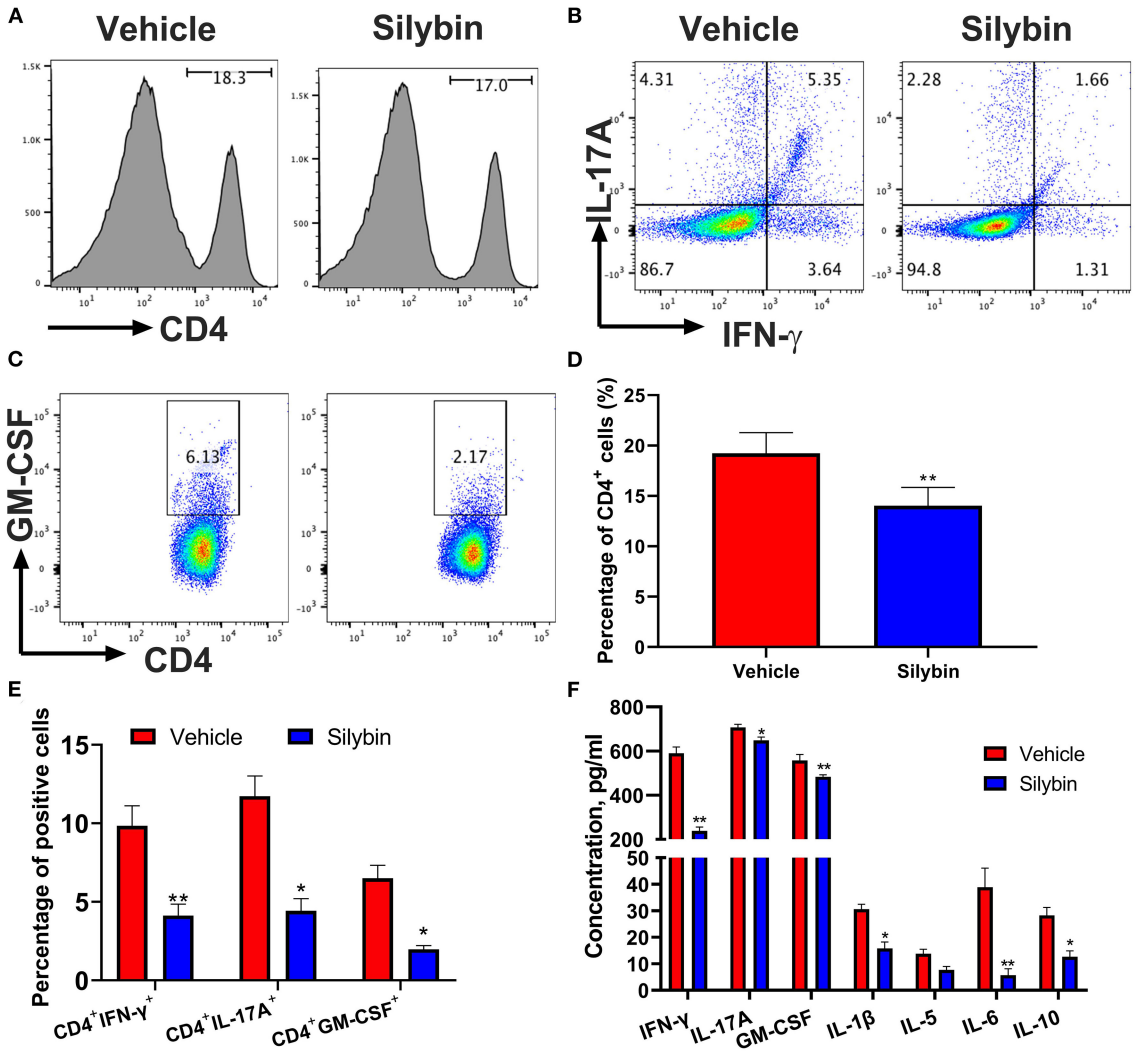

FIGURE 3 | Silybin treatment suppressed inflammatory response in the periphery. mononuclear cells (MNCs) of the spleen harvested from experimental autoimmune encephalomyelitis (EAE) mice described in **Figure 1A** (10 mg/kg/day) and isolated ($n = 3$ mice each group) at the day of 21 p.i. **(A)** The percentage of $CD4^+$ in lymphocyte gate of the above mice was analyzed by flow cytometry. Percentages of positive cells for these markers in the periphery are expressed as mean ± SD. **(B,C)** Subsets of Th1, Th17, and GM-CSF^+ cells in $CD4^+$ gate were analyzed by intracellular staining of IFN-γ^+, IL-$17A^+$, and GM-CSF^+ from the periphery. Percentages of positive cells for **(D)** $CD4^+$ and $CD8^+$ and **(E)** Th1, Th17, and GM-CSF^+CD4^+ T cells in the periphery are expressed as mean ± SD. **(F)** Supernatants derived from splenocyte cultures described in the section Material and Methods were analyzed for the level of indicated cytokines. Data are mean ± SD ($n = 3$). Statistical significance was determined by unpaired Student's *t*-test (*$p < 0.05$ and **$p < 0.01$).

the vehicle-treated mice (**Figures 4B–E**). The gating strategies are shown in **Supplementary Figure 2**. Furthermore, to clarify how silybin administration suppressed inflammatory cell response, we determined the inflammatory molecules' expression level in the SC of vehicle- and silybin-treated mice. As shown in **Figure 4F**, expression of inflammatory cytokine, comprising IFN-γ, IL-1β, IL-6, IL-17A, and GM-CSF, significantly decreased in mice treated with silybin. These results demonstrated that silybin considerably blocks inflammatory cell response and infiltration in the CNS.

Silybin Inhibits Bone Marrow-Derived Dendritic Cell Activation *in vitro*

Because the silybin possessed the most significant therapeutic effect in the prophylactic disease stage, and ELISA and RT-PCR data show that the expression of IL-1β and IL-6 is remarkably downregulated, we speculate whether silybin has a therapeutic effect on DCs. Here, we tested its direct effects on DC activation in culture. For this reason, BM-DCs were extracted and cultivated. DCs were stimulated by LPS and expressed relatively high levels of CD11b, CD11c, CD80, CD86, and MHC class II markers of DC activation; and the level of these molecules was

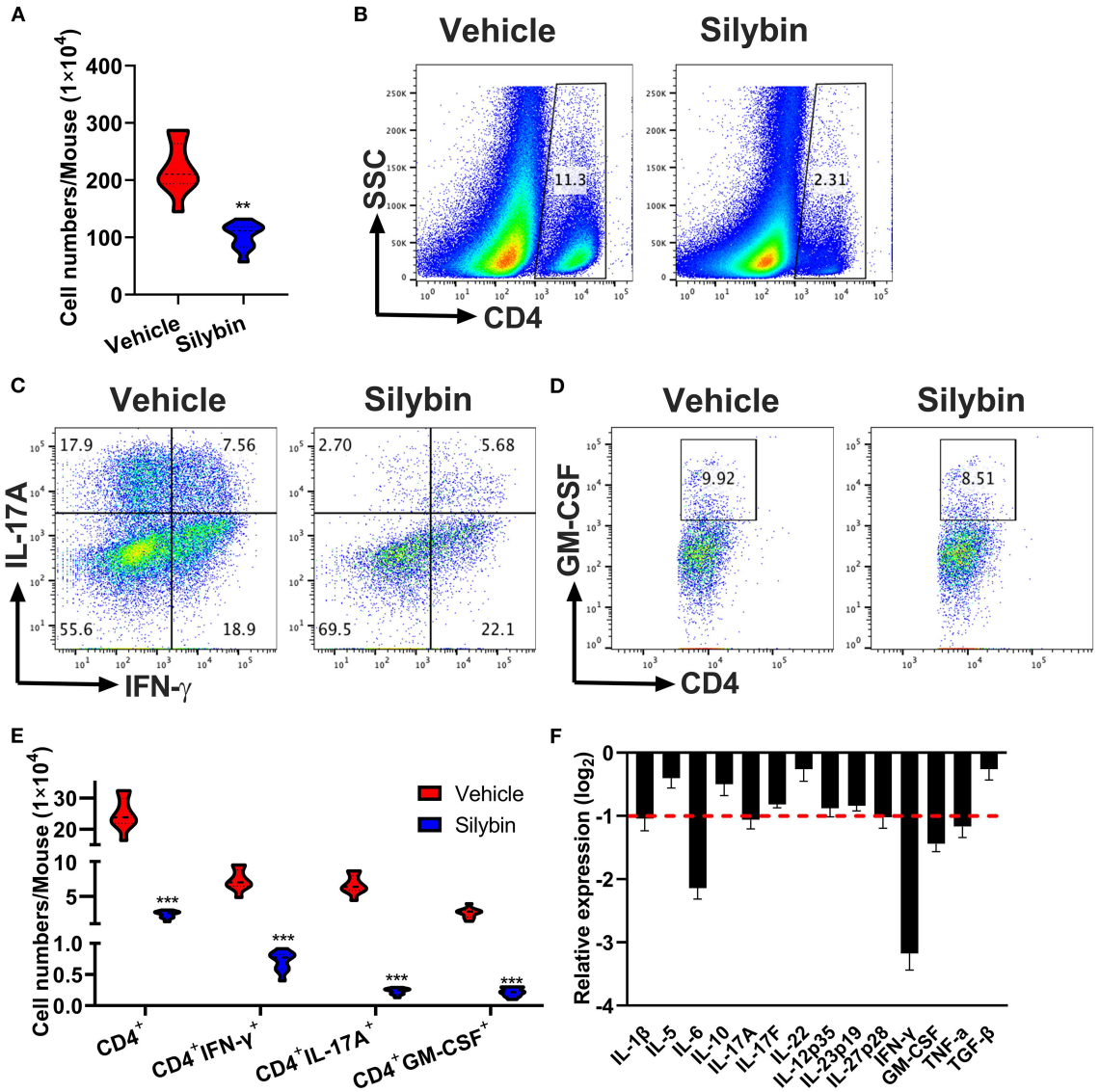

FIGURE 4 | Silybin therapeutic blocked inflammatory infiltration in the central nervous system (CNS). Mice were treated with vehicle or silybin at the day of experimental autoimmune encephalomyelitis (EAE) induction and sacrificed at day 21 p.i. Spinal cords and brain were collected and mononuclear cells (MNCs) separated ($n = 5$ each group). **(A)** Total MNC numbers in CNS were counted by a light microscope. **(B)** The percentage of $CD4^+$ T cells was measured by flow cytometry. **(C,D)** Frequencies of IFN-γ^+, IL-17A$^+$, and GM-CSF$^+$ cells among $CD4^+$ cells were assessed by flow cytometry. **(E)** Absolute numbers of infiltrated $CD4^+$ T and Th subsets were calculated by multiplying the percentages of these cells with total numbers of MNCs in each CNS tissue. **(F)** The expression of cytokine genes was determined using real-time RT-PCR analysis, and their relative expression was calculated by $\log_2$ of $-\Delta\Delta$Ct values from triplicate of PCR. More than two-fold changes ($\log_2 < -1$) were considered significant between groups (red dotted line). Symbols represent mean ± SD ($n = 5$ each group). $^{**}p < 0.01$ and $^{***}p < 0.001$. Student's *t*-test. One representative of three independent experiments is shown.

greatly suppressed by silybin administration (**Figures 5A–J**). The gating strategies are shown in **Supplementary Figure 3**.

To further examine the inhibitory effect of silybin on BM-DC activation, we then determined the mRNA levels of multiple inflammatory-associated genes expressed by BM-DCs. Kim et al. have reported that silybin has a significant inhibitory effect on Th1 cells (17) but that its effect on Th17 is unknown. Previous studies showed that IL-1β, IL-6, IL-23, and TGF-β are crucial to the Th17 differentiation (18). In this study, we focused on testing the effect of silybin on these cytokines. The data showed that silybin mainly inhibited the expression of IL-1β and IL-6. It is suggested that silybin may indirectly block the polarization of Th17 cells by regulating the activity of DCs (**Figure 5K**).

Silybin Treatment Blocks T-Cell Proliferation and Polarization

To study *ex vivo* proliferation response affected by autoantigen MOG_{35-55} in splenocytes of the vehicle and silybin-treated mice

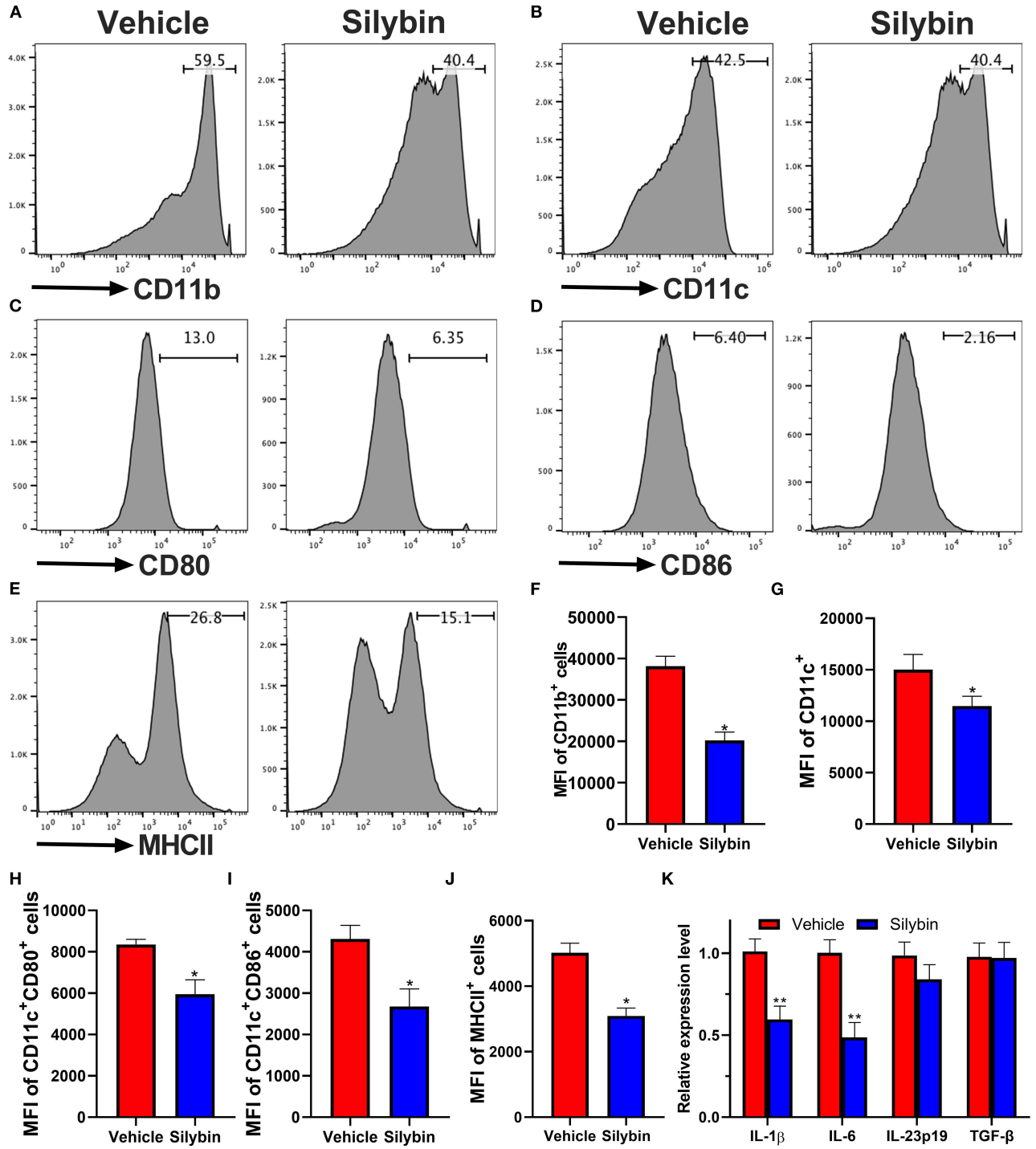

FIGURE 5 | Silybin inhibited the activation of bone marrow-derived dendritic cells (BM-DCs) *in vitro*. BM-DCs were generated and stimulated with 100 ng/μl of lipopolysaccharide (LPS), and simultaneously, silybin at a dose of 50 μM was added into the culture medium. After 18 h, expression of **(A)** CD11b, **(B)** CD11c, **(C)** CD80, **(D)** CD86, and **(E)** MHC II (the positive cells of CD80, CD86, and MHC II are gated below the CD11c-positive cells) was measured by flow cytometry following overnight incubation and treatment with or without silybin. **(F–J)** Percentages of each molecule were counted. **(K)** Figures **(A–E)** are representatives of three independent experiments. Statistical data are expressed as mean ± SD of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ by Student's *t*-test.

starting from day 0 p.i., the mice were euthanized on day 21 p.i., and low proliferation efficiency was observed in both vehicle- and silybin-treated groups without antigen stimulation. While cells were pulsed with MOG_{35-55}, a stronger response was recorded in the vehicle-treated group compared with the silybin-treated group. By contrast, no significant difference was observed in both vehicle- and silybin-treated groups, which responded to Con A stimulation (**Supplementary Figure 4**). This result indicated that silybin administration suppressed the MOG_{35-55}-induced proliferation response.

To elucidate the mechanism underlying the activities of silybin on various Th17 cell populations, the differentiation efficiency of T-cell subsets was tested. Under Th17-polarization media, approximately 25% of cells were IL-17A positive in the vehicle-treated group, though silybin (50 μM) exhibited great activity to decrease IL-17A expression by T cells (24.80 ± 1.253% vs. 18.66 ± 0.794%, $p < 0.01$) (**Figures 6A,E**). We then studied the activities of silybin on Th1, Th2, and Treg cell polarization. Unlike the Th17 cell differentiation result, IFN-γ, IL-4, and Foxp3 expression under Th1, Th2, and Treg differentiation conditions were not effectively suppressed by silybin (**Figures 6B–D,F–H**). The gating strategies are shown in **Supplementary Figure 5**. Interestingly, although the expression of IFN-γ increased slightly under the Th17 polarization condition in the silybin-treated group, no significant difference was observed compared with the vehicle-treated group (**Supplementary Figure 6A**). Also, we found that silybin only specifically suppressed ROR-γt expression in $CD4^+$ T cells under Th17-polarizing conditions. In contrast, T-bet, Gata-3, and Foxp3 expression in $CD4^+$ T cells under Th1, Th2, or Treg cell conditions were not significantly decreased compared with those in vehicle-treated groups (**Supplementary Figure 6B**). Altogether, these data indicated that silybin suppressed Th17 differentiation.

Silybin-Conditioned Bone Marrow-Derived Dendritic Cells Have a Reduced Ability to Initiate Th1 and Th17 Polarization

Then, we investigated whether BM-DCs treated by silybin inhibited the differentiation of T cells. We tried to determine whether the differential expression of MHC II and other costimulatory molecules affected the polarization of Th17. Comparison of surface marker expression of silybin- and vehicle-treated DC demonstrated apparent differences in expression of MHC II, the costimulatory molecule CD80 or CD86, indicating that there are underlying differences between the activating signals to T cells. Therefore, we conducted a co-culture test of DC and T cells. After DC was treated with silybin, the activation of T cells and the differentiation tendency of Th1 and Th17 were inhibited (**Figures 7A–D**). In addition, we found that silybin suppressed GM-CSF and ROR-γt expression level but not T-bet from the T cell, which was polarized by MOG_{35-55}-pulsed DCs (**Figure 7E**). Based on this result, we speculate that silybin inhibits the polarization of Th1 mainly by blocking the activity of DC, which is different from inhibiting the polarization of Th17. This result indicates that silybin can indirectly inhibit the differentiation of T cells and affect the polarization of T cells by regulating DC.

DISCUSSION

In this study, we demonstrate the promising effects of silybin on both prophylactic and onset phases of EAE. Silybin blocks the migration of inflammatory cells into the CNS and inhibits the myelin damage process remarkably, thus relieving the disorder development. The function of silybin on EAE is mainly due to its repressive effects on Th17 cell polarization. Furthermore, silybin suppresses the T-cell polarization, which is dependent on the inhibition of the activation of DCs, a critical underlying mechanism of silybin for the treatment of autoimmune disease.

Silybin is a flavonoid, a primary component of silymarin, extracted from the seed of species derived from *S. marianum* (19). *S. marianum* has been used in traditional medicine for many years. In China, owing to its specific characteristics in treating liver diseases, it has been widely used for more than 2,000 years. Ancient herbalists described silybin as possessing nephron-, neuro-, hepato-, and cardio-protective activities due to its antioxidant, anti-inflammatory, and regenerative effects (20, 21). In recent years, the antioxidant activity of silybin has been reported. It can directly act on the scavenging of free radicals and block the specific enzyme generators of free radicals. Moreover, it induces non-enzymatic antioxidant defenses, such as glutathione or transcription factors (Nrf2 and NF-κB) (22, 23). Studies have shown that silybin treatment attenuates the production of prostaglandin E2, IL-1β, and major chemotactic protein-1 (MCP-1), suggesting that silybin has a significant anti-inflammatory effect via suppressed NF-κB activity (24). Based on this theory, Min et al. used silybin to treat EAE mice and found that it can inhibit the disease development significantly (25). These results are consistent with our conclusion and encourage us to identify the possible mechanism of action; we found the inhibitory effects of silybin on DC activation in an inflammatory state. Moreover, the results also showed that silybin possessed significant inhibitory activity on Th17 differentiation. However, there are also some conflicting conclusions. For example, we found that silybin does not exhibit a comprehensive suppression effect and immunomodulatory activity on the immune response, because silybin does not alter the differentiation efficiency of Th2 and Treg cells significantly. For autoimmune diseases, candidate drugs can usually show a significant inhibitory effect on autoreactive T cells and can exhibit a certain immunomodulatory effect. However, in this study, our results showed that silybin not only inhibited pro-inflammatory cytokines but also blocked the production of anti-inflammatory cytokines. This may be related to the use dosage of silybin, and it will need further test in the future experiments. We have also noticed that the differences in some details of the experiment may lead to the opposite results, such as the amount of PT injection, the manner of drug administration, and the sacrifice time of mice.

Previous studies reported that silybin also possessed tissue regeneration functions. Tabandeh et al. found that

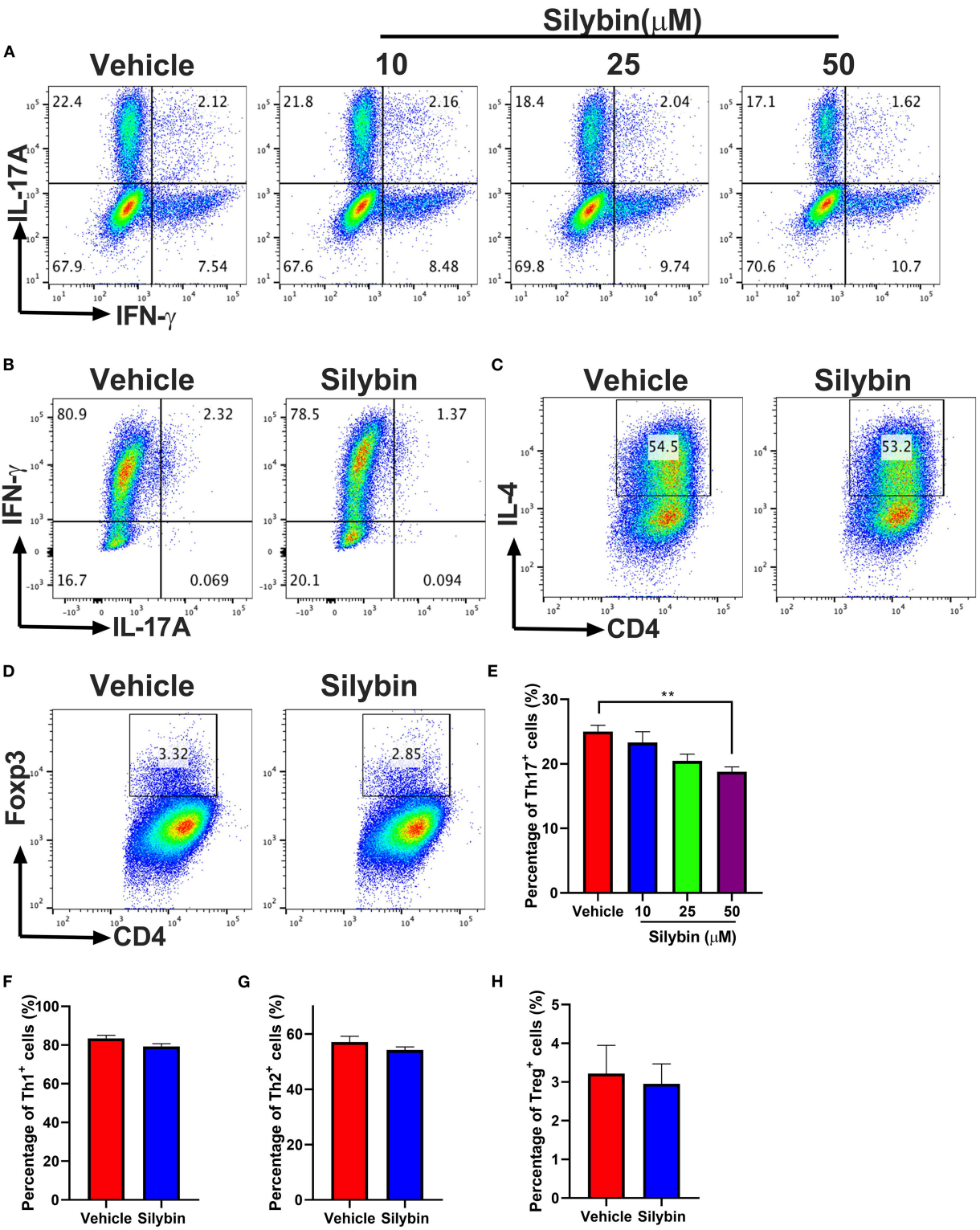

FIGURE 6 | Silybin inhibited Th cell subset differentiation. **(A)** $CD4^+$ cells were isolated from mice and cultured under the Th17-polarizing condition with different concentrations of silybin for 3 days. Percentage of IL-$17A^+$ cells was analyzed by intracellular staining of IL-17A. **(B–D)** $CD4^+$ cells were cultured under the Th1, Th2, and Treg-polarizing conditions with silybin (50 μM) for 3 days. Percentages of Th1, Th2, and Treg cells were analyzed by intracellular staining of IFN-γ^+, IL-4^+, and $Foxp3^+$, respectively. The RNA extracted from the vehicle- or silybin-treated DCs and RT-PCR was performed to determine the mRNA expression level of IL-1β, IL-6, IL-23, and TGF-β. **(E–H)** Statistical analysis of **(A–D)**. Data represent mean ± SD ($n = 3$ each group). $^{**}p < 0.01$. Student's *t*-test. One representative of three independent experiments is shown.

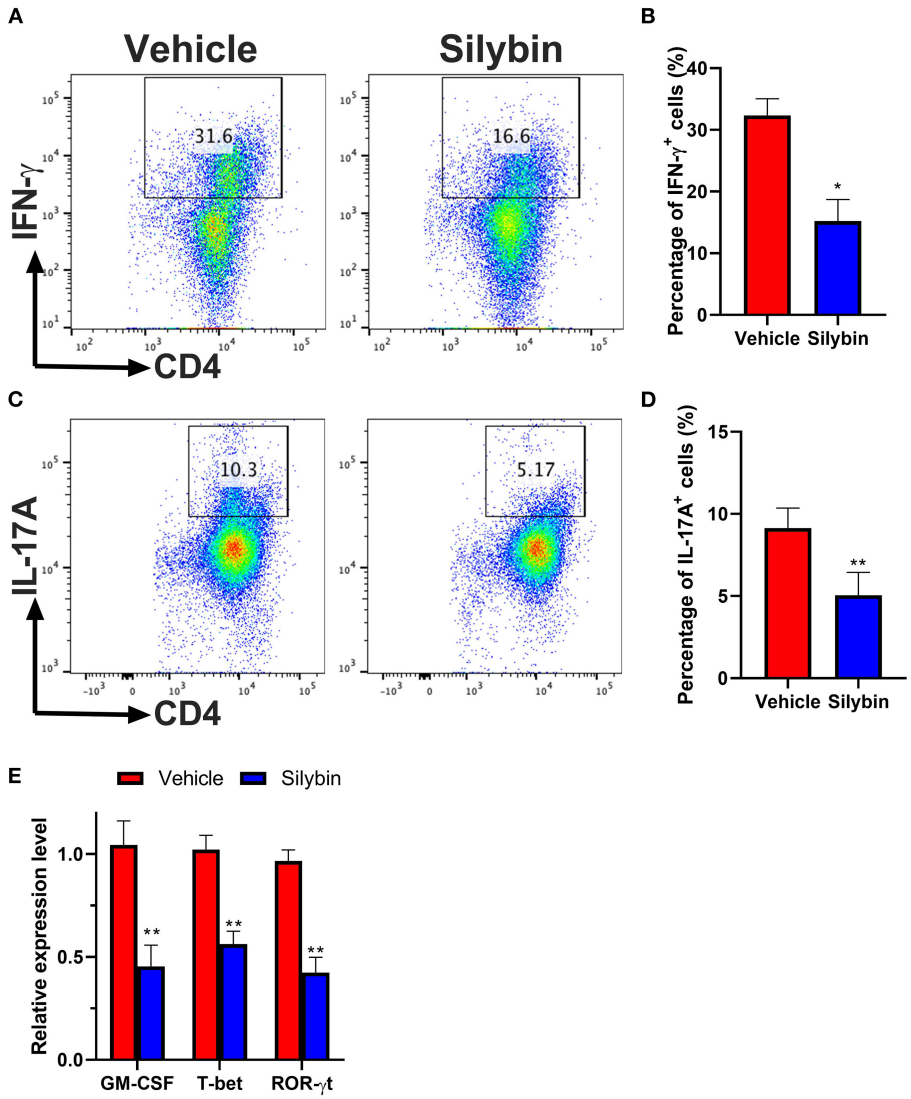

FIGURE 7 | Silybin-treated bone marrow-derived dendritic cells (BM-DCs) induce Th1 and Th17 CD4+ T-cell polarization *in vitro*. **(A,C)** Intracellular staining for IFN-γ and IL-17A of naïve $CD4^+$ T cells after 3 days of co-culture with lipopolysaccharide (LPS)-activated BM-DCs. **(B,D)** Analysis of IFN-γ- and IL-17A-positive $CD4^+$ T cells by division number as assessed by intracellular staining. **(E)** RNA extraction of from the vehicle- or silybin-treated T cells and RT-PCR was performed to determine the mRNA expression level of granulocyte/macrophage colony-stimulating factor (GM-CSF), T-bet, and ROR-γt. Statistical data are expressed as mean ± SD of three independent experiments. $^*p < 0.05$ and $^{**}p < 0.01$ by paired *t*-test.

silybin treatment could increase stromelysin-1 expression and extracellular matrix constituents, thus promoting the wound healing process (26). Silybin complex with copper(II) ion stimulates the expression of osteoblastic marker genes, such as Runx2, ALP, type 1 collagen, and OCN at the molecular level, and enhances osteoblast differentiation (27). In addition, in Alzheimer's disease, silybin-treated APP/PS1 transgenic mice showed higher numbers of newly generated microglia, astrocytes, neurons, and neuronal precursor cells, indicating its positive effects on neuro-regeneration. Based on these data, we speculate whether silybin also has functions for the treatment of the CNS demyelinating diseases. We tested its effects on the differentiation of oligodendrocytes, but no significant differences were observed (data not shown). In another study, Tsai et al. showed that silymarin has a better protective effect than silybin in the SC injury model. It is indicated that the regenerative function might be an indirect effect, which is achieved through the inhibition of peroxide-induced reactive oxygen species (ROS) (28).

So far, there are few reports on the effects of silybin on DCs. As early as 2007, Lee et al., for the first time, reported silybin on the phenotypic and functional maturation of murine BM-derived DCs. Silybin was shown to strongly inhibit CD80, CD86, MHC class I, and MHC class II expression on the surface of DCs and was also related to the impairments of LPS-induced IL-12

expression of DCs (17). In this study, based on previous work, we further tested the activity of silybin on DC activation in the EAE model. The co-culture system of DCs and T cells was employed to test that the inhibitory effects of silybin on Th1 and Th17 differentiation. The results indicated that the inhibiting effects of silybin are partially dependent on the DCs.

A number of studies have proved that silybin significantly promoted T-cell activation and proliferation (8, 25). In other words, silybin has also exhibited immunomodulatory effects (6). However, in our study, we did not observe that silybin treatment significantly altered the percentage of Th1, Th2, and Treg. Similar to the previous results, silybin did not significantly affect Th2 cells (25). In addition, although Min's research showed that silybin inhibited Th1-related cytokine production, such as IL-2 and IL-12, Min did not test the expression of IFN-γ (25). In another study, Lee et al. showed that silybin inhibited the polarization of Th1 cells. Nevertheless, this result comes from LPS-treated chronic inflammation mice with distinct pathogenesis of MOG_{35-55}-induced EAE mice. For the first time, our study used anti-CD3/28 and various cytokines to polarize the T subset *in vitro*, and we tested the effects of silybin on these populations. Interestingly, the polarization experiment of silybin on Th1 cells is not consistent with the results of DC and T-cell co-culture. We speculate that it may be due to the dosage of silybin. Perhaps silybin possesses immunomodulatory capabilities at low doses. This hypothesis will be further verified in our subsequent experiments.

The structure of silybin can be depicted as two sections with carbo- and heterocycles. One section of the molecule is a flavonol group, taxifolin; another is a unit of coniferyl alcohol phenylpropanoid; and they are linked by an oxirane ring (29). It is highly soluble in polar aprotic solutions such as DMSO, acetone, tetrahydrofuran (THF), and *N*, *N*-dimethylformamide (DMF). It is hardly soluble in ethanol or methanol and is insoluble in non-polar solvents such as chloroform and petroleum ether. In addition, because the molecular weight of silybin is high, it cannot be absorbed by simple diffusion, and the oral bioavailability is low (30). Therefore, the solubility of silybin as a drug limits its therapeutic effects. At present, there are some targeted drug carriers, such as the use of nanoparticles or exosomes (31, 32), which may contribute to the clinical therapeutic effect of silybin.

In conclusion, our study demonstrated that silybin is a valuable anti-inflammatory agent to treat autoimmune disease and therapeutically manage such as MS.

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 animal study was reviewed and approved by Animal Ethics Committee of Xian Yang Central Hospital.

AUTHOR CONTRIBUTIONS

H-LY and X-WS conceived and designed the experiments, carried out the experiments, and wrote the manuscript. All authors read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.659678/full#supplementary-material

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CASE REPORT
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Case Report: Covid-19 in Multiple Sclerosis Patients Treated With Ocrelizumab: A Case Series

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Introduction: Limited data are available on the course of Coronavirus disease 2019 (COVID-19) in people with Multiple Sclerosis (MS). More real-world data are needed to help the MS community to manage MS treatment properly. In particular, it is important to understand the impact of immunosuppressive therapies used to treat MS on the outcome of COVID-19.

Methods: We retrospectively collected data on all confirmed cases of COVID-19 in MS patients treated with ocrelizumab, followed in two MS Centers based in University Hospitals in Northern Italy from February 2020 to June 2021.

Results: We identified 15 MS patients treated with ocrelizumab with confirmed COVID-19 (mean age, 50.47 ± 9.1 years; median EDSS, 3.0; range 1.0–7.0). Of these, 14 were confirmed by nasal swab and 1 was confirmed by a serological test. COVID-19 severity was mild to moderate in the majority of patients (n = 11, 73.3%; mean age, 49.73; median EDSS 3.0). Four patients (26.7%; mean age, 52.5 years; median EDSS, 6) had severe disease and were hospitalized; one of them died (age 50, EDSS 6.0, no other comorbidities). None of them had underlying respiratory comorbidities.

Conclusion: This case series highlights the large variability of the course of COVID-19 in ocrelizumab-treated MS patients. The challenges encountered by the healthcare system in the early phase of the COVID-19 pandemic might have contributed to the case fatality ratio observed in this series. Higher MS-related disability was associated with a more severe COVID-19 course.

Keywords: multiple sclerosis, ocrelizumab, COVID-19, SARS-CoV-2, disease-modifying treatment

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a contagious respiratory disease caused by Severe Acute Respiratory Syndrome CoronaVirus 2 (SARS-CoV2), first identified in Wuhan, China in 2019, and responsible for the ongoing pandemic (1, 2). According to epidemiological data, about 80% of patients with COVID-19 develop a self-limiting illness, while 20% need hospitalization, and around 5% need ventilatory support (3, 4). In this last group, death occurs in about half of the cases (1, 5, 6). In the months following the pandemic outbreak, efforts have been made to identify risk factors associated with the worst outcomes: death has been related to older age and comorbidities such as cardiovascular and lung diseases, obesity, diabetes, and smoking habit (2, 7).

In this scenario, the management of people with MS (PwMS) has become more challenging: MS disease-modifying treatments (DMTs) may interfere with the immune system and increase the risk of infections (8, 9), with potential safety issues in the case of COVID-19 infection. As the pandemic situation is still very serious and widespread worldwide, more real-world data on COVID-19 in PwMS, particularly those receiving DMTs, are needed to manage MS treatment properly. In this case series, we will focus on one specific DMT, ocrelizumab. Ocrelizumab (Ocrevus®, Roche) is authorized in Europe for the treatment of adults with relapsing-remitting MS (RR-MS) and with early primary progressive MS (PP-MS) (10).

Ocrelizumab is a humanized monoclonal antibody that targets CD20 on B lymphocytes, with immunosuppressive effects due to peripheral B lymphocyte depletion (11). Ocrelizumab can also reduce T cells and has a slight effect on monocytes (12, 13). Infections occurring during ocrelizumab treatment can be serious (14, 15), due to persistent B cell depletion (14) and hypogammaglobulinemia (16). As of July 31, 2020, over 170,000 MS patients worldwide have been treated with ocrelizumab (17). Data on COVID-19 infection in these patients are controversial (17, 18). Spontaneous and detailed case reports may improve our knowledge of the impact of COVID-19 on ocrelizumab-treated patients.

Here, we describe a case series of confirmed COVID-19 infection in MS patients treated with ocrelizumab, in two MS Centers based in University Hospitals in Northern Italy, one of the first areas of Europe to experience the breakout of the COVID-19 pandemic at the beginning of 2020.

METHODS

We retrospectively collected data on all the cases of confirmed COVID-19 in ocrelizumab-treated MS patients, from February 2020 to June 2021, followed in two MS Centers based in University Hospitals in Northern Italy: (1) Multiple Sclerosis Center, Neurologia I U—AOU Città della Salute e della Scienza di Torino; (2) S.S.D. Patologie Neurologiche Specialistiche—AOU San Luigi Gonzaga. The total number of ocrelizumab-treated MS patients, as of June 2021, was 110 patients in Center 1 and 25 patients in Center 2.

All patients signed an informed consent for the data collection and publication in an anonymized form.

RESULTS

We identified 15 ocrelizumab-treated MS patients with confirmed COVID-19 (mean age, 50.47 ± 9.1 years; median EDSS 3.0, range 1.0–7.0). None of them was vaccinated before the infection since the majority of them contracted the disease before the vaccine was available. Anagraphic and anamnestic data on all the patients included in the case series are shown in **Table 1**.

COVID-19 severity was mild to moderate in the majority of patients ($n = 11$, 73.3%; mean age 49.73; median EDSS 3.0). Four patients (26.7%; mean age 52.5 years; median EDSS 6) had severe disease and were hospitalized, and one of them died (age 50, EDSS 6.0, no other comorbidities). Three patients developed neurological symptoms during COVID-19 such as dizziness, worsening of pre-existing neurological symptoms, and increased leg stiffness. Eight patients had respiratory signs/symptoms (radiologically documented pneumonia, dyspnea, low blood oxygen saturation), but only three of them received oxygen therapy (one received mechanical ventilation, one received assisted non-invasive ventilation, and one received home oxygen therapy) (**Table 2**).

In nine patients, there was at least one risk factor associated with a more severe COVID-19 disease course: age > 50 years ($n = 9$); obesity ($n = 3$); hypertension ($n = 2$); diabetes ($n = 1$). None of them had underlying respiratory comorbidities; one patient had epilepsy since childhood and two patients had hyperthyroidism. The proportion of patients with an EDSS > 3 was 53.3%. In the last blood tests within 3 months before the onset of COVID-19, total leukocytes count and total lymphocyte counts were within normal limits in all patients. Likewise, serum Ig levels were within the normal range in all patients (for one patient this data was not available) (**Table 1**).

Comparing the patients with and without respiratory signs/symptoms during COVID-19 (radiologically-documented pneumonia, dyspnea, low blood oxygen saturation), the patients showing respiratory signs/symptoms showed a higher median EDSS than the patients without respiratory signs/symptoms (EDSS 5.0 vs. EDSS 3.0; Mann-Whitney U-test, $p = 0.0150$).

After COVID-19 recovery, six patients underwent a serological test to detect the presence of antibodies against SARS-CoV2 (quantitative test with chemiluminescent immunoassays): one patient tested positive for both IgM and IgG; three patients tested positive for IgG against SARS-CoV2; and two tested negatives (**Table 2**).

Four patients showed EDSS progression in the months following COVID-19 (**Table 2**). For two of them, this could be related to a delayed infusion and for one patient, this could be related to the prolonged hospitalization during and after COVID-19 (**Table 2**).

Over the following months, the majority of patients fully recovered. However, four patients were still symptomatic even after several months from COVID-19, presenting increased fatigue, worsening of neurological symptoms and, in one case, need for chronic oxygen therapy (**Table 2**).

Five patients received SARS-CoV2 vaccines based on mRNA technology, at least 3 months after COVID-19 recovery (**Table 2**).

Case 1

A 50-year-old woman with a history of RR-MS since 1989 was treated with interferon beta 1a and teriflunomide before switching to ocrelizumab due to disease activity. On February 20, 2020, she underwent the most recent administration and her EDSS score was 6.0. On March 19, 2020, the patient presented to the emergency room (ER) with complaints of fever with syncope. Chest X-ray showed bronchopneumonia foci of inflammatory condensation, a swab for COVID-19 was carried out and, pending the outcome, the patient was discharged with amoxicillin-azithromycin antibiotic therapy. On

TABLE 1 | Anagraphic and anamnestic data.

Case	Year of Birth	Age	Sex	BMI*	MS° course	MS duration (year)	EDSS£	Comorbidities	Previous DMT$	Number of Ocrelizumab cycles before COVID-19 infection	Time between last Ocrelizumab cycle and COVID-19 symptoms (days)	White blood cells	Total lymphocytes	Ig&G	IgA	IgM
1	1969	50	F	21.5	R	30	6	None	Teriflunomide, beta interferon 1a	3	36	7.26 × 1,000 uL	1.84 × 1,000 uL	NA	NA	NA
2	1980	40	M	25	R	6	1	Epilepsy	Teriflunomide, beta interferon 1a	2	73	4.42 10∧9/L	1.97 10∧9/L	879	168	55
3	1979	41	M	20.7	R	1	2	None	Natalizumab, Dimethylfumarate	1	10	10.23	3.69	751	89	159
4	1967	53	F	30.5	R	17	3.5	None	Dimethylfumarate, glatiramer acetate	4	11	5.84	1.2	968	353	207
5	1951	69	F	31.6	R	5	4	Hypertension, sick sinus syndrome	Teriflunomide	3	116	7.91	1.23	1,232	220	139
6	1970	49	F	19.1	R	1	2.5	Type 1-diabetes	None	1	27	5.72	1.3	1,264	335	45
7	1968	52	F	22.9	R	15	3	None	Beta interferone 1 a	3	137	7.05	2.1	868	147	145
8	1970	50	F	24.1	R	5	3	None	Natalizumab, teriflunomide	3	26	5.34	1.71	1,354	241	249
9	1968	52	F	19.5	R	1	2	None	None	3	136	5.5	1.2	1,403	250	107
10	1962	58	F	37.5	R	7	7	Graves' disease	Beta interferone 1 a	4	113	8.09	1.47	921	108	123
11	1959	62	M	23	R	36	5.5	Benign prostatic hyperplasia	Natalizumab, beta interferon 1 a	4	22	7.59	1.88	975	387	17
12	1980	41	F	24.5	R	12	4.5	None	Natalizumab, fingolimod	4	125	5.81	1.12	856	115	60
13	1967	53	F	17.2	R	16	5,5	HBV	Natalizumab, mitoxantrone, betaIFN1a	5	22	4.75	1.5	917	107	29
14	1968	54	M	27	R	1	4	Hypertension	None	1	48	5.94	1.1	1,083	97	74
15	1987	33	F	22.6	R	10	3	Hypertyroidism	Natalizumab, fingolimod, alemtuzumab	3	184	5.53	1.22	1,037	149	31
1	1969	50	F	21.5	R	30	6	None	Teriflunomide, beta interferon 1a	3	36	7.26 × 1,000 uL	1.84 × 1,000 uL	NA	NA	NA
2	1980	40	M	25	R	6	1	Epilepsy	Teriflunomide, beta interferon 1a	2	73	4.42 10∧9/L	1.97 10∧9/L	879	168	55

(Continued)

TABLE 1 | Continued

Case	Year of Birth	Age	Sex	BMI*	MS° course	MS duration (year)	EDSS£	Comorbidities	Previous DMT$	Number of Ocrelizumab cycles before COVID-19 infection	Time between last Ocrelizumab cycle and COVID-19 symptoms (days)	White blood cells	Total lymphocytes	Ig&G	IgA	IgM
3	1979	41	M	20.7	R	1	2	None	Natalizumab, Dimethylfumarate	1	10	10.23	3.69	751	89	159
4	1967	53	F	30.5	R	17	3.5	None	Dimethylfumarate, glatiramer acetate	4	11	5.84	1.2	968	353	207

**BMI, Body Mass Index.*
°MS, Multiple Sclerosis.
£ EDSS, Expanded Disability Status Scale.
$DMT, Disease Modifying Treatment.
& Ig, Immunoglobulin.
NA, not available.

March 26, due to the worsening of dyspnea, the patient came back to the ER with subsequent hospitalization. Chest X-ray showed multiple confluent parenchymal thickenings and the second swab for COVID-19 tested positive. Assisted non-invasive ventilation (NIV) was started, together with hydroxychloroquine, ceftriaxone, and antiviral therapy (darunavir and ritonavir). Due to the worsening of the general conditions and respiratory exchanges, on the second day after admission, the patient was transferred to the Intensive Care Unit (ICU) and underwent mechanical ventilation. After 4 days, for further worsening of respiratory exchanges, the patient received nitric oxide and steroid therapy. Because of increased platelets and hypertension, antithrombotic, and antihypertensive therapy with acetylsalicylic acid and analytics was added. A Chest CT scan showed bilateral pneumonia. The slow but progressive clinical and radiological improvement led on April 7 to suspend invasive ventilation and to start physiotherapy. On April 20, a new febrile episode occurred with negative blood cultures. Due to persistent anemia, the patient received a periodic blood transfusion. Bone marrow biopsy showed a hyporegenerative marrow, the absence of blasts, and the absence of viruses; in peripheral blood, no CD19+ lymphocytes were found at immunotyping. A Chest CT scan performed on May 13 showed a worsening of the pulmonary infiltrates on the left fields. The bronchoalveolar lavage showed persistence of positivity for SARS-CoV2. The patients had persistent hyperpyrexia up to 40°C; suspecting a persistence of COVID-induced interstitial pneumonia in a patient with a poor immune response, on May 18, an off-label treatment with ozone was carried out. This treatment was initially well-tolerated with an improvement of respiratory exchanges; however, 2 h after the end of the second ozone session, the patient died due to acute pulmonary edema with cardiorespiratory arrest.

Casr 2

A 40-year-old male was diagnosed with RR-MS in 2014; epilepsy was also reported in his medical history. He was initially treated with beta-interferon and then switched to teriflunomide. In July 2019, therapy with ocrelizumab was started because of disease activity. EDSS score was 1.0. On April 18, 2020, he developed a fever and cough. He presented to the ER, a CT scan showed ground-glass opacity and interstitial abnormalities, and he was hospitalized. Chest X-ray showed inflammatory condensation. The patient underwent three nasal swabs for SARS-CoV2: all of them were negative, but the suspicion of COVID-19 pneumonia was high. He was treated with hydroxychloroquine, antimicrobial therapy, and steroids. On May 9, due to mild residual symptomatology, he was discharged and completed therapy at home. He completely recovered over the following days and, on May 12, a chest CT scan was normal. On May 27, he underwent a quantitative serological test that detected the presence of antibodies against SARS-CoV2, both immunoglobulin-M (IgM) and IgG (IgM 5.47 U/L and IgG 42.6 U/L).

Case 3

A 41-year-old man was diagnosed with RR-MS in May 2019. He had no other medical illnesses and he started di-methyl-fumarate

TABLE 2 | Data about COVID-19.

Case	Nasal swab positive/negative	Respiratory symptom	Delayed infusion Yes/NO	Days of delayed infusion	Fever Yes/No	COVID-19 treatment	Hospitalization Yes/NO	Antibodies against SARS-CoV2	Serological test type	Covid-19 outcome	COVID-19 sequelae	Vaccination Yes/No
1	pos	Pneumonia			Y	Hydroxychloroquine, darunavir, ritonavir, ceftriaxone, steroids, nitric oxide, ozone, antitrombotic, NIV	Y	Not performed		Deceased		/
2	neg	Pneumonia	N		Y	Hydroxychloroquine, azithromycin, steroid	Y	IgM and IgG pos (27/5/2021)	ChLIA	Recovered		
3	pos (15/9/2020)		N		N	Paracetamol	N	IgG neg, IgM neg (18/5/2021)	ChLIA	Recovered		
4	pos (28/10/2020)		N		Y	Steroid	N	IgG pos, IgM neg /5/3/21)	ChLIA	Recovered	Increased fatigue	
5	pos (22/10/21)	SpO_2 reduction	Y	30	N	Steroid, LMWH, azithromycin	N	IgM neg, IgG pos (20/4/2021), 12/20, 01/21, 02/21.	ChLIA	Recovered		Y
6	pos (7/11/20)		N		Y	/	N	Not performed		Recovered		Y
7	pos (23/10/20)		N		Y	Steroid therapy, amoxicilline	N	IgG neg (2/21)	ChLIA	Recovered		Y
8	pos (30/10/2020)		N		Y	Steroid therapy, amoxicilline	N	Not performed		Recovered		
9	pos (23/10/2020)		N		Y	/	N	Not performed		Recovered		
10	pos (9/12/20)	SpO_2 reduction	Y	280	Y	Steroid therapy, antibiotic	Y	Not performed		Recovered		Y
11	pos (4/1/2021)	SpO_2 reduction	N		Y	Steroid, heparin, antibiotic coverage, oxygen therapy	Y	Not performed		Recovered	Dyspnea, cycle of O_2 therapy, worsening of neurological symtoms	Y
12	pos (14/2/2021)	Dyspnea, cough	Y	35	Y	Antibiotic therapy, steroid	N	IgG pos, IgM neg (1/6/2021)	ChLIA	Recovered	Cough, dyspnea, headache (improving)	
13	pos (20/3/21)	Dyspnea, SpO_2 reduction (88%)	N		Y	Steroid, oxygen therapy	N	Not performed		Recovered		N

(Continued)

TABLE 2 | Continued

Case	Nasal swab positive/negative	Respiratory symptom	Delayed infusion Yes/NO	Days of delayed infusion	Fever Yes/No	COVID-19 treatment	Hospitalization Yes/NO	Antibodies against SARS-CoV2	Serological test type	Covid-19 outcome	COVID-19 sequelae	Vaccination Yes/No
14	pos (2/4/2021)	Dyspnea	N		Y	Steroid therapy, antibiotic therapy	N	Not performed		Recovered	Ipogeusia, fatigue, increased legs stifness	N
15	pos (03/06/2021)		Y	38	Y	Heparin, antibiotic therapy	N	Not performed		Recovered		N
Case #	Nasal swab pos/neg	Type of symptom	Delayed infusion Y/N	Days of delayed infusion	Fever Y or N	Covid-19 treatment	Hospitalization	Antibodies against SARS-CoV2	Serological test type	Covid-19 outcome	Type of symptoms	vaccino Y/N
1	pos	Pneumonia			Y	Hydroxychloroquine, darunavir, ritonavir, ceftriaxone, steroids, nitric oxide, ozone, antitrombotic, NIV	y	Not performed		Deceased		/
2	neg	Pneumonia	N		Y	Hydroxychloroquine, azithromycin, steroid	Y	IgM and IgG pos (27/5/2021)	ChLIA	Recovered		
3	pos (15/9/2020)		N		N	Paracetamol	N	IgG neg, IgM neg (18/5/2021)	ChLIA	Recovered		

but switched to Natalizumab because of disease activity. In August 2020, a brain MRI showed disease activity and antibodies against Natalizumab tested positive. Therefore, Natalizumab was stopped and ocrelizumab was started in September 2020. After 10 days, he developed fatigue and nasal congestion. His cohabitant partner also developed similar symptoms and both of them tested positive by nasal swab for COVID-19. They were quarantined at home; the symptoms of the patient rapidly improved over the following days with no need for specific therapies. The patient tested negative by nasal swab on November 15. On May 18, 2021, he underwent a quantitative serological test that did not detect antibodies against SARS-CoV2.

Case 4

A 53-year-old woman was diagnosed with RR-MS in 2003. She was treated with different DMTs, such as glatiramer acetate and dimethyl-fumarate, but she switched to ocrelizumab in February 2019 due to disease activity (fourth cycle on October 8, 2020). Her last EDSS score was 3.5 and she had no other serious medical illnesses. On October 19, 2020, she presented with vomit, diarrhea, headache, and fever. She tested positive by nasal swab for SARS-CoV2 on October 28. Due to mild symptomatology, she was self-quarantined at home, she was prescribed oral steroid therapy and gradually recovered over the following days. She tested negative by nasal swab on November 17, and she underwent a quantitative serological test in March 2021 that detected the presence of antibodies against SARS-CoV2.

Case 5

A 69-year-old woman was diagnosed with RR-MS in 2015 and started teriflunomide in February 2016. Her comorbidities included hypertension and sick sinus syndrome (pacemaker wearer). Due to a persistent disease activity, she switched to ocrelizumab in March 2019. Her last EDSS score was 4.0.

On October 11, 2020, she developed dizziness, nausea, vomit, and fatigue, but no fever or cough. She underwent an urgent neurological visit, in suspicion of an MS relapse. Brain MRI was stable, and she tested positive for SARS-CoV2 by nasal swab on October 22. Steroid therapy, azithromycin, and low molecular weight heparin were started, and her symptoms remained stable over the following days while she was self-quarantined at home. She recovered in 2 weeks, but nasal swabs in the following month were still positive. She tested negative on January 20, 2021. One month later, she tested positive for IgG to SARS-CoV2 (detected with a quantitative test), and she also tested positive in the following test performed in April 2021.

Case 6

A 49-year-old woman was diagnosed with aggressive RR-MS in June 2020, with a high lesion burden on MRI. Her EDSS was 2.5 and she suffered from type1-diabetes since childhood, treated with insulin therapy. She had a positive serology for the JC virus. After a careful discussion of the pro and cons of the available treatments, she started ocrelizumab on September 16, 2020. On October 29, 2020, she developed fever, cough, headache, and nasal congestion and tested positive for COVID-19 by nasal swab on November 7. Her symptoms improved over the following days without any specific treatment and were fully resolved.

Case 7

A 52-year-old woman was diagnosed with RR-MS in October 2005. In 2019, she switched from beta-interferon to ocrelizumab (last cycle on June 8, 2020) because of evidence of disease activity. Her last EDSS score was 3.0 and she had no other medical illnesses. On October 23, 2020, she developed fever, cough, shortness of breath, anosmia, and ageusia. She started amoxicillin and prednisone following the advice of her doctor. Nasopharyngeal swab, performed on October 25, was positive for SARS-CoV2 and, due to mild symptoms, she was self-quarantined at home. She completely recovered over the following weeks except for a persisting anosmia and ageusia, and she tested negative by the nasal swab on November 16, 2020. In February 2021, she underwent a quantitative serological test that detected the presence of IgG against SARS-CoV2.

Case 8

A 50-year-old woman was diagnosed with RR-MS in 2015. She was treated with teriflunomide and natalizumab before switching to ocrelizumab on September 16, 2019 due to the persistence of disease activity (third cycle on October 1, 2020). She had no other medical illness and her EDSS score was 3.0. On October 27, 2020, she reported fever, headache, nasal congestion, hyposmia, and ageusia. After a few days, a nasal swab confirmed the diagnosis of COVID-19. She started steroid therapy and gradually recovered without the need for hospitalization.

Case 9

A 52-year-old woman was diagnosed with aggressive RR-MS in 2019 and was treated with ocrelizumab on May 23, 2019 (last cycle June 19, 2020). She had no other comorbidities and her EDSS score was 2.0. On October 23, 2020, she developed fever, fatigue, and bone pain. She rapidly recovered without therapy over the following days. Two nasal swabs were still positive for SARS-CoV2 (October 28 and November 11); she tested negative on January 27, 2021.

Case 10

A 58-year-old woman was diagnosed with an active PP-MS in 2014. Her comorbidities included obesity and Graves' disease. Ocrelizumab therapy was started in September 2018. In June 2020, she was hospitalized with the suspicion of clinical and radiological relapse and treated with steroid therapy. Her EDSS score was 7.0. On August 18, 2020, she received the fourth cycle of ocrelizumab. On December 9, 2020, during a hospitalization in a rehabilitation facility, she developed cough, high fever, and vomit and tested positive for SARS-CoV2 by the nasal swab. She was immediately transferred to a dedicated ward and was treated with intravenous steroids and antibiotics for 3 weeks; she also needed oxygen support because of hypoxemia. She gradually and completely recovered over the following weeks and tested negative by nasal swab on January 20, 2021.

Case 11

A 62-year-old man with a diagnosis of RR-MS was treated with several DMTs, such as beta-interferon and Natalizumab, before switching to ocrelizumab in April 2019. He received his last cycle on December 17, 2020. EDSS was 5.5 and he had no other medical illnesses except for benign prostatic hyperplasia. In December 2020, his cohabiting partner tested positive for SARS-CoV2 by nasal swab. For this reason, he performed various nasal swabs and, on January 4, 2021 he tested positive. He developed fever, nasal congestion, and cough on January 8, 2021. On January 20, due to the worsening of symptoms and the appearance of dyspnea, the patient was hospitalized. Steroids, low molecular weight heparin, and antibiotics (with three different therapeutic regimens) were used, and due to the worsening of respiratory exchanges, he received NIV. Over the following days his symptoms remained stable, he tested negative by nasal swab on February 4, 2021, and he was switched to oxygen therapy before being discharged on February 12, 2021. He gradually recovered over the following weeks and gradually tapered oxygen therapy at home.

Case 12

A 40-year-old woman was diagnosed with RR-MS in 2008. She was treated with natalizumab but switched to fingolimod in 2013 due to a positive serology for the JC virus. Due to the persistent disease activity, she switched to ocrelizumab in February 2019 and she received her last cycle on September, 30, 2020. Her EDSS score was 4.5 and she had no other medical illnesses. On February 14, 2021, she developed fever (39.5°C), cough, headache, shortness of breath, nasal congestion, nausea, and lymph nodes enlargement. The next day, she tested positive for SARS-CoV2 by nasal swab. She gradually recovered over the following weeks, with a persistent cough, headache, and thoracic pain. After 2 months, she underwent a chest X-ray that showed thickened bronchovascular markings. She received steroid and antibiotic therapy, and she fully recovered over the following weeks. She received her fifth cycle of Ocrelizumab on May 5, 2021 and, on June 1, 2021, she underwent a serological test that detected the presence of antibodies (IgG) against SARS-CoV2.

Case 13

A 53-year-old woman with a diagnosis of RR-MS was treated with several DMTs (beta-interferon, mitoxantrone, and natalizumab) before switching to ocrelizumab on February 13, 2019. She had no other medical illnesses (except for HBV positivity) and her EDSS score was 5.5. On March 22, 2021, 22 days after her fifth cycle of ocrelizumab, she developed fatigue, sore throat, headache, shortness of breath, and loss of taste, and a nasal swab tested positive for COVID-19. Over the following days, her oxygen blood saturation dropped below 90% and she received oxygen therapy for 10 days. She also received steroid therapy and paracetamol, fully recovered over the following days, and tested negative by nasal swab on April 16, 2021.

Case 14

A 54-year-old man was diagnosed with an aggressive RR-MS in January 2021. He had no other medical illnesses, his EDSS score was 4.0, and he received his first cycle of ocrelizumab on January 28, 2021. On April 1, 2021 he developed leg stiffness, headache, fever (40°C), loss of taste and smell, and on the next day, he tested positive for COVID-19 by nasal swab. He received steroid and antibiotic therapy, but he did not fully recover despite testing negative on May 6, 2021. He still is symptomatic, with increased fatigue, persisting reduction of taste, and leg stiffness.

Case 15

A 33-year-old woman with a 10-year history of MS disease was treated with several DMTs such as beta-interferon, natalizumab, fingolimod, and alemtuzumab, before switching to ocrelizumab on October 16, 2019. Her EDSS score was 3.0, and she suffered from hyperthyroidism. She received her last cycle on November 20, 2020 and 6 months later, few days before receiving the next cycle, she developed fever, cough, headache, and fatigue. She tested positive by nasal swab for COVID-19 on June 3, 2021, and she fully recovered over the following days with heparin and antibiotic therapy. She tested negative by nasal swab on June 19, and she received her fourth cycle of ocrelizumab on July 8, 2021.

DISCUSSION

Here, we describe an incident case series of 15 ocrelizumab-treated MS patients with confirmed COVID-19, followed at two MS Centers in Northern Italy, from the beginning of the pandemic in continental Europe in February 2020 to June 2021. Knowledge about the SARS-CoV2 virus itself and how it may affect MS patients is still limited (6, 18–20). The available data regarding COVID-19 in people with MS show that, in general, the MS population does not seem to be at a higher risk of death from COVID-19 (18–21). Major risk factors identified for a severe/fatal COVID-19 in the MS population are the same factors identified in the general population: older age (>50 years old), higher levels of disability, MS progressive course, and presence of comorbidities (6, 7, 18–20). The role of single DMTs on the COVID-19 disease course is not completely clear yet: mortality rates do not differ when considering specific DMT use (18, 19), but the frequency of severe COVID-19 could be higher in anti-CD-20 therapies compared to other DMTs (18) and an increased risk of hospitalization was reported in rituximab-treated patients (20).

The pathophysiology of SARS-CoV-2 is complex, with aggressive inflammatory responses implicated in the resulting damage to the airways; therefore, disease severity in patients is due to not only the viral infection but also to the host response which may cause a systemic involvement (22). In most individuals, recruited cells clear the infection in the lung, the immune response recedes, and the patient recovers. The adaptive immune response is necessary to eliminate the virus: this requires the presence of an appropriate genetic background in the host that activates specific antiviral immunity. In this phase of the infection, efficient immune responses are essential to avoid disease progression and virus propagation (23, 24).

However, in some patients, a dysfunctional immune response occurs, which triggers a cytokine storm that can lead to cytokine release syndrome (CRS) and that mediates widespread lung

inflammation (22, 25). In this phase, strategies to suppress inflammation are required (24); immunosuppression might prevent the overly active immune response that drives tissue damage (26).

The initial responses against viruses are led mainly by T-lymphocytes and natural killer cells, and, to a lesser extent, by B-cells. This may be the reason why patients on anti-CD20 therapies cope generally well with viral infections. Anti-CD20 therapies have a relatively minor impact on T-cell counts and have not been associated with severe viral infections (27). These findings and the outcome observed in most of our patients suggest that the presence of circulating B cells is not necessarily required for recovery from COVID-19. Accordingly, we observed that four out of six tested cases showed the presence of IgG to SARS-COV2. Following these premises, many patients described in this case series had received the last infusion of ocrelizumab shortly before the onset of COVID-19 symptoms and, despite this, most of them developed a self-limiting disease that did not require hospitalization.

Patients with a higher MS-related disability in this series were likely to develop a more severe COVID-19 course, in line with the data reported from the French and Italian cohorts (18, 19).

Some of our patients reported prolonged and persistent symptoms even after several months of recovery. The time to symptoms resolution has been related to the severity of the acute illness and premorbid risk factors (28, 29). Some patients may also report prolonged neurological symptoms even without a premorbid neurological condition (30). Neurologic complications may be the consequences of the direct effects of the virus or the systemic response to the infection (31).

Moreover, given this possibility of long-term symptoms, many patients may require rehabilitation services, such as pulmonary and cardiac rehabilitation and physical therapy (32).

At the beginning of 2020, northern Italy was one of the first regions in Europe to be heavily hit by the first wave of the COVID-19 pandemic. The difficult challenges and uncertainties encountered by the healthcare system in the early phase of the COVID-19 pandemic might have contributed to the case fatality ratio observed in this series.

Registry-based studies suggested a higher risk of a more severe COVID-19 disease course in ocrelizumab-treated (18) and rituximab-treated MS patients (20). Recently, a report on COVID-19 in ocrelizumab-treated PwMS has been published by Roche clinical researchers. They concluded that COVID-19 severity in PwMS was in line with that of the general population and that case fatality rates were comparable with other MS cohorts (17). Potential limitations of this study are the lack of untreated MS controls matched to ocrelizumab-treated MS patients, the relatively low number of patients included, and the unavailability of serological data on the SARS-CoV2 antibody status for some of the patients.

In conclusion, this real-life case series highlights the variability of the course of COVID-19 in ocrelizumab-treated MS patients. The decision of treating a patient with ocrelizumab during the COVID-19 pandemic needs to be discussed between the patient and the treating neurologist, based on a benefit/risk assessment specific to the individual patient. A position paper was recently issued by expert clinicians regarding ocrelizumab use during the Covid-19 pandemic: considering that experiences coming from the first pandemic wave have not been brought to different risk stratification in terms of DMTs, these Authors suggest maintaining pre-pandemic criteria in the therapeutic choice (33); it is also suggested that these patients are immunized with seasonal-flu and COVID-19 vaccination (33).

The MS International Federation spread global advice to patients with MS, with measures to minimize the infection risk and implications associated with DMTs use (34). Recommendations from several scientific societies strongly urge to consider patients with MS patients treated with a disease-modifying treatment as a priority group for COVID-19 vaccination (35).

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

SD, MV, and AA: data collection and paper writing. CB and VS: data collection. MC and PC: data collection, paper writing, and supervision. All authors contributed to the article and approved the submitted version.

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Clinical Pathway for the Diagnosis and Management of Patients With Relapsing–Remitting Multiple Sclerosis: A First Proposal for the Peruvian Population

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Background: Relapsing–remitting multiple sclerosis (RRMS) is a subtype of degenerative inflammatory demyelinating disease of multifactorial origin that affects the central nervous system and leads to multifocal neurological impairment.

Objectives: To develop a clinical pathway (CP) for the management of Peruvian patients with RRMS.

Methods: First, we performed a literature review using Medline, Embase, Cochrane, ProQuest, and Science direct. Then, we structured the information as an ordered and logical series of five topics in a defined timeline: (1) How should MS be diagnosed? (2) How should a relapse be treated? (3) How should a DMT be initiated? (4) How should each DMT be used? and (5) How should the patients be followed?

Results: The personnel involved in the care of patients with RRMS can use a series of flowcharts and diagrams that summarize the topics in paper or electronic format.

Conclusions: We propose the first CP for RRMS in Peru that shows the essential steps for diagnosing, treating, and monitoring RRMS patients based on an evidence-based medicine method and local expert opinions. This CP will allow directing relevant clinical actions to strengthen the multidisciplinary management of RRMS in Peru.

Keywords: multiple sclerosis, relapsing-remitting, patient care management, critical pathways, Peru

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INTRODUCTION

Multiple sclerosis (MS) is a degenerative inflammatory demyelinating disease of multifactorial origin that affects the central nervous system (CNS) and leads to multifocal neurological impairment. It occurs more frequently in young adults aged between 15 and 35, being more frequent in women (1). It is currently considered a complex disease influenced by genetic, epigenetic, and environmental factors (2).

The overall prevalence has been estimated at 30 per 100,000 inhabitants (3). The regions with the highest prevalence of MS are North America with 191.2 cases per 100,000 inhabitants, and Europe, with 96 to 200 cases per 100,000 inhabitants. On the other hand, Asia and sub-Saharan Africa have a lower prevalence with <0.22 per 100,000 inhabitants. In South America, a prevalence of 5.24 cases per 100,000 inhabitants was recorded, particularly in Panama and Argentina (Patagonia), with an estimated prevalence and incidence of 17.2/100,000 and 1.4/100,000, respectively (4, 5). In Peru, the prevalence calculated for Lima was 7.69 per 100,000 inhabitants (6). The current perception is that there is an increase in the prevalence and incidence of this disease that could be explained by increased disease awareness, better access to diagnostic tools, longer survival, and more sensitive diagnostic criteria resulting in better case detection (7, 8).

MS has a varied clinical presentation in which two recognized clinical phenotypes have been described and are characterized by their activity and progression: (1) relapsing MS and (2) progressive MS. However, a clinically isolated syndrome (CIS) and a radiologically isolated syndrome (RIS) have also been described and should be taken into account (9).

Clinical pathways (CP) are a helpful tool for continuous quality improvement in healthcare and facilitate the integration of clinical practice guidelines, protocols, and local algorithms. The advantages of CP are based on optimizing integrated mechanisms that include the appropriate activities necessary to manage specific medical problems (10, 11). Therefore, they allow standardizing diagnosis and treatment while always prioritizing common sense and clinical experience, positively influencing the best professional training, and facilitating teamwork (12, 13).

In 2019, the Peruvian Society of Neurology published a clinical guideline for managing patients with MS to provide neurologists with a valid, updated tool to treat these patients in a comprehensive manner (14). However, a more practical and user-friendly tool was needed to achieve greater acceptance among Peruvian neurologists, thus standardizing the management of Peruvian patients based on quality external information adapted to our context by a group of thematic experts.

Therefore, we developed a CP to diagnose and manage patients with relapsing–remitting MS (RRMS) with summary versions of the recommendations through evidence-based algorithms.

MATERIALS AND METHODS

We developed a CP route with the following methodological design criteria aimed at (1) developing a structured multidisciplinary care plan; (2) channeling the translation of guides or tests to local structures; (3) describing the steps of the therapeutic course using a route, an algorithm, a guide, a protocol, or another "inventory of actions"; and (4) standardizing care for a specific clinical problem, procedure, or episode of care in a specific population (15).

To achieve this, we recruited a group of Peruvian neurologists working at public hospitals with over 5 years of experience managing MS and a methodological team with experience in synthesizing evidence. Then, we conducted a review of the literature using different sources (Medline, Embase, Cochrane, ProQuest, and Science direct) with "Multiple Sclerosis, Relapsing–Remitting" as the MeSH term. We identified relevant evidence that covers issues related to the care of patients with RRMS, and during six meetings, we planned and designed five key topics developed in this CP: (1) diagnosis, (2) relapse treatment, (3) initiation of disease-modifying treatment (DMT), (4) use of each DMT, and (5) follow-up.

RESULTS

We organized the present CP as an ordered and logical series of resolved topics in a consecutively defined timeline (**Figure 1**). In addition, we accompanied this CP with a series of flow diagrams that neurologists can use. This CP for the care of patients with MS could be used in paper or electronic format and consists of the following questions:

How Should MS Be Diagnosed?

The initial evaluation must define whether the patient presents with a typical CIS (9) (**Additional File 1**). Patients with ≥2 lesions on magnetic resonance imaging (MRI) have a high probability of developing MS (16, 17). The McDonald 2017 criteria must be applied to confirm the diagnosis of MS. There are no specific considerations for the Peruvian population except ruling out tuberculosis (18) (**Additional File 2**). Finally, the prognosis should be assessed according to the following unfavorable outcome factors: age >40 years, male sex, African American or Latin American ethnicity, polyfocal presentation, involvement of the afferent system, and partial or no recovery, all of which can increase the risk of developing aggressive forms of MS (19).

How Should a Relapse Be Treated?

In patients presenting moderate to severe relapse (20–22) (**Additional File 3**), the first option is intravenous high-dose methylprednisolone pulse therapy (6, 23, 24) (**Figure 2**). Alternatively, oral methylprednisolone could be used since evidence of a similar effect exists (25–28), but tablets >8 mg are not available in Peru. Another alternative could be oral prednisone 1,250 mg daily; however, 25 tablets per day of prednisone 50 mg make this alternative not suitable. If the patient does not respond favorably or cannot comply with methylprednisolone therapeutic protocol, treatment with therapeutic plasma exchange should be considered (23, 29) (**Figure 3**).

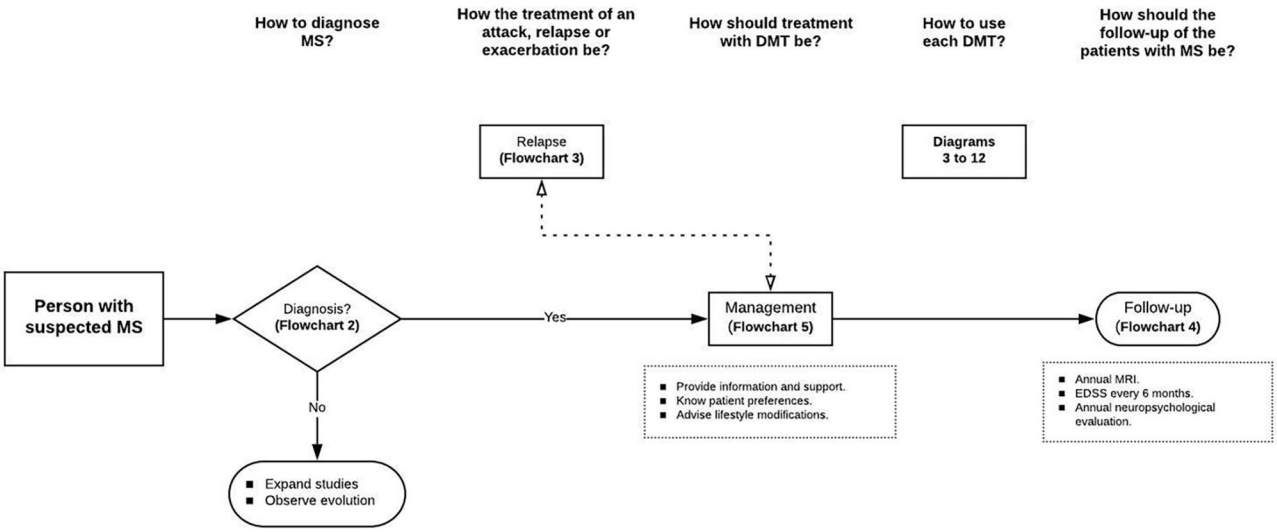

FIGURE 1 | Clinical pathway for diagnosis and treatment of RRMS. Source: Authors.

Formulation:
- ***Methylprednisolone*** (500mg)

Prior to treatment initiation	Treatment Initiation	During Treatment
Previous analysis: ■ CBC ■ Urinalysis *Contraindications:* ■ Hypersensitivity to methylprednisolone or any component of the formulation	*Route of administration*: ■ **IV infusion** *Dosage and timing*: ■ **Infusion:** Dilute 2 vials (1g) in 100ml of 0.9% NaCl and administer in 1 to 2 hours every day for 5 days *Considerations*: ■ Ranitidine 50mg IV during infusion ■ Enoxaparin 40mg SC during infusion	*Monitor:* ■ BP ■ Glycemia ***Adverse events:*** ◦ Gastric discomfort ◦ Venous thrombosis ◦ Hiperglycemia ◦ BP increase

CBC: Complete blood count; IV: Intraveous ; NaCl: Sodium chloride; SC: Subcutaneous; BP: Blood pressure;. * Treatment with oral MTP of IV has the same benefit. In our country there is no oral presentation. ** Sensitive relapses are not treated with IV MTP

Replacement fluids:
- Of choice: ***Human albumin***
- Alternative: ***Fresh Frozen Plasma***

Prior to treatment intiation	Treatment Initiation	During Treatment	
Previous analysis: ■ CBC ■ Hct ■ Seric calcium ■ PT ■ aPTT ■ Fibrinogen *Contraindications:* ■ Hemodinamically unstable ■ Not possible to use a CVC ■ Albumin or FFP allergy	*Route of administration*: ■ **CVC** *Plasma volume to replace*: ***EPV (Liters)= 0.07 x Weight (kg) x (1- Hct)*** *Frequency*: ■ 5 sesions of TPE every other day during 10 to 14 days	*Monitor:* ■ CBC ■ Hct ■ Seric calcium ■ PT ■ aPTT ■ Fibrinogen	***Adverse events:*** ◦ CVC infectios ◦ Thrombosis ◦ Hypotensión ◦ Muscle cramps ◦ Paresthesia ◦ Urticaria ◦ Vertigo ◦ Headaches ◦ Sickness ◦ Angina ◦ Arrhytmia

FIGURE 2 | Therapeutic protocol for relapses: Methylprednisolone and therapeutic plasma exchange. Source: Authors.

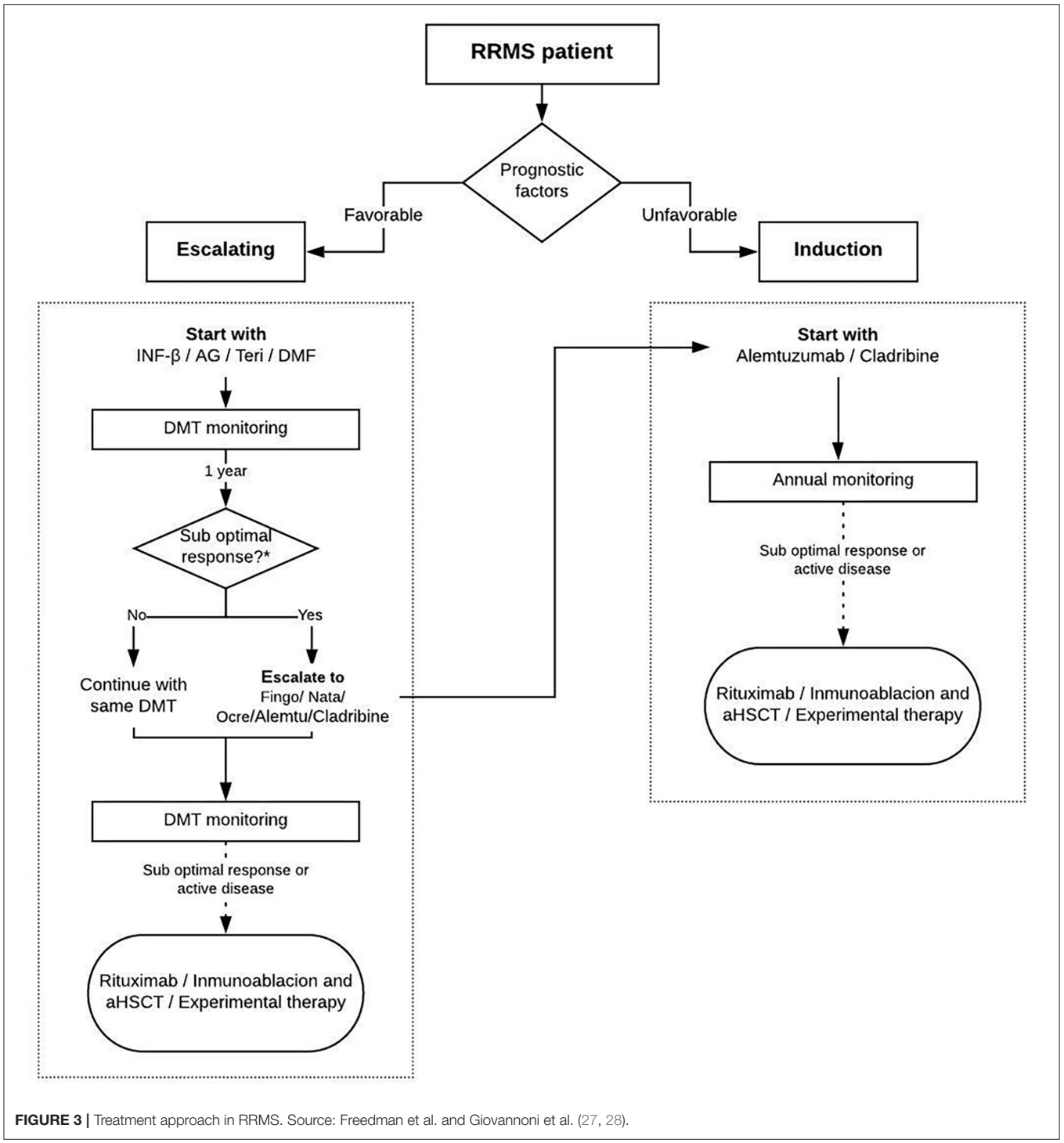

FIGURE 3 | Treatment approach in RRMS. Source: Freedman et al. and Giovannoni et al. (27, 28).

How Should a DMT Be Initiated?

The choice of a DMT should be made according to the patients' characteristics, the evaluation of prognostic factors, the risk–benefit balance of the treatment options, and the experience of the treating neurologist (30–32) (**Figure 4**). It should be noted that this algorithm is only a reference since high efficacy can also be achieved with first-line therapies depending on the clinical factors. The Modified Rio score should be used to evaluate the treatment response with IFN, teriflunomide, and glatiramer acetate at 12 months later (33–35). For the remaining DMTs, clinical and imaging assessments should be performed every year (**Additional File 4**).

Formulation:
- ***INTERFERON-β***

Prior to treatment Initiation	Treatment Initiation	During Treatment	//	Treatment Discontinuation
Within 3 months prior: ■ CBC ■ MRI ■ Liver function test ■ Thyroid function test *Contraindications:* ■ Hypersensitivity to natural or recombinant beta interferon, human albumin or any other component of the formulation ■ Cardiovascular disease ■ Hepatic disease ■ History of alcoholism ■ History of seizures ■ Thyroid dysfunction ■ Renal disease	*Route of administration*: ■ **IM, SC** *Dosage and timing*: ■ ***IFN-β 1a SC*** : 44 µg 3 times a week ■ ***IFN-β 1a IM*** : 30ug every week ■ ***IFN-β 1b SC***: 0.25mg every other day	*Considerations:* ■ Discontinue therapy if pregnancy occurs. ■ Perform an annual MRI ■ Escalate to a more effective treatment if sub optimal response occur ■ Treat flu-like syndrome with paracetamol of ibuprofen		*For alternative treatment:* ■ No wash-out period ***Adverse events:*** ◦ Inflammation at the injection site ◦ Flu-like syndrome ◦ Leukopenia ◦ Thrombocytopenia ◦ Anemia ◦ Increased transaminase ◦ Thyroid dysfunction: Hypo o hyperthyroidism ◦ Depression or other serious psychiatric symptoms

MRI: Magnetic resonance imaging; IM: Intra-muscular; SC: Subcutaneous; IFN-β : Interferon-β

Formulation:
- ***GLATIRAMER ACETATE*** (20mg/ml; 40mg/ml)

Prior to treatment Initiation	Treatment Initiation	During Treatment	//	Treatment Discontinuation
Within 3 months prior: ■ CBC ■ MRI *Contraindications:* ■ Hypersensitivity to glatiramer acetate, manitol or any component of the formulation.	*Route of administration*: ■ **SC** *Dosage and timing*: ■ 20 mg everyday ■ 40 mg three times a week	■ Discontinue therapy if pregnancy occurs ■ Perform annual MRI ■ Escalate to a more effective treatment if sub optimal response occur		*For alternative treatment:* ■ No wash-out period ***Adverse events:*** ◦ Lipodystrophy ◦ Inflammation at the injection site ◦ Infection ◦ Vasodilation ◦ Angina ◦ Palpitations ◦ Acne ◦ Nausea ◦ Anxiety ◦ Hypersensitivity

CBC: Complete blood count; MRI: Magnetic resonance imaging; SC: Subcutaneous

Formulation:
- ***TERIFLUNOMIDE*** (14 mg)

Prior to treatment Initiation	Treatment Initiation	During Treatment	//	Treatment Discontinuation
Within 3 months prior: ■ CBC ■ Liver function test ■ MRI ■ Negative pregnancy test in women of childbearing age ■ Negative CXR and PPD or negative quantiferon *Contraindications:* ■ Hypersensitivity to teriflunomide, leflunomide or any component of the formulation ■ Severe liver damage ■ Active severe infections ■ Pregnancy or non-effective contraceptive method	*Route of administration:* ■ **Oral** *Dosage and timing:* ■ 14 mg tablet every day	*Monitor:* ■ Complete blood count and liver profile every 02 weeks for 6 months ■ Wait 2 years or until plasma level is < 0.02 mg/l - alternative treat with cholestyramine or active charcoal for 11 days if pregnancy is desired ■ Discontinue therapy if pregnancy occurs ■ Escalate to a more effective treatment if sub optimal response occur		*For alternative treatment:* ■ Accelerated elimination or 3.5 months of wash-out period ■ Eleven days of cholestyramine ***Adverse events:*** ◦ Diarrhea ◦ Alopecia ◦ Headache ◦ Hypophosphatemia ◦ Increase in transaminases ◦ BP increase ◦ Leukopenia: Neutropenia and lymphopenia ◦ Infections

CBC; Complete blood count; MRI: Magnetic resonance imaging; CXR: Chest X-ray; PPD: Tuberculine purified protein derivative; BP: Blood pressure

Formulation:
- ***DIMETHYL FUMARATE*** (240mg)

Prior to treatment initiation	Treatment Initiation	During Treatment	//	Treatment Discontinuation
Within 3 months prior: ■ CBC ■ MRI ■ Negative pregnancy test in women of childbearing age ■ Negative CXR and PPD or negative quantiferon ■ HIV serology ■ HBV, HCV, VZV serology *Contraindications:* ■ Hypersensitivity to dimethyl fumarate or any component of the formulation.	*Route of administration*: ■ **Oral** *Dosage and timing*: ■ 120mg tablet twice a day for a weeks and then 240mg tablet twice a day *Considerations*: ■ Administer with food ■ Administration with high proteins foods reduce flushing and gaastrointestinal effects ■ Flushing can be reduce administering up to 325mg of acetylsalicylic acid	*Monitor:* ■ CBC and hepatic profile every 3 months ■ Lymphocyte count every 3 months. Discontinue if lymphocyte count <0.5x10^9/l for 6 months ■ Escalate to a more effective treatment if sub optimal response occur		*For alternative treatment:* ■ Wait for lymphocyte levels return to normal values ***Adverse events:*** ◦ Flushing ◦ Increase in transaminases ◦ Abdominal pain ◦ Diarrhea ◦ Nausea ◦ Leukopenia: Lymphopenia ◦ Infection ◦ PML ◦ Allergic reactions

FIGURE 4 | Therapeutic protocol for induction: Interferon-β, glatiramer acetate, teriflunomide, and dimethyl fumarate. Adapted from Sorensen et al. (33).

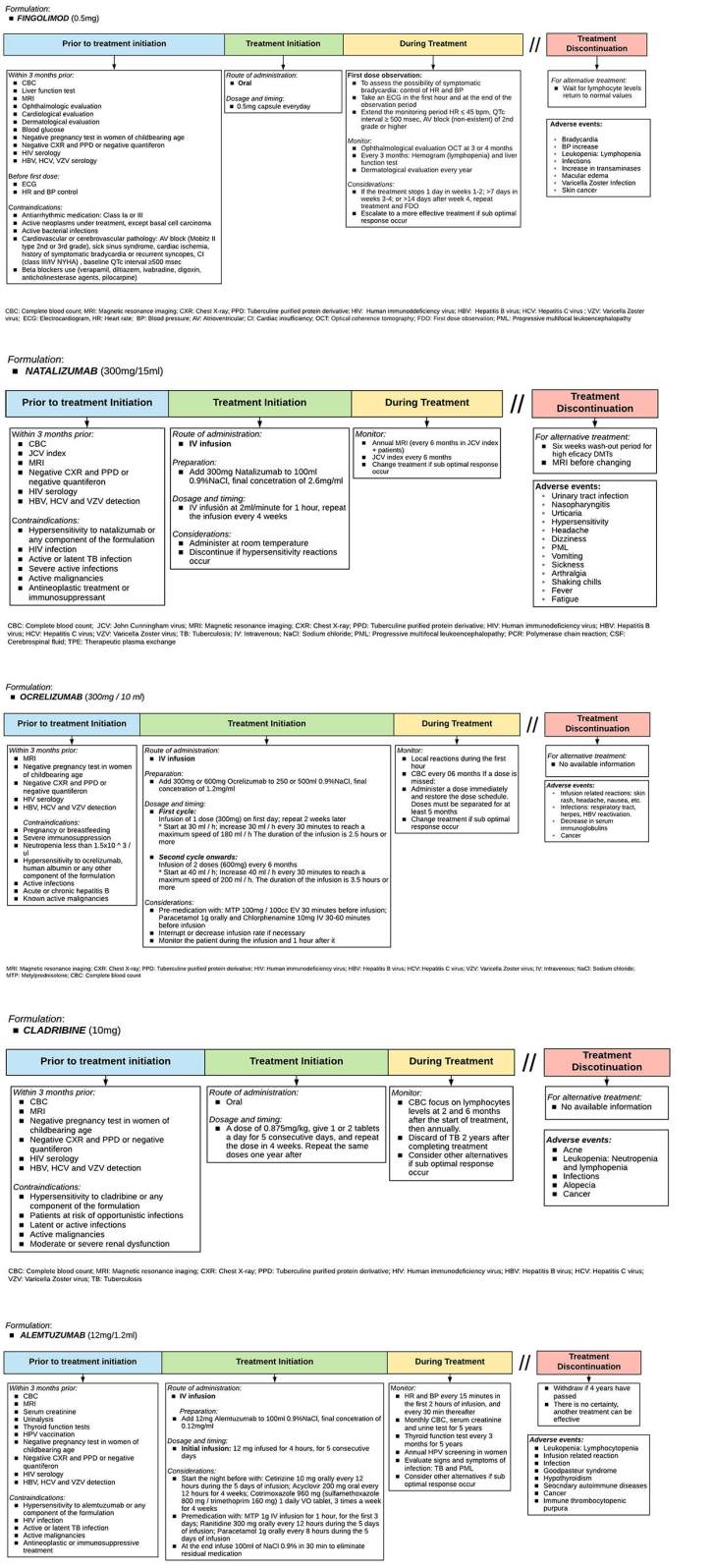

FIGURE 5 | Therapeutic protocol for escalating: Fingolimod, natalizumab, ocrelizumab, cladribine, and alemtuzumab. Adapted from Sorensen et al. (33).

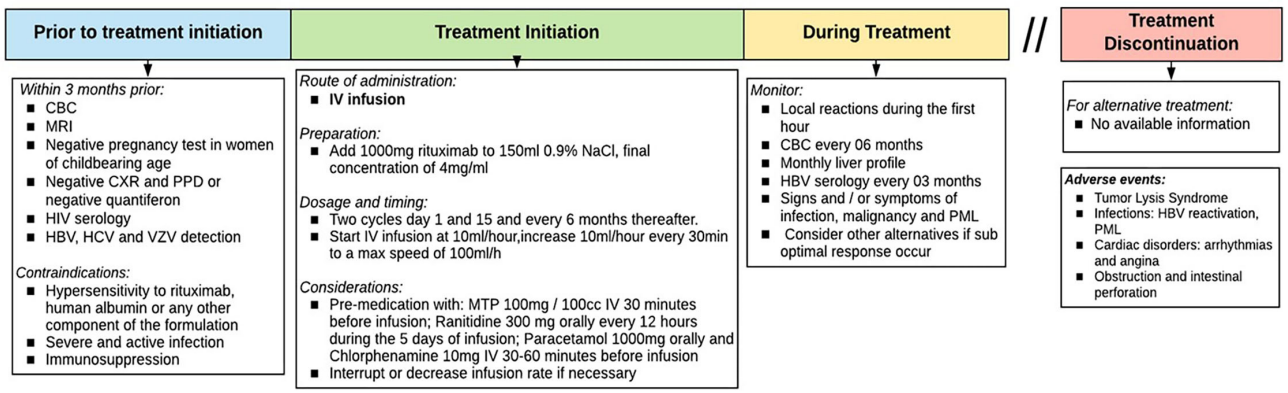

FIGURE 6 | Therapeutic protocol for failure: Rituximab. The indication of rituximab as a DMT in RRMS has not yet been approved in Peru and is used as an off-label indication. Source: Authors.

How Should Each DMT Be Used?

There are several DMT for RRMS treatment (14, 36, 37). However, specific tests are needed before initiating treatment. It is also important to know patient preferences and provide advice on individual general recommendations (38). **Figures 5**, **6** show diagrams describing the use of each DMT (1, 38–50).

How Should the Patients Be Followed?

After treatment initiation, a brain MRI must be obtained annually, and a spinal MRI should be requested only if spinal cord symptoms occurred (51). In addition, experts suggest a comprehensive clinical assessment including biannually Expanded Disability Status Scale (EDSS) and annually neuropsychological evaluations (Expert consensus) (33, 34).

No evidence of disease activity (NEDA) considering no relapses, no increase of disability (as measured with EDSS), and no new or active MRI lesions can also be used as a treatment objective (52, 53).

DISCUSSION

Patients with RRMS are young and present a chronic and disabling evolution, making it necessary to perform a multidisciplinary approach. This disease is characterized by an often-unpredictable course making diagnosis difficult and the choice of the adequate DMT for each patient challenging (1, 14, 54).

The clinical variability of RRMS requires a multidisciplinary intervention by healthcare professionals, making adequate resource management a necessity to reduce morbidity and disability, and thereby improve the quality of life of individuals with this disease. The use of the proposed CP will allow patients to receive relevant, timely clinical interventions and significantly reduce the use of hospital resources, without negatively affecting the length of stay and hospital costs (54–56).

Our CP indicates the steps to be followed in the initial phase of diagnosis, then in the treatment and monitoring phase, and finally during patient follow-up. We propose a current and adapted list of diagrams to guide DMT use in the Peruvian population based on the previous proposal by Sorensen et al. (38), which explains the tests to be made before, during, and after initiating treatment in chronological order as well as possible treatment schemes that neurologists can choose and how to perform monitoring.

There are limitations for MS diagnosis in Peru due to the difficult access to specialists access and MRI (16); therefore, there is a delay between the first clinical outbreak and the confirmatory diagnosis of up to 3.2 years (57). In addition, there are difficulties in accessing timely treatment because public institutions only have interferon and glatiramer acetate as the DMT scale. Moreover, to access induction DMT, it is necessary to evaluate a case for at least 12 months, classify it as a therapeutic failure and make a request for the new treatment that takes an average of 4–6 months, delaying the start of treatment with more significant disability and lower quality of life (16).

We organized several meetings with methodologists and neurologists to adapt the selected external information on the management of RRMS to the national context to resolve this. Our CP is innovative and is the first approach to integrating processes oriented at the diagnostic and therapeutic resources available for RRMS in Peru.

CONCLUSIONS

We have proposed the first CP for RRMS in Peru with a chronological description of the steps to follow for the diagnosis, treatment, and follow-up of RRMS patients. This will be a helpful tool for Peruvian neurologists in order to carry out a systematic process for the care of persons with MS.

We hope that the use of this CP will have a real impact on continuous improvement in the care and quality of health

provided by neurologists, which will be reflected by the satisfaction perceived by Peruvian RRMS patients. Finally, we believe that this CP for diagnosing and managing patients with RRMS will be an essential tool for encouraging correct and methodical approaches to the disease based on quality scientific-technical evidence, generating standard use of treatments and rational use of health resources.

AUTHOR CONTRIBUTIONS

CA-D, VV-R, NM, CC-Z, and WD-P participated in meetings to plan, design, and elaborate on the clinical pathway. VV-R, AH-R, RM, and CF-G identified relevant studies to support the clinical pathway. VV-R, AH-R, and CA-D participated in the writing of the manuscript. All authors designed the study and approved the final version of the 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.2021.667398/full#supplementary-material

Additional File 1 | MS diagnostic approach. Source: Huamani et al. (16) and Kuhler et al. (17).

Additional File 2 | Diagnostic protocol: Mc Donald criteria 2017. Source: Thompson et al. (18).

Additional File 3 | Relapse treatment of patients with MS. Source: Berkovich et al. (23, 26) and Correale et al. (21).

Additional File 4 | Assessment of treatment response modified Rio score. Source: Sormani et al. (33, 34).

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CASE REPORT
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Opportunities and Obstacles Associated With Sequential Immune Reconstitution Therapy for Multiple Sclerosis: A Case Report

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Cladribine is an effective disease-modifying treatment for relapsing-remitting multiple sclerosis that acts as an immune reconstitution therapy and is administered in a pulsed manner. Despite its efficacy, severe disease reactivation early after treatment represents a serious clinical problem, and clear evidence to guide the management of such a situation is lacking. Here, we describe the case of a patient experiencing considerable disease activity during the 1st year after the initiation of cladribine treatment. The patient was switched to alemtuzumab and, therefore, received double immune reconstitution therapy. Data regarding this approach are lacking, and real-world observations may be of interest. Despite achieving good control of disease activity, we observed several serious infectious complications. Our results suggest that sequential immune reconstitution therapies may be effective; however, at the price of higher susceptibility to infections.

Keywords: multiple sclerosis, cladribine, alemtuzumab, immune reconstitution therapy, case report

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INTRODUCTION

Cladribine and alemtuzumab have proven to be effective treatments for relapsing-remitting multiple sclerosis (RRMS), and both act as immune reconstitution therapies administered in a pulsed manner (1–3). Disease activity may occur early after the first course of treatment. However, this does not necessarily imply a treatment failure that requires further modifications to the treatment strategy. For this reason, drug response evaluation is generally performed at least a few months after the second drug course (4). Nevertheless, relevant disease activity early after a treatment course of one of these drugs may sometimes represent a serious clinical problem, potentially leading to permanent disability. In the CLARITY trial, interferon beta-1a rescue therapy was used (1). However, evidence of managing such a problem is scarce, subsequently leading to different clinical choices in a real-world setting (5). Here, we report a case of considerable ongoing disease activity after the first course of cladribine treatment, which was managed with alemtuzumab administration. Data regarding this sequence of therapies, which act through immune system depletion and reconstitution, are lacking, and real-world observations are, therefore, of interest. After alemtuzumab treatment, the patient achieved disease stability; however, several infectious complications were observed. This suggests that this sequential treatment strategy can be applied but warrants caution and careful monitoring.

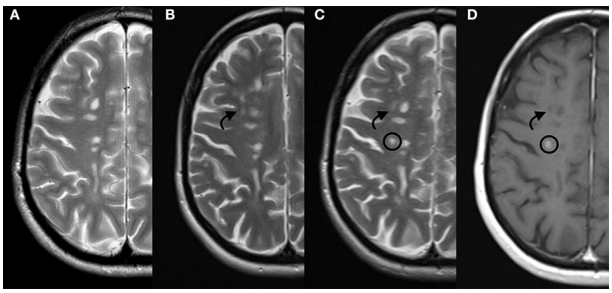

FIGURE 1 | Patient's disease progression on MRI. From the left to the right: T2 weighted sequences respectively on June 2018 **(A)**, February 2019 **(B)**, and July 2019 **(C)**, and; contrast enhanced T1 weighted sequence on July 2019 **(D)**. Arrow indicates a new demyelinating lesion and circle indicates a new demyelinating lesion presenting contrast enhancement. MRI, magnetic resonance imaging.

CASE DESCRIPTION

Here, we report the case of a 42-year-old patient diagnosed with RRMS at the age of 24 years, which was treated with different disease-modifying therapies. In 2012, after 2 years of natalizumab treatment, the patient was switched to fingolimod because of the high risk of progressive multifocal leukoencephalopathy. The patient remained stable until March 2017, when MRI progression was observed followed by a clinical relapse during the subsequent year.

Considering the presence of relevant disease activity, after discussing possible alternatives with the patient and considering an anti-JC virus (JCV) antibody index of 3.72, in May 2018, fingolimod therapy was discontinued and 9 weeks later, after lymphocyte count (ALC) recovery, oral cladribine was started. The patient received 1.75 mg/kg of cladribine and completed the first treatment course. The expanded disability status scale (EDSS) score at therapy initiation was 2.0, the ALC was 1,380 cells/μl, and baseline control MRI did not show any new lesions or contrast enhancement.

The patient consulted us in February 2019, reporting a slowly progressive somatosensory symptomatology over the previous month, which was considered as a relapse with no impact on permanent disability. However, MRI was performed and revealed four new demyelinating lesions, two of which presented with contrast enhancement. In July 2019, a new MRI scan was obtained, revealing five new cerebral enhancing lesions (**Figure 1**). We decided to switch therapy from cladribine to alemtuzumab. Therefore, the patient did not receive the second course of cladribine. The first course of alemtuzumab was administered in September 2019, when the ALC returned to the normal range (1,060/μl). The patient received 200 mg oral acyclovir twice daily for a month after infusions.

After starting alemtuzumab, the patient did not present any clinical relapses, radiologic signs of disease activity, or worsening EDSS score until January 2021. Despite good disease control, the patient experienced various infectious complications. In November 2019, she was treated with oral amoxicillin/clavulanate to address an upper airway infection. In December 2019, the patient was hospitalized on a precautionary basis because of A/H3 influenza infection; however, she did not require treatment. At the end of January, she received oral antibiotic treatment for upper airway infection. In February 2020, the patient presented with dermatomal varicella zoster virus reactivation, which required hospitalization and intravenous acyclovir.

The second course of alemtuzumab was administered in September 2020. A few days after treatment, the patient was again hospitalized for *Escherichia coli*-related left pyelonephritis, with findings of a duplicated ureter, and was successfully treated with antibiotics. Case timeline is provided in **Figure 2**.

Written informed consent was obtained from the patient for the use of clinical data and imaging studies.

DISCUSSION

In the current report, we describe the management of ongoing disease activity in the 1st year after cladribine initiation in a patient previously treated with fingolimod. Although cladribine has been proved to be effective in highly active multiple sclerosis (6, 7), it might not be sufficient to control inflammatory activity after fingolimod withdrawal, as recently reported in other cases (8–10). Some indirect comparisons suggest that cladribine and fingolimod have similar efficacy (7, 11). However, it has been postulated that lymphocytes entrapped in the lymph nodes due to fingolimod action could evade depletion provoked by subsequent immune reconstitution therapies (9). In our case, cladribine was initiated only after ALC recovery.

Management of considerable disease activity that appears early after the administration of an immune reconstitution therapy course is challenging without strong evidence to guide clinical decisions. Regarding alemtuzumab, some cases of severe reactivation after the first treatment course have been described with different management strategies, including continuation of scheduled therapy (12) or administration of a B-cell depleting agent, such as rituximab (13, 14) or ocrelizumab (15). Both these strategies have been proven to be effective and safe. In more aggressive cases, autologous stem cell transplantation could be an option as well (16). Regarding cladribine, very scarce data are available in the literature, and switching to another highly effective therapy, as in our case, is thought to be a reasonable option (17). In the CLARITY trial, rescue therapy with interferon beta-1a could be applied for patients with highly active disease (1), and 2.5% of the patients in the cladribine 3.5 mg/kg group received this treatment (18). In the reported case, interferon therapy was not considered as the patient had already received it in the past, without successful disease activity control.

Treatment with natalizumab, fingolimod, rituximab, ocrelizumab, and autologous stem cell transplantation has also been reported in the 1st year after cladribine initiation, but without outcome details (5, 8, 9).

Both alemtuzumab and cladribine cause lymphocyte depletion. The extent of B cell reduction is quite similar among the two treatments, but with a slower repopulation rate under

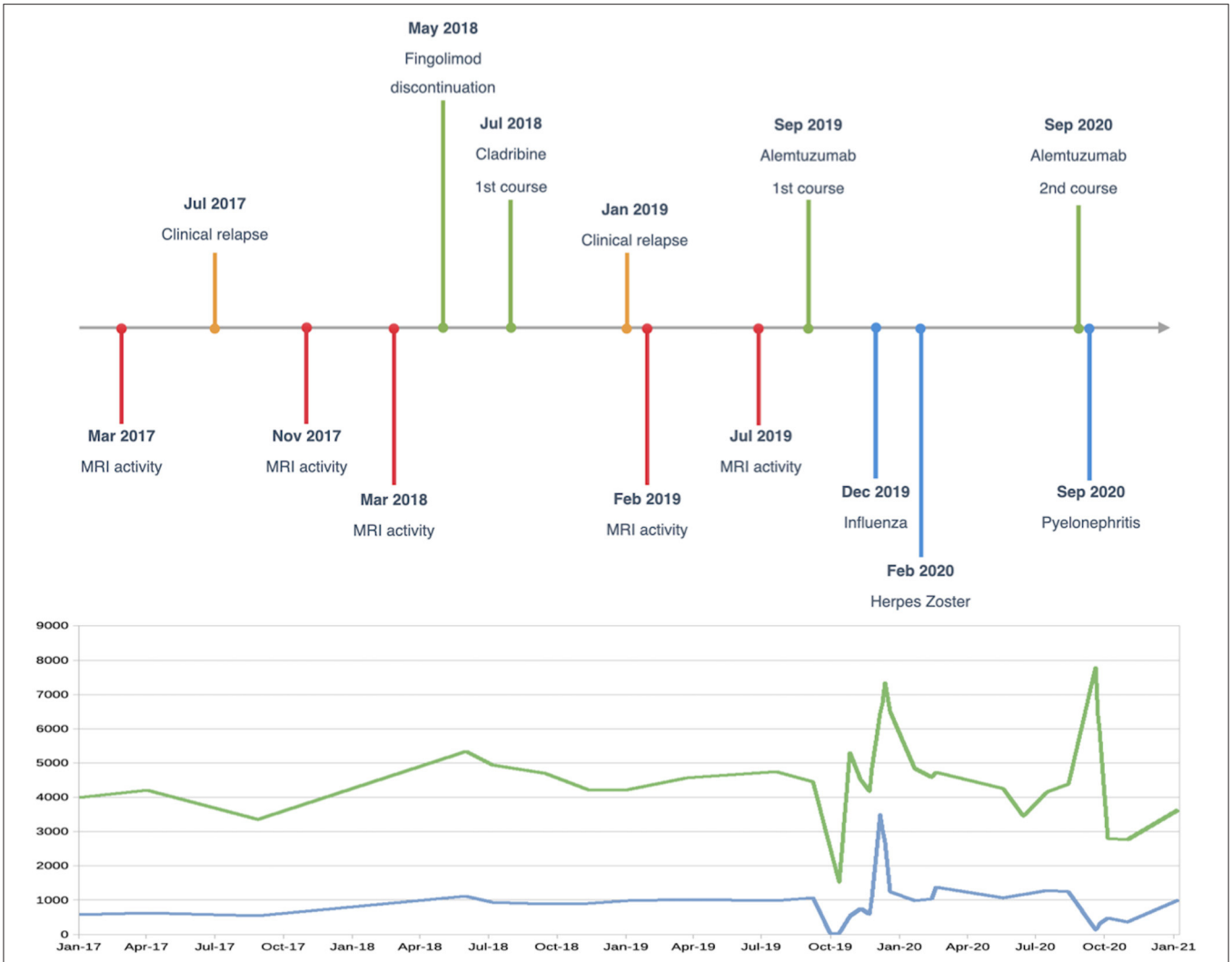

FIGURE 2 | Case timeline, and white blood cell (green line) and lymphocyte (blue line) changes over time (cells/μl).

cladribine administration (19). Alemtuzumab provokes a more rapid lymphocyte depletion, has a broader degree of action, and causes a more profound and durable reduction of CD4+ and CD8+ T cells compared to cladribine (19). Unfortunately, since lymphocyte subset monitoring is not routinely required in clinical practice, we measured them only at a few time points. This makes these measurements of scarce interest, as no trend after treatments or correlation with disease activity or infectious complications could be identified. However, given the previously mentioned pharmacodynamics of these treatments along with other multiple sclerosis treatments, ALC may be of limited utility, and immunophenotyping may be helpful in guiding treatment decisions in the future.

Regarding efficacy, no head-to-head comparisons exist between cladribine and alemtuzumab, and the results from a network meta-analysis did not reveal any differences in the outcome measures (6). Longer follow-up will be required to assess the long-term efficacy of alemtuzumab in the reported case. However, breakthrough disease activity observed after cladribine initiation was rapidly and effectively controlled with the new subsequent immune reconstitution treatment. With this approach, there may be an augmented risk of side effects due to the additional action on the immune system. In trials of alemtuzumab, the more frequently observed infections included upper airway infections, influenza, herpetic virus infections, and urinary tract infections, as observed in our present case (20). Other opportunistic infections have been observed mostly within months after treatment initiation (21). In addition, an increased risk of herpes zoster infection has been reported in association with cladribine (18). Along with the infections reported during alemtuzumab treatment, an additional risk caused by previous cladribine exposure should also be considered in our patient. We waited for ALC normalization before alemtuzumab administration, but ALC was anyway lower than the levels observed before cladribine initiation. However, the status of ALC before an alemtuzumab treatment course does not

predict any subsequent infection risk (20). Depletion of CD8+ T cells has been suggested to be associated with an increased risk of viral infection after alemtuzumab treatment (22). Although cladribine has a small effect on naive and memory CD8+ T cell counts, recovery at week 48 was minimal for naïve CD8+ T-cells and did not occur for memory CD8+ T cells in clinical trials (23). However, this aspect could be negligible considering the more profound T cell depletion induced by alemtuzumab.

In conclusion, alemtuzumab proved to be effective at controlling severe disease activity that appeared early after cladribine administration. However, the observation of different infectious complications warrants caution and a discussion about pharmacological prophylaxis for intercurrent infections. A longer follow-up and the description of similar cases may be helpful in the assessment of the efficacy and safety of sequential immune reconstitution therapies.

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/s.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

RG: investigation and writing the original draft. DC: investigation, conceptualization, writing, reviewing, and editing. SL: conceptualization, writing, reviewing, and editing. GG: supervision, writing, reviewing, and editing, as well as resources. DB: visualization. MV: supervision, writing, reviewing, and editing. All authors contributed to the manuscript and approved the submitted version.

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Cladribine Treatment for MS Preserves the Differentiative Capacity of Subsequently Generated Monocytes, Whereas Its Administration *In Vitro* Acutely Influences Monocyte Differentiation but Not Microglial Activation

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Cladribine (2-chlorodeoxyadenosine, 2CdA) is one of the most effective disease-modifying drugs for multiple sclerosis (MS). Cladribine is a synthetic purine nucleoside analog that induces cell death of lymphocytes and oral cladribine treatment leads to a long-lasting disease stabilization, potentially attributable to immune reconstitution. In addition to its effects on lymphocytes, cladribine has been shown to have immunomodulatory effects on innate immune cells, including dendritic cells and monocytes, which could also contribute to its therapeutic efficacy. However, whether cladribine can modulate human macrophage/microglial activation or monocyte differentiation is currently unknown. The aim of this study was to determine the immunomodulatory effects of cladribine upon monocytes, monocyte-derived macrophages (MDMs) and microglia. We analyzed the phenotype and differentiation of monocytes from MS patients receiving their first course of oral cladribine both before and three weeks after the start of treatment. Flow cytometric analysis of monocytes from MS patients undergoing cladribine treatment revealed that the number and composition of CD14/CD16 monocyte subsets remained unchanged after treatment. Furthermore, after differentiation with M-CSF, such MDMs from treated MS patients showed no difference in gene expression of the inflammatory markers compared to baseline. We further investigated the direct effects of cladribine *in vitro* using human adult primary MDMs and microglia. GM-CSF-derived MDMs were more sensitive to cell death than M-CSF-derived MDMs. In addition, MDMs treated with cladribine showed increased expression of costimulatory molecules CD80 and CD40, as well as expression of anti-inflammatory, pro-trophic genes IL10 and MERTK, depending on the differentiation condition. Cladribine treatment *in vitro*

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did not modulate the expression of activation markers in human microglia. Our study shows that cladribine treatment *in vitro* affects the differentiation of monocytes into macrophages by modulating the expression of activation markers, which might occur similarly in tissue after their infiltration in the CNS during MS.

Keywords: multiple sclerosis, cladribine, 2-chlorodeoxyadenosine (2-CdA), innate immunity, neuroinflammation, monocyte, macrophage, microglia

1 INTRODUCTION

Multiple sclerosis (MS) is an immune-mediated demyelinating disease of the central nervous system (CNS) and the leading non-traumatic cause of disability in young adults (1). Current MS therapies can reduce the frequency of relapses mainly by suppressing the immune response (2). Cladribine (2-chlorodeoxyadenosine, 2CdA) is a synthetic purine nucleoside analog that was first designed to treat hematological cancers (3) and is now used to treat several diseases (4), including MS (5). The main effect of oral cladribine administration is lymphocyte depletion (6). However, cladribine treatment leads to drug-free remission in MS patients (7), suggesting the mechanism of action includes potential long-term immune modulation, rather than only cell death.

Cladribine's impact on the immune system is not limited to lymphocyte depletion, as cladribine also affects innate immune cells. Monocyte-derived dendritic cells are sensitive to death induced by cladribine *in vitro* (8). Moreover, cladribine oral administration leads to a slight reduction in the numbers of circulating NK cells and monocytes in the blood (6). Other than inducing immune cell death, cladribine also exerts immunomodulatory effects in dendritic and T cells (9, 10), and also inhibits cytokine response (11) and migration (12) of mononuclear cells.

Since cladribine crosses the blood-brain barrier (BBB) (13–15), it could also have direct effects on central nervous system (CNS)-resident immune cells (16). As the resident macrophages of the CNS parenchyma, microglia are essential regulators of CNS homeostasis and are implicated in MS (17). Microglia sense their microenvironment and respond with a broad range of activation states, which are thought to play diverse roles in MS pathology (18, 19). On the one hand, scavenger microglia can promote remyelination and tissue repair by phagocytosis of debris and secretion of anti-inflammatory and growth factor molecules (20). On the other hand, inflammatory microglia can secrete pro-inflammatory cytokines and generate reactive oxygen and nitrogen species, which can promote neuroinflammation and neuro-axonal damage (21) and induce neurotoxic reactive astrocytes (22).

Other than the tissue-resident microglia, monocyte-derived macrophages (MDMs) that infiltrate the CNS during inflammation can also contribute to disease progression and appear to play different roles than microglia (23). Studies show that the prevention of monocyte infiltration by CCR2 knockout in mice confers protection against experimental autoimmune encephalomyelitis (EAE) (24–26) and that these infiltrating monocytes do not contribute to the pool of resident microglia (27). Monocytes present distinct phenotypic subsets based on their expression of CD14 and CD16 (28). These subsets show distinct potentials of differentiation (29, 30), and have been suggested to play different roles in MS (26, 31, 32). Moreover, non-classical monocytes ($CD14^{+}$ $CD16^{++}$) have been proposed as novel therapeutic targets in MS (33).

The possibility of drug-free remission in MS patients treated with cladribine suggests a potential long-term immune modulation of microglia and monocytes and their derivatives, rather than simply cell death. However, knowledge on whether cladribine treatment can induce immunomodulatory effects in human microglia and monocytes and their macrophage derivatives is still lacking or limited (34). Therefore, the aim of this study was to determine the immunomodulatory effect of cladribine upon microglia and monocytes and their macrophage-derivatives in the context of MS. Thus, our study had two objectives: to assess the *ex vivo* differentiation potential of monocytes and monocyte-derived macrophages (MDMs) from MS patients treated with cladribine; and to assess how the *in vitro* administration of cladribine influenced the viability and activation profile of primary human adult MDMs and microglia. To this end, we isolated and differentiated monocytes from MS patients before and 19-21 days after treatment with cladribine, as a proxy of how cladribine affects the activation and differentiation of monocytes after their infiltration into the CNS. We also isolated and treated primary human adult MDMs and microglia with cladribine *in vitro* to assess the direct effects of cladribine on the activation of tissue macrophages. Our results suggest that cladribine treatment for MS has limited effects on the subsequent differentiation of circulating monocytes into macrophages. Our data also suggest that during administration, cladribine does not directly activate microglia but can directly affect MDMs differentiation by enhancing the expression of anti- or pro-inflammatory markers, depending on the microenvironment in which they differentiate.

2 MATERIAL AND METHODS

2.1 Study Design

We isolated and analyzed *ex vivo* monocytes and MDMs from MS patients under cladribine treatment, which may indicate how these monocytes behave after infiltration in the CNS during MS pathology. We isolated and treated primary human adult MDMs and microglia with cladribine *in vitro*, to directly assess the effects

of cladribine challenge in macrophage differentiation and (microglia) activation. We analyzed the expression of activation markers *via* flow cytometry and RT-qPCR and cell viability *via* flow cytometry and fluorescence microscopy (**Figure 1**).

2.2 Cladribine Treatment of MS Patients

To investigate the effects of cladribine treatment, blood samples of diagnosed relapsing-remitting (RR) MS patients receiving oral cladribine (Mavenclad®) were collected before (baseline) and 19-21 days after (follow up) starting their first course of cladribine treatment. The recommended cumulative dose of cladribine is 3.5 mg/kg body weight over two years, administered as one treatment course of 1.75 mg/kg per year. The standard treatment course consists of two treatment weeks, one at the beginning of the first month, and one at the beginning of the second month of the respective treatment year. Each treatment week consists of five days on which the patient receives 10 mg or 20 mg as a single daily oral dose, depending on body weight (7). At the time of sample collection, the patients had only taken the first week of the first course of their standard cladribine treatment. Relevant clinical data from patients, including the expanded disability status scale (EDSS) (35), is summarized in **Table 1**.

2.3 Cell Isolation, Culturing, and Differentiation

2.3.1 Monocyte Isolation

Briefly, blood samples collected in EDTA tubes (BD, #366643) were diluted 1:1 in FACS buffer (Dulbecco's phosphate-buffered solution without magnesium or calcium ($DPBS^{-/-}$; Gibco, #14190136) containing 2 mM EDTA and 2% heat-inactivated fetal calf serum (HI-FCS; Scientifix life, #FFBS-500)). Peripheral blood mononuclear cells (PBMCs) were obtained by centrifugation [1200 xg, 10 min, room temperature (RT)] using 50 mL SepMate™ tubes (Stemcell technologies, #85460) containing 15 mL Histopaque-1077 (#10771-100, Sigma-Aldrich, St. Louis, MO, USA). PBMCs were then centrifuged (350 xg, 10 min) and washed with FACS buffer. Monocytes were isolated from PBMCs *via* MACS using the QuadroMACS Starting Kit (#130-091-051, Miltenyi Biotec, Macquarie Park, NSW, Australia), which includes CD14 MicroBeads (Miltenyi Biotec, #130-050-201), LS columns (Miltenyi Biotec, #130-042-401) and QuadroMACS™ Separator (Miltenyi Biotec, #130-090-976), according to the manufacturer's protocol. The purity of MACS-isolated monocytes was determined by the expression of CD14.

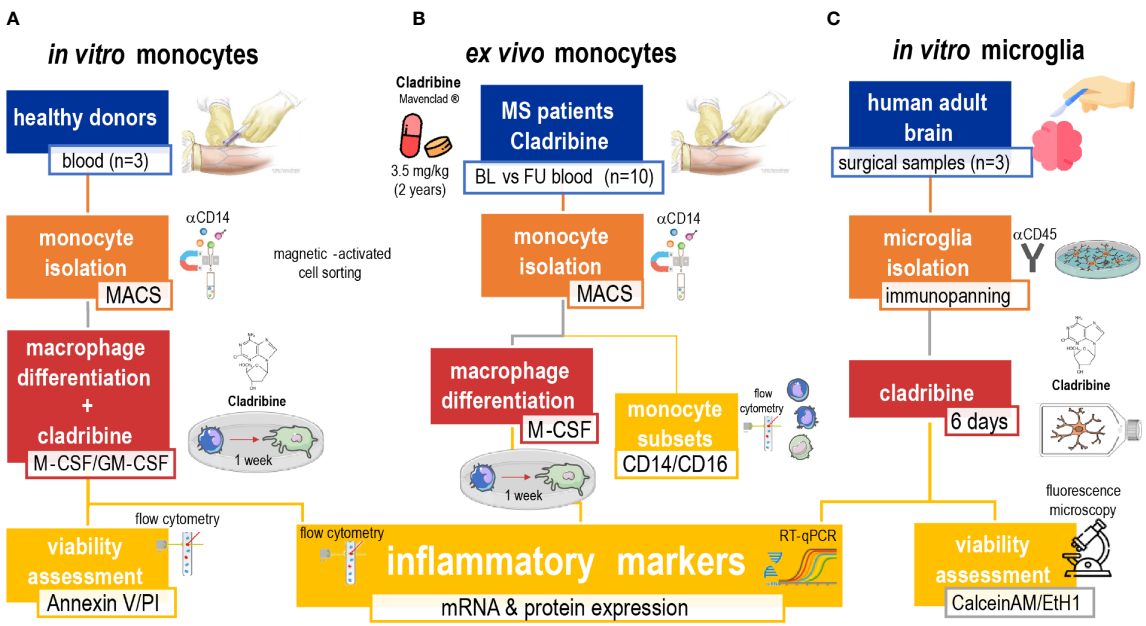

FIGURE 1 | Schematic of the experimental approach. The study was composed of three arms aimed to investigate the effects of cladribine in monocytes, monocyte-derived macrophages (MDMs) and microglia. The first *in vitro* **(A)** approach comprised the isolation of monocytes from healthy donors' PBMCs by MACS, and M-CSF and/or GM-CSF-induced MDM differentiation. MDMs in differentiation were treated with cladribine for 6 days (total culture period of 7 days). Their viability was analyzed with the annexin V/PI assay by flow cytometry and their activation profile was analyzed by flow cytometry and RT-qPCR. The *ex vivo* **(B)** approach comprised monocytes isolated *via* magnetic-activated cell sorting (MACS) from PBMCs of MS patients undergoing cladribine treatment. These monocytes were analyzed *via* flow cytometry and used to generate M-CSF-induced MDMs *in vitro*, whose activation profile was further analyzed by flow cytometry and RT-qPCR. The second *in vitro* **(C)** approach comprised the isolation of microglia from adult surgical brain samples from patients with temporal lobe epilepsy by immunopanning. Microglia were then treated with cladribine for 6 days. Their viability was assessed by fluorescence microscopy with calcein AM and ethidium homodimer-1 staining, and their activation profile was analyzed by flow cytometry and RT-qPCR. Blue: origin of cells. Orange: isolation technique. Red: *in vitro* culturing and treatment. Yellow: analysis. MS, multiple sclerosis; MACS, magnetic-activated cells sorting; M-CSF, macrophage colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; RT-qPCR, quantitative reverse transcription-polymerase chain reaction; PI, propidium iodide.

TABLE 1 | Summary of MS patients' clinical data.

Patient	MS type	Age	Gender	Disease duration	EDSS	Medication during study	Previous immunomodulatory treatments (in years)
#1	RR	38	F	17	0	N/A	β-interferon (12)
#2	RR	50	F	9	0	Coversyl	Fingolimod (7)
#3	RR	54	F	17	4	Oxybutynin	Copaxone (1) and Tysabri (7)
#4	RR	35	F	1	2	N/A	Nil
#5	RR	50	F	13	1.5	N/A	Interferon (3) and Fingolimod (8)
#6	RR	57	M	22	1	N/A	β-interferon (17) and Dimethyl fumarate (4)
#7	RR	47	F	13	1	N/A	β-interferon (12)
#8	RR	45	M	11	2	N/A	β-interferon (11)
#9	RR	46	M	23	3	N/A	β-interferon (3)
#10	RR	50	M	1	2	N/A	Nil

EDSS, expanded disability status scale [ref (35)], RR, relapsing-remitting; N/A, not applicable.

2.3.2 Differentiation of Monocyte-Derived Macrophages (MDMs)

To generate MDMs, MACS-isolated monocytes were seeded in 24-well plates at $5x10^5$ cells/well and cultured (at 37°C, 5% CO_2) for 7 days in RPMI 1640 without L-glutamine (Gibco, #11875085), supplemented with 10% HI-FCS (Scientifix life, FFBS-500), 100 units(µg)/mL penicillin/streptomycin (Gibco, #15140122), MEM-NEAA (Gibco, #11140050), Glutamax (Gibco, #35050061), 100 ng/mL macrophage colony-stimulating factor (M-CSF) (Peprotech, #300-25), and/or 50 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech, #300-03). GM-CSF was used only for experiments with monocytes isolated from healthy donors. Media were not changed for the duration of the experiments.

2.3.3 Microglia Isolation *via* Immunopanning

Primary human adult microglia were isolated for *in vitro* experiments from surgical brain tissue resected for epilepsy treatment. The epileptic focus was not used for the microglia isolation and was separated at the time of resection by the neurosurgeon.

Microglia were isolated *via* immunopanning after tissue enzymatic digestion (as previously described (36, 37), with adaptations). Specifically, 500 mg of brain tissue was placed in a 6-well plate well with 500 µL $DPBS^{-/-}$ (Gibco, #14190136), and minced to ~1 mm^3 pieces with a scalpel blade. In each well, 10 mL of 95% O_2 5% CO_2-equilibrated papain solution was added. The papain solution consisted of 1x Earle's balanced salt solution [EBSS; made in house 10x concentrated: 1.16 M NaCl, 54 mM KCl, 10 mM $NaH_2PO_4•H_2O$, 1% D(+)-glucose and 0.005% phenol red (Sigma-Aldrich-Aldrich P0290)] containing 30% D (+)-glucose (Sigma-Aldrich, #G7021), 1M $NaHCO_3$ (Sigma-Aldrich, #S-5761), 50 mM EDTA, 0.2 mg/mL L-cysteine (Sigma-Aldrich, #C7880), 20 U/mL papain (Worthington, #LS03126), and 125 U/mL DNase I (Worthington, #LS002007). A hole was drilled into the plate lid and connected to a CO_2 gas supply to maintain pH equilibrium. The plate was placed on a 34°C heat block and incubated for 100 minutes with manual shakes every 15 minutes. Digested brains were transferred to 15 mL tubes and, after the tissue had settled, the supernatant was aspirated and replaced by 4.5 mL of low-ovomucoid (low-ovo) solution (1x EBSS containing 30% D(+)-glucose, 1M $NaHCO_3$, 1.5 mg/mL BSA (Sigma-Aldrich, #A8806), 1.5 mg/mL trypsin inhibitor (Worthington, LS003086) and 62.5 U/mL DNase I). The tissue was allowed to settle again, and the supernatant was replaced by more 4.5 mL low-ovo solution. This step was done four times in total. Four mL of low-ovo solution was added and the tissue chunks were triturated 40 times using a 5 mL serological pipette. The tissue was allowed to settle, and the supernatant removed with a 1 mL pipette. This trituration step was repeated until almost all tissue had been dissociated (3-5 times in total). Dissociated cells were layered on top of 10 ml of high-ovomucoid (high-ovo) solution (1x EBSS containing 30% D (+)-glucose, 1M $NaHCO_3$, 5 mg/mL BSA, 5 mg/mL trypsin inhibitor and 25 U/mL DNase I) and centrifuged at 220 xg for 15 min. The cell pellet was then resuspended in 9 ml of $DPBS^{+/+}$ (Gibco, 14040133) containing 0.02% BSA and applied directly to a positive-selection immunopanning dish, previously coated with rat anti-mouse CD45 monoclonal antibodies (30-F11 clone, BD Pharmingen #550539).

To coat the immunopanning dish, a 10 cm sterile petri dish was incubated overnight at 4°C with 10 mL of 50 mM Tris-HCl (pH 9.5) containing 30 µL goat anti-rat IgG (#112-005-167, Jackson ImmunoResearch, Baltimore, PA, USA). The dish was rinsed three times with $DPBS^{+/+}$ (Gibco, #14040133) and incubated with 12 mL of 0.2% BSA containing 20 µL of rat anti-mouse CD45 monoclonal antibodies (clone 30-F11, BD Pharmingen #550539) for 3-5 h at room temperature. The coated dish was rinsed three times with $DPBS^{+/+}$ immediately before incubation with cell suspension.

Cell suspensions (from up to 500 mg of tissue digestion per dish) were allowed to interact with immunopanning dishes coated with rat anti-mouse CD45 monoclonal antibodies (clone 30-F11, BD Pharmingen #550539) for 30 min at room temperature, with gentle shakes every 10 min. Dishes were washed with $DPBS^{+/+}$ five consecutive times to remove unbound cells and debris. Bound cells were trypsinized (10 min at 37°C) with 12 mL $DPBS^{-/-}$ (Gibco, #14190136) containing 500 units/mL trypsin (Sigma-Aldrich, #T9935) [made up in $EBSS^{-/-/-}$ (Sigma-Aldrich, #E7510)]. Because microglia were still strongly adherent, the trypsin solution was discarded, the panning dish was rinsed twice with $DPBS^{-/-}$ and incubated for 1 min on ice with cold microglia culture medium (Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM/F12; Gibco, #11330032) supplemented with 10% heat-inactivated FCS (Scientifix life, FFBS-500), 100 units(µg)/mL

penicillin/streptomycin (Gibco, #15140122) and 1x minimum essential medium non-essential amino acids (MEM-NEAAs; Gibco, #11140050)), to weaken cell interaction with the dish surface. The microglia were recovered by repeated pipetting and were centrifuged (400g, 10 minutes at RT). Pelleted microglia were resuspended in 0.5 mL of microglia medium supplemented with 40 ng/mL of the CSF1R ligand IL-34 (R&D SYSTEMS, #5265-IL-010), and counted using a hemocytometer. Trypan blue staining (Gibco, #15250061) was used for discrimination of dead cells.

2.3.4 Microglia Culture

For the *in vitro* experiments, immunopanning-isolated microglia were seeded in 96-well plates at $5x10^4$ cells/well, and cultured (at 37°C, 5% CO_2) for 7 days in DMEM/F12 supplemented with 10% HI-FCS, 100 units(μg)/mL penicillin/streptomycin and 1x MEM-NEAAs in addition to 40 ng/mL IL-34 (R&D SYSTEMS, #5265-IL-010). No media changes were performed for the duration of the experiments. The culture was supplemented with 40 ng/mL IL-34 every second day (days 2, 4 and 6).

2.4 *In Vitro* Cladribine Treatment

To assess the effects of cladribine *in vitro*, monocytes isolated on day 1 of differentiation into MDMs, and isolated microglia on day 1 of culture, were treated with cladribine at either 0.01 μM, 0.05 μM or 0.25 μM for 6 days (cultured for 7 days in total). Cladribine active pharmaceutical ingredient (API) (kindly provided by Merck KGaA, Darmstadt, Germany) was reconstituted in DMSO (Sigma-Aldrich, #D2650100) at a concentration of 10 mM, and serially diluted to working concentrations (10 μM or 1 μM) in culture media. The same volumes of DMSO as the highest concentration of cladribine (either 0.05 μM or 0.25 μM) were serially diluted in culture media and added to cells as the vehicle control for the respective experiment.

2.5 Flow Cytometry

To determine the purity and immunophenotype of monocytes, the expression of CD14 and CD16 was assessed in PBMCs and MACS-isolated monocytes by flow cytometry. Briefly, cells were resuspended in FACS buffer ($DPBS^{-/-}$ (Gibco, #14190136) containing 2 mM EDTA and 2% HI-FCS and were stained for 15 min at RT with the PE-conjugated monoclonal antibody mouse anti-human CD14 (Miltenyi,130-091-242), and the FITC-conjugated mouse monoclonal anti-human CD16 antibody (Miltenyi, 130-091-244) at a 1:500 and 1:50 final dilution, respectively. For the isotype controls, cells were stained with mouse PE-conjugated IgG2a isotype control (clone S43.10, Miltenyi, 130-113-834) or mouse FITC-conjugated IgM isotype control (clone IS5-20C4, Miltenyi, 130-113-834) at 1:500 and 1:50 final dilution, respectively. Cells were washed once with FACS buffer, centrifuged (350 xg, 5 min) and the pellet was resuspended in FACS buffer for acquisition in a flow cytometer analyzer.

The expression of surface activation markers was determined by flow cytometry in monocyte-derived macrophages at day 7 of culture. Briefly, the MDMs were detached by incubation with pre-warmed (37°C) $DPBS^{-/-}$ containing 5 mM EDTA for 20 min at 37°C, and were recovered by repeated pipetting, pooled with respective supernatant, and centrifuged (350 xg, 5 min). The pellet was resuspended in FACS buffer, and cells were blocked with fragment crystallizable region (Fc) receptor (FcR) human blocking reagent (Miltenyi, #130-059-901) at a 1:5 final dilution, for 10 min at 4-8°C. Blocked cells were stained for 15 min at RT with anti-human CD80 PE-conjugated antibody (clone REA661; Miltenyi, #130-110-270), anti-human CD163 VioBlue-conjugated antibody (clone REA812; Miltenyi #130-112-134) and anti-human Mer APC-conjugated antibody (R&D SYSTEMS, #FAB8912A) at a final dilution of 1:100, 1:50 and 1:11, respectively. Cells were washed once with FACS buffer, centrifuged (350 xg, 5 min), and the pellet resuspended in FACS buffer for acquisition with a flow cytometer analyzer. Dead cells were identified by propidium iodide (PI; Sigma-Aldrich, #P4864-10) (immediately before acquisition, 1 μL of PI solution was added per mL of sample).

To assess microglia enrichment after isolation, the expression of surface markers in immunopanning-isolated microglia was analyzed by flow cytometry. Primary human microglia were immediately detached from immunopanning dishes *via* trypsinization and washed once with FACS buffer. The cells were then centrifuged (350 xg, 5 min) and resuspended in FACS buffer. Cells were blocked for 10 min at 4-8°C with Human TruStain FcX™ (Biolegend, #422302) Fc receptor blocking solution, at a final dilution of 1:20. Cells were subsequently stained with PE-conjugated anti-human CD11b antibody (clone ICFR44, Biolegend, #301306), APC/Cy7-conjugated anti-human CD45 antibody (clone HI30, Biolegend, #304014), APC-conjugated anti-human CD64 antibody (clone 10.1, Biolegend, #305014), and Brilliant Violet™ 421-conjugated anti-human CX3CR1 antibody (clone 2A9-1, Biolegend, #341620) for 15-30 min at 4-8°C, with each antibody at a final dilution of 1:50.

UltraComp eBeads™ (#01-2222-42, Invitrogen, Carlsbad, CA, USA) stained with the conjugated antibodies were used for compensation. All samples were acquired with CytoFLEX S flow cytometer analyzer (Beckman Coulter, Brea, California, United States) and data were processed using FlowJo (version 10.6.1, Becton Dickinson, Ashland, OR).

2.5.1 Annexin V/PI Viability Assay

To assess cell viability *via* flow cytometry, we performed the annexin V/PI assay. Monocyte-derived macrophages at day 7 of culture (day 6 post cladribine treatment) were detached from wells by incubation with $DPBS^{-/-}$ containing 5 mM EDTA and 0.25% trypsin (Gibco, #15090-046) for 5-10 min at 37°C. Trypsin was neutralized by adding culture media (supplemented with FCS) at 4 times the volume of trypsin solution. Cells were harvested by repeated pipetting, combined with supernatant, and centrifuged (350 xg, 5 min, 4°C). The pellet was resuspended in 1x binding buffer, and annexin V and PI staining was performed with the Annexin V-FITC Kit (Miltenyi, 130-092-052), according to the manufacturer's protocol. All samples were acquired with a CytoFLEX S flow cytometer analyzer (Beckman Coulter), and data were processed using FlowJo V10 software (Treestar).

2.6 Immunocytochemistry

Surface marker expression was visualized *via* immunostaining using fluorescence microscopy. Primary human microglia seeded on coverslips in 24-well plates at day 1 of culture were washed once with $DPBS^{+/+}$ and fixed in 4% paraformaldehyde (PFA) for 5 min at RT. Wells were washed three times with MT-PBS (16.3 mM $Na_2HPO{\bullet}H_2O$, 62.7 mM $NaH_2PO_4{\bullet}H_2O$, 148 mM NaCl (Chem-Supply, Brisbane, Queensland, Australia) in MilliQ H_2O) for 5 min at RT on platform shaker. Next, cells were blocked and permeabilized with blocking buffer [MT-PBS containing 0.3% Triton X-100 and 10% normal goat serum (NGS; Merck Millipore, #S26-100)] for 1 h at RT. Cells were incubated overnight at 4°C with blocking buffer or anti-Iba1 primary antibody (Wako, #019-19741, RRID: AB_839504) diluted 1:1000 in blocking buffer. Coverslips were washed three times with MT-PBS for 5 min on a platform shaker, and incubated with FITC-conjugated goat anti-rabbit secondary antibody (Jackson Immuno, #111-005-144) (1:200 dilution) and Hoechst 33342 nuclear dye (Invitrogen, H3570) (1:1000 dilution) for 1 h at RT on platform shaker in the dark. After staining, coverslips were washed three times with MT-PBS for 5 min on a platform shaker and mounted on microscopy slides using Dako fluorescence mounting medium (Dako, #S3023). Microscopy pictures were acquired with an Axio Imager.M2 (Zeiss) fluorescence microscope, using ApoTome.2 optical sectioning (Zeiss) and an Axiocam 506 mono camera (Zeiss) with ZEN software (Zeiss). Images were processed and analyzed with Fiji software (38).

2.7 Calcein AM/Ethidium Homodimer-1 Viability Assay

The viability of primary human microglia at day 7 of culture (day 6 post cladribine treatment) was determined by fluorescence microscopy using the LIVE/DEAD™ Viability/Cytotoxicity Kit (Invitrogen, L3224), according to the manufacturer's protocol. Briefly, wells containing cells were incubated with 1 mM calcein-AM (Invitrogen, #C3099) and 2 mM ethidium homodimer-1 (EthD-1; Invitrogen, L3224) diluted in $DPBS^{+/+}$ for 30 min at RT in the dark. Microscopy pictures were acquired with an IX81 inverted fluorescence microscope (Olympus) and processed and analyzed with Fiji software.

2.8 Gene Expression Analysis (RT-qPCR)

2.8.1 RNA Extraction

Total RNA was extracted from patient MDMs at day 7 of culture (day 6 post cladribine treatment) using the RNeasy Mini Kit (Qiagen, #74104) with on-column DNase digestion (Qiagen, #79254), as per manufacturer's instructions. Total RNA was extracted from primary human microglia and MDMs from healthy donors at day 7 of culture (day 6 post cladribine treatment) using the RNeasy Plus Micro Kit (Qiagen, #74034). The concentration and purity of the RNA were determined using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific), as per manufacturer's instructions.

2.8.2 cDNA Synthesis

Total RNA was reverse transcribed to complementary DNA (cDNA) using the TaqMan Reverse Transcription kit (Applied Biosystems, #N8080234), as per manufacturer's protocol. Reactions of 40 µL were prepared using RNA template (0.5 µg for patients' MDMs), (0.25 µg for healthy donors' MDMs), or (0.225 µg for primary human microglia). Random hexamers (at 2.5 µM) were used as primers for the cDNA synthesis. The reaction was incubated in a Peltier thermal cycler (DNA Engine Tetrad 2 Thermal Cycler, Bio-Rad Laboratories) at the following conditions: 25°C for 10 min, 37°C for 30 min, 95°C for 5 min.

2.8.3 Relative Quantification by qPCR

cDNA was amplified using SYBR green PCR master mix (Applied Biosystems, #4309155), as per manufacturer's protocol. Reactions of 10 µL were prepared using either 12.5 ng (for patients' samples), or 6.25 ng (for healthy donors' and microglia samples) of previously generated cDNA (equivalent RNA quantity), and gene-specific primers (Integrated DNA Technologies, see **Table 2**) at final concentrations of 0.8 µM each. Samples were run in triplicates or duplicates, based on sample availability. qPCR was performed using the ViiA 7 Real-Time PCR System (Applied Biosystems) at the following incubation conditions: hold stage 50°C for 2 min, 95°C for 10 min; PCR stage 95°C for 15 sec, 60°C for 1 min, repeat PCR stage for an additional 39 cycles; melt curve stage 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec.

Relative gene expression was determined by the comparative 'delta CT' (ΔCT) analysis (39), where an internal control (*18S* rRNA as reference house-keeping gene) was run for each sample for normalization of target gene expression. For data analysis, the 'delta delta CT' (ΔΔCT) analysis, i.e. $\log_2$ fold change (40), was performed using a reference control (patients' baselines or vehicle).

2.9 Statistical Analyses

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc.). The statistical tests used for each experiment are detailed in their respective figure. For patient experiments, paired t-tests were performed with *post-hoc*

TABLE 2 | List of primer pairs used for qPCR reactions.

Gene	NCBI accession number	Forward primer (5'➔3')	Reverse primer (5'➔3')
18S	NR_003286.4	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT
IL1B	NM_000576.2	TACTCACTTAAAGCCCGCCT	ATGTGGGAGCGAATGACAGA
TNF	NM_000594.3	AGGACGAACATCCAACCTTC	GTGTCTGAAGGAGGGGGTAA
IL10	NM_000572.3	TTAAGGGTTACCTGGGTTGC	TGTCTGGGTCTTGGTTCTCA
MERTK	NM_006343.2	ACATCGACCCTGACTCTATAATTGC	TGAACTTCTGCTGTGACCACACT
CD40	NG_007279.1	CAGACACCATCTGCACCTGT	AATTGATCTCCTGGGGTTCC

Bonferroni correction for multiple testing where indicated (p^{adj}). For *in vitro* experiments with healthy donor's monocyte-derived macrophages, ordinary two-way ANOVA with either Tukey's or Sidak's *post-hoc* test was performed. "n" refers to biological replicate, i.e., each n represents data from a different individual. Only relevant p-values are depicted in figures.

2.10 Ethical Statement

Brain tissue and blood samples were obtained with informed consent under the protocol HREC Project 2018.197 (HREC/18/MH/259) Assessment of whether cladribine reconfigures mononuclear cells – approved by the Melbourne Health Human Research Ethics Committee (HREC). The HREC is constituted and operated in accordance with the National Statement on Ethical Conduct in Human Research 2007 (developed jointly by the National Health and Medical Research Council, the Australian Research Council and Universities Australia).

3 RESULTS

3.1 Effect of Cladribine Treatment in MS Patients

3.1.1 Cladribine Treatment *In Vivo* Does Not Affect Monocyte Numbers or Subsets

To determine the influence of Clabribine administered to patients with RRMS on monocytic populations and their derivatives, monocytes were isolated before and after the therapeutic intervention (0.875 mg/kg *per os* (P.O.) distributed over 5 consecutive days) and then studied *ex vivo*. Human monocytes subsets are categorized according to their surface expression of CD14 and CD16, as classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and non-classical ($CD14^{+}CD16^{+}$). These subpopulations reflect distinct functional roles and differentiation potential (26, 27). To assess the potential influence of cladribine on monocytes and macrophages, monocytes were isolated by magnetic-activated cell sorting (MACS) (**Supplementary Figure 1**) from peripheral blood mononuclear cells (PBMCs) of MS patients before and 19-21 days after the start of cladribine treatment. We analyzed the subsets of these monocytes based on their surface expression of CD14 and CD16 (**Supplementary Figure 2**). Cladribine treatment significantly reduced the concentration of PBMCs in the patients post-cladribine by $1.06x10^6$ [95% confidence interval (C.I.) $0.48x10^6$-$1.64x10^6$] cells per ml, representing a 1.72-fold (95% C.I. 1.30-fold to 2.27-fold) reduction (**Figure 2A**). However, cladribine did not significantly alter the concentrations of monocytes (**Figure 2B**) or the proportion between the CD14/CD16 monocytic subsets (**Figure 2C**).

3.1.2 Monocytes Isolated From MS Patients Previously Treated With Cladribine Do Not Exhibit Significantly Altered Differentiative Capacity

Although cladribine treatment did not significantly alter the monocytic population, it could still have influenced their differentiative potential and, therefore, the phenotype of MDMs in the target tissue. To assess this possibility, we differentiated monocytes purified from both the pre and post-cladribine samples *ex vivo* and compared the phenotype of these derivatives. Differentiation was effected using M-CSF, followed by analysis of the surface expression of CD80 as a pro-inflammatory macrophage marker and CD163 together with MERTK as anti-inflammatory markers, quantitated by flow cytometry (**Supplementary Figure 3**). No significant differences in the expression profile of any of these markers were identified amongst the pre and post-cladribine samples (paired t-test, P>0.05) (**Figure 2D**).

To further investigate MDM activation, we analyzed mRNA expression of genes related to pro-inflammatory or anti-inflammatory macrophage activation. We used the gene expression of the pro-inflammatory cytokines *TNF, IL1B*, and the costimulatory molecule *CD40* as markers of pro-inflammatory activation, while the expression of the anti-inflammatory cytokine *IL10*, and the efferocytosis-related receptor *MERTK* were used as markers of anti-inflammatory activation. TNF expression was downregulated 1.5 fold in MDMs derived from MS patients treated with cladribine compared with baseline, although this did not reach significance following correction for multiple testing (p=0.0138, p adj. = 0.064) (**Figure 2E**), while the expression of the other genes remained unchanged (**Figures 2F–I**). These results show that five days of cladribine treatment (+15 days without cladribine, in total 19-21 days) for MS does not modulate the differentiation of monocytes into macrophages with M-CSF.

3.2 Cladribine Treatment *In Vitro* Affects the Differentiation of Primary Human Monocyte-Derived Macrophages

Cladribine treatment could also exert a direct effect on monocyte differentiation. Therefore, we investigated the effects of cladribine treatment on monocytes *in vitro*, using primary monocytes isolated from healthy donors. We used M-CSF and/or granulocyte-macrophage colony stimulation factor (GM-CSF, also known as colony-stimulating factor 2; CSF2) to generate anti-inflammatory and pro-inflammatory monocyte-derived macrophages, respectively. The M-CSF-differentiated MDMs, but not GM-CSF-differentiated MDMs, developed a spindle-shaped morphology (**Figure 3A**, **Supplementary Figure 4A**), higher surface expression of MERTK and CD163 (**Supplementary Figure 4B**), increased gene expression of *TNF, MERTK*, *IL10*, and reduced gene expression of the costimulatory molecule *CD40* (**Supplementary Figure 4C**). MDMs differentiated with both M-CSF and GM-CSF presented a round and ovoid-shaped morphology (**Figure 3A** and **Supplementary Figure 4A**), and expression profiles similar to GM-CSF differentiated macrophages, as previously described (41), showing reduced expression of MERTK, CD163, and *IL10*, and increased expression of *CD40* (**Supplementary Figures 4B, C**).

To determine the optimal concentration of cladribine for MDM treatment during differentiation *in vitro*, we assessed the influence of two different cladribine concentrations [0.05 μM and 0.25 μM, based on previous studies (8–12) and

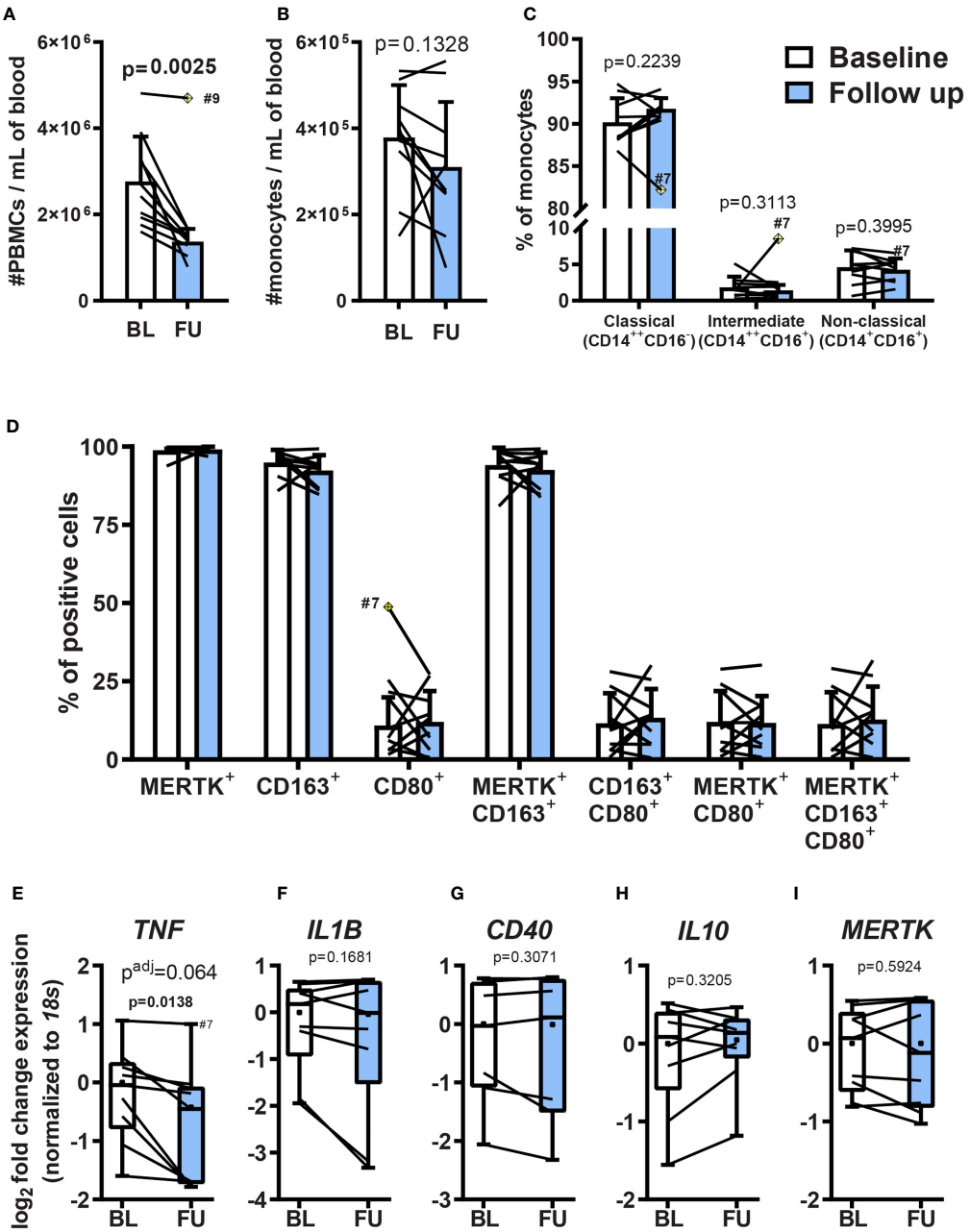

FIGURE 2 | Effects of oral cladribine treatment on circulating PBMCs from MS patients and monocyte *ex vivo* differentiation. Monocytes isolated from MS patients' PBMCs before (white) and 19-21 days after (blue) the beginning of cladribine treatment were differentiated to MDMs for 7 days with M-CSF. **(A)** The density of peripheral blood mononuclear cells (PBMC) in blood collected from MS patients before (BL; baseline) and 19-21 days after (FU; follow up) the beginning of cladribine treatment. **(B)** The density of MACS-isolated monocytes from freshly isolated PBMCs of MS patients before (BL; baseline) and 19-21 days after (FU; follow up) the beginning of cladribine treatment. **(C)** Monocyte subsets from cladribine-treated MS patients before (white) and 19-21 days after (blue) the beginning of cladribine treatment were determined by CD14 and CD16 surface expression, as analyzed by flow cytometry. **(D)** MDMs of cladribine-treated MS patients were differentiated for 7 days with M-CSF and the expression of pro-inflammatory (CD80) and anti-inflammatory (CD163 and MERTK) activation markers were analyzed by flow cytometry. **(E–I)** Gene expression of activation markers in monocyte-derived macrophages (MDM) from cladribine-treated MS patients. RT-qPCR gene expression analysis of pro-inflammatory **(E–G)** and anti-inflammatory **(H, I)** activation markers. Positive cells were determined according to **(C)** isotype controls and **(D)** FMO controls. **(A–D)** Data depicted as mean (with SD) and lines indicate before-after cladribine treatment, **(A, C)** n=9, **(B–I)** n=10. The yellow crossed diamond shows the statistically significant outlier (ESD, extreme studentized deviate method), which was excluded from the analysis. **(A–D)** Statistical significance was calculated with paired t-test. **(E–I)** Data depicted as median (with quartiles, dot indicates mean), lines indicate before-after cladribine treatment, n=9 (patient #9 was not included due to technical problems). **(E)** n=8, the outlier value #7 follow up (ESD method) was not included in the analysis. **(E–I)** p-values between baseline and follow up were determined by paired t-test and the adjusted p-value (p^{adj}) was calculated using the posthoc Holm-Sidak correction for multiple testing.

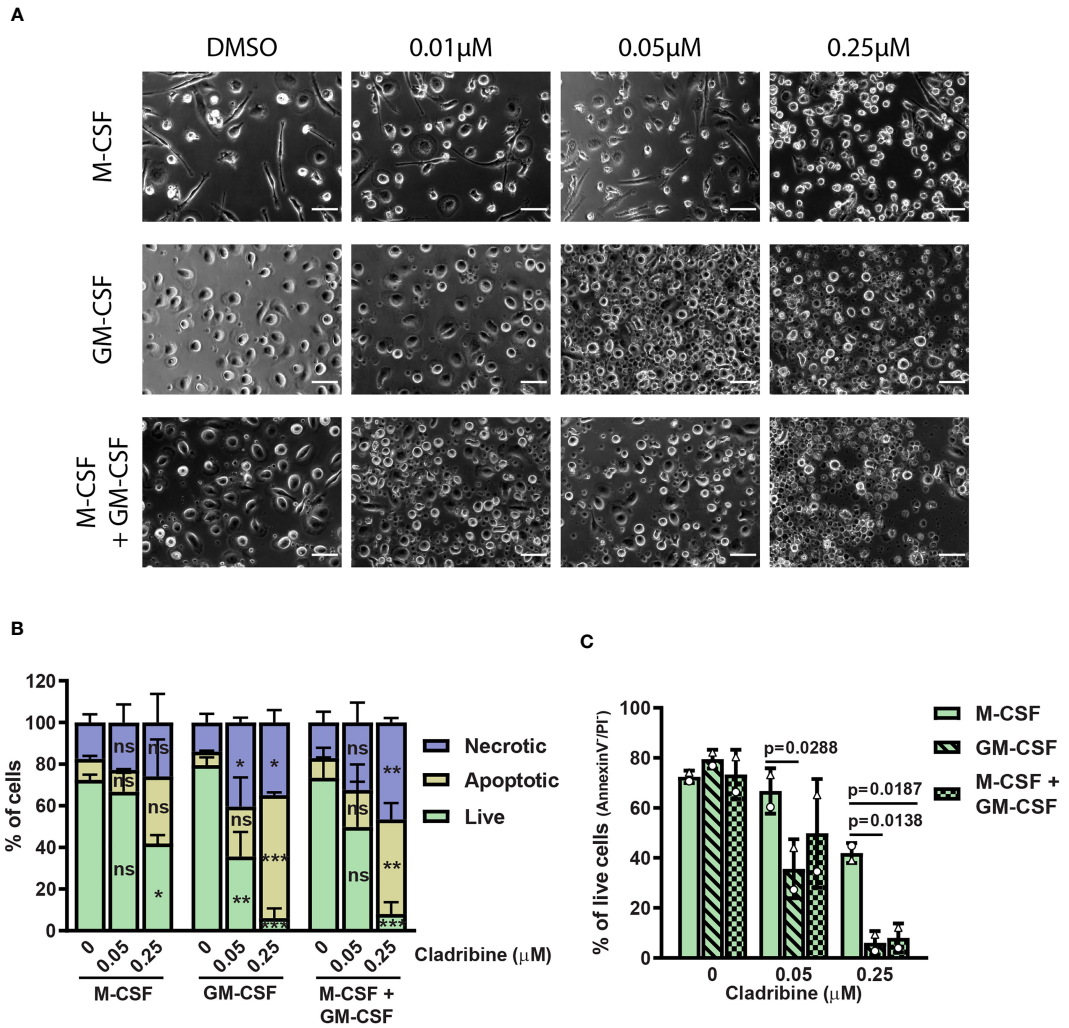

FIGURE 3 | Morphology and viability of monocyte-derived macrophages (MDMs) treated with cladribine *in vitro*. Monocytes isolated from healthy donors were differentiated to MDMs with M-CSF and/or GM-CSF in the presence of cladribine. **(A)** Light microscopy images (phase contrast) of MDMs differentiated with M-CSF and/or GM-CSF with DMSO (vehicle), 0.01μM, 0.05μM or 0.25μM cladribine. Scale bar: 50 μm. **(B)** MDMs viability at day 6 of cladribine treatment *in vitro* was determined by Annexin V/PI staining and analyzed by flow cytometry. Necrotic cells are PI-positive, while apoptotic cells are Annexin V-positive only and live cells **(C)** are negative for both Annexin V and PI. Data depicted as mean (with SD) and symbols in **(C)** (triangle and circle) indicate matched experiments from the same healthy donor's sample. n=2, **(B)** ns: $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared to vehicle (DMSO, 0 μM) from the same differentiation condition (CSF group). **(C)** p-values lower than 0.05 between M-CSF and other two differentiation conditions are shown. P-values were determined by ordinary two-way ANOVA with Tukey's posthoc test.

physiologically-relevant concentrations (13–15)] on cell viability 6 days post-treatment (**Supplementary Figure 5** and **Figures 3B, C**). Cladribine reduced the cell viability in all MDMs, independent of their cytokine exposure, at the concentration of 0.25 μM, while only GM-CSF exposed MDMs had reduced cell viability at the concentration of 0.05 μM (mean difference of 43.90% of live cells, 95% C.I. 16.26% to 71.54%) (**Figures 3B, C**). MDMs differentiated with only M-CSF had a significantly higher proportion of live cells upon 0.05 μM cladribine treatment compared to MDMs differentiated with only GM-CSF (mean difference of 31.15% of live cells, 95% C.I. 3.512% to 58.79%) (**Figures 3B, C**). Upon 0.25 μM cladribine treatment, MDMs differentiated with only M-CSF had a significantly higher proportion of live cells compared to both MDMs differentiated with GM-CSF alone or in combination with M-CSF (respectively, a difference of 35.89% of live cells, 95% C.I. 8.252% to 63.53% and a difference of 33.92% of live cells, 95% C.I. 6.282% to 61.56%) (**Figures 3B, C**).

3.2.1 Cladribine Treatment *In Vitro* Increased the Expression of CD80, CD40, *IL10*, and *MERTK* in Monocyte-Derived Macrophages

To assess the direct effect of cladribine treatment on differentiation and activation of MDMs *in vitro*, we treated monocytes

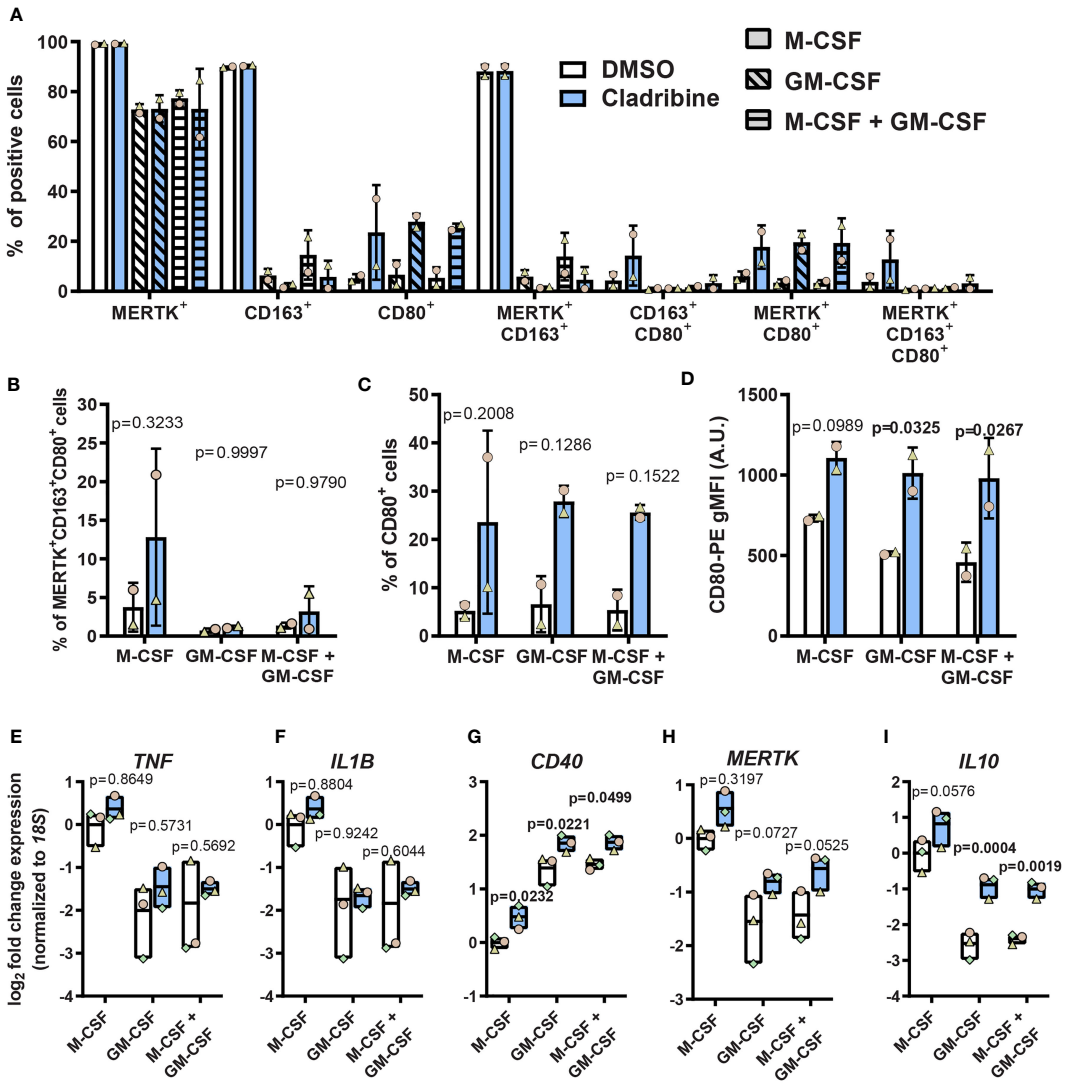

FIGURE 4 | Expression of activation markers in monocyte-derived macrophages (MDMs) treated with cladribine *in vitro*. Monocytes isolated from healthy donors were differentiated to MDMs with M-CSF and/or GM-CSF in the presence of cladribine. **(A–C)** Percentage of MDMs expressing pro-inflammatory (CD80) and anti-inflammatory (CD163 and MERTK) activation markers at day 6 of cladribine treatment *in vitro* as analyzed by flow cytometry. Positive cells were determined according to their fluorescent minus one (FMO) control. **(D)** Levels of CD80 surface protein expression on MDMs treated with cladribine during differentiation *in vitro*, as analyzed by flow cytometry. **(E–I)** RT-qPCR gene expression analysis of **(E–G)** pro-inflammatory and **(H, I)** anti-inflammatory activation markers at day 6 post cladribine treatment *in vitro*. **(A–C)** Positive cells were determined according to FMO controls. **(A–D)** Data depicted as mean (with SD) and symbols indicate matched experiments from the same healthy donor's sample, n=2. p-values between cladribine 0.05 μM and vehicle (DMSO) were determined by ordinary two-way ANOVA with Sidak's posthoc test. **(E–I)** Data depicted as mean (with max-min) and symbols indicate matched experiments from the same healthy donor's sample, n=3. p-values between cladribine 0.05 μM and vehicle (DMSO) were determined by ordinary two-way ANOVA with Sidak's posthoc test.

differentiating into MDMs with 0.05 μM cladribine and analyzed the protein expression (**Figures 4A–D**) and gene expression (**Figures 4E–I**) of surface activation markers and cytokines 6 days post-treatment. Cladribine treatment in MDMs differentiated with GM-CSF alone or in combination with M-CSF significantly increased the surface expression of the costimulatory molecule CD80 (**Figure 4D**). Moreover, it significantly induced the expression of the gene encoding the costimulatory molecule *CD40*, regardless of the differentiation factor (fold-change≥1.35 relative to the respective vehicle, $p \leq 0.0499$) (**Figure 4G**). On the other hand, cladribine treatment in MDMs differentiated with GM-CSF alone or in combination with M-CSF resulted in significantly increased expression of the anti-inflammatory cytokine *IL10* (fold-change ≥ 2.60 relative to the respective vehicle, $p \leq 0.0019$) (**Figure 4I**). There were no significant differences in expression of the other analyzed markers (**Figures 4A, E, F, H**).

In summary, cladribine induced the expression of costimulatory molecules CD80 and *CD40* and the anti-

inflammatory cytokine *IL10* in the presence of GM-CSF while inducing only *CD40* expression in M-CSF-differentiated MDMs. These data suggest that cladribine affects macrophage differentiation, depending on the differentiation-inducing factor and, therefore, possibly depending on the microenvironment in which these macrophages are generated.

3.3 Effect of Cladribine on Primary Human Microglia *In Vitro*

Given the aforementioned results suggested a potential role of cladribine in modulating the activation of monocyte-derived macrophages, we argued that cladribine might likely also directly affect the activation of tissue-resident innate immune

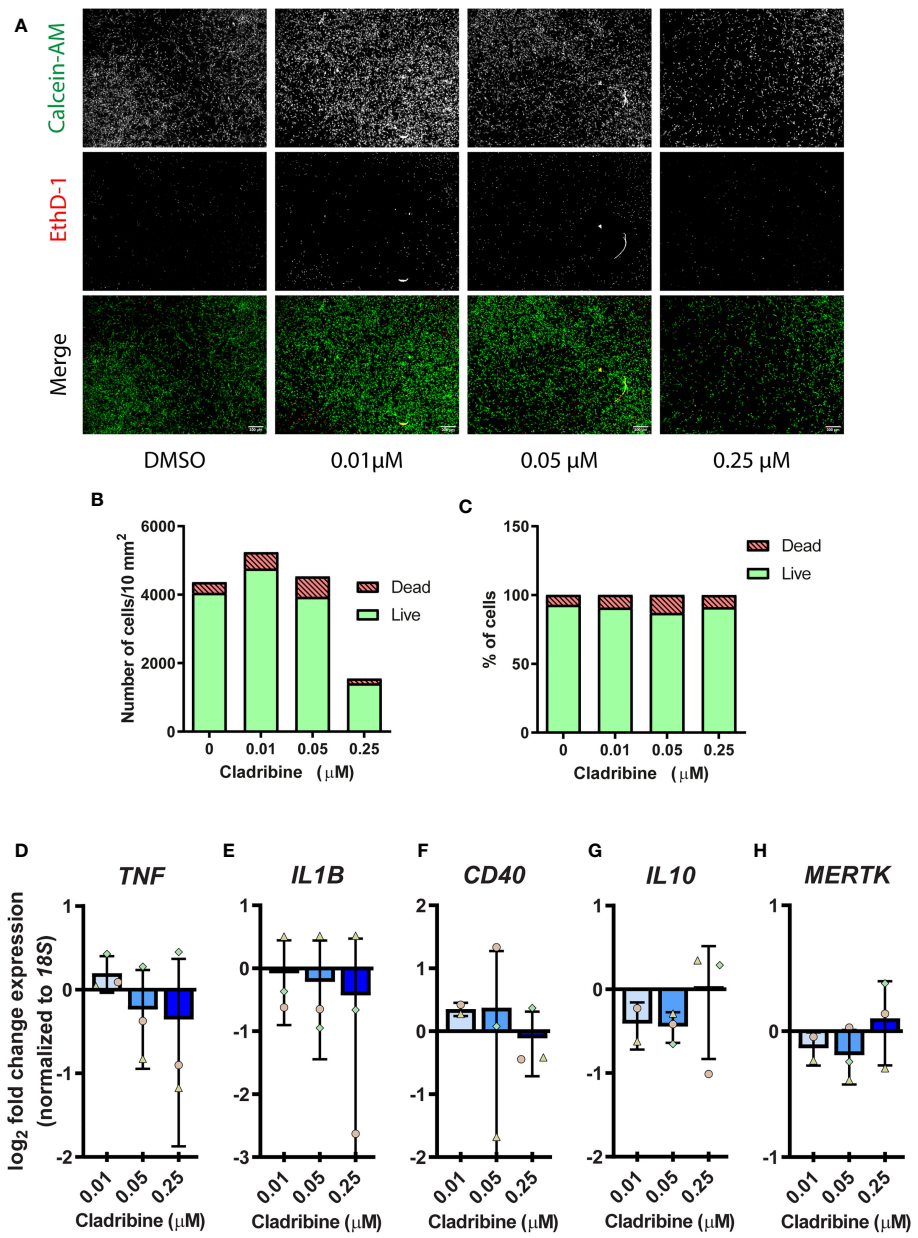

FIGURE 5 | Cell viability and gene expression of activation markers in primary human microglia treated with cladribine. Microglia isolated from temporal lobe surgical resections were treated with cladribine *in vitro* for 6 days. **(A)** Fluorescence microscopy images of human adult microglia stained with calcein-AM (green, live cells) and ethidium homodimer-1 (EthD-1) (red, dead cells) at day 6 post cladribine treatment *in vitro*. **(B, C)** Cell viability of cladribine-treated microglia as assessed by fluorescence microscopy from panel **(A)**, cells positive for calcein-AM are counted as live and positive for EthD-1 are counted as dead. **(B, C)** Data depicted as value, n=1. **(D–H)** RT-qPCR gene expression analysis of **(D–F)** pro-inflammatory and **(G, H)** anti-inflammatory activation markers in microglia at 6 days post cladribine treatment. **(D–H)** Data depicted as mean (+ min/max) and symbols indicate matched experiments from the same brain donor. The fold change expression was calculated for the respective control of each donor. n=3 (except for 0.01 µM for genes *IL10*, *CD40* and *MERTK*, which n=2). Statistical analysis: one-way ANOVA.

cells. Therefore, we investigated the effects of cladribine on microglia, from the CNS parenchyma. We isolated $CD11b^{+}CD45^{low}CD64^{+}$ adult human microglia from surgical brain dissections, obtaining a highly pure and viable (PI^{-}) population of cells (**Supplementary Figure 6A**), which were also $Iba1^{+}$ (**Supplementary Figures 6B, C**). To determine the range of cladribine concentration for microglia *in vitro* treatment, we first assessed the effect of three cladribine concentrations (0.01 μM, 0.05 μM, 0.25 μM) on microglial viability by fluorescence microscopy using calcein-AM/ethidium homodimer-1 staining (**Figure 5A**). The highest cladribine concentration induced a reduction of 65.23% in the number of live cells as compared to vehicle (DMSO) (**Figure 5B**), but there was no difference in the proprotion between dead and live cells (**Figure 5C**), likely due to the fact that dead cells eventually detach from the plate, as evidenced by the difference in cell density (**Figures 5A, B**). We then assessed the effect of the different cladribine concentrations on microglia activation *in vitro*, based on their gene expression. None of the cladribine concentrations significantly changed the expression of *TNF*, *IL1B*, *CD40*, *IL10*, *MERTK* after 6 days of treatment (**Figures 5D–H**).

4 DISCUSSION

Current MS therapies can reduce the frequency of relapses of MS by suppressing the immune response, and the main effect of oral cladribine treatment is lymphocyte depletion. Cladribine treatment leads to drug-free remission (7), and this long-lasting effect cannot be attributed only to lymphocyte depletion. Cladribine has been shown to exert immunomodulatory effects in dendritic and T cells (9, 10), also inhibiting mononuclear cell cytokine response (11) and migration (12), and such effects are thought to contribute to its efficacy in reducing neuroinflammatory relapses. However, the immunomodulatory effects of cladribine treatment on monocyte-derived macrophage differentiation, and whether cladribine can directly modulate the phenotype of human microglia is unknown. Here we have shown that oral cladribine administration to MS patients does not affect monocyte phenotypes or their differentiation to monocyte-derived macrophages (MDMs), corroborating previous data on MDMs differentiation (34). Still, such MDMs showed a moderate downregulation in *TNF* expression compared to baseline. It has been shown that cladribine treatment in combination with lipopolysaccharide (LPS) increases the expression of *TNFR2* in primary neonatal mouse microglia (42), and decreases the secretion of TNF-α in GM-CSF-derived MDMs (34), which suggests that cladribine affects the *TNF* pathway. Nonetheless, our data show that cladribine treatment *in vitro* does not modulate the expression of inflammatory markers, including *TNF*, in primary adult microglia. Moreover, our results also show that cladribine treatment during macrophage differentiation leads to an upregulation of pro-inflammatory or anti-inflammatory activation markers, depending on the differentiation agent (GM-CSF or M-CSF) to which they are exposed. These data suggest that during the administration period and while cladribine is available in tissue, the interaction between cladribine and the CNS microenvironment could determine the inflammatory potential of MDMs that are generated.

Monocytes are short-lived cells derived from hematopoietic precursors and play roles in MS by infiltrating the CNS and differentiating into macrophages (43). In accordance with previous studies (44–46), our results show that cladribine treatment does not alter monocyte total numbers or their subsets (classical, intermediate, and non-classical). Our data shows that monocytes isolated 19-21 days after the start of cladribine treatment generate similar macrophages to the baseline, as evidenced by the comparable expression of the selected surface markers and inflammatory genes. Being short-lived cells, it is reasonable to assume that the analyzed monocytes were most likely generated after treatment and were probably not directly exposed to cladribine. This would imply that any observed effects upon the phenotype of these cells and of their derivatives would downstream to direct effects exerted upon the long-lived progenitor cells in the bone marrow from which they were derived. As shown by previous studies, hematopoietic stem cells can be epigenetically and metabolically reprogrammed after stimulation and retain memory-like features termed innate immune memory (47–49) [reviewed in (50)]. It is possible that similar mechanisms take place during cladribine treatment and lead to the generation of reprogrammed monocytes (51), which would explain the moderate downregulation in basal *TNF* expression in the MDMs. However, challenging such MDMs with inflammatory stimuli such as LPS and cytokines would be necessary to better understand the MDMs inflammatory potential before and after treatment. Further signaling, epigenetic, metabolic analysis, and unbiased approaches covering the whole genome would be necessary to fully determine the potential of cladribine on the reprogramming of monocytes.

The lesions in the MS brain are heterogeneous and dynamic, ranging from pre-active and chronic active to inactive, displaying different levels of neuroinflammation, demyelination and remyelinative potential (52). Mononuclear cells in these lesions also display a range of phenotypes that can contribute to disease pathogenesis or repair. Consistent with this dichotomy, monocyte differentiation can be induced experimentally using either M-CSF or GM-CSF, which are known to generate anti-inflammatory and pro-inflammatory polarized macrophages, respectively (41, 53, 54). For the differentiation of monocytes from MS patients, we used M-CSF because both of the CSF1 receptor ligands, M-CSF and IL-34, are expressed in the brain (55), and CSF1R signaling is necessary for the maintenance of microglia (56). Therefore, using M-CSF to differentiate MDMs would model conditions found in non-inflammatory MS lesions, including those undergoing repair. In contrast, GM-CSF participates in CNS inflammation and autoimmunity (57, 58), and exposure of monocytes and MDMs to this cytokine can be used to model environments in which pathogenesis is active. Therefore, investigating the effects of cladribine on the differentiation of both M-CSF and GM-CSF-derived MDMs could reveal the effects of cladribine on monocytes and their derivatives present in different types of MS lesions. Our

data show that cladribine treatment during differentiation induces contrasting outcomes on the expression of activation markers between M-CSF- and GM-CSF-generated macrophages. These results suggest that cladribine effects on monocyte differentiation depend on the microenvironment to which they are exposed. Such heterogeneous effects have implications for MS since neuroinflammation and de/remyelination are dynamic processes occurring throughout disease progression and lesion stages (52).

Macrophages and microglia are plastic and sentinel cells that activate in response to changes in their microenvironment, and their activation phenotype influences their role in disease. Indeed, the adoptive transfer of anti-inflammatory modulated microglia has been shown to be protective in both autoimmune and demyelination mouse models of MS (59), and clearance of debris by microglia is known to be essential for the remyelination process (60). Additionally, microglia-specific knockout of a molecule in the NF-κB inflammatory pathway, and the expansion of neuroprotective microglia *via* CSF1R stimulation, have both been reported to reduce CNS inflammation in the EAE MS model (61, 62). Thus, macrophage/microglial immunosuppressive and phagocytic activities are considered to be neuroprotective (63), while pro-inflammatory and antigen-presenting activation is thought to promote autoimmune inflammation and demyelination.

Two markers of anti-inflammatory and phagocytic activation are MERTK and IL10. The MERTK receptor plays major roles in myelin phagocytosis (64) and efferocytosis (65, 66), and MERTK expression, together with anti-inflammatory activation, which is induced by IL-10, is important for apoptotic cell clearance (66). Our data show that cladribine treatment *in vitro* during GM-CSF-induced MDM differentiation significantly upregulated the expression of the *IL10* but not the *MERTK* gene. However, during differentiation with GM-CSF, cladribine treatment also significantly induced the expression of costimulatory molecules *CD40* and CD80, which are markers of inflammatory activation. In contrast, cladribine treatment during M-CSF-induced differentiation only significantly upregulated the expression of *CD40*, but not of the other tested genes or surface markers. These results indicate that cladribine treatment might influence macrophage activation in different environmental circumstances but in disparate ways, specific to each tissue microenvironment. Interestingly, our data show that GM-CSF-induced MDMs are more susceptible to cell death induced by cladribine compared to M-CSF-derived MDMs, which indicates a possible mechanism that might contribute to cladribine therapeutic efficacy even in a pro-inflammatory environment: selectively reducing the viability of pro-inflammatory MDMs.

A recent study by Mathieson et al. (31) has also investigated the effects of cladribine treatment on MDMs and monocyte-derived dendritic cells generated from healthy volunteers. This study showed that the pre-treatment with 60 nM cladribine significantly reduced the secretion of IL-6 and TNF-α (but not other analyzed cytokines) and also the phagocytic activity of M-CSF-derived MDMs when challenged with LPS. In contrast to our findings, Mathieson et al. identified no significant reduction in cell viability or significant changes in surface expression of, among other markers, CD40 and CD80. Possible reasons for the disparities in the two studies could lie in the: treatment regimens; for the Mathieson study, cladribine was added at day 2 and 5 of differentiation; cladribine was used at 5, 20 and 60 nM, GM-CSF was used at 100 ng/mL, CD40 levels were assessed by cell surface protein expression and cell viability was assessed using a live/dead cell stain, which preferentially stains necrotic but not apoptotic cells. Of particular note, we had identified that cladribine induced significant apoptosis in MDMs (**Figure 4A**), which would be undetected if Annexin V stain had not been used.

Since cladribine can cross the blood-brain barrier (13), cladribine treatment could have direct effects on microglia, the tissue-resident innate immune cells within the CNS parenchyma. Microglial numbers in the healthy CNS are maintained by self-renewal without contribution from cells in the blood; although, as indicated above, monocytes do enter the MS brain to generate MDMs, which exhibit and maintain a phenotype distinct to that exhibited by the resident microglia (23). When microglia die, the adjacent sentinel microglia undergo mitosis, and in this manner, microglial numbers in the CNS are tightly regulated. Previous studies showed that primary rodent microglia are sensitive to cell death by cladribine *in vitro* (67, 68), and another study showed that cladribine treatment does not alter microglial proliferation in the striatum of mice in the EAE model of MS (69). Regardless of whether cladribine treatment affects microglia numbers, it may still affect their activation and, thus, still be of importance for the therapeutic efficacy of cladribine treatment for MS.

In this study, primary adult human microglia did not show any significant modulation of gene expression upon six days of cladribine treatment. However, it is important to note that the high donor variation due to the nature of the brain tissue (from temporal lobe epilepsy patients) can influence these results and will require replication. For instance, the microglia from two out of three donors showed a dose-dependent downregulation of inflammatory genes, which is in line with the *ex vivo* results of modest *TNF* downregulation in patient-derived MDMs. Other studies have also investigated the effect of cladribine on murine microglia activation. One study has shown that cladribine treatment in primary neonatal rat microglia does not change nitric oxide (NO) generation or TNF-α secretion in response to LPS (67). Additionally, another recent study showed that cladribine, in combination with LPS, but not alone, decreases the phagocytic activity and motility of microglia. The same study showed that the higher concentration of 10 μM cladribine for 24h, in combination with LPS, alters the gene expression of inflammatory cytokines (42). However, there was no difference in the protein secretion of the inflammatory cytokines, and the gene expression changes were only observed if in combination with LPS; cladribine did not modulate these inflammatory genes alone or in combination with IL-4. A more recent study showed that cladribine can inhibit cytokine secretion in primary mouse microglia, albeit in high concentrations (10-200 μM) (68). Altogether, our data and these studies suggest that, at physiologically relevant concentrations for the CNS (13–15) cladribine's immunomodulatory effect on microglia is limited.

Moreover, there are significant differences in the these studies: the time of treatment, the drug concentration, and, most importantly, the origin of cells (murine/humans and neonatal/adult). Of note, an ongoing clinical trial (NCT04239820) (70) will help elucidate the clinical implications of cladribine treatment on human microglia of MS patients by TSPO-PET imaging. Finally, the immunomodulatory effects of cladribine upon human microglia might become evident when cells are treated in combination with inflammatory challenges, such as LPS or cytokines, and with analysis of a larger array of microglia activation genes which were not assessed in our study.

Some limitations should be considered in our study. Cladribine treatment *in vitro* most likely does not represent physiological conditions related to apoptotic cell clearance *in vivo* since dead cells are not quickly cleared as in tissue. Therefore, the observation that cladribine upregulates pro- and anti-inflammatory in MDMs *in vitro* might be a result of increased accumulation of dead cells, which induces the expression of pro-phagocytic genes in M-CSF-differentiated MDMs and the expression of costimulatory molecules in GM-CSF-differentiated MDMs. The accumulation of dead cells can be inferred from the flow cytometry data of cladribine-treated GM-CSF-differentiated MDMs, which are not as phagocytic as M-CSF-differentiated MDMs, and showed a higher percentage of apoptotic/necrotic cells at day seven compared to cladribine-treated M-CSF macrophages (**Figure 3B**). Importantly, this limitation is not present in the *ex vivo* differentiation of monocytes from MS patients since the cells were not directly treated with cladribine *in vitro*, and there was no significant loss of cell viability.

Altogether, our results indicate that cladribine treatment for MS has a limited, if any, indirect effect on the differentiation potential of monocytes and a pronounced direct effect on MDMs differentiation but not on the activation of human microglia. More studies contemplating the functionality of such cells are necessary to assess if the alteration in MDMs gene expression leads to an altered phenotype and whether the phenotype of activated human microglia is affected in other ways. In particular, phagocytosis assays could be of great value in assessing these potential effects since macrophage and microglia phagocytotic activity is an important influence upon CNS physiology and disease (71). Indeed, a recent study showed that cladribine reduced the phagocytic activity of GM-CSF-derived MDMs, but only when these cells were challenged with LPS (34). Future studies further elucidating the immunomodulatory effects of cladribine will provide insight into the role of innate immune cells in CNS inflammatory diseases and could potentially indicate further applications of cladribine beyond immune cell depletion.

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 the Melbourne Health Human Research Ethics Committee (HREC). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

TK conceived the project. TK, MB, TM-F, and SA designed the experiments. SA optimized preliminary protocols, and TM-F carried out the experiments, with support from LJ for flow cytometry and from AA and EN for microglia isolation. TM-F analyzed the data and performed statistical analysis. TM-F, TK, and MB wrote the manuscript. 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/fimmu.2022.678817/full#supplementary-material

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