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Factors associated with dimethyl fumarate-induced lymphopenia

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ABSTRACT

Background: Lymphopenia is a major concern in MS patients treated with dimethyl-fumarate (DMF) as it increases the risk of progressive multifocal leukoencephalopathy.
Objective: To identify factors associated with lymphopenia in DMF-treated patients and explore changes in blood lymphocyte subsets associated with DMF-induced lymphopenia.
Methods: Prospective longitudinal study including 106 patients initiating DMF treatment followed for a median time of 24.67 months. Blood lymphocyte subsets were studied in 64 patients by flow cytometry at baseline and 6 months after.
Results: Mean absolute lymphocyte counts (ALCs) decreased by 29% during the first year of DMF-treatment. Patients developing lymphopenia showed a faster decline within the three first months. A reduction of ALCs higher than 38% at this time was associated to subsequent development of grade 2–3 lymphopenia (OR = 5.93, 95% CI: 1.9–18.6, $p = 0.002$). All patients showed a significant decrease in different T and B lymphocyte subsets upon DMF therapy. In addition, lymphopenic patients experienced a selective decrease in natural killer T (NKT) cell percentages ($p = 0.01$), and a high drop in NKT total counts ($p < 0.0001$).
Conclusions: Patients who experience a drop in ALCs by > 38% at three months of DMF-treatment are about 6-times more likely to develop significant lymphopenia. This decrease is clearly associated with a considerable loss of NKT cells.

1. Introduction

Dimethyl fumarate (DMF; Tecfidera, Biogen, Weston, MA) is an oral disease-modifying drug indicated for the treatment of relapsing-remitting multiple sclerosis (RRMS) with potential anti-inflammatory and cytoprotective effects [1,2]. In phase III placebo-controlled studies, mean lymphocyte counts decreased by approximately 30% during the first year of DMF treatment, and 6% of patients receiving DMF experienced a decline in absolute lymphocyte counts (ALC) below 500/μl [3,4]. Although there was no increased incidence of serious infections observed in patients with ALC < 800 or 500/μl in clinical trials, rare cases of progressive multifocal leukoencephalopathy (PML) have occurred in the postmarketing setting in the presence of prolonged lymphopenia [5–7]. Current prescribing information recommends monitoring ALC during the treatment, and considering interruption of DMF in patients with ALC < 500/μl persisting for > 6 months [1]. Predicting which patients are at greater risk for developing severe lymphopenia may have important implications for clinical decisions, including disease-modifying therapy (DMT) selection and early treatment discontinuation for prevention of PML [8].

Lymphopenia occurs more likely in older patients, with lower baseline ALC, and those previously treated with natalizumab [8,9]. We aimed to explore additional risk factors for developing lymphopenia and to evaluate the effect of DMF on blood lymphocyte subsets as related to decline in ALC in patients with RRMS.

Abbreviations: DMF, dimethyl-fumarate; ALC, absolute lymmphocyte count

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2. Methods

2.1. Study design and population

This was a prospective longitudinal study of 106 consecutive RRMS patients treated with DMF at the MS Comprehensive Care Center at the Hospital Universitario Ramón y Cajal in Madrid, Spain. The study was approved by the local ethics committee. All patients signed a written informed consent form before inclusion.

2.2. Evaluation and follow-up

Clinical, MRI, and routine laboratory data, including ALC, were recorded at baseline. Patients were evaluated every 3 months thereafter to assess both the ocurrence of relapses and the EDSS score. MRI scans were performed yearly after DMF initiation. Complete blood counts were compiled at each visit. Blood lymphocyte subsets were examined at baseline and six months thereafter, in a representative group of 64 patients.

Lymphopenia was classified according to NIH common terminology criteria for adverse events (CTCAE v4.0) as follows: grade 1 (ALC 800–999/μl), grade 2 (ALC 500–799/μl) and grade 3 (ALC < 500/μl) [10]. DMF was discontinued if ALCs fell below 500/μl for > 3 months, and lymphocyte counts were monitored until their recovery.

2.3. Flow cytometry studies

Heparinized whole blood was collected from each patient immediately before DMF initiation and after six months of treatment. Peripheral blood mononuclear cells (PBMC) were obtained within 2 h by Ficoll density gradient centrifugation (Fresenius Kabi, Norway) and cryopreserved in aliquots of $5\text{-}6 \times 10^6$cells until studied.

The following monoclonal antibodies were used in the blood lymphocyte subsets study: CD8-FITC, CD27-FITC, CD24-PE, CD197-PE (CCR7-PE), CD3-PerCP, CD38-PE-Cy5.5, CD19-PE-Cy7, CD25-PE-Cy7, CD45RO-APC, CD56-APC, CD4-APC-H7, CD8-APC-H7, CD3-BV421, CD127-BV421, CD45-V500 (all from BD Biosciences, San Diego, CA).

Blood leukocyte subsets were studied by flow cytometry as previously described [11]. Briefly, for membrane antigen staining, 10^6 PMBC were labelled with the appropriate amounts of monoclonal antibodies, washed with PBS, and analyzed in a FACSCanto II flow cytometer (BD Biosciences). According to the differential expression of several antigens, CD4+ and CD8+ T cells were classified as: naïve (CCR7+ CD45RO−); central memory (CM) (CCR7+ CD45RO+); effector memory (EM) (CCR7− CD45RO+); terminally differentiated (TD) (CCR7− CD45RO−). Regulatory CD4 T cells (Treg) were defined as CD3+ CD4+ CD25hi CD127low. B cells were classified as: memory (CD19+ CD27dim CD38dim), plasmablasts (CD19+ CD27hi CD38hi); and CD27- regulatory cells (Breg) (CD19+ CD27- CD24hi CD38hi); CD56+ cells were subdivided into natural killer (NK) cells (CD56dim CD3−), natural killer T (NKT) cells (CD56dim CD3+), and CD56bright NK cells (CD3− CD56bright).

2.4. Statistical analysis

Analyses were performed using the Stata/IC Version 12.1 (StataCorp LP, College Station, TX, USA).

We used Fisher's exact test for categorical variables, and the Mann–Whitney *U* test for continuous ones. Wilcoxon matched pair tests were used to assess differences between baseline and follow-up data. *p* values were corrected using Bonferroni method.

A receiver operating characteristic (ROC) curve was generated to assess association between the decline in ALCs at three months of treatment and the subsequent development of lymphopenia. The optimal cut-off point was identified as the maximum point of the Youden index (specificity + sensitivity − 1). *p* values below 0.05 were considered as significant.

3. Results

3.1. Patients

106 consecutive patients (75.5% female) who received DMF treatment for 24.67 [6.03–34.63] months (median [range]) were included in the study. Age at treatment initiation was 41.5 ± 8.9 and the duration of the disease was 11.1 ± 8.2 years. Relapses in the last 12 months were 0.77 ± 0.73 and the EDSS score at baseline was 1.5 [0–7]. Seventy four patients received other previous disease modifying treatments (DMT).

3.2. Incidence of lymphopenia

A total of 37 (34.5%) patients developed some grade of lymphopenia, considering worst post-baseline ALC. The incidence of grade 1, 2 and 3 lymphopenia was 17.9% (19/106), 13.2% (14/106), and 5.7% (6/106), respectively. Baseline demographic and clinical characteristics of patients classified according to the development of lymphopenia are shown in Table 1. Patients who experienced lymphopenia were older at DMF initiation (45.2 vs. 39.5 years; $p = 0.001$) and had lower ALCs prior to treatment (1681 vs. 2195 cells/μl; $p = 0.002$). There were no other differences in basal characteristics of patients classified according to lymphopenia status.

Table 1
Baseline characteristics of patients treated with DMF classified according to the development of lymphopenia.

Variable	No lymphopenia ($n = 69$)	Lymphopenia ($n = 37$)	*p*
Age, m ± SD (years)	39.5 ± 9.9	45.2 ± 5.0	0.001
Females, n (%)	50 (72.5)	30 (81.1)	NS
Time since first MS symptoms, m ± SD (years)	10.4 ± 8.0	12.4 ± 8.1	NS
Previous DMT, n (%)			NS
None (treatment-naïve patients)	23 (33.3)	9 (24.3)	
Interferon beta/Glatiramer acetate	34 (49.3)	9 (24.3)	
Teriflunomide	2 (2.9)	0 (0)	
Natalizumab	5 (7.2)	2 (5.4)	
Fingolimod	4 (5.8)	2 (5.4)	
Others	1 (1.4)	2 (5.4)	
N. of relapses in the previous 12 months, m ± SD	0.85 ± 0.75	0.62 ± 0.68	NS
EDSS score at baseline, median [range]	2.0 [0–6,5]	1.5 [1–7]	NS
N. of gadolinium-enhancing T1-weighted lesions, m ± SD	1.47 ± 3.89	0.34 ± 0.84	NS
N. of hyperintense T2-weighted lesions, m ± SD	25.3 ± 15.2	24.1 ± 14.9	NS
Baseline ACLs, m ± SD (cells/μl)	2195 ± 951	1681 ± 477	0.002

ALCs: absolute lymphocyte counts; DMF: dimethyl fumarate; DMT: disease modifying therapy; m ± SD: mean ± standard deviation; MRI: magnetic resonance imaging; n: number; NS, non significant.

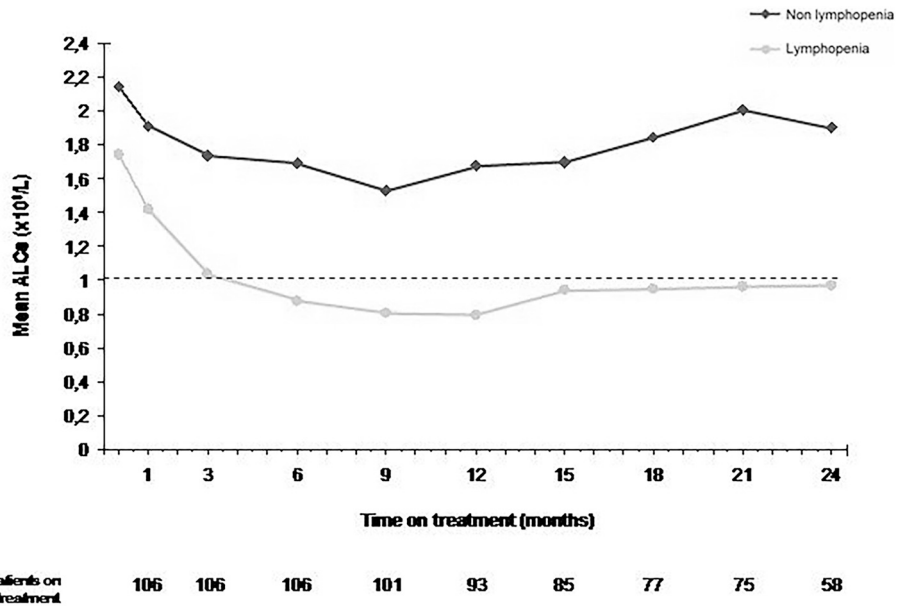

Fig. 1. Mean absolute lymphocyte count (ALCs) changes along dimethyl fumarate treatment in patients who developed (grey line) or not (black line) lymphopenia. It should be taken into account that dimethyl fumarate was discontinued in patients with grade 3 lymphopenia before month 12, so mean ALCs increase thereafter.

3.3. ALCs evolution during DMF treatment

Mean ALCs decreased by approximately 29% during the first year of treatment and then plateaued. Time until lymphopenia was indentified ranged between one month to one year (median six months). Patients who subsequently developed lymphopenia experienced a higher decline in mean ALCs at three months of treatment than those who did not develop lymphopenia (41.4% vs. 15.5% decrease, $p = 0.0001$; Fig. 1). According to the range of lymphopenia the patients developed later, the rate of decrease in mean ALCs by month-3 was 25.6%, 38.1% and 50.7% for grade 1, grade 2 and grade 3 lymphopenia, respectively. Since DMF-associated PML has been described mainly in patients with grade 2–3 lymphopenia [5–7], we studied this group of patients separately. We found that they experienced a significantly greater decline in mean ALCs within three months of treatment than the rest of patients (44.9% vs. 19.9% decrease, $p = 0.001$). We analyzed this data using a ROC curve and established a cut-off value of 38% decrease in ALCs. Higher values were associated with subsequent appearance of grade 2–3 lymphopenia (OR = 5.93, 95% CI: 1.9–18.6, $p = 0.002$). DMF was discontinued in six patients with grade 3 lymphopenia. After that, lymphocyte counts gradually rose in all patients, but did not reach normal range until a median time of 150.5 days [range 76–256].

3.4. Effectiveness and safety of DMF according to the developement of lymphopenia

Although assessment of effectiveness of DMF was not the main objective of the study, data on clinical and radiological disease activity was collected as part of routine clinical practice. In the course of the study, 6/37 (16.2%) lymphopenic patients and 13/69 (18.8%) non-lymphopenic patients experienced breakthrough disease activity, defined as a clinical relapse or appearance of new T2 or gadolinium enhancing lesions on follow-up MRI. No significant difference was observed between both groups.

Infections were not systematically queried, as evaluation of infection risk was not a part of the study. However, two of the six patients who developed grade 3 lymphopenia incidentally reported an infection while ALC < 500/μl; one patient developed herpes zoster involving right T5 dermatome, and the other patient developed bacterial pneumonia. Both of these were resolved after specific antimicrobial therapy.

3.5. Lymphocyte subsets in patients classified according to lymphopenia status

Percentages of blood lymphocyte subsets were studied in a representative group of 64 patients. Baseline clinical and demographic data of patients developing ($n = 24$) or not ($n = 40$) lymphopenia were similar to those observed in the whole group of patients (data not shown). There were no differences in basal leukocyte subsets between both groups of patients. Percentages of the different leukocyte subpopulations in basal and six months samples are shown in Table 2. All patients experienced a decrease in effector memory CD4+ and CD8+ T cells, in terminally differentiated CD8+ T cells, in total CD8+ T cells and in memory B cells, with no differences according to lymphopenia status. In addition, patients developing lymphopenia experienced a selective decrease in NKT cells ($p = 0.01$) which corresponded with a substantial decrease in the absolute numbers of these cells ($p < 0.0001$; Fig. 2A, B). Representative dot plots are shown in Fig. 2C and D.

In contrast, after 6 months of treatment non-lymphopenic patients experienced a relative increase in naïve CD4+ T cells ($p = 0.009$) not confirmed when absolute cell numbers were evaluated (data not shown).

4. Discussion

DMF is a new oral therapy recently approved for RRMS that induces a shift in the abnormal immune response observed in peripheral blood; it decreases effector subsets and increases regulatory ones, which is associated with an improvement of the disease course [11–17]. The most concerning adverse event of DMF is the decrease in lymphocyte counts, as prolonged severe lymphopenia has been related with rare cases of PML [5–7]. Thus, the identification of patients at risk of lymphopenia and the analysis of changes in the immune system associated with this phenomenon are of high clinical interest.

We studied 106 patients treated with DMF during a mean time of about two years in a university hospital. The reduction in mean ALCs (29%) and the percentage of patients with grade 3 lymphopenia (5.7%) were consistent with DMF placebo-controlled trials [3,4]. Patients who developed lymphopenia were older at DMF initiation and had lower ALCs prior to treatment, thus confirming previous reports [8,9]. We

Table 2
DMF induced changes in lymphocyte blood subsets.

Variable		No lymphopenia (n = 40)			Lymphopenia (n = 24)		
		Basal (M ± SEM)	6 Mo (M ± SEM)	p	Basal (M ± SEM)	6 Mo (M ± SEM)	p
Effector and memory subsets	CD4 + T	35.6 ± 1.5	37.9 ± 1.6	NS	31.4 ± 2.9	31.3 ± 2.9	NS
	CD4 + N	17.0 ± 1.4	22.1 ± 1.8	0.009	14.0 ± 2.2	21.1 ± 2.9	NS
	CD4 + CM	8.2 ± 0.6	7.8 ± 0.8	NS	6.8 ± 1.0	4.7 ± 0.7	NS
	CD4 + EM	8.1 ± 0.6	5.5 ± 0.5	< 0.0001	8.3 ± 1.0	3.9 ± 0.6	< 0.0001
	CD4 + TD	2.4 ± 0.2	2.4 ± 0.4	NS	2.3 ± 0.4	1.6 ± 0.3	NS
	CD8 + T	16.7 ± 1.2	13.9 ± 0.9	< 0.0001	14.9 ± 1.5	10.5 ± 1.1	< 0.0001
	CD8 + N	5.4 ± 0.7	6.5 ± 0.6	NS	3.8 ± 0.7	4.2 ± 0.5	NS
	CD8 + CM	0.7 ± 0.08	0.6 ± 0.08	NS	0.6 ± 0.1	0.5 ± 0.1	NS
	CD8 + EM	4.3 ± 0.6	2.3 ± 0.3	< 0.0001	4.1 ± 0.8	1.9 ± 0.4	0.015
	CD8 + TD	5.6 ± 0.9	4.3 ± 0.6	0.0015	5.6 ± 1.1	2.9 ± 0.7	0.0015
	NKT	2.5 ± 0.4	2.2 ± 0.4	NS	2.3 ± 0.5	1.1 ± 0.3	0.0105
	NK	12.2 ± 1.3	14.6 ± 1.7	NS	13.8 ± 2.7	11.0 ± 1.8	NS
	CD19+ T	8.5 ± 0.7	7.5 ± 0.6	NS	10.1 ± 1.5	10.6 ± 1.8	NS
	Bmem	1.1 ± 0.1	0.7 ± 0.1	< 0.0001	1.3 ± 0.2	0.5 ± 0.08	< 0.0001
	PB	0.06 ± 0.01	0.08 ± 0.03	NS	0.08 ± 0.02	0.09 ± 0.02	NS
Regulatory subsets	Treg	1.5 ± 0.1	1.5 ± 0.1	NS	1.2 ± 0.2	1.2 ± 0.2	NS
	Breg	0.1 ± 0.04	0.1 ± 0.02	NS	0.1 ± 0.02	0.2 ± 0.04	NS
	$CD56^{bright}$	1.2 ± 0.2	1.5 ± 0.2	NS	1.2 ± 0.2	1.7 ± 0.2	NS

Values are expressed as percentages of total peripheral blood mononuclear cells.
Breakdown of lymphopenic patients (n): Grade 1: 14; Grade 2: 7; Grade 3: 3. Bmem: Memory B cells; Breg: Regulatory B cells; CM: Central memory; DMF: Dimethyl fumarate; EM: Effector memory; M ± SEM: Mean ± Standard error of mean; Mo: Months; N: Naïve; NK: Natural Killer cells; NKT: Natural Killer T cells; NS: Not significant; PB: Plasmablast; T: Total; TD: Terminally differentiated; Treg: Regulatory T cells.

explored additional risk factors for DMF-related lymphopenia, and observed that patients who had > 38% reductions in ALCs at three months of treatment were 5.93-times more likely to develop subsequent grade 2–3 lymphopenia. Patients not experiencing a rapid decrease in ALCs during first three months of treatment are unlikely to have persisting lymphopenia thereafter. When validated in independent cohorts, this may be a useful and easy-to-use tool for early indentification of patients at a higher risk of subsequently developing lymphopenia.

Different investigations explored the mechanism of action of DMF by studying changes in peripheral blood cell profiles. It has been sugested that differential susceptibility of distinct lymphocyte subpopulations to DMF-induced apoptosis may contribute to efficacy and safety profile of this drug [18]. It was reported that patients treated with DMF showed a more significant reduction of CD8+ T cells as

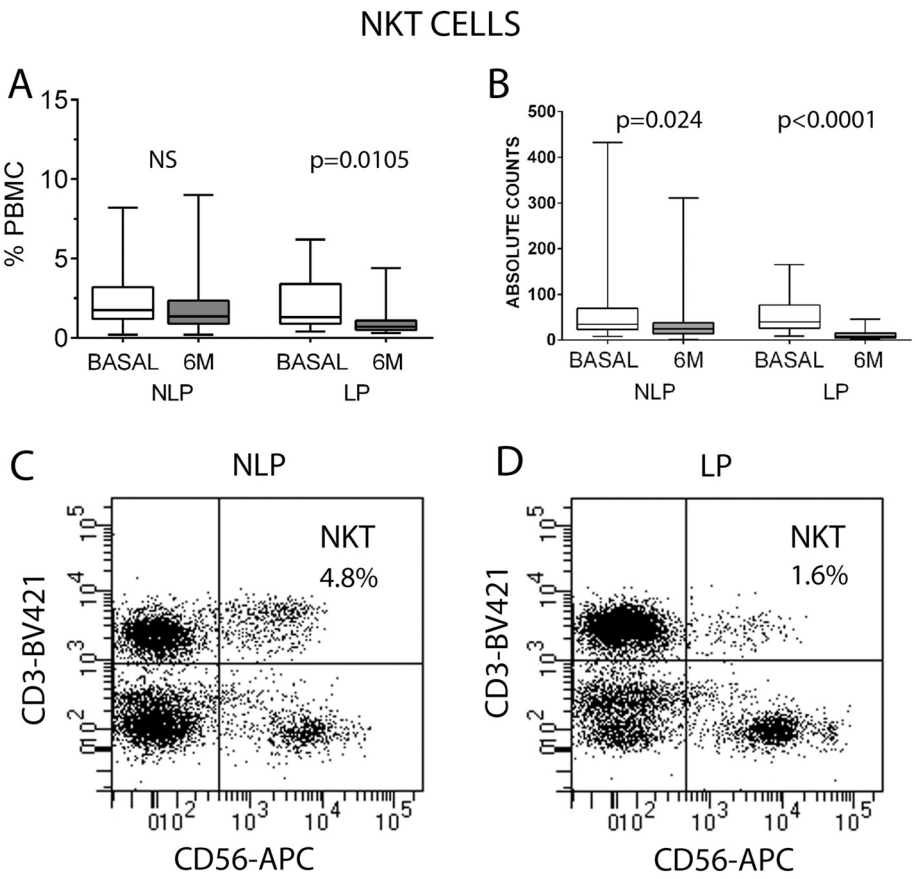

Fig. 2. A, B: Percentages (A) and absolute numbers (B) of Natural Killer T cells (NKT) shown by non-lymphopenic (NLP) or lymphopenic (LP) patients before (basal) and after 6 months (6 M) of dimethyl fumarate treatment. C, D: Representative dot plots showing NKT cells from a non-lymphopenic (NLP, C) and from lymphopenic patient (LP, D) after six months of treatment. Plots are gated on total peripheral blood mononuclear cells. Wilcoxon matched pair tests were used to assess differences between basal and 6 M results. Breakdown of lymphopenic patients (n): Grade 1: 14; Grade 2: 7; Grade 3: 3. M: Months; NS: Non significant; PBMC: Peripheral blood mononuclear cells.

compared to CD4+ T cells, and it is thought that viral infections may become more prevalent in the setting of this CD8+ T cells reduction [19–21]. In patients showing optimal response to this drug, a decrease in the percentage different effector lymphocyte subsets has been reported (mainly occurring in central memory T cells, memory B cells, and T and B cells producing IFNγ and TNFα respectively), as well as an increase in regulatory NK cells [11,15]. The lymphocyte subpopulations that diminish in lymphopenic patients were also studied, and significant reductions in CD4+ and CD8+ T cells have been observed [22].

We aimed to further characterize changes in lymphocyte subsets in DMF-treated patients who developed lymphopenia and observed that both, lymphopenic and non lymphopenic patients, showed a significant reduction in the percentages of some effector and memory lymphocyte subsets. Of note, a decrease in central memory CD4+ T cells or memory B cells, previously associated with optimal response to DMF [11], was similar in both groups of patients. This strongly suggests that lymphopenia status is independent of patient response to this drug, as we have observed in our study. However, some differences were found between lymphopenic and non lymphopenic individuals. The first group of patients experienced a specific decrease in NKT cell percentages which resulted in a large reduction of their absolute cell numbers. These cells can have a potential role in MS, since they can rapidly produce a variety of proinflammatory cytokines [23] and exhibit cytotoxic activity after activation [24]. However, their reduction is not associated with an optimal response to the drug [11]. It was described that CD8+ T cells clearly diminished in patients showing DMF associated lymphopenia [18,19]. We did not appreciate these variations in these cells within lymphopenic and non lymphopenic patients since both showed a considerable decrease. The great decrease on the NKT cell subset, which mostly expresses CD8, shown by lymphopenic patients may account for the differences in CD8 expressing T cells previously associated with this condition. Further research is needed to explore the clinical significance of this NKT reduction. Another difference we found in the population analysis was a relative increase of naïve CD4+ T cells after 6 months of treatment restricted to non lymphopenic patients, not confirmed when exploring absolute cell numbers, which shows that this relative increase may be a consequence of the decrease of other lymphoid populations.

The main limitation of our study is the low number of patients with grade 3 lymphopenia which makes it difficult to identify significant differences in the lymphopenic group. Nevertheless, our data provides an easy tool for the early identification of patients at risk of developing lymphopenia under DMF therapy. It shows that lymphopenia under this treatment is not associated with an indiscriminate reduction of B and T cells or with the efficacy of the drug, but probably relates to a loss of CD8+ expressing T cells, mostly NKT cells.

Conflicts of interest

LMV, LCF, SSM and JCA-C received payment for giving conferences, or travel expenses or research grants from Merck-Serono, Biogen, Sanofi-Genzyme, Roche, Bayer and Novartis. The remaining authors declare no conflicts of interest.

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