


ORIGINAL ARTICLE

Predicting therapeutic response to fingolimod treatment in multiple sclerosis patients

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Summary

Aims: Fingolimod, an orally active immunomodulatory drug for relapsing-remitting multiple sclerosis (RRMS), sequesters T cells in lymph nodes through functional antagonism of the sphingosine-1-phosphate receptor, reducing the number of potential autoreactive cells that migrate to the central nervous system. However, not all RRMS patients respond to this therapy. Our aim was to test the hypothesis that by immune-monitoring RRMS patient's leukocyte subpopulations it is possible to find biomarkers associated with clinical response to fingolimod.

Methods: Prospective study. Analysis of peripheral blood mononuclear cell subpopulations by multiparametric flow cytometry, at baseline and +1, +3, +6, +12 months of follow-up in 40 RRMS patients starting fingolimod therapy.

Results: Fingolimod treatment induced a severe lymphopenia affecting mainly T and B cells. A relative increase in T_{reg} (memory T_{reg} : $3.8 \pm 1.0\%$ baseline vs $8.8 \pm 4.4\%$ month +1; activated T_{reg} : $1.5 \pm 0.7\%$ baseline vs $3.7 \pm 2.1\%$ month +1, $P < 0.001$) as well as transitional B cells ($10.5 \pm 12.3\%$ baseline vs $18.7 \pm 14.6\%$ month +1, $P < 0.001$) was observed.

Interestingly, lymphocyte subpopulations were already at baseline significantly different in responder patients. The percentage of recent thymic emigrants (RTE) used to stratify fingolimod responder, and no responder patients was the best biomarker ($4.0 \pm 1.4\%$ vs $7.4 \pm 1.9\%$, respectively [$P < 0.001$]).

Conclusion: The results support that immune-monitoring of lymphocyte subpopulations in peripheral blood is a promising tool to select RRMS candidate for fingolimod treatment.

KEYWORDS

fingolimod, flow cytometry, multiple sclerosis, predictive biomarker, recent thymic emigrants

1 | INTRODUCTION

Fingolimod is an immune-modulatory treatment for relapsing-remitting multiple sclerosis (RRMS) patients, reducing significantly disease activity.^{1,2} This drug embodies and degrades the sphingosine-1-phosphate (S1P) receptor on leukocytes, inhibiting the egress of lymphocytes from lymph nodes. Fingolimod acts specifically on cells expressing CCR7 on their surface, which is the main chemokine receptor involved in homing to lymph nodes.^{3,4} This molecule is mainly expressed by naïve and central memory T and B cells. Hence, effector memory cells, which lack CCR7, remain unaffected.⁵ Patients under fingolimod treatment present peripheral blood lymphopenia due to the redistribution of leukocytes, which are being trapped inside lymph nodes. Consequently, this treatment reduces the migration of potential inflammatory cells to the central nervous system. Its use in Europe has been restricted to highly active RRMS patients or as escalation after failure to first-line disease modifying therapies (DMT).⁶ Previous studies by Cohen et al¹ found that 83% of patients under fingolimod treatment remain relapse free after the first year, and Kappos et al² a 70, 4% after 2 years of follow-up. However, failure mechanisms or suboptimal response to fingolimod were not addressed in those studies.

In a preliminary longitudinal study, we showed that a higher percentage of recent thymic emigrants (RTE) and transitional B cells at baseline correlate with a suboptimal response to fingolimod,⁷ suggesting that it is possible to identify biomarkers of treatment response in peripheral blood. The purpose of this study was to analyze the possible relationship between the immune profile of leukocyte subpopulations in peripheral blood and the clinical response to fingolimod in RRMS patients during 1 year follow-up.

2 | METHODS

2.1 | Patients

A total of 44 RRMS patients under fingolimod treatment from 5 distinct MS units in Catalonia (Spain) and who were selected according to McDonald's criteria 2010⁸ were included. Patients were monitored for 12 months. This cohort is an extension of our pilot study.⁷

The study protocol was approved by the local ethics committees of the participating centers, and the patients signed the informed consent prior to their inclusion in the study.

Eligible MS patients were aged between 18 and 59 and had Expanded Disability Status Scale (EDSS) scores ≤ 6.0 . Noneligible patients either had progressive forms of MS or were receiving interferon beta (IFN β) or glatiramer acetate (GA) within at least the previous 24 hours or natalizumab (NTZ) or metilprednisolone within the previous 30 days.

The cohort was monitored for 12 months.

During follow-up, immunological and clinical parameters (adverse events, relapses, and EDSS score) were recorded at baseline, +1, +3, +6, and +12 months after starting the treatment.

A relapse of MS was defined as occurrence, recurrence, or worsening of symptoms of neurological dysfunction lasting over 24 hours without infection or fever. The annualized relapse rate (ARR) was defined as the total number of relapses divided by the number of patients/y. The ARR was assessed in the 12 months prior to the initiation of treatment (baseline), and after 12-month follow-up.

Disability progression was defined as an increase of at least 1 point in the EDSS score sustained over at least 6 months.

Brain magnetic resonance imaging (MRI) scans were performed at baseline and at +12 months and the number of new/enlarged lesions on T2-weighted MRI recorder for all patients. Both brain MRI scans of each patient were carried out with the same equipment. Additionally, MRI with gadolinium (Gd) administration was performed in 22 patients and gadolinium-enhancing (Gd+) T1 lesions recorded.

Patients were divided into those with good response to treatment (R) or those with suboptimal response to treatment (NR) according to the Rio Score⁹: A patient was considered a NR patient when at least 2 of the following 3 criteria were met: (i) 1 or more clinical relapse(s) during the first 12 months of treatment, (ii) an increase of at least 1 point in the EDSS score sustained over at least 6 months, and (iii) the presence at month +12 of active lesions in brain MRI (ie, >2 new or enlarging T2-weighted lesions or at least 1 Gd-enhancing T1-weighted lesion).

2.2 | Flow cytometry analysis

Peripheral blood samples were collected in ethylene diamine tetra acetic acid (EDTA) at baseline and at +1, +3, +6, and +12 months of treatment. Analysis of leukocyte subpopulations was evaluated in patients with at least 6 months of follow-up.

Samples of whole blood were incubated with monoclonal antibodies for 20 minutes at room temperature and in darkness. Samples were washed and acquired on LSRFortessa flow cytometer (BD Biosciences, San José, CA, USA) after erythrocyte lysis.

Leukocyte subpopulations were defined over 4 panels using the markers specified in Table 1, containing the following monoclonal antibodies per panel: (i) T-cell panel: CD3-V450, CD4-PerCP-Cy5.5, CD45RA PE-Cy7, CCR7 PE, CD38 APC, CD8 APC-H7, HLA-DR V500 (BD Bioscience), CD183 AF488, CD196 BV605 and CD45 AF700 (Biolegend, San Diego, CA, USA); (ii) T_{reg} panel: CD4 PerCP-Cy5.5, CD25 PE, CCR4 PE-Cy7, CD127 AF647, CD45RO APC-H7, CD3 V450, HLA-DR V500 (BD Bioscience) and CD45 AF700 (Biolegend); (iii) B-cell panel: CD24 FITC, CD19 PerCP-Cy5.5, CD38 APC, CD20 APC-H7, CD3V500 (BD Bioscience), IgD PE-Cy7, CD27 BV421 and CD45 AF700 (Biolegend); (iv) DC/monocyte/NK panel: CD3⁺ CD19 APC-H7, CD56 PE, CD16 APC, CD14 V450, CD123PerCP-Cy5.5, CD11c PE-Cy7, HLA-DR V500 (BD Bioscience) and Slan FITC (Miltenyi Biotec, Bergisch Gladbach, Germany). All panels were analyzed with FACSDiva software (BD Biosciences).

The gating strategy to analyze the desired leukocyte subpopulations (Figures S1-S4) was based on international consensus.¹⁰

TABLE 1 Leukocyte subpopulations and phenotype

Leukocyte subpopulations	Phenotype
T-cell subsets (CD3 ⁺)	
CD4 ⁺ naïve T cell	CD4 ⁺ CCR7 ⁺ CD45RA ⁺
CD8 ⁺ naïve T cell	CD8 ⁺ CCR7 ⁺ CD45RA ⁺
CD4 ⁺ central memory (CD4 ⁺ T _{CM})	CD4 ⁺ CCR7 ⁺ CD45RA ⁻
Th1 central memory (Th1 _{CM})	CD4 ⁺ CCR7 ⁺ CD45RA ⁻ CCR6 ⁻ CXCR3 ⁺
Th2 central memory (Th2 _{CM})	CD4 ⁺ CCR7 ⁺ CD45RA ⁻ CCR6 ⁻ CXCR3 ⁻
Th17 central memory (Th17 _{CM})	CD4 ⁺ CCR7 ⁺ CD45RA ⁻ CCR6 ⁺ CXCR3 ⁻
Th1Th17 central memory (Th1Th17 _{CM})	CD4 ⁺ CCR7 ⁺ CD45RA ⁻ CCR6 ⁺ CXCR3 ⁺
CD8 ⁺ central memory T cell (CD8 ⁺ T _{CM})	CD8 ⁺ CCR7 ⁺ CD45RA ⁻
CD4 ⁺ effector memory T cell (CD4 ⁺ T _{EM})	CD4 ⁺ CCR7 ⁻ CD45RA ⁻
Th1 effector memory (Th1 _{EM})	CD4 ⁺ CCR7 ⁻ CD45RA ⁻ CCR6 ⁻ CXCR3 ⁺
Th2 effector memory (Th2 _{EM})	CD4 ⁺ CCR7 ⁻ CD45RA ⁻ CCR6 ⁻ CXCR3 ⁻
Th17 effector memory (Th17 _{EM})	CD4 ⁺ CCR7 ⁻ CD45RA ⁻ CCR6 ⁺ CXCR3 ⁻
Th1Th17 effector memory (Th1Th17 _{EM})	CD4 ⁺ CCR7 ⁻ CD45RA ⁻ CCR6 ⁺ CXCR3 ⁺
CD8 ⁺ effector memory T cell (CD8 ⁺ T _{EM})	CD8 ⁺ CCR7 ⁻ CD45RA ⁻
Terminal differentiated effector memory CD4 ⁺ T cell (T _{EMRA})	CD4 ⁺ CCR7 ⁻ CD45RA ⁺
Terminal differentiated effector memory CD8 ⁺ T cell (T _{EMRA})	CD8 ⁺ CCR7 ⁻ CD45RA ⁺
Recent thymic emigrants (RTE)	CD4 ⁺ CD27 ⁺ CCR7 ⁺ CD45RA ⁺ CD31 ⁺ PTK7 ⁺
Double negative T cell	CD4 ⁻ CD8 ⁻
Memory T _{reg}	CD4 ⁺ CD127 ⁻ CD25 ⁺ CCR4 ⁺ CD45RO ⁺
Activated T _{reg}	CD4 ⁺ CD127 ⁻ CD25 ⁺ CCR4 ⁺ CD45RO ⁺ HLA-DR ⁺
B-cell subsets (CD19 ⁺)	
Naïve B cell	CD27 ⁻ IgD ⁺
Transitional B cells	CD24 ^{hi} CD38 ^{hi} CD27 ⁻ IgD ⁺
T1 transitional B cells	CD24 ^{hihi} CD38 ^{hihi} CD27 ⁻ IgD ⁺
T2 transitional B cells	CD24 ^{hi} CD38 ^{hi} CD27 ⁻ IgD ⁺
Unswitched memory	CD27 ⁺ IgD ⁺
Switched memory	CD27 ⁺ IgD ⁻
Plasma cells	CD38 ^{hi} CD27 ⁺ IgD ⁻
Exhausted memory	CD27 ⁻ IgD ⁻

(Continues)

TABLE 1 (Continued)

Leukocyte subpopulations	Phenotype
Nk cells	CD3 ⁻ CD19 ⁻ CD56 ⁺
CD56 ^{bright} CD16 ⁻ cells	CD3 ⁻ CD19 ⁻ CD56 ^{bright} CD16 ⁻
CD56 ^{dim} CD16 ⁺ cells	CD3 ⁻ CD19 ⁻ CD56 ^{dim} CD16 ⁺
Monocytes	CD3 ⁻ CD19 ⁻ CD14 ⁺
Classical monocyte	CD3 ⁻ CD19 ⁻ CD14 ⁺ CD16 ⁻
Nonclassical monocyte	CD3 ⁻ CD19 ⁻ CD14 ^{low} CD16 ⁺
Dendritic cells	CD3 ⁻ CD19 ⁻ CD56 ⁻ CD14 ⁻ HLA-DR ⁺
Myeloid DC	CD3 ⁻ CD19 ⁻ CD56 ⁻ CD14 ⁻ HLA-DR ⁺ CD11c ⁺
Plasmacytoid DC	CD3 ⁻ CD19 ⁻ CD56 ⁻ CD14 ⁻ HLA-DR ⁺ CD123 ⁺

Recent thymic emigrants subpopulation analysis and absolute cell number quantification were performed as previously reported.⁷

2.3 | Statistical analysis

Two approaches were conducted: (i) clinical and immunological descriptive analysis of the effect of fingolimod and (ii) correlation between leukocyte subpopulations and clinical response to fingolimod.

Data were expressed as the mean (standard deviation) for quantitative variables or percentages and numbers (n [%]) for qualitative variables. General characteristics were calculated in total cohort and according response to fingolimod. Univariate comparison was made using *t* test and paired *t* test or nonparametric Wilcoxon, paired Wilcoxon, or Kruskal-Wallis test when appropriate. *P*-values <0.05 were considered significant. Figures show means ± SEM. The Statistical Package for Social Sciences (SPSS/Windows version 15.0; SPSS Inc, Chicago, IL, USA) and the software program GraphPad Prism (5.0 version; GraphPad, La Jolla, CA, USA) were used to perform statistical analyses.

3 | RESULTS

3.1 | Patients

Forty-four RRMS patients were selected for the study, but 4 of them were not eligible for further analysis due to atrioventricular (AV) block after the first dose of fingolimod (2 patients) or recurrent infections during the first 3 months of follow-up (2 patients).

Additionally, before the end of the study 6 patients discontinued treatment due to a pregnancy wish (1 patient), hepatotoxicity (1 patient), lymphopenia (1 patient), unclassified (1 patient), or lack of effectiveness (2 patients). Nonetheless, the latter 2 patients were classified as NR complying with our response criteria. As a result, a total of 36 patients were analyzed in relation to clinical response: R (28 patients) and NR (8 patients). There were no differences in gender, age, disease duration, previous immunomodulatory treatments,

TABLE 2 Demographic and clinical characteristics of the patients before fingolimod treatment (n = 36)

	Total cohort (n = 36)	Responders (INR) (n = 28)	Nonresponders (NR) (n = 8)	P-value R/NR patients
Female sex, n. of patients (%)	20 (56)	17 (61)	3 (38)	0.2
Age (y), mean (SD)	33.8 ± 8.3	29.6 ± 7.3	34.9 ± 8.3	0.68
First symptoms to fingolimod start (y), mean (SD)	6.6 ± 5.6	7.4 ± 6.1	4.1 ± 5.6	0.85
Previous immunomodulating drugs	15 naïve 10 IFNb (6IFNb-1a IM, 3 IFNb 1a sc, 1 IFNb1b sc) 1 GA 9 NTZ 1 diazoxide	10 naïve 8 IFNb (4IFNb-1a IM, 3 IFNb 1a sc, 2 IFNb1b sc) 1 GA 1 NTZ 8 NTZ 1 diazoxide	5 naïve 2 IFNb (2IFNb-1a IM) 1 NTZ	0.56
Number of previous treatment (y), mean (SD)	1 ± 1.1	1.1 ± 1.1	0.5 ± 0.8	
0 treatment	15	10	5	0.73
1 treatment	10	8	2	
2 treatments	8	7	1	
≥3 treatments	3	3	0	
Washout period (mo), mean (SD)	2.7 ± 4.1			
Injectable drugs ^a	2.7 ± 5.5	3 ± 6.1	1 ± 0	0.6
NTZ	2.4 ± 1.5	2.1 ± 1.2	5	
Diazoxide	5	5	–	

GA, glatiramer acetate; IFNb, interferon beta; NR, nonresponder; NTZ, natalizumab.

^aInjectable drugs: Interferon beta + glatiramer acetate.

EDSS score, or ARR in the 12 months prior to fingolimod treatment between groups (Table 2).

Regarding the previous MS treatment, patients were classified as naïve (n = 15) and patients who had received previous treatment, (i) Betaferon®, Rebif®, Avonex®, Copaxone®, or Extavia® (injectable drugs) (n = 12), or (ii) NTZ, who had stopped due to risk of progressive multifocal leukoencephalopathy (n = 9). The demographic and clinical characteristics of patients analyzed are shown in Table 2.

3.2 | Treatment efficacy

The ARR was significantly reduced during the follow-up compared to the 12 months prior to fingolimod treatment (0.6 ± 0.8 vs 1.6 ± 1.2 , $P < 0.001$). The distribution of patients depending on the previous treatment showed a reduction in ARR of injectable drugs (from 1.5 ± 1.4 to 0.5 ± 0.7 ; $P < 0.01$) and naïve patients (from 1.7 ± 1.1 to 0.9 ± 0.9 ; $P < 0.05$). However, in patients who had received NTZ, the ARR remained unchanged during fingolimod treatment (from 0.6 ± 0.9 to 0.7 ± 1.1 ; $P = 0.625$). A significant reduction in the ARR was also observed when patients were stratified according to the clinical response. R patients showed 0.4 ± 0.7 ARR compared to the 12 months prior to fingolimod treatment (1.6 ± 1.5) ($P < 0.001$).

No significant reduction in the ARR was observed for NR ($P = 0.73$) (Table 3). NR group only included 1 patient with previous NTZ treatment (Table 3).

No statistically significant differences were observed in EDSS score after 12 months of treatment in the R and NR group ($P = 0.77$; $P = 0.6$; respectively) (Table 3).

The proportion of patients free of new/enlarged T2 lesions in R patients was 52% and in NR patients was 13% at month 12. A total of 8 patients had new Gd+ T1 lesions at month +12 (24% of R patients and 80% of NR patients) (Table 3).

3.3 | Adverse effects

Fingolimod treatment caused at least 1 adverse effect during the follow-up in 35 patients (80%) (Table 4). Most of the adverse effects were considered mild or moderate in severity.

A total of 29 patients (66%) presented lymphopenia, being counts ≤ 200 lymphocytes/ μ L in 5 of them (Table 4). Infections (especially upper respiratory tract and urinary tract) were observed in 51% of the patients and were either mild or moderate. No correlation was found between peripheral leukocyte distribution before starting treatment and a higher infection risk (data not shown).

TABLE 3 Analysis of clinical characteristics of the patients stratified according response to fingolimod at month +12 after treatment (n = 36)

	Responders (R) (n = 28)			Nonresponders (NR) (n = 8)		
	Baseline	+12 mo	P-value	Baseline	+12 mo	P-value
ARR mean (SD)	1.6 (1.5)	0.4 (0.7)	0.001	1.5 (0.5)	1.6 (0.7)	0.73
Total patients						
Naïve patients	1.7 (1.3)	0.6 (0.8)	0.048	1.6 (0.5)	1.7 (0.5)	1
Treated patients	1.5 (1.5)	0.2 (0.5)	0.008	1.3 (0.6)	1.8 (1.2)	0.74
Injectable drugs ^a patients	2 (1.2)	0.1 (0.3)	0.003	1.5 (0.7)	1 (0)	0.5
NTZ patients	0.5 (0.9)	0.4 (0.7)	0.8	1	1.7	–
EDSS (median ± SD)	2.9 ± 1.6	2.7 ± 1.8	0.77	2.5 ± 1.9	3.2 ± 1.9	0.6
Relapses-free patients, n. of patients (%)		21 (75)			0 (0)	
Progression-free patients, n. of patients (%)		26 (93)			3 (38)	
New/enlarged T2 lesions, n. of patients (%)						
0		14 (52)			1 (13)	
1		10 (37)			2 (25)	
2		2 (7)			0 (0)	
≥3		1 (4)			5 (63)	
Gd enhanced lesions						
Gd+		4 (24)			4 (80)	

ARR, annualized relapse rate; EDSS, Expanded Disability Status Scale; Gd, gadolinium contrast; NR, nonresponder; NTZ, natalizumab.

^aInjectable drugs (Interferon beta + glatiramer acetate).

TABLE 4 Patients' adverse effects after start fingolimod treatment

Adverse effects	n. of patients, (%)
AV block	2 (5)
Hepatotoxicity ^a	4 (9)
Lymphopenia	29 (64)
Lymphopenia >200 and <500/μL	24 (53)
Severe lymphopenia ≤200/μL	5 (11)
Infections	23 (51)
≥500 lymphocytes/μL	9 (20)
<500 lymphocytes/μL	14 (31)
≤200 lymphocytes/μL	3 (7)

AV, atrioventricular.

^aHepatotoxicity (alanine aminotransferase level increase more than 5 times the upper limit of the normal range).

3.4 | Fingolimod treatment affects mainly T and B lymphocytes

Total lymphocyte counts were drastically reduced after the first month of treatment compared to baseline data. These differences remained stable until the end of follow-up (baseline:

1890 ± 957 cells/μL; month +1: 569 ± 280 cells/μL; mean during the follow-up: 520 ± 182 cells/μL [$P < 0.001$]) (Table S3). The main changes in lymphocyte subpopulations also occurred after the first month of fingolimod treatment. Fingolimod treatment induced already in month +1 a decrease in percentages of (i) CD3⁺ T cells (baseline: 74.94 ± 6.8%; month +1: 53.68 ± 16.1% [$P < 0.001$]), (ii) CD4⁺ T cells (baseline: 60.06 ± 9.4%; month +1: 29.03 ± 14.2% [$P < 0.001$]), and (iii) CD19⁺ cells (baseline: 12.77 ± 6%; month +1: 6.09 ± 3.5% [$P < 0.001$]). The percentages of CD8⁺ T cells, however, significantly increased from month +1 (baseline: 33.82 ± 8.8%; month +1: 54.38 ± 14.1% [$P < 0.001$]). Differences remained stable until 12 months after treatment (Tables S1 and S2).

An in-depth analysis of CD4⁺ and CD8⁺ T lymphocyte subsets showed that from month +1 fingolimod treatment induced (i) a significant decrease in the percentage of central memory (T_{CM}, CCR7⁺ CD45RA[−]) (CD4⁺ T_{CM}: baseline: 39.27 ± 11.3%; month +1: 31.47 ± 12.3%; CD8⁺ T_{CM}: baseline: 10.78 ± 7.4%; month +1: 6.09 ± 5.1% [$P < 0.001$]) and naïve (CCR7⁺ CD45RA⁺) T cells (CD4⁺ naïve: baseline: 36.85 ± 12.93%; month +1: 12.8 ± 8.56%; CD8⁺ naïve: : baseline: 38.67 ± 18.5%; month +1: 5.65 ± 4.9% [$P < 0.001$]), and (ii) a significant increase in the percentage of effector memory (T_{EM}, CCR7[−] CD45RA[−]) (CD4⁺ T_{EM}: baseline: 21.26 ± 9.64%; month +1: 49.07 ± 15.53%; CD8⁺ T_{EM}: baseline: 26.69 ± 11.4%; month +1: 38.89 ± 17% [$P < 0.001$]) and terminal

differentiated effector memory (T_{EMRA}^+ , $CCR7^- CD45RA^+$) T cells ($CD4^+ T_{EMRA}^+$: baseline: $1.77 \pm 4.44\%$; month +1: $6 \pm 11.1\%$; $CD8^+ T_{EMRA}^+$: baseline: $20.68 \pm 18.1\%$; month +1: $43.43 \pm 22.6\%$ [$P < 0.001$]) (Table S1).

Analysis of B lymphocytes ($CD19^+$ cells) revealed a decrease in both percentage and absolute number of B cells from the start of fingolimod treatment. However, the percentage of naïve B cells ($CD27^- IgD^+$) showed a significant increase from month +3 until the end of follow (baseline: $59.28 \pm 16.6\%$; month +3: $63.83 \pm 19.7\%$ ($P < 0.05$) (Table S2).

Our results also showed a significant increase in transitional ($CD24^{hi} CD38^{hi} CD27^- IgD^+$) and exhausted ($CD27^- IgD^-$) B-cell percentages ($P < 0.001$). In contrast, a decrease in percentages of unswitched ($CD27^+ IgD^+$) and switched memory ($CD27^+ IgD^-$) B cells was observed, starting in month +3 and lasting until month +12 ($P < 0.01$). No differences in the percentage of plasma cells ($CD38^{hi} CD27^+ IgD^-$) were found during follow-up (Table S2).

Fingolimod treatment also altered natural killer cells (NK), monocytes, and dendritic cells (DC) populations. Our results showed that from month +1 after starting treatment, the percentages of NK cells, monocytes, and myeloid DC (mDC) were increased ($P < 0.001$). No differences were observed in minor subpopulations of NK cells ($CD56^{bright} CD16^-$ and $CD56^{dim} CD16^+$) and monocytes ($CD14^+ CD16^-$ and $CD14^{low} CD16^+$) (Table S2).

3.5 | Pro- and antiinflammatory balance in lymphocytes subsets following fingolimod treatment

While analyzing specific Th subpopulations, we observed that the percentages of $Th1_{EM}$ ($CD4^+ CCR7^- CD45RA^- CCR6^- CXCR3^+$) increased and of $Th1_{CM}$ ($CD4^+ CCR7^+ CD45RA^- CCR6^- CXCR3^+$) decreased compared with baseline data from patients under fingolimod treatment (Figure 1A,B) (Table S1). These changes were already present at the first month of treatment ($P < 0.001$). When analyzing the number of cells, $Th1_{EM}$ remained unchanged until month +12 of treatment (Table S3) although there was an important decrease in $Th1_{CM}$ counts.

Data also revealed that fingolimod induced a significant increase in percentage of $Th17_{EM}$ ($CD4^+ CCR7^- CD45RA^- CCR6^+ CXCR3^+$) from month +1 while $Th17_{CM}$ ($CD4^+ CCR7^+ CD45RA^- CCR6^+ CXCR3^+$) were significantly reduced (compared with baseline levels) from month +3 ($P < 0.01$) until the end of follow-up (Figure 1C,D and Table S1).

$Th1Th17$ lymphocytes—another Th subset not formerly studied in patients under fingolimod treatment—were analyzed too. Whereas $Th1Th17_{EM}$ ($CD4^+ CCR7^- CD45RA^- CCR6^+ CXCR3^+$) percentages were increasing from month +1, $Th1Th17_{CM}$ ($CD4^+ CCR7^+ CD45RA^- CCR6^+ CXCR3^+$) percentages were decreased progressively, being these differences statistically significant from month +3 until the end of follow-up ($P < 0.01$) (Figure 1E,F and Table S1).

Fingolimod treatment induced a significant increase in the percentage of several regulatory subpopulations: activated T_{reg} ($CD4^+ CD127^- CD25^+ CCR4^+ CD45RO^+ HLA-DR^+$), memory T_{reg} ($CD4^+ CD127^- CD25^+ CCR4^+ CD45RO^+$) (Figure 1G), and transitional B cells

($CD24^{hi} CD38^{hi} CD27^- IgD^+$) (Figure 1H), from month +1 until the end of follow-up ($P < 0.001$) (Tables S1 and S2).

3.6 | Baseline differences in lymphocyte subpopulations in fingolimod R patients

After analyzing data from 36 RRMS patients to find correlation between percentages and absolute number of leukocyte subpopulations and clinical response, we found that at baseline, fingolimod responders showed (i) a lower percentage of RTE lymphocytes ($CD4^+ CD27^+ CCR7^+ CD45RA^+ CD31^+ PTK7^+$) (R: $4.0 \pm 1.4\%$ vs NR: $7.4 \pm 1.9\%$, $P < 0.001$) (Figure 2A), (ii) lower T1 transitional B-cell numbers (R: 4.2 ± 5.1 cells/ μ L vs NR: 9.4 ± 7.0 cells/ μ L, $P < 0.01$) (Figure 2B), and (iii) a higher percentage of $CD8^+$ T cells (R: $36.1 \pm 8.8\%$ vs NR: $27.2 \pm 5.2\%$, $P = 0.013$) (Figure 2C) similarly to our preliminary study.⁷

This study revealed also differences in other lymphocyte subpopulations at baseline between R and NR patients. R patients showed (i) a lower percentage of double negative T cells (R: $4.0 \pm 2.4\%$ vs NR: $8.2 \pm 5.9\%$, $P = 0.015$) (Figure 2D), (ii) a lower CD4/CD8 ratio (R: 1.8 ± 0.72 vs NR: 2.40 ± 0.54 , $P < 0.05$) (Figure 2E), and (iii) a lower percentage of $Th1Th17_{CM}$ (R: $11.8 \pm 5.3\%$ vs NR: $18.5 \pm 4.5\%$, $P < 0.05$) (Figure 2F).

Although RTE lymphocytes and transitional B cells were significantly different at baseline and remained different until the end of follow-up in both groups, differences in other subpopulations found at baseline between R and NR patients disappeared along treatment.

4 | DISCUSSION

Clinical heterogeneous response to different treatments exists in MS patients,¹¹ possibly partially due to the heterogeneity of MS pathology. This is why an exhaustive analysis of the immunological background of the patients and knowledge on their ability to respond to different therapeutic alternatives is still needed.^{12–16} We examined in the present study changes in leukocyte subpopulations and their association with clinical and brain MRI activity in RRMS patients before and during 1 year of follow-up under fingolimod treatment. Our analysis revealed lymphocyte subpopulations that can be used as biomarkers to predict the clinical response to fingolimod at baseline, even before starting treatment. From these biomarkers, the percentage of RTE was the best parameter to differentiate R and NR patients.

Some research groups have described changes in peripheral blood subpopulations from MS patients caused by fingolimod treatment.^{17–24} Due to S1P receptor being blocked in the surface of lymphocytes, fingolimod triggers the lymphocytes to be confined in lymph nodes and induces a peripheral lymphopenia, affecting mainly naïve and central memory, but not effector memory cells. Data collected from their studies support the idea that the beneficial effect of fingolimod may be due to an increase in regulatory T cells (T_{reg}) and/or a decrease in Th17 cells in peripheral blood.^{17,20,24} In our longitudinal study, we observed major changes

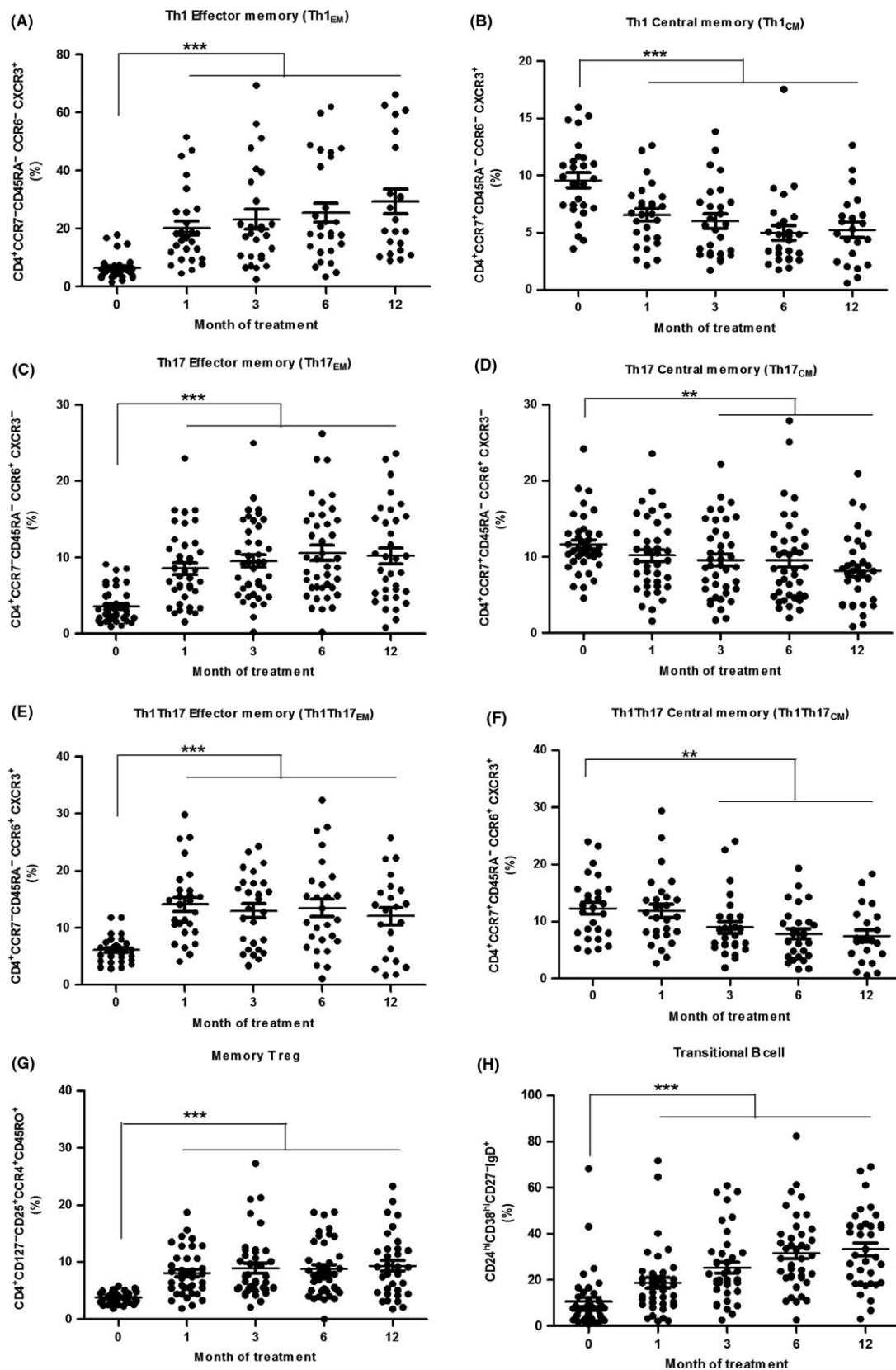


FIGURE 1 Changes in T- and B-cell subpopulations induced by fingolimod treatment in relapsing-remitting multiple sclerosis (RRMS) patients. Percentages of (A) Th1 effector memory (Th1_{EM}) (n = 26), (B) Th1 central memory (Th1_{CM}) (n = 26), (C) Th17 effector memory (Th17_{EM}) (n = 40), (D) Th17 central memory (Th17_{CM}) (n = 40), (E) Th1Th17 effector memory (Th1Th17_{EM}) (n = 26), (F) Th1Th17 central memory (Th1Th17_{CM}) (n = 26), (G) memory regulatory T cells (memory T_{reg}) (n = 40), and (H) transitional B cells (n = 40). Each dot represents the value of an individual patient. **P < 0.01; ***P < 0.001

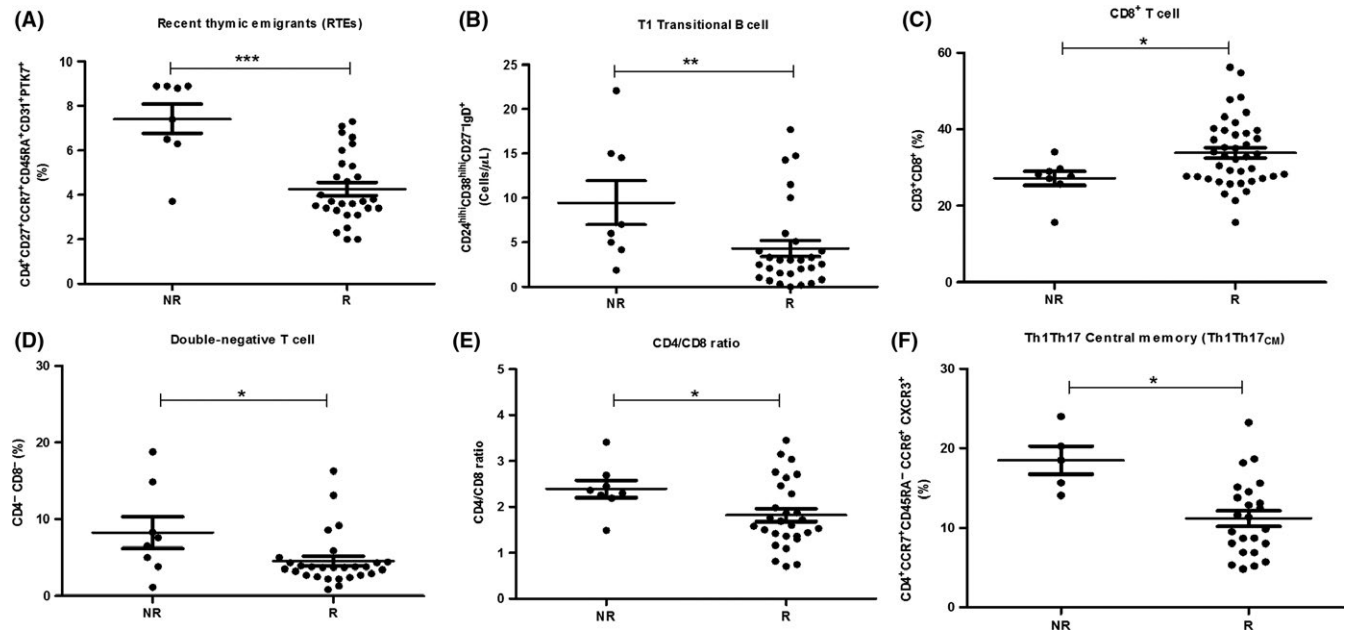


FIGURE 2 Different distribution of lymphocyte subpopulations at baseline in fingolimod responder (R) and nonresponder (NR) relapsing-remitting multiple sclerosis (RRMS) patients. (A) Percentage of recent thymic emigrants (RTEs) ($n = 36$), (B) number of T1 transitional B cells ($n = 36$), (C) percentage of CD8⁺ T cells ($n = 36$), (D) percentage of CD4⁺ CD8⁻ (double negative) T cells ($n = 36$), (E) CD4/CD8 ratio ($n = 36$), and (F) percentage of Th1Th17 central memory (Th1Th17_{CM}) ($n = 22$). Each dot represents the value of an individual patient. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

induced in leukocyte subpopulations already 1 month after starting the fingolimod treatment. As expected, the percentage and absolute number of naïve and T_{CM} cells were reduced and remained stable until the end of follow-up. In accordance with other studies,^{7,20} we also found an increase in regulatory cells (transitional B cell and T_{reg}). However, and with the exception of our preliminary work⁷, there are no studies that correlate leukocyte subpopulations and the clinical outcome in MS patients before starting fingolimod treatment.

The results of the present multicenter study confirm our preliminary results pointing out different percentages of RTE at baseline among R (exhibiting similar values to healthy donors²⁵) and NR patients (presenting higher values).⁷ The RTE lymphocytes are the most immature and recently released cells to periphery from thymus. Our hypothesis is that NR patients may show alterations in the process of negative selection of T cells in thymus, thus leading to an increase in autoreactive cells and RTE in periphery. The peripheral lymphopenia induced by fingolimod might decrease the capacity of the peripheral mechanisms of tolerance to overcome this central failure. Moreover, recent *in vitro* experiments have shown that, in an inflammatory environment, RTE lymphocytes can be converted into competent effectors cells.²⁶

In the past years, it was quite difficult to compare results from studies analyzing RTE due to the lack of specific markers for this immature stage of T cells. Recently, PTK7 (protein tyrosine kinase 7) was identified and reported as the best surrogate marker of RTE.²⁷ PTK7⁺ cells are CD31⁺ and have high levels of signal joint TCR gene excision circles (sjTREC), the most frequently used molecular marker to define RTE lymphocytes.²⁷

When the age of healthy donors was taken into account, a decrease in the percentage of RTE was reported, probably due to a less effective thymopoiesis.²⁸ Importantly, while using the same markers, R and NR patients in our study did not show any differences related to age, excluding it from being the cause of differences found in the percentages of RTE. The influence of previous NTZ treatment on the baseline levels of RTE remains to be clarified. Our group found in a previous work that NTZ stimulates the release of hematopoietic precursors and that patient under NTZ treatment shows an increase in RTE and transitional B cells.²⁹ In the present study, we analyzed differences in RTE percentage at baseline between naïve and treated patients (either with injectable drugs or with NTZ), to discard a possible influence of former treatments on RTE baseline levels. Remarkably, the NR group only included 1 patient who was previously treated with NTZ, and it had a washout period of 5 months, discarding in this way influence of former NTZ treatment on higher levels of RTE found in the NR.

The main limitation of this study is the small cohort of nonresponders with different treatment histories and washout periods. In order to use this biomarker in clinical practice—alone or in combination with others—analysis of a large cohort of patients is required to define the optimal RTE threshold able to discriminate between R and NR.

In conclusion, the results of this study strongly suggest that RRMS patients can be stratified in R and NR to fingolimod before starting the treatment. We found the RTE percentage as the best predictor of clinical response. And finally, these results also show the relevance of immune-monitoring MS patients. Identifying biomarkers of clinical response permits to know in advance to which

treatment MS patients will respond or not and will be a big step toward personalizing medicine in MS.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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