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Induction of apoptosis in CD4(+) T-cells is linked with optimal treatment response in patients with relapsing-remitting multiple sclerosis treated with Glatiramer acetate

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ABSTRACT

Background: Induction of T-cell apoptosis constitutes a mechanism of action for Glatiramer Acetate (GA). We investigated whether activation of apoptotic T-cell death may be indicative of optimal treatment response in patients with relapsing-remitting Multiple Sclerosis (RRMS), with respect to radiological activity.

Methods: We studied apoptotic markers on blood T-cells of forty patients with RRMS, 19 patients under GA and 21 patients under interferon-β (IFNβ), by flow cytometry. Patients were relapse-free and were further classified into optimal and sub-optimal responders based on radiological activity. Eighteen patients (8 patients under GA and 10 patients under IFNβ were additionally evaluated at a 12-month follow-up and were relapse- and radiological activity-free. For these patients, apoptosis was also evaluated by molecular techniques.

Results: At inclusion, optimal responders to GA exhibited increased (23.6 ± 1.976) relative % frequency of CD4(+)AnnexinV(+)7AAD(−) T-cells, compared to sub-optimal responders (14.478 ± 1.204, $p = 0.001$). Similarly, relative % frequency of caspase-3(+) T-cells was 1.517 ± 0.436 versus 0.45 ± 0.149 ($p = 0.041$), respectively. Anti-apoptotic molecule bcl-2 showed an inverse pattern 4.532 ± 1.321 versus 13.094 ± 3.987, $p = 0.044$, respectively. These differences were not evident for IFNβ-treated patients.

Conclusions: T-cell apoptotic markers may be applied as a biomarker useful in evaluating optimal treatment response under GA, thus allowing for personalized treatment decisions.

1. Introduction

Altered apoptotic profile has recently been reported in the T-cells of patients with multiple sclerosis (MS). Resistance of T-cells towards Fas-mediated apoptosis has been implicated in MS pathogenesis [1] and disease exacerbation [2]. Increased Fas/Fas-L expression levels in peripheral blood mononuclear cells (PBMCs) from patients with Relapsing-Remitting MS (RRMS) and Secondary Progressive MS (SPMS) have been linked with more favorable outcome with respect to long-term disability worsening/progression [3]. Moreover, the potential of disease modifying treatments (DMTs) to interfere with T-cell apoptotic pathways has been proposed as a mechanism of action mediating disease remission. Induction of tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL)-mediated apoptotic pathway has been associated with optimal treatment response in patients with RRMS receiving Interferon-β (IFNβ) treatment [4], a long-used DMT.

Glatiramer acetate (GA), previously known as copolymer 1, structurally imitates myelin basic protein (MBP), a well characterized myelin

Abbreviations: GA, Glatiramer acetate; IFNβ, interferon-β; PBMCs, peripheral blood mononuclear cells; NEDA, No Evidence of Disease Activity; EDSS, Expanded Disability Status Scale; MSFC, Multiple Sclerosis Functional Composite; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis inducing ligand; MBP, myelin basic protein; Th1, T-helper type-1; Th2, T-helper type-2; Bax, Bcl-2-associated X protein; Card4, caspase activation and recruitment domain family - member 4; Casp3, caspase 3; Casp9, caspase 9; Cse1L, cellular apoptosis susceptibility gene; Dapk3, death-associated protein kinase 3; DR3, death receptor 3; DR6, death receptor 6; Gorasp1, golgi reassembly-stacking protein 1; Madd, MAP-kinase activating death domain

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antigen. GA's potential to attenuate the production of autoreactive T-cells specific for myelin antigens has been demonstrated in vitro [5], as well as its capacity to enhance a shift from T-helper (Th) type-1 (Th1) to Th type-2 (Th2) effector profile [6], a cell population with suppressive properties [7]. With respect to peripheral immune responses, GA is known to modify the function of antigen-presentating cells (APCs), namely, dendritic cells and monocytes, in order to shift cytokine production from APCs towards anti-inflammatory repertoire [8,9]. Moreover, by acting as altered peptide ligand (APL) showing structural homology to the encephalitogenic myelin antigen MBP, GA antagonizes T-cell receptor (TCR) recognition, thus inhibiting the activation of autoreactive T-cell clones [10]. GA exhibits the ability to induce anti-inflammatory T-effector profile, as means of “bystander suppression mechanisms” [11]. GA-treated mice and humans produce modulatory suppressor cells of the Th-2/3 subtype [6,7,12]. Moreover, GA treatment leads in increased production of T-regulatory cells, characterized by activation of the transcription factor forkhead box P3 (Foxp3) [13]. The effect of GA treatment on B-cells has also been recently evaluated, especially in the context of the newly acknowledged role of B-cell mediated CNS autoimmune responses. Long-term GA treatment has been described by numerous researchers to induce the development of anti-GA antibodies, with isotype switching from IgG1 to IgG4 [14–20]. Although the clinical relevance of GA-speacific IgG4 is being disputed [15,19], a shift towards IgG1 production relative to IgG2 indicates the induction of Th2 anti-inflammatory immune response, following GA treatment [16]. Moreover, GA treatment was shown to induce a decrease in the relative frequency of circulating B cells in patients with RRMS [21]. As B-cells are often recruited as antigen-presenting cells, this observation poses prominent implication for GA-mediated antigen-presentation.

Evidence of GA-mediated neuroprotective effect has also been recently provided, by the demonstration that T-cells stemming from GA-treated patients secrete brain derived neurotrophic factor (BDNF) [22,23]. GA-treated mice also exhibit increased levels of BDNF in the CNS [23], as well as other neurotrophic and growth factors, such as neurotrophin (NT)-3 and NT4 [23], insulin-like growth factor (IGF)-1 [24] and IGF-2 [25] and GA treatment has been shown to lead in rescuing BDNF levels in the serum and the cerebral spinal fluid (CSF) of MS patients [26].

However, the molecular pathways involved in T-cell proliferation and function in the context of GA treatment remain to be fully elucidated. Apoptosis seems to constitute a central mechanism in T-cell modulation by GA. GA was found to increase T-cell apoptosis in patients with RRMS over the first year of treatment, compared to pre-treatment conditions [27]. Increased expression of several apoptotic markers has been reported in T-cells of patients with RRMS receiving GA, such as bcl-2, bax and Cyt-c [28]. Moreover, a panel of genes implicated in several intracellular apoptotic pathways ultimately leading in caspase 9 and caspase 3 activation was found to be primarily upregulated in patients with RRMS under GA [29].

Gene expression analysis has been used in order to elucidate sets of biomarkers with the potential to predict relapse probability in RRMS [30]. However, approximately 10 new clinically silent new MRI T2 lesions correspond in one clinical relapse [31–33] and the discrepancy between T2 lesion burden and clinical disability seems to increase for disability estimates higher than that corresponding to Expanded Disability Status Scale (EDSS) score 4.0 [34]. New or enlarging T2 lesions, as well as gadolinium (Gd) enhancement account for radiological activity in RRMS [35,36]. The importance of radiological activity with respect to long-term disability outcome, even in the absence of clinical relapses is underlined by the recommendation for annual MRI evaluation in all patients with RRMS [37,38] and by its consideration when contemplating DMT escalation [39–41].

The present study aims to investigate whether activation of apoptotic cell death in T-cells, in the context of GA treatment, may be indicative of optimal treatment response in patients with RRMS, compared to sub-optimal response, as indicated by the presence of radiological activity.

2. Materials and methods

2.1. Patients

Forty patients with RRMS, 19 patients under GA and 21 patients under IFNβ, fulfilling the 2010 revised McDonald's diagnostic criteria [42] were included in the present study. All patients were followed by the Multiple Sclerosis Center of the B′ Neurological Department of the AHEPA University Hospital in Thessaloniki. The protocol for the present study was approved by the Hospital's Scientific Committee and was according to the ethical principles defined by the Declaration of Helsinki. All patients were informed by the treating neurologist regarding the aim and procedures of the present study and signed an informed consent form prior to their inclusion. All patients needed to receive the respective DMT for at least 12 months prior to their inclusion to the study. Steroid treatment within 6 months and immunosuppressive treatment at any point prior to the inclusion to the study served as exclusion criteria. Demographics, EDSS assessment and other clinical disease parameters were collected at inclusion to the study. All patients included in the study underwent routine blood analysis and absolute counts of white blood cells, as well as lymphocytes, were comparable and within normal limits. Radiological activity was defined according to Rio et al. [43] as at least two new T2 and/or at least one Gd(+) MRI lesion at inclusion, compared to routine MRI evaluation 12 months before. Clinical activity was defined as either at least one relapse and/or disability progression during the last 12 months. Therefore, the 12 months prior to the inclusion were used in order to evaluate clinical and radiological activity at inclusion, as suggested in [44]. Based on their MRI at inclusion, all patients were classified as either optimal responders, further mentioned as “responders”, or as sub-optimal responders, in the presence of radiological activity. Sub-optimal responders exhibited only radiological activity, thus not reaching the threshold, according to the Rio score, in order to be classified as non-responders. Moreover, the radiological burden alone in the absence of clinical relapse or increase in disability did not dictate treatment escalation. All patients in the present study remained free of relapses and free of disability worsening. Therefore, in the absence of radiological activity, patients classified as responders are the ones that practically achieved the No Evidence of Disease Activity (NEDA) criterion. At the end of a 12-month follow-up, EDSS assessment, as well as brain and cervical MRI evaluation were repeated. All MRI evaluations were conducted at a 1.5 Tesla scanner.

2.2. Flow cytometry

For analysis of markers of apoptosis by means of flow cytometry, EDTA blood was collected at inclusion and at the end of the follow-up. PBMCs isolation was conducted immediately by Ficoll gradient centrifugation and cells were stained for flow cytometry. Monoclonal antibodies and reagents used are shown in Supplementary Table 1. Flow cytometry analysis evaluated relative % frequency of caspase3(+), bcl-2(+), Annexin V(+) 7AAD(−) [early apoptotic] and Annexin V(+) 7AAD(+) [late apoptotic/necrotic] CD4(+) T-cells. Gating strategy is illustrated in Supplementary Fig. 1. Samples were acquired at the level of at least 50×10^3 cells. All samples were acquired in a FACSCalibur (BD) by the use of CellQuest software and analyzed by FlowJo software (TreeStar).

2.3. Real-time polymerase chain reaction (RT-PCR)

For analysis of markers of apoptosis by means of molecular techniques, whole blood was collected into PAXgene™ Blood RNA Tubes (Qiagen) at inclusion and at the end of the follow-up, and retained at

−20 °C until further processing. RNA was extracted using PAXgene RNA extraction kit (Qiagen) and was reverse transcribed to cDNA using Iscript cDNA Synthesis Kit (Biorad) following manufacturers' instructions. Real-time PCR was performed using iTaq Universal SYBR Green supermix (Biorad) in the IQ5 ICycler Multicolor Detection System (Biorad). The relative gene expression was normalized to β-actin which served as a housekeeping gene. The results were analyzed using the ΔΔCt method. Primers were designed for B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax), caspase activation and recruitment domain family - member 4 (Card4), caspase 3 (Casp3), caspase 9 (Casp9), cellular apoptosis susceptibility gene (Cse1L), death-associated protein kinase 3 (Dapk3), death receptor 3 (DR3), death receptor 6 (DR6), Fas, golgi reassembly-stacking protein 1 (Gorasp1), MAP-kinase activating death domain (Madd) and b-actin using Primer3 (Supplementary Table 2).

2.4. Statistical analysis

Results were presented as mean ± standard error (SE) of mean. Fisher's exact test was used for the relative frequency comparison of discrete variables. For continuous variables, normality was assessed by the use of Kolmogorov-Smirnov Test. For the comparison of means, either Independent – Samples' *t*-test and One-Way Analysis of Variance (ANOVA), or the non-parametric equivalents Mann-Whitney and Kruskal-Wallis tests were used followed by Bonferroni's or Dunn's Post-Hoc comparisons, respectively. Statistical analysis was conducted by the use of IBM SPSS Software 16.0 and GraphPad Prism 5.0 (GraphPad Software). Graphs were illustrated by the use of GraphPad Prism. Statistical significance was set at the level of $p < 0.05$.

3. Results

3.1. Patients' characteristics

Table 1 shows demographics, clinical and radiological characteristics at inclusion. Overall, 40 patients participated in the present study, 19 patients under GA, of which 10 responders and 9 sub-optimal responders and 21 patients under IFNβ, of which 10 responders and 11 sub-optimal responders. Twelve-month follow-up data were available for 8 patients under GA, of which 4 initially responders and 5 initially sub-optimal responders and 10 patients under IFNβ, of which 5 initially responders and 5 initially sub-optimal responders. All patients that underwent the follow-up evaluation proved to be responders at this stage. With respect to the patients that did not complete their participation in the study, twelve were switched to an oral first-line DMT, and ten were lost to follow-up. Low adherence to clinical follow-up is common among patients under long-term treatment with first-line DMTs that exhibit sustained clinical remission, in spite of the detailed information provided by the treating neurologist regarding the indicated routine clinical and laboratory assessment. Molecular analysis for apoptotic markers was conducted only for patients who completed the follow-up.

Patients' groups were matched for age ($p = 0.06$) and gender ($p = 0.427$). Overall, patients across groups exhibited low levels of disability, as indicated by low (< 3.0) mean EDSS scores ($p = 0.878$). With respect to treatment duration in months, the majority of patients exhibited long-term treatment with the respective DMT of comparable duration, as indicated in Table 1 (65 ± 13.91 for responders under GA, 47 ± 11.668 for sub-optimal responders under GA, 54.6 ± 12.938 for responders under IFNβ and 89.091 ± 15.841 for sub-optimal responders under IFNβ, $p = 0.168$).

Brain T2 burden at inclusion was comparable across patients' groups (13.7 ± 2.902 for responders under GA, 21 ± 3.753 for sub-optimal responders under GA, 17.2 ± 2.01 for responders under IFNβ and 14.909 ± 1.928 for sub-optimal responders under IFNβ, $p = 0.234$), as was the distribution of lesions in periventricular (8.4 ± 1.74 for responders under GA, 8.778 ± 2.08 for sub-optimal responders under GA, 7.4 ± 0.718 for responders under IFNβ and 6.909 ± 1.179 for sub-optimal responders under IFNβ, $p = 0.864$), juxtacortical (3 ± 0.83 for responders under GA, 6.222 ± 1.605 for sub-optimal responders under GA, 3.7 ± 0.7 for responders under IFNβ and 3.273 ± 0.469 for sub-optimal responders under IFNβ, $p = 0.091$) and infratentorial areas (2.2 ± 0.49 for responders under GA, 2.111 ± 0.754 for sub-optimal responders under GA, 1.8 ± 0.467 for responders under IFNβ and 2.091 ± 0.436 for sub-optimal responders under IFNβ, $p = 0.897$). As by definition, sub-optimal responders under GA and sub-optimal responders under IFNβ exhibited increased new T2 lesion burden (2.556 ± 0.294 and 2.456 ± 0.207, respectively) compared to responders under GA and IFNβ who did not show evidence of new T2 lesions in brain MRI ($p < 0.001$). Although a similar pattern was observed with respect to Gd enhancing lesions, the difference across groups was not significant ($p = 0.22$).

As expected by the low levels of disability, also spinal T2 lesion burden was relatively low and comparable across patients' groups

Table 1
Demographics, clinical and radiological characteristics at inclusion.

	GA responders ($N = 10$)	GA sub-optimal responders ($N = 9$)	IFNβ responders ($N = 10$)	IFNβ sub-optimal responders (N = 11)	p
Age	44.4 ± 2.377	34.778 ± 1.588	38.8 ± 1.548	39.455 ± 3.522	0.06
Gender (m/f)	2/8	5/4	3/7	4/7	0.427
EDSS score	2.5 ± 0.289	2.333 ± 0.612	2.65 ± 0.914	2.636 ± 0.37	0.878
Treatment duration[a]	65 ± 13.91	47 ± 11.668	54.6 ± 12.938	89.091 ± 15.841	0.168
Brain MRI lesion count					
T1 total[b]	7.1 ± 1.636	5.667 ± 1.462	5 ± 0.931	4.273 ± 0.864	0.596
T2 total	13.7 ± 2.902	21 ± 3.753	17.2 ± 2.01	14.909 ± 1.928	0.234
Periventricular	8.4 ± 1.74	8.778 ± 2.08	7.4 ± 0.718	6.909 ± 1.179	0.864
Juxtacortical	3 ± 0.83	6.222 ± 1.605	3.7 ± 0.7	3.273 ± 0.469	0.091
Infratentorial	2.2 ± 0.49	2.111 ± 0.754	1.8 ± 0.467	2.091 ± 0.436	0.897
New T2	0	2.556 ± 0.294	0	2.456 ± 0.207	< 0.001
Gd(+)	0	0.222 ± 0.147	0	0.182 ± 0.122	0.22
Cervical MRI lesion count					
T1 total	0.2 ± 0.422	0.778 ± 0.222	0.3 ± 0.153	0.273 ± 0.195	0.101
T2 total	2 ± 0.422	2.667 ± 0.236	2.4 ± 0.34	1.727 ± 0.384	0.277
New T2	0	0	0	0.182 ± 0.122	0.144
Gd(+)	0	0	0	0.091 ± 0.091	0.451

GA, glatiramer acetate; IFNβ, interferon-β; EDSS, Expanded Disability Status Score; m/f, male/female; Gd(+), gadolinium enhancing lesion; N/A, non-applicable.
[a] Treatment duration in months.
[b] Black holes. Values represent mean ± standard error of mean.

(2 ± 0.422 for responders under GA, 2.667 ± 0.236 for sub-optimal responders under GA, 2.4 ± 0.34 for responders under IFNβ and 1.727 ± 0.384 for sub-optimal responders under IFNβ, $p = 0.277$).

Upon follow-up evaluation all patients remained relapse-free and exhibited < 2 new T2 lesions in brain MRI (0.25 ± 0.16 for responders under GA, 0.1 ± 0.1 for responders under IFNβ, $p = 0.19$), whereas none exhibited new or Gd(+) lesions on cervical MRI (Supplementary Table 3).

3.2. Patients with RRMS under GA with evidence of radiological activity exhibit lower expression levels of T-cell apoptotic markers, compared to patients under GA with clinical and radiological remission

Supplementary Fig. 2 exhibits representative FACS plots of patients under GA and IFNβ, optimal and sub-optimal responders. Table 2 and Fig. 1 refer to the relative % expression of apoptotic markers on T-cells by means of flow cytometry at inclusion. At inclusion, patients under GA, responders (38.46 ± 1.386) and sub-optimal responders (36.7 ± 2.53), exhibited low relative % frequency of CD4(+) T-cells compared to patients under IFNβ, responders (46.43 ± 2.061, $p = 0.026$ and $p = 0.016$, respectively) and sub-optimal responders (45.582 ± 2.708, $p = 0.049$ and $p = 0.048$, respectively). With respect to DNA fragmentation analysis at inclusion, optimal responders to GA exhibited increased (23.6 ± 1.976) relative % frequency of CD4(+)AnnexinV(+)7AAD(−) T-cells (early apoptotic), compared to sub-optimal responders to GA (14.478 ± 1.204, $p = 0.001$), optimal responders to IFNβ (14.863 ± 1.17, p = 0.001) and sub-optimal responders to IFNβ (14.476 ± 1.089, $p < 0.001$). A similar pattern of expression was observed with respect to caspase-3 at inclusion. Optimal responders to GA exhibited increased relative % frequency of caspase-3(+) T-cells (1.517 ± 0.436) compared to sub-optimal responders to GA (0.45 ± 0.149, $p = 0.041$). Also in comparison to optimal responders to IFNβ (0.645 ± 0.14) and sub-optimal responders to IFNβ (0.641 ± 0.289), optimal responders to GA exhibited increased relative % frequency of caspase-3(+) T-cells, a difference that showed a trend towards an effect, although it did not reach statistical significance ($p = 0.073$ and p = 0.073, respectively). Anti-apoptotic molecule bcl-2 showed an inverse pattern, according to which optimal responders to GA at inclusion exhibited reduced relative % frequency of bcl-2(+) T-cells (4.532 ± 1.321) compared to sub-optimal responders to GA (13.094 ± 3.987, $p = 0.044$). Also in comparison to optimal responders to IFNβ (12.91 ± 4.822) and sub-optimal responders to IFNβ (15.555 ± 3.802), optimal responders to GA exhibited reduced relative % frequency of bcl-2(+) T-cells, a difference that showed a trend, although it did not reach statistical significance ($p = 0.123$ and $p = 0.073$, respectively). Due to the fact that relative % frequency of early apoptotic T-cells, of caspase-3(+) T-cells and of bcl-2(+) T-cells did not differ between optimal and sub-optimal responders under IFNβ (for early apoptotic T-cells, $p = 0.811$; for caspase-3(+) T-cells, $p = 0.573$; for bcl-2(+) T-cells, $p = 0.526$), modulation of T-cell apoptotic pathways is likely to be a GA-mediated effect.

These results were, at least in part, confirmed by molecular analysis at a transcriptional level (Table 3 and Fig. 2). At inclusion, caspase 3 relative expression was increased in optimal responders under GA compared to sub-optimal responders (1.753 ± 0.108 vs. 0.689 ± 0.253, respectively; $p = 0.029$). Also sub-optimal responders under GA exhibited reduced caspase 3 relative expression in comparison to optimal responders under IFNβ (0.689 ± 0.253 vs. 1.942 ± 0.229, respectively; $p = 0.016$). Similarly, DR3 relative expression was increased in optimal responders under GA compared to sub-optimal responders at inclusion (0.137 ± 0.048 and 0.003 ± 0.001, respectively; p = 0.029). Also sub-optimal responders under GA exhibited reduced DR3 relative expression in comparison to optimal responders under IFNβ (0.003 ± 0.001 vs. 0.163 ± 0.066, respectively, p = 0.016). In accordance to caspase-3 and DR3 association, Gorasp1 relative expression, a substrate protein for caspase-3 which, upon cleavage by caspase-3, mediates Golgi fragmentation in the frame of apoptosis, was reduced in GA responders compared to sub-

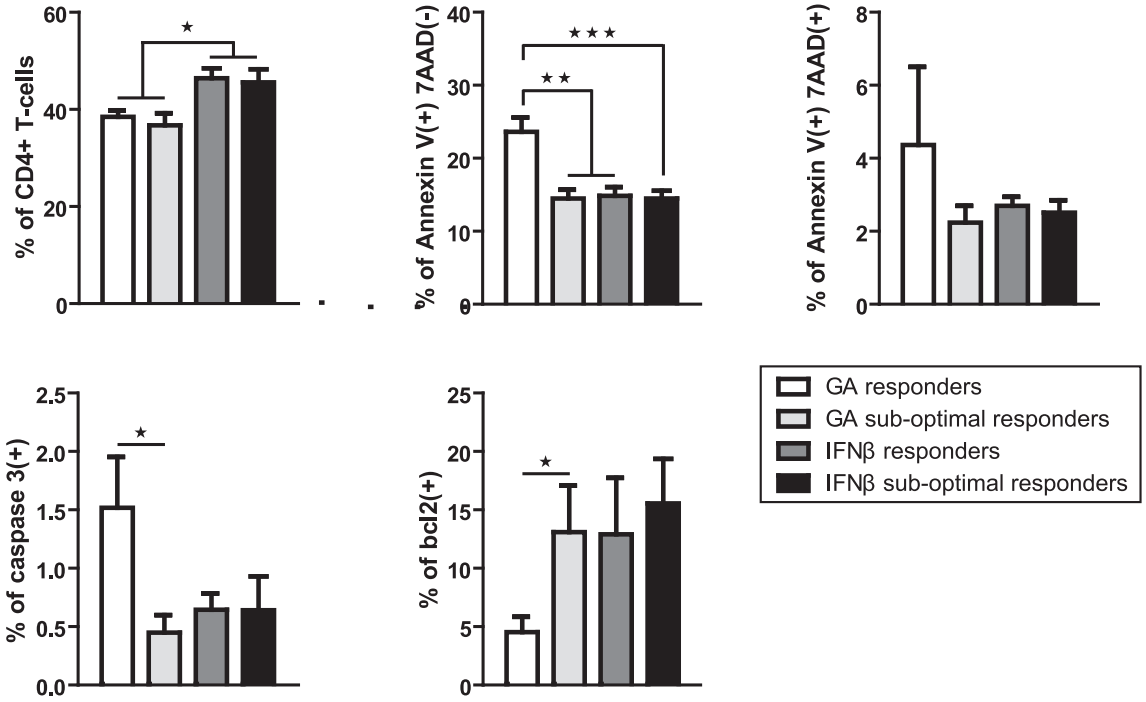

Fig. 1. Quantification of the relative % frequency of CD4(+) T-cells and the relative % expression of apoptotic markers on CD4(+) T-cells, by means of flow cytometry at inclusion. Relative % frequency of CD4(+) T-cells, of AnnexinV(+)7AAD(−) [early apoptotic], AnnexinV(+)7AAD(+) [late apoptotic/necrotic], caspase-3(+) and bcl-2(+) T-cells in patients under GA (optimal and sub-optimal responders; white and light grey bars, respectively) and IFNβ (optimal and sub-optimal responders; dark grey and black bars, respectively). Bars and error bars represent mean ± standard error of mean. GA = glatiramer acetate; IFNβ = interferon-β; Bcl-2, B-cell lymphoma 2. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Table 2
Relative % frequency of CD4(+) T-cells and CD4(+) T-cells positive for apoptotic markers at inclusion.

	GA responders (N = 10)	GA sub-optimal responders (N = 9)	IFNβ responders (N = 10)	IFNβ sub-optimal responders (N = 11)	p
CD4(+)[f]	38.46 ± 1.386	36.7 ± 2.53	46.43 ± 2.061	45.582 ± 2.708	0.019; 0.026[b]; 0.049[c]; 0.016[d]; 0.048[e]
Annexin V(+) 7AAD(−)[g]	23.6 ± 1.976	14.478 ± 1.204	14.863 ± 1.17	14.476 ± 1.089	< 0.001; 0.001[a]; 0.001[b]; < 0.001[c]
Annexin V(+) 7AAD(+)[g]	4.364 ± 2.138	2.239 ± 0.458	2.694 ± 0.248	2.507 ± 0.341	0.713
Caspase3(+)[g]	1.517 ± 0.436	0.45 ± 0.149	0.645 ± 0.14	0.641 ± 0.289	0.167; 0.041 [a]
Bcl2(+)[g]	4.532 ± 1.321	13.094 ± 3.987	12.91 ± 4.822	15.555 ± 3.802	0.143; 0.044[a]

GA, glatiramer acetate; IFNβ, interferon-β; Bcl2, B-cell lymphoma 2.
[a] GA responders vs. GA sub-optimal responders.
[b] GA responders vs. IFNβ responders.
[c] GA responders vs. IFNβ sub-optimal responders.
[d] GA sub-optimal responders vs. IFNβ responders.
[e] GA sub-optimal responders vs. IFNβ sub-optimal responders. Values represent mean ± standard error of mean.
[f] Gated on lymphocytes.
[g] Gated on CD4(+) T-cells.

optimal responders (4.976 ± 3.534 vs. 181.931 ± 65.675, respectively). Caspase-3, DR3 and Gorasp1 relative expression did not differ between optimal and sub-optimal responders under IFNβ (for caspase-3, 1.942 ± 0.229 vs. 2.322 ± 1.37, respectively, $p = 0.792$; for DR3, 0.163 ± 0.066 vs. 16.377 ± 16.299, respectively, $p = 0.421$; for Gorasp1 98.219 ± 39.643 vs. 357.541 ± 164.646, respectively, $p = 0.31$). Moreover, we did not observe significant difference between responders and sub-optimal responders under IFNβ for neither of the other apoptosis-related molecules under study. DR6 relative expression was found to be increased in responders under IFNβ compared to responders under GA (4280.746 ± 4278.615 vs. 0.021 ± 0.008, $p = 0.016$), however, this increase was not significant when compared to sub-optimal responders under IFNβ (4280.746 ± 4278.615 vs. 2.553 ± 2.106, $p = 0.222$). Similarly, although responders under IFNβ exhibited increased relative expression of caspase-3 and DR3 compared to sub-optimal responders under GA (for caspase-3 1.942 ± 0.229 vs. 0.689 ± 0.253, respectively, p = 0.016; for DR3 0.163 ± 0.066 vs. 0.003 ± 0.001, respectively, p = 0.016), they did not exhibit a significant difference with respect to these pro-apoptotic markers when compared to sub-optimal responders under IFNβ (for caspase-3 1.942 ± 0.229 vs. 2.322 ± 1.37, respectively, $p = 0.791$; for DR3 0.163 ± 0.066 vs. 16.377 ± 16.299, respectively, $p = 0.421$). We therefore conclude that modulation of T-cell apoptotic pathways is likely to be a GA-mediated effect.

3.3. Absence of radiological activity at follow-up evaluation in responders under GA is accompanied by increased expression of T-cell apoptotic markers

Upon follow-up evaluation all patients under GA and IFNβ did not exhibit radiological activity and were therefore, classified as responders. Relative % frequency of CD4(+) T-cells was comparable between patients under GA and patients under IFNβ ($p = 0.958$) (Supplementary Table 3 and Supplementary Fig. 3). Responders under GA at follow-up evaluation exhibited increased relative % frequency of early apoptotic T-cells compared to responders under IFNβ (24.98 ± 1.8 vs. 16.47 ± 0.74, $p = 0.002$), as well as increased relative % frequency of caspase-3(+) T-cells (1.35 ± 0.1 vs. 0.54 ± 0.07, $p < 0.001$). With respect to the anti-apoptotic molecule bcl-2 we observed an inverse effect, as responders under GA exhibited reduced relative % frequency of bcl-2(+) T-cells compared to responders unders IFNβ (6 ± 1.4 vs. 14.66 ± 3.26, $p = 0.031$). Moreover, responders under GA exhibited comparable expression of T-cell apoptotic markers between the inclusion and follow-up evaluation (for relative % frequency of early apoptotic T-cells $p = 0.622$; for relative % frequency of late apoptotic T-cells $p = 0.31$; relative % frequency of caspase-3(+) T-cells $p = 0.738$ and for relative % frequency of bcl-2(+) T-cells $p = 0.461$). This result was verified by molecular techniques (for caspase 3 $p = 0.467$; for caspase 9 $p = 0.576$; for Bax $p = 0.546$; for Card4 $p = 0.413$; for Cse1l $p = 0.161$; for DR3 $p = 0.59$; for DR6 $p = 0.427$; for Dapk3 $p = 0.574$; for Fas $p = 0.506$; for Corasp1 $p = 0.438$ and for Madd $p = 0.485$).

4. Conclusions

In the present study we investigated the assumption that the relative activation T-cell apoptotic death in patients with RRMS receiving first-line DMTs may determine treatment response. By combining techniques that allow assessment of T-cell apoptotic profile at both transcriptional and post-transcriptional level, we were able to correlate increased activation of apoptotic cell death in T-cells of patients with RRMS exhibiting optimal treatment response to GA administration, in terms of radiological activity, in comparison to IFNβ, thus indicating a treatment-specific effect. Activation of T-cell apoptosis in optimal responders under GA was evidenced by relative DNA fragmentation quantification, as well as by analysis at a post-transcriptional level,

Table 3
Relative expression of apoptotic markers in whole peripheral blood at inclusion.

	GA responders (*N* = 4)	GA sub-optimal responders (N = 4)	IFNβ responders (N = 5)	IFNβ sub-optimal responders (*N* = 5)	p
Caspase 3	1.753 ± 0.108	0.689 ± 0.253	1.942 ± 0.229	2.322 ± 1.37	0.175; 0.029[a]; 0.016[b]
Caspase 9	95.762 ± 70.589	264.866 ± 218.918	319.022 ± 281.173	49.947 ± 27.226	0.829
Bax	227.318 ± 220.387	2644.666 ± 1855.36	896.041 ± 461.793	4122.81 ± 3299.681	0.199
Card4	2.003 ± 1.181	1.483 ± 1.331	7.59 ± 2.786	88.655 ± 85.14	0.467
Cse1l	0.014 ± 0.005	0.565 ± 0.502	0.647 ± 0.566	0.5 ± 0.267	0.714
DR3	0.137 ± 0.048	0.003 ± 0.001	0.163 ± 0.066	16.377 ± 16.299	0.051; 0.029 [a]; 0.016 [b]
DR6	0.021 ± 0.008	128.446 ± 127.262	4280.746 ± 4278.615	2.553 ± 2.106	0.057; 0.016[c]
Dapk3	3.887 ± 3.497	2.861 ± 2.627	1338.475 ± 1338.208	14.215 ± 12.191	0.874
Fas	0.368 ± 0.153	36.626 ± 24.961	0.544 ± 0.384	7.606 ± 5.096	0.797
Gorasp1	4.976 ± 3.534	181.931 ± 65.675	98.219 ± 39.643	357.541 ± 164.646	0.039; 0.029[a]; 0.016[d]
Madd	4.596 ± 2.137	1.668 ± 1.192	5.143 ± 1.721	30.471 ± 22.253	0.417

[a] GA responders vs. GA sub-optimal responders.
[b] GA sub-optimal responders vs. IFNβ responders.
[c] GA responders vs. IFNβ responders.
[d] GA responders vs. IFNβ sub-optimal responders. GA, glatiramer acetate; IFNβ, interferon-β; Bax, B-cell lymphoma 2 (Bcl-2)-associated X protein; Card4, caspase activation and recruitment domain family - member 4; Casp3, caspase 3; Casp9, caspase 9; Cse1L, cellular apoptosis susceptibility gene; Dapk3, death-associated protein kinase 3; DR3, death receptor 3; DR6, death receptor 6; Gorasp1, golgi reassembly-stacking protein 1; Madd, MAP-kinase activating death domain. Values represent mean ± standard error of mean.

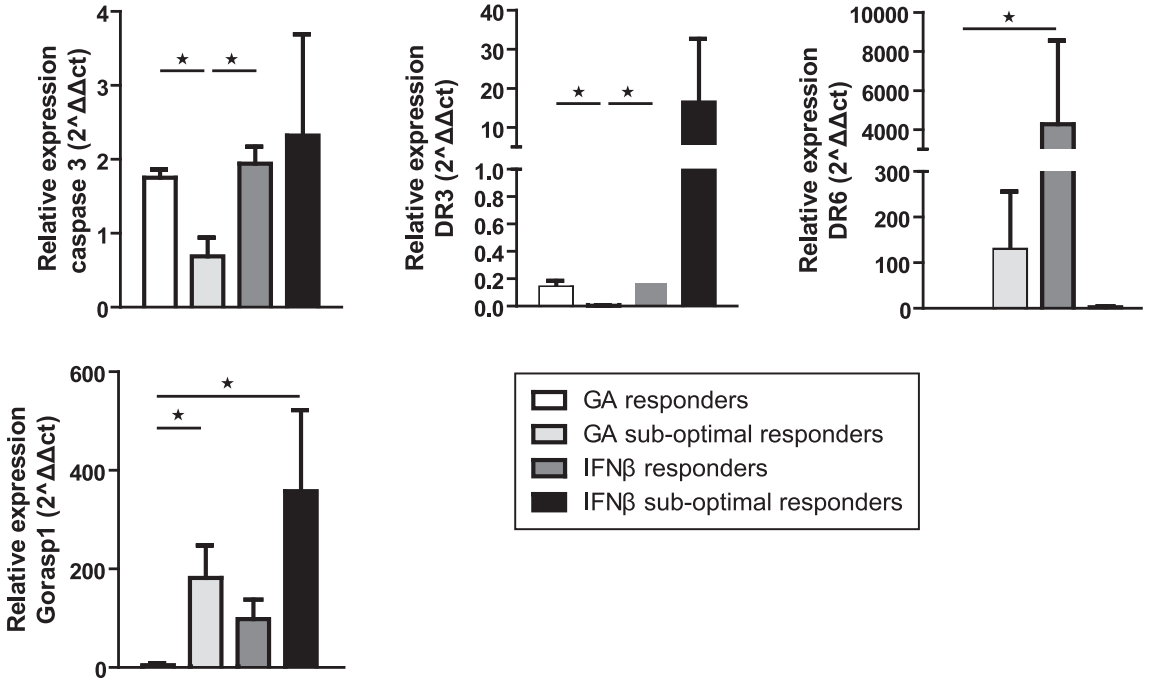

Fig. 2. Quantification of the relative expression of apoptotic markers in whole peripheral blood, by means of molecular techniques at inclusion. Relative expression of caspase-3, DR3, DR6 and Gorasp1 in patients under GA (optimal and sub-optimal responders; white and light grey bars, respectively) and IFNβ (optimal and sub-optimal responders; dark grey and black bars, respectively). Bars and error bars represent mean ± standard error of mean. GA = glatiramer acetate; IFNβ = interferon-β; DR3, death receptor 3; DR6, death receptor 6; Gorasp1, golgi reassembly-stacking protein 1. * = $p < 0.05$. Only markers for which significant associations were observed are presented.

showing increased caspase-3 and concurrent decreased bcl-2 relative expression. These differences were eliminated upon follow-up evaluation a setting allowing for direct comparison between different time-points and disease activity status in the same patient. Resolution of radiological activity in patients initially classified as sub-optimal responders under GA was linked with increased activation of T-cell apoptotic death pathways compared to inclusion, at levels comparable with optimal responders under GA, thus indicating correlation of GA's mechanism of action with MRI parameters of disease activity. Our results were, at least in part, verified by molecular analysis with respect to caspase-3 and DR3, an indirect upstream activator of caspase-3 [45], expression levels, as well as expression levels of Gorasp1, a known substrate for caspase-3 which is cleaved upon Golgi fragmentation in the frame of apoptosis and is linked, therefore, with low expression levels in apoptotic cells [46]. Our results do not indicate a GA-related effect on late apoptotic and/or necrotic cells. It is likely that the contribution of different cell death pathways for these cells is responsible for the lack of association with treatment-specific effects.

With respect to the expression of apoptosis markers in IFNβ-treated patients, both responders and non-responders exhibited comparable expression levels, similar to the sub-optimal responders to GA at inclusion. Therefore the results of the present study do not provide evidence that enhancement of apoptosis in autoreactive T-cells is a prominent mechanism of action for IFNβ. These results were also verified by molecular techniques according to which, due to large value distribution, mean difference in apoptosis expression markers did not

reach significance between IFNβ responders and sub-optimal responders.

Our study is subjected to potential limitations: first, small sample size may account for not elucidating correlation of optimal treatment response to GA with other pro-apoptotic markers under investigation, by molecular techniques. Second, additional measures, such as the Multiple Sclerosis Functional Composite (MSFC) and MRI volumetry in terms of whole brain and lesion volume may add valuable information with respect to the evaluation of overall disability. Similarly, future studies may address apoptosis profiling in additional immune cell populations known to contribute to MS pathogenesis, such as CD8(+) T-cells and B-cells. Finally, although it would be valuable to include analysis of T-cell markers of apoptosis for treatment-naïve patients with similar disease duration to the patients under GA or IFNβ, the recruitment of this group of patients poses a significant challenge, since almost all patients with RRMS are under DMTs, as suggested by the international treatment guidelines for the disease [47].

The assumption that defective apoptotic regulation of T-cell survival may be implicated in CNS inflammation in the frame of MS was suggested by an early report of reduced Fas-mediated T-cell death in patients with MS compared to healthy controls, and in patients with SPMS compared to patients with RRMS [1]. Moreover, increased expression of apoptosis inhibitor FLICE/caspase-8-Inhibitory Protein (FLIP) was found to correlate with disease activity [48]. A study on kinetics of T-cell apoptosis in patients with RRMS under GA treatment showed that Fas-mediated induction of T-cell apoptotic death may be included in the drug's immunomodulative properties [27], although other molecules modifying apoptosis mechanisms, such as Bcl-2, Bax and Cyt-c have also been implicated [28]. Moreover, clinical remission in the context of recent corticosteroid treatment in patients with RRMS was linked with increased activation of T-cell apoptosis [49] and increased PBMCs Fas/FasL expression has been associated with milder disability deterioration and progression in patients with RRMS and SPMS, respectively [3]. By the use of molecular T-cell expression profiling Achiron et al. suggested a panel of apoptotic molecules that may be used in order to evaluate optimal GA treatment response [29] within 3 months of treatment onset. Although the study provided a detailed and systematic approach in profiling the drug's immunomodulatory mode of action, it did not provide correlations with respect to optimal vs. sub-optimal treatment response in the long-term.

This is the first study to assess the effect of apoptosis-mediated T-cell death to optimal treatment response in terms of radiological activity in patients with RRMS who receive GA, compared to IFNβ. Radiological activity early in the disease course is regarded as a significant predictor of poor long-term prognosis with respect to disability progression [50]. Combined with clinical relapses and EDSS score increase, radiological activity is taken into account upon evaluation of treatment response and the need for therapy escalation [41,43]. Biomarkers of optimal treatment response have been the focus of extensive research in MS, in the scope of the variety of DMTs available to date, and the advocated potential to achieve No-Evidence of Disease Activity (NEDA) status [51–53]. However, newer DMT treatments pose significant health risks [54–56]. GA is one of the earliest first-line DMT treatments available worldwide and long-term follow-up of patients under GA indicates a low-risk safety profile [57]. The identification of optimal treatment responders based on DMT-specific mechanisms may therefore facilitate more targeted and personalized assessment of the necessity for treatment escalation, thus allowing for reduced risk exposure of patients with RRMS. Based on the present study, we hereby suggest that T-cell profiling with respect to apoptotic mechanisms may be used as a marker for optimal treatment response to GA, with implication for long-term disability outcomes.

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References

[1] C. Comi, M. Leone, S. Bonissoni, S. DeFranco, F. Bottarel, C. Mezzatesta, et al., Defective T cell fas function in patients with multiple sclerosis, Neurology 55 (7) (Oct 10, 2000) 921–927.
[2] Y. Okuda, B.R. Apatoff, D.N. Posnett, Apoptosis of T cells in peripheral blood and cerebrospinal fluid is associated with disease activity of multiple sclerosis, J. Neuroimmunol. 171 (1–2) (Feb, 2006) 163–170.
[3] L. Lopatinskaya, J. Zwemmer, B. Uitdehaag, K. Lucas, C. Polman, L. Nagelkerken, Mediators of apoptosis Fas and FasL predict disability progression in multiple sclerosis over a period of 10 years, Mult. Scler. 12 (6) (Dec, 2006) 704–709.
[4] K.P. Wandinger, J.D. Lunemann, O. Wengert, J. Bellmann-Strobl, O. Aktas, A. Weber, et al., TNF-related apoptosis inducing ligand (TRAIL) as a potential response marker for interferon-beta treatment in multiple sclerosis, Lancet 361 (9374) (Jun 14, 2003) 2036–2043.
[5] B. Gran, L.R. Tranquill, M. Chen, B. Bielekova, W. Zhou, S. Dhib-Jalbut, et al., Mechanisms of immunomodulation by glatiramer acetate, Neurology 55 (11) (Dec 12, 2000) 1704–1714.
[6] P.W. Duda, M.C. Schmied, S.L. Cook, J.I. Krieger, D.A. Hafler, Glatiramer acetate (Copaxone) induces degenerate, Th2-polarized immune responses in patients with multiple sclerosis, J. Clin. Invest. 105 (7) (Apr, 2000) 967–976.
[7] O. Neuhaus, C. Farina, A. Yassouridis, H. Wiendl, F. Then Bergh, T. Dose, et al., Multiple sclerosis: comparison of copolymer-1- reactive T cell lines from treated and untreated subjects reveals cytokine shift from T helper 1 to T helper 2 cells, Proc. Natl. Acad. Sci. U. S. A. 97 (13) (Jun 20, 2000) 7452–7457.
[8] P.L. Vieira, H.C. Heystek, J. Wormmeester, E.A. Wierenga, M.L. Kapsenberg, Glatiramer acetate (copolymer-1, copaxone) promotes Th2 cell development and increased IL-10 production through modulation of dendritic cells, J. Immunol. 170 (9) (May 1, 2003) 4483–4488.
[9] M.S. Weber, T. Prod'homme, S. Youssef, S.E. Dunn, C.D. Rundle, L. Lee, et al., Type II monocytes modulate T cell-mediated central nervous system autoimmune disease, Nat. Med. 13 (8) (Aug, 2007) 935–943.
[10] R. Aharoni, D. Teitelbaum, R. Arnon, M. Sela, Copolymer 1 acts against the immunodominant epitope 82-100 of myelin basic protein by T cell receptor antagonism in addition to major histocompatibility complex blocking, Proc. Natl. Acad. Sci. U. S. A. 96 (2) (Jan 19, 1999) 634–639.
[11] E.J. McMahon, S.L. Bailey, C.V. Castenada, H. Waldner, S.D. Miller, Epitope spreading initiates in the CNS in two mouse models of multiple sclerosis, Nat. Med. 11 (3) (Mar, 2005) 335–339.
[12] R. Aharoni, D. Teitelbaum, M. Sela, R. Arnon, Copolymer 1 induces T cells of the T helper type 2 that crossreact with myelin basic protein and suppress experimental autoimmune encephalomyelitis, Proc. Natl. Acad. Sci. U. S. A. 94 (20) (Sep 30, 1997) 10821–10826.
[13] J. Hong, N. Li, X. Zhang, B. Zheng, J.Z. Zhang, Induction of CD4+CD25+ regulatory T cells by copolymer-I through activation of transcription factor Foxp3, Proc. Natl. Acad. Sci. U. S. A. 102 (18) (May 3, 2005) 6449–6454.
[14] E. Basile, E. Gibbs, T. Aziz, J. Oger, During 3 years treatment of primary progressive multiple sclerosis with glatiramer acetate, specific antibodies switch from IgG1 to IgG4, J. Neuroimmunol. 177 (1–2) (Aug, 2006) 161–166.
[15] R. Bomprezzi, R. Schaefer, V. Reese, A. Misra, T.L. Vollmer, M. Kala, Glatiramer acetate-specific antibody titres in patients with relapsing/remitting multiple sclerosis and in experimental autoimmune encephalomyelitis, Scand. J. Immunol. 74 (3) (Sep, 2011) 219–226.
[16] T. Brenner, R. Arnon, M. Sela, O. Abramsky, Z. Meiner, R. Riven-Kreitman, et al., Humoral and cellular immune responses to Copolymer 1 in multiple sclerosis patients treated with Copaxone, J. Neuroimmunol. 115 (1–2) (Apr 2, 2001) 152–160.
[17] C. Farina, V. Vargas, N. Heydari, T. Kumpfel, E. Meinl, R. Hohlfeld, Treatment with glatiramer acetate induces specific IgG4 antibodies in multiple sclerosis patients, J. Neuroimmunol. 123 (1–2) (Feb, 2002) 188–192.
[18] D. Karussis, D. Teitelbaum, C. Sicsic, T. Brenner, Long-term treatment of multiple sclerosis with glatiramer acetate: natural history of the subtypes of anti-glatiramer acetate antibodies and their correlation with clinical efficacy, J. Neuroimmunol. 220 (1–2) (Mar 30, 2010) 125–130.
[19] F. Sellebjerg, C.J. Hedegaard, M. Krakauer, D. Hesse, H. Lund, C.H. Nielsen, et al., Glatiramer acetate antibodies, gene expression and disease activity in multiple sclerosis, Mult. Scler. 18 (3) (Mar, 2012) 305–313.
[20] D. Teitelbaum, T. Brenner, O. Abramsky, R. Aharoni, M. Sela, R. Arnon, Antibodies to glatiramer acetate do not interfere with its biological functions and therapeutic efficacy, Mult. Scler. 9 (6) (Dec, 2003) 592–599.
[21] P.B. Carrieri, F. Carbone, F. Perna, D. Bruzzese, C. La Rocca, M. Galgani, et al., Longitudinal assessment of immuno-metabolic parameters in multiple sclerosis patients during treatment with glatiramer acetate, Metabolism 64 (9) (Sep, 2015) 1112–1121.
[22] J. Kipnis, E. Yoles, Z. Porat, A. Cohen, F. Mor, M. Sela, et al., T cell immunity to copolymer 1 confers neuroprotection on the damaged optic nerve: possible therapy for optic neuropathies, Proc. Natl. Acad. Sci. U. S. A. 97 (13) (Jun 20, 2000) 7446–7451.
[23] R. Aharoni, R. Eilam, H. Domev, G. Labunskay, M. Sela, R. Arnon, The immunomodulator glatiramer acetate augments the expression of neurotrophic factors in brains of experimental autoimmune encephalomyelitis mice, Proc. Natl. Acad.

Sci. U. S. A. 102 (52) (Dec 27, 2005) 19045–19050.
[24] V. Skihar, C. Silva, A. Chojnacki, A. Doring, W.B. Stallcup, S. Weiss, et al., Promoting oligodendrogenesis and myelin repair using the multiple sclerosis medication glatiramer acetate, Proc. Natl. Acad. Sci. U. S. A. 106 (42) (Oct 20, 2009) 17992–17997.
[25] Y. Zhang, F. Jalili, N. Ouamara, A. Zameer, G. Cosentino, M. Mayne, et al., Glatiramer acetate-reactive T lymphocytes regulate oligodendrocyte progenitor cell number in vitro: role of IGF-2, J. Neuroimmunol. 227 (1–2) (Oct 8, 2010) 71–79.
[26] D. Azoulay, V. Vachapova, B. Shihman, A. Miler, A. Karni, Lower brain-derived neurotrophic factor in serum of relapsing remitting MS: reversal by glatiramer acetate, J. Neuroimmunol. 167 (1–2) (Oct, 2005) 215–218.
[27] M. Rieks, V. Hoffmann, O. Aktas, M. Juschka, I. Spitzer, N. Brune, et al., Induction of apoptosis of CD4+ T cells by immunomodulatory therapy of multiple sclerosis with glatiramer acetate, Eur. Neurol. 50 (4) (2003) 200–206.
[28] M. Ruggieri, C. Avolio, S. Scacco, C. Pica, A. Lia, G.B. Zimatore, et al., Glatiramer acetate induces pro-apoptotic mechanisms involving Bcl-2, Bax and Cyt-c in peripheral lymphocytes from multiple sclerosis patients, J. Neurol. 253 (2) (Feb, 2006) 231–236.
[29] A. Achiron, A. Feldman, M. Gurevich, Molecular profiling of glatiramer acetate early treatment effects in multiple sclerosis, Dis. Markers 27 (2) (2009) 63–73.
[30] M. Gurevich, T. Tuller, U. Rubinstein, R. Or-Bach, A. Achiron, Prediction of acute multiple sclerosis relapses by transcription levels of peripheral blood cells, BMC Med. Genet. 2 (Jul 22, 2009) 46.
[31] M. Filippi, D.W. Paty, L. Kappos, F. Barkhof, D.A. Compston, A.J. Thompson, et al., Correlations between changes in disability and T2-weighted brain MRI activity in multiple sclerosis: a follow-up study, Neurology 45 (2) (Feb, 1995) 255–260.
[32] M. Filippi, M.A. Rocca, MRI evidence for multiple sclerosis as a diffuse disease of the central nervous system, J. Neurol. 252 (Suppl. 5) (Nov, 2005) v16–v24.
[33] M. Filippi, M.A. Rocca, MR imaging of gray matter involvement in multiple sclerosis: implications for understanding disease pathophysiology and monitoring treatment efficacy, AJNR Am. J. Neuroradiol. 31 (7) (Aug, 2010) 1171–1177.
[34] D.K. Li, U. Held, J. Petkau, M. Daumer, F. Barkhof, F. Fazekas, et al., MRI T2 lesion burden in multiple sclerosis: a plateauing relationship with clinical disability, Neurology 66 (9) (May, 2006) 1384–1389.
[35] G. Giovannoni, D. Tomic, J.R. Bright, E. Havrdova, "No evident disease activity": the use of combined assessments in the management of patients with multiple sclerosis, Mult. Scler. 23 (9) (Aug, 2017) 1179–1187.
[36] L. Kappos, N. De Stefano, M.S. Freedman, B.A. Cree, E.W. Radue, T. Sprenger, et al., Inclusion of brain volume loss in a revised measure of 'no evidence of disease activity' (NEDA-4) in relapsing-remitting multiple sclerosis, Mult. Scler. 22 (10) (Sep, 2016) 1297–1305.
[37] J. Imitola, M.K. Racke, Is no evidence of disease activity a realistic goal for patients with multiple sclerosis? JAMA Neurol. 72 (2) (Feb, 2015) 145–147.
[38] F.D. Lublin, S.C. Reingold, J.A. Cohen, G.R. Cutter, P.S. Sorensen, A.J. Thompson, et al., Defining the clinical course of multiple sclerosis: the 2013 revisions, Neurology 83 (3) (Jul 15, 2014) 278–286.
[39] M.P. Sormani, Prognostic factors versus markers of response to treatment versus surrogate endpoints: three different concepts, Mult. Scler. 23 (3) (Mar, 2017) 378–381.
[40] M. Sormani, A. Signori, M. Stromillo, N. De Stefano, Refining response to treatment as defined by the modified Rio score, Mult. Scler. 19 (9) (Aug, 2013) 1246–1247.
[41] M.P. Sormani, J. Rio, M. Tintore, A. Signori, D. Li, P. Cornelisse, et al., Scoring treatment response in patients with relapsing multiple sclerosis, Mult. Scler. 19 (5) (Apr, 2013) 605–612.
[42] C.H. Polman, S.C. Reingold, B. Banwell, M. Clanet, J.A. Cohen, M. Filippi, et al., Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria, Ann. Neurol. 69 (2) (Feb, 2011) 292–302.
[43] J. Rio, J. Castillo, A. Rovira, M. Tintore, J. Sastre-Garriga, A. Horga, et al., Measures in the first year of therapy predict the response to interferon beta in MS, Mult. Scler. 15 (7) (Jul, 2009) 848–853.
[44] J. Rio, A. Rovira, M. Tintore, J. Sastre-Garriga, J. Castillo, C. Auger, et al., Evaluating the response to glatiramer acetate in relapsing-remitting multiple sclerosis (RRMS) patients, Mult. Scler. 20 (12) (Oct, 2014) 1602–1608.
[45] A.M. Siwkowski, L.A. Madge, S. Koo, E.L. McMillan, B.P. Monia, J.S. Pober, et al., Effects of antisense oligonucleotide-mediated depletion of tumor necrosis factor (TNF) receptor 1-associated death domain protein on TNF-induced gene expression, Mol. Pharmacol. 66 (3) (Sep, 2004) 572–579.
[46] J.D. Lane, J. Lucocq, J. Pryde, F.A. Barr, P.G. Woodman, V.J. Allan, et al., Caspase-mediated cleavage of the stacking protein GRASP65 is required for Golgi fragmentation during apoptosis, J. Cell Biol. 156 (3) (Feb 4, 2002) 495–509.
[47] X. Montalban, R. Gold, A.J. Thompson, S. Otero-Romero, M.P. Amato, D. Chandraratna, et al., ECTRIMS/EAN guideline on the pharmacological treatment of people with multiple sclerosis, Mult. Scler. 24 (2) (Feb, 2018) 96–120.
[48] Y.K. Semra, O.A. Seidi, M.K. Sharief, Overexpression of the apoptosis inhibitor FLIP in T cells correlates with disease activity in multiple sclerosis, J. Neuroimmunol. 113 (2) (Feb 15, 2001) 268–274.
[49] Z. Petelin, V. Brinar, D. Petravic, N. Zurak, K. Dubravcic, D. Batinic, CD95/Fas expression on peripheral blood T lymphocytes in patients with multiple sclerosis: effect of high-dose methylprednisolone therapy, Clin. Neurol. Neurosurg. 106 (3) (Jun, 2004) 259–262.
[50] J. Rio, A. Rovira, M. Tintore, S. Otero-Romero, M. Comabella, A. Vidal-Jordana, et al., Disability progression markers over 6-12 years in interferon-beta-treated multiple sclerosis patients, Mult. Scler. 24 (3) (Mar, 2018) 322–330.
[51] G. Giovannoni, Personalized medicine in multiple sclerosis, Neurodegener. Dis. Manag. 7 (6s) (Nov, 2017) 13–17.
[52] P.K. Coyle, Pharmacogenetic biomarkers to predict treatment response in multiple sclerosis: current and future perspectives, Mult. Scler. Int. 2017 (2017) 6198530.
[53] D.L. Rotstein, B.C. Healy, M.T. Malik, T. Chitnis, H.L. Weiner, Evaluation of no evidence of disease activity in a 7-year longitudinal multiple sclerosis cohort, JAMA Neurol. 72 (2) (Feb, 2015) 152–158.
[54] C. Warnke, G. von Geldern, P. Markwerth, T. Dehmel, R. Hoepner, R. Gold, et al., Cerebrospinal fluid JC virus antibody index for diagnosis of natalizumab-associated progressive multifocal leukoencephalopathy, Ann. Neurol. 76 (6) (Dec, 2014) 792–801.
[55] R. Gold, L. Kappos, D.L. Arnold, A. Bar-Or, G. Giovannoni, K. Selmaj, et al., Placebo-controlled phase 3 study of oral BG-12 for relapsing multiple sclerosis, N. Engl. J. Med. 367 (12) (Sep 20, 2012) 1098–1107.
[56] J.A. Cohen, B. Khatri, F. Barkhof, G. Comi, H.P. Hartung, X. Montalban, et al., Long-term (up to 4.5 years) treatment with fingolimod in multiple sclerosis: results from the extension of the randomised TRANSFORMS study, J. Neurol. Neurosurg. Psychiatry 87 (5) (May, 2016) 468–475.
[57] K.P. Johnson, B.R. Brooks, J.A. Cohen, C.C. Ford, J. Goldstein, R.P. Lisak, et al., Extended use of glatiramer acetate (Copaxone) is well tolerated and maintains its clinical effect on multiple sclerosis relapse rate and degree of disability. 1998 [classical article], Neurology 57 (12 Suppl 5) (Dec, 2001) S46–S53.