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Lymphocyte populations and their change during five-year glatiramer acetate treatment

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ABSTRACT

Background: The goal of this study was to determine the characteristics that are affected in patients treated with glatiramer acetate (GA).
Methods: A total of 113 patients were included in this study. Patients were treated with glatiramer acetate (subcutaneous injection, 20 mg, each day). Peripheral blood samples were obtained just prior to treatment as well as 5 years after GA treatment. All the calculations were performed with the statistical system R (r-project.org).
Results: After 5 years of treatment, a significant decrease was found in the absolute and relative CD3+/CD69+ counts, the absolute and relative CD69 counts, the relative CD8+/CD38+ count and the relative CD38 count. A significant increase was found in the absolute and relative CD5+/CD45RA+ counts and the absolute CD5+/CD45RO+ count after 5 years of treatment.
Conclusion: This study presents some parameters that were affected by long-term GA treatment.

1. Introduction

Multiple sclerosis (MS) is a disease characterized by both relapses and insidious progression and is notably heterogeneous in clinical course, symptomatology, and severity [1]. Despite considerable progress in the diagnosis and treatment of this serious disease, the aetiology of MS remains unknown. Genetic and environmental factors are assumed to play a role in the pathogenesis of the disease [2]. Environmental influences include vitamin D deficiency, insufficient exposure to sunlight, viral infections, stress, smoking and intestinal microbiota [3–7].

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Immune responses caused by genetic dispositions are likely to have a primary role in the pathogenesis of MS. A ''genome-wide association study'' clearly proved that there is a genetically determined dysfunction of T and B cells [8,9]. The HLA (human leucocyte antigen) system demonstrably influences the development of MS. The carriers of HLA-DRB5*0101, HLA-DRB1*1501, HLA-DQA1*0102 and HLA-DQB1*0602 alleles are at increased risk [10]. In an individual with genetic predisposition, the influence of external factors causes the development of inflammatory responses leading to demyelination and axonal and neuronal loss [11,12].

MS is one of the autoimmune diseases with a well-explored pathogenesis. The reasons for such research are the extraordinary clinical seriousness of the disease and the experimental animal model for MS that has existed for several decades. The experimental animal model is called experimental autoimmune encephalomyelitis (EAE). According to classic conceptions of immune system functions, the onset and development of the autoimmune immunopathological reaction are caused by disruption of the recognition and self-tolerating mechanisms. Although T-lymphocytes are considered the key component of autoimmune disease pathogenesis (and thus MS), the role of humoral immunity components in damaging inflammatory reactions cannot be ignored.

One of the basic medications for treating clinically isolated syndrome (CIS) or relapsing-remitting MS is glatiramer acetate (GA). GA, originally named Copolymer 1, was developed in the 1960s in the Weizmann Institute of Science in the Israeli town of Rechovot. GA is a mixture of synthetic polypeptides containing L-amino acids, glutamic acid, alanine, lysine and tyrosine. Paradoxically, GA was originally synthesised with intentions to induce EAE. In 1971, Teitelbaum et al. showed that GA suppressed the induction of acute EAE. The mechanism of action of GA is not precisely understood. The effect of GA is immunomodulatory [13]. GA relieves inflammation and has neuroprotective properties. GA causes rearrangement from activity of the Th1 subset of T-lymphocytes to activity of the Th2 subset of T-lymphocytes [14]. The Th1 subset is responsible for the development of cytotoxic reactions. Th1 T-lymphocytes produce proinflammatory cytokines such as interferon γ (INFγ), tumour necrosis factor β (TNFβ) and interleukin 2 (IL-2). In contrast, the Th2 subset of T-lymphocytes acts antagonistically to the Th1 subset and produces anti-inflammatory cytokines such as IL-4 and IL-13. With antigen-presenting cells such as monocytes and dendritic cells, it changes the function of CD4+ and CD8+ T-lymphocytes. It binds with high affinity to MHC (major histocompatibility complex) class II molecules on cells presenting MBP (myelin basic protein) and thus prevents the advancement of antigen fragments derived from MBP. In other words, it competes with MBP immunogen fragments for receptors on autoreactive T-lymphocytes and presumably energises them or causes their apoptosis [15]. GA-reactive T cells can deliver brain-derived neurotrophic factor (BDNF) to neurons, which upregulates the corresponding full-length signalling receptor tyrosine kinase gp145 trkB in multiple sclerosis lesions [16,17].

In this study, individual populations of lymphocytes in MS patients (CIS and relapsing-remitting MS) were examined. The goal was to determine the populations that are affected in patients treated with GA.

2. Material and methods

2.1. Population sample

All the subjects (aged from 17 to 55, mean age 35 ± 9) were of Caucasian origin and fulfilled the McDonald criteria or revised McDonald criteria for RR MS [18–20]. Between 2008–2017, a total of 113 patients (25 men and 88 women) were included in the study. The patients were treated by GA (subcutaneous injection, 20 mg per day). Seventy-two patients completed the study (12 men and 60 women), and 41 patients dropped out prematurely because of treatment ineffectiveness or intolerance (13 men and 28 women).

All the participants were recruited during their hospital visit, during which all relevant MS information (Expanded Disability Status Scale (EDDS), disease duration, MS treatment history) was obtained. Clinical evaluations were performed by an attending neurologist. Peripheral blood samples were obtained just prior to treatment and 5 years after GA treatment.

All the participants gave written informed consent.

2.2. Sample collection and sample processing

Blood samples were collected from the antecubital fossa vein. Relative numbers of clusters of differentiated CD3+, CD4+, CD8+, CD19+, CD3-/CD16+56+, CD3+CD69+, CD3+CD25+, CD4+/CD45RA+, CD4+/CD45RO+, CD8+/CD38+, CD19+/CD5+, CD40 and CD40L lymphocytes were analysed by two-colour flow cytometry. For surface staining, 100 l of blood was added to tubes containing 10 l of a cocktail of fluorochrome-labelled mAbs. mAbs used included fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (clone UCHT1), anti-CD4 (clone 13B8.2), anti-CD45RA (clone ALB11), anti-CD8 (clone B9.11) and anti-CD19 (clone J3-119) and phycoerythrin (PE)-conjugated anti-CD25 (clone B1.49.9), anti-CD69 (clone TP1.55.3), anti-CD4 (13B8.2), anti-CD45RO (clone UCHL1), anti-CD38 (clone LS198-4-3), anti-CD5 (clone BL1a), anti-CD40 (clone MAB89) and anti-CD40L (clone TRAP-1) all supplied by Beckman Coulter (Miami, FL, USA). Class-matched isotype immunoglobulin FITC- and PE-conjugated negative control monoclonal antibodies were added simultaneously to separate tubes for all the samples to detect nonspecific binding.

Subsequently, 100 l of heparinised peripheral blood was mixed with the monoclonal antibody cocktail and was incubated for 15 min at room temperature. After the incubation, lysing solution (OptiLyse C, Beckman Coulter) was added, and the mixture was incubated for a further 10 min. Flow cytometric analysis was performed using Cytomics FC 500 cytometer (Beckman Coulter) equipped with a 15-mW air-cooled 488-nm argon laser and a 625-nm neon diode laser, and the data were analysed using the CXP Analysis Software (Beckman Coulter). Data on at least 10,000 events were acquired for each staining and stored as list mode.

2.3. Statistical analysis

We compared paired values of parameters of peripheral blood samples at baseline and the end of the follow-up (after five years

of treatment), and we used running paired *t*-test for normally distributed variables and Wilcoxon signed-rank test for non-normally distributed variables. The Lilliefors normality test was used to assess whether the variable is normally or non-normally distributed. The effect size was assessed by Cohen's *d*, which is the standardized difference between paired means. To control the false discovery rate, the Benjamini–Hochberg procedure with $p < 0.05$ was applied. All the calculations were performed in the statistical system R (r-project.org) [21].

3. Results

During the observation, multiple peripheral blood parameters were examined in absolute and relative values. These parameters were lymphocytes, CD4+T lymphocytes, CD8+T lymphocytes, CD 19 (B lymphocytes), natural killer cells (CD3-/CD16+56+), CD3 +/CD69+ cells, CD5 cells, CD25 cells, CD3+/CD25+ cells, CD5+/CD19 + cells, CD4+/CD45RO+ cells, CD8+CD38+ cells, CD4+/CD45RA+ cells, CD 69 protein, CD 40 protein, CD 40L protein in absolute and relative values and the absolute leukocyte (white blood cell) count.

Table 1 presents the statistical significance for the changes in the measured value levels from the treatment. The data denoted with ab represent an absolute value, and the data without ab represent a relative value.

At the 5% significance level, statistically significant differences (changes) during follow-up were identified for the following parameters (sorted by effect size): CD3+CD69+ (2.00 vs. 1.25, *p*-value = 0.0002, effect size = 0.63) (Fig. 1), CD3 +CD69+ ab (0.04 vs. 0.03, 0.0011, 0.5) (Fig. 2), CD8+CD38+ (11.45 vs. 7.75, 0.0077, 0.42), CD4+CD45RA+ ab (0.41 vs. 0.48, 0.009, 0.41), CD69ab (0.08 vs. 0.06, 0.0081, 0.4), CD38 (53.63 vs. 47.96, 0.0132, 0.39), CD4+CD45RA+ (19.72 vs. 21.33, 0.014, 0.37), CD4

Table 1 – Comparison of parameters (baseline and after 5 years of treatment.

Parameter	At baseline (before treatment)		After 5 years of treatment		Paired comparison	
	Mean (SD)	Median [IQR]	Mean (SD)	Median [IQR]	*p*-value	Effect size
Absolute lymphocyte count	2.04 (0.68)	2.08 [1.52, 2.35]	2.20 (0.64)	2.13 [1.73, 2.48]	0.166	0.20
CD3	76.27 (7.19)	77.40 [71.58, 82.15]	76.15 (7.02)	76.10 [71.58, 81.35]	0.8934	0.01
CD3 ab	1.56 (0.56)	1.49 [1.22, 1.81]	1.69 (0.55)	1.61 [1.30, 1.93]	0.166	0.20
CD4	48.29 (8.67)	49.30 [42.30, 54.20]	49.21 (8.24)	49.35 [43.27, 55.02]	0.3759	0.13
CD4 ab	0.99 (0.41)	0.92 [0.70, 1.20]	1.09 (0.38)	1.07 [0.85, 1.25]	0.0918	0.25
CD8	26.90 (6.52)	25.85 [22.00, 30.52]	25.51 (5.93)	25.25 [21.53, 28.25]	0.166	0.21
CD8ab	0.54 (0.21)	0.51 [0.42, 0.64]	0.56 (0.22)	0.52 [0.41, 0.67]	0.6304	0.07
CD19	9.75 (4.44)	9.25 [6.52, 12.33]	10.66 (3.94)	10.75 [7.55, 12.62]	0.166	0.21
CD19 ab	0.20 (0.11)	0.20 [0.12, 0.26]	0.24 (0.12)	0.22 [0.15, 0.29]	0.0918	0.25
Natural killer[a]	11.52 (6.90)	9.60 [6.22, 15.58]	10.60 (5.27)	9.75 [6.55, 14.32]	0.4594	0.14
Natural killer ab[a]	0.23 (0.15)	0.20 [0.13, 0.32]	0.22 (0.12)	0.21 [0.14, 0.27]	0.7642	0.08
Absolute total leukocyte count	7.19 (2.30)	6.70 [5.71, 8.15]	7.23 (1.67)	6.96 [5.86, 8.35]	0.8934	0.01
Relative lymphocyte count	29.45 (8.68)	29.15 [23.75, 35.78]	30.94 (7.78)	30.10 [25.53, 36.15]	0.33	0.15
CD3+CD69+[a]	2.84 (2.26)	2.00 [1.28, 3.85]	1.38 (0.61)	1.25 [1.00, 1.63]	**0.0002**	0.63
CD3+CD69+ ab[a]	0.06 (0.06)	0.04 [0.02, 0.07]	0.03 (0.02)	0.03 [0.02, 0.04]	**0.0011**	0.50
CD40 ligand[a]	0.34 (0.57)	0.10 [0.00, 0.30]	0.39 (1.53)	0.20 [0.10, 0.30]	0.8687	0.20
CD40 ligand ab[a]	0.01 (0.02)	0.00 [0.00, 0.01]	0.01 (0.03)	0.00 [0.00, 0.01]	0.4594	0.18
CD38	53.63 (12.92)	53.20 [48.97, 61.18]	47.96 (9.36)	47.60 [40.22, 55.92]	**0.0132**	0.39
CD38 ab	1.09 (0.49)	1.12 [0.77, 1.33]	1.06 (0.38)	0.98 [0.74, 1.29]	0.6866	0.06
CD3+CD25+	5.73 (3.63)	4.90 [3.10, 7.58]	5.22 (2.06)	5.15 [3.77, 6.12]	0.4876	0.11
CD3+CD25+ ab	0.12 (0.09)	0.09 [0.06, 0.17]	0.11 (0.06)	0.10 [0.08, 0.14]	0.9657	0.05
CD5+CD19+[a]	1.60 (1.69)	1.10 [0.50, 2.05]	1.55 (1.05)	1.35 [0.80, 1.90]	0.8035	0.04
CD5+CD19+ ab[a]	0.03 (0.03)	0.02 [0.01, 0.04]	0.03 (0.03)	0.03 [0.02, 0.04]	0.4594	0.08
CD4+CD45RA+	19.72 (8.06)	18.30 [13.85, 27.60]	21.33 (8.76)	20.45 [14.70, 27.45]	**0.014**	0.37
CD4+CD45RA+ ab[a]	0.41 (0.24)	0.40 [0.24, 0.56]	0.48 (0.27)	0.43 [0.29, 0.64]	**0.009**	0.41
CD5[a]	76.80 (7.95)	78.20 [72.95, 82.90]	75.60 (10.91)	77.05 [72.58, 81.75]	0.9756	0.09
CD5 ab	1.58 (0.57)	1.52 [1.23, 1.81]	1.66 (0.56)	1.60 [1.30, 2.00]	0.5313	0.10
CD25[a]	6.33 (3.94)	5.75 [3.30, 8.60]	5.55 (2.25)	5.35 [4.10, 6.55]	0.7169	0.16
CD25 ab[a]	0.13 (0.10)	0.10 [0.06, 0.18]	0.12 (0.06)	0.11 [0.09, 0.15]	0.9494	0.09
CD40	9.43 (4.34)	9.00 [6.30, 11.72]	10.55 (4.44)	10.30 [7.45, 13.83]	0.0738	0.28
CD40 ab	0.20 (0.11)	0.17 [0.10, 0.26]	0.24 (0.13)	0.22 [0.15, 0.28]	0.0738	0.28
CD69[a]	5.47 (7.23)	4.30 [2.48, 6.62]	3.03 (1.69)	2.75 [1.90, 3.82]	**0.0011**	0.32
CD69 ab[a]	0.11 (0.10)	0.08 [0.05, 0.14]	0.06 (0.03)	0.06 [0.04, 0.08]	**0.0081**	0.40
CD4+CD45RO+	22.20 (6.37)	21.10 [17.40, 25.68]	23.79 (6.11)	23.20 [19.32, 27.65]	0.0738	0.28
CD4+CD45RO+ ab[a]	0.44 (0.15)	0.41 [0.36, 0.54]	0.51 (0.18)	0.46 [0.39, 0.57]	**0.012**	0.35
CD8+CD38+[a]	12.05 (6.42)	11.45 [7.35, 15.65]	9.03 (4.66)	7.75 [6.10, 11.10]	**0.0077**	0.42
CD8+CD38+ ab[a]	0.25 (0.16)	0.23 [0.13, 0.31]	0.20 (0.12)	0.17 [0.12, 0.23]	0.0584	0.26

Parameters at baseline and after 5 years of treatment were compared by parametric paired *t*-test.
p-values <0.05 are emphasized in bold.
[a] For non-normally distributed variables was used nonparametric Wilcoxon signed-rank test.

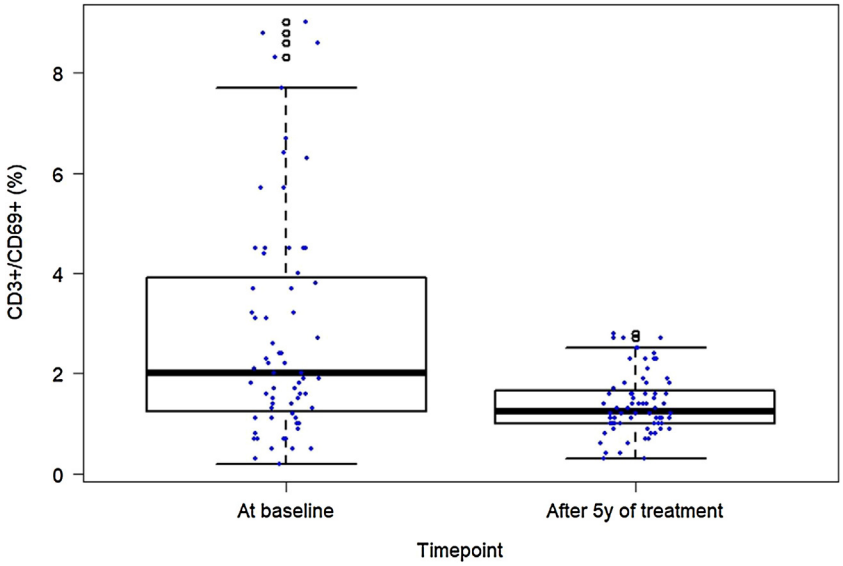

Fig. 1 – Boxplots of CD3+/CD69+ (%) at baseline and after 5 years of treatment.

+CD45RO+ ab (0.41 vs. 0.46, 0.012, 0.35), and CD69 (4.3 vs. 2.75, 0.0011, 0.32). A Cohen's D value of greater than 0.5 is perceived as a large effect size. In this study, large changes were observed for CD3+CD69+ and CD3+CD69+ ab. Medium changes (effect size greater than 0.3) were observed for the rest of parameters mentioned above.

4. Discussion

Autoimmune immunopathological reactivity, based on components of natural and specific immunity, plays a key role in the pathogenesis of MS. The activation of autoreactive T-lymphocytes occurs in peripheral lymphatic nodes. Antigen-presenting dendritic cells, which expose antigen peptides on their surface using MHC (major histocompatibility complex) class II, are involved in this process. Afterwards, these MHC complexes are identified by the T-cell receptor on T-lymphocytes. Activated T-lymphocytes then migrate to the central nervous system (CNS) where the inflammatory reaction occurs.

It was previously proven in the EAE model that some CNS structures are probable targets of autoreactive T-lymphocytes. Myelin sheaths of nerve fibres are the key structures. The actions of Th1 T-lymphocytes, Th17 T-lymphocytes and activated macrophages dominate in the inflammatory reaction.

In patients with MS, an intrathecal production of antibodies occurs. The increased level of immunoglobulins in the cerebrospinal fluid (CSF) of patients with MS is mainly caused

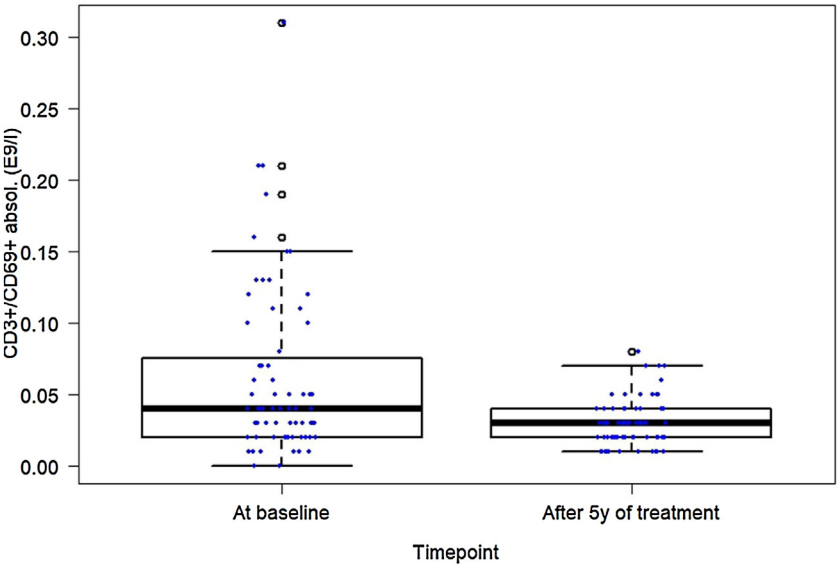

Fig. 2 – Boxplots of CD3+/CD69+ absol. (E9/l) at baseline and after 5 years of treatment.

by antibodies of the IgG class. Using immunoelectrophoretic analysis of CSF samples, it is possible to find so called oligoclonal bands in patients with MS. These bands are considered proof that B-lymphocyte oligoclonal expansion occurs in the CNS. Autoantibodies have an opposing role in MS pathogenesis. Binding of specific antibodies on immunodominant epitopes of CNS molecules can modulate their function in a negative way. The immunoglobulin–antigen complexes represent one of the outcomes of complement system activation, which can contribute to cell structure damage in the CNS with its cytolytic activity. Specific antibodies play a role in antibody-dependent cellular cytotoxicity (ADCC). However, the specific antibodies can bind and can thus neutralize autoantigens released during neural structure impairment. Antigens facilitate the removal of damaged structures in the CNS and positively modulate the reparative processes.

The goal in this study was to determine which immunological parameters are affected by long-term GA treatment in peripheral blood.

A significant decrease in CD69 was found. CD69, one of the earliest specific antigens acquired during lymphoid activation, acts as a signal-transducing receptor involved in cellular activation events, including proliferation and induction of specific genes. CD69 belongs to a family of receptors that modulate the immune response and whose genes are clustered in the natural killer (NK) gene complex [22]. The recent discovery of a CD69 ligand expressed on dendritic cells, Galectin-1, has confirmed the immunoregulatory role of CD69 mainly through the inhibition of Th17 differentiation and function in mice and humans. In this regard, the expression of CD69, both in Th17 lymphocytes and in a subset of regulatory T cells, has an important role controlling the immune response and inflammatory phenomenon [23]. There is an integral membrane interaction between CD69 and S1P(1); CD69 induces an S1P(1) conformation that shares some properties with the ligand-bound state, thereby facilitating S1P(1) internalisation and degradation [24]. Lymphocyte egress requires sphingosine 1-phosphate receptor-1 (S1P1), and IFN-alpha/beta was found to inhibit lymphocyte responsiveness to S1P [25]. Furthermore, CD69 was identified as a downregulated transcript after VCAM1 knockdown in oligodendrocytes. Knockdown of CD69 in mice indicates the role of CD69 in myelination. Therefore, VCAM1 contributes not only to the initiation of myelination but also to its regulation by controlling the abundance of CD69, demonstrating that an intercellular molecule with primarily immune roles can also play an unexpected role in the CNS [26]. CD25 and CD69 are two additional markers of monocyte activation. CD25 and CD69 are induced both by Toll-like receptor ligands and inflammatory cytokines. This work validates the results of Weber et al. which show that GA consistently reduces the induction of CD69 [27]. However, it does not agree with GA reducing the induction of CD25 (significant changes in relative and absolute counts of CD25 were not proven).

CD3+/CD69+ cells represent early-activated T-lymphocytes. One study showed that, during long-term chronic exposure to solar radiation, a decrease in CD3+/CD69+ cells occurred [28]. A decrease in the CD3+CD69+ count was also seen during GA treatment.

Bahri et al. identified CD8+CD38+ T-cells as potential inhibitors of excessive immune responses. CD8+CD38+ lymphocytes suppress CD4+ effector T-cell proliferation in an antigen non-specific manner via interferon gamma. In vivo, CD8+CD38+ T cells mitigate EAE by reducing the clinical score and delaying disease occurrence [29]. Although we expected an increase in this parameter, we observed a decrease in absolute and relative CD8+CD38+ counts.

Blanco et al. observed an increase in the CD4+CD45RA+ count in GA-treatment responders. Upregulation of CD4 +CD45RA+ seems to be one of the mechanisms by which GA inhibits MS activity [30]. An increase in CD4+CD45RA+ was also observed in this study.

During GA treatment, a decrease in the relative CD38 count was observed. Loss of CD38 function is associated with impaired immune responses [8].

CD4+CD45RO+ are memory helper T-lymphocytes, i.e., lymphocytes that contact antigens. This population changes with age with the highest levels later in life. CD4+CD45RO+ memory T-cells from MS patients showed a reduced ability to suppress NLRP3 inflammasome activation. In the aforementioned study, a significant decrease in absolute and relative CD4+CD45RO+ counts was observed. After GA therapy, a decrease in central memory T-cells was also observed by Praksova et al. [31]. The findings contradict the results of Carrieri et al. Authors proved that naïve-to-treatment RRMS patients showed a significantly higher number of CD4+T cells with a memory phenotype (CD4+CD45RO+) whose peripheral frequency was not affected by GA treatment [32].

Many of the other examined parameters play a role in MS pathogenesis. However, the findings did not confirm that they were affected by long-term GA treatment.

5. Conclusion

Treatment with GA reduces clinical exacerbations of multiple sclerosis (MS) through several known immunomodulatory mechanisms. However, the exact mechanism of action of this medication is not known. This study presents some parameters that were affected by long-term GA treatment.

Conflict of interest

None declared.

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