

## INTERFERON- $\beta$ 1A, AN IMMUNOMODULATOR IN RELAPSING REMITTING MULTIPLE SCLEROSIS PATIENTS. THE EFFECT ON PRO-INFLAMMATORY CYTOKINES

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### Abstract

Interferon- $\beta$  was the first disease-modifying therapy used for recurrent-relapsing multiple sclerosis, with an intricate mechanism of action. The objectives of the current study were identifying the cytokine profile in recurrent-relapsing multiple sclerosis serum samples, both naïve and after one year of Interferon- $\beta$ 1a treatment, in order to study the mechanism of action. 37 recurrent-relapsing multiple sclerosis patients and 37 healthy subjects were included. Serum levels of 15 cytokines were evaluated for the recurrent-relapsing multiple sclerosis patients in the beginning of the study and after one year of treatment, respectively at the beginning for healthy subjects. The recurrent-relapsing multiple sclerosis lot had at the beginning significantly higher levels of interleukin (IL)-10, IL-17F, IL-23, IL-31, sCD40L, TNF- $\alpha$  and "cytokine signature" compared to healthy controls. Treatment with Interferon- $\beta$ 1a significantly reduced the levels of IL-23, IL-31, sCD40L, tumour necrosis factor (TNF)- $\alpha$  and cytokine signature. IL-21 and TNF- $\alpha$  positively correlated with the activity of the disease (relapses, disability). The serum levels of the pro-inflammatory cytokines are higher in naïve recurrent-relapsing multiple sclerosis patients compared to healthy controls. Treatment with Interferon- $\beta$  significantly decreases the inflammatory profile.

### Rezumat

Interferonul  $\beta$  (Interferon- $\beta$ ), primul imunomodulator utilizat în tratamentul sclerozei multiple recurent remisive (SM-RR), prezintă un mecanism de acțiune intricat. Studiul își propune identificarea profilului de citokine în serul pacienților cu SM-RR naivi și după 1 an de tratament cu Interferon- $\beta$ 1a, în vederea aprofundării mecanismului de acțiune. Au fost incluși 37 pacienți cu SM-RR și 37 martori sănătoși. S-au determinat nivelurile serice a 15 citokine pentru lotul SM-RR la debutul și la finalul studiului, respectiv pentru martori la debutul studiului. Lotul SM-RR a avut la debut valori semnificativ mai mari ale interleukinei (IL)-10, IL-17F, IL-23, IL-31, sCD40L, factorului de necroză tumorală (TNF) $\alpha$  și "cytokine signature" comparativ cu subiecții sănătoși. Tratamentul a determinat scăderea semnificativă a valorilor serice ale IL-23, IL-31, sCD40L, TNF $\alpha$  și "cytokine signature". Nivelurile serice ale IL-21 și TNF- $\alpha$  s-au corelat pozitiv cu activitatea bolii (recurențe, dizabilitate). Tratamentul cu Interferon- $\beta$ 1a scăzut semnificativ acest profil proinflamator.

**Keywords:** multiple sclerosis, treatment, immunomodulator

### Introduction

Multiple sclerosis is a chronic, inflammatory, progressive and neurodegenerative disorder of the central nervous system. It affects 0.2% of the population in Europe and North America and appears more frequently in females. Multiple sclerosis is the main cause of neurological disability in young adults,

with an onset ranging between 20 and 40 years of age [15, 31, 43, 48].

The aetiology of multiple sclerosis is still unclear. The involvement of genetic and environmental factors builds the etiopathogenic basis of the disease and it is considered a possible autoimmune disorder. It is characterized by a high interindividual variability

regarding the clinical evolution, symptoms and treatment's response [43, 49].

The most frequent evolutive form is relapsing-remitting multiple sclerosis (recurrent-relapsing multiple sclerosis). Regarding the pathogenesis, multiple sclerosis is characterized by immune mediated-inflammation - proinflammatory T lymphocytes are activated in the periphery by the antigen-presenting cells, they pass through the blood-brain barrier and breach into the central nervous system where they secrete pro-inflammatory cytokines, followed by demyelination, axonal loss and neurodegeneration in the brain and in the spinal cord [31, 33, 34, 37, 43, 57].

Regarding the immunopathogenesis of multiple sclerosis, high levels of anti-inflammatory cytokines, secreted by Th2 lymphocytes, such as interleukin IL-4, IL-10 and IL-5, are associated with a lesser inflammation and a favourable clinical outcome, while high levels of pro-inflammatory cytokines secreted by Th1 lymphocytes, IFN- $\gamma$  and TNF- $\alpha$ , are associated with an increase in inflammation, a worse clinical outcome and a marked progression of the disease. An important role in the immunopathogenesis of multiple sclerosis is played by Th17 lymphocytes, notably by the main cytokine secreted by this cell line, IL-17, with strong pro-inflammatory properties [14, 43, 45]. Various studies also described a deficit in the T regulatory cells in multiple sclerosis patients, a subtype of T lymphocytes that normally reduce the activity of pro-inflammatory T cells and induce regeneration in the inflammatory lesions [33]. The clinical evolution of multiple sclerosis is dependent on the equilibrium between Th1 and Th2 lymphocytes, therefore, one of the main mechanisms of action of disease modifying therapies should imply the immune shift towards a Th2 response, with a predominance of the anti-inflammatory cytokines [45, 51]

Interferon- $\beta$  1a is a natural cytokine produced by the fibroblasts, a type I interferon with antiviral, anti-proliferative and immunomodulatory effects [52]. International studies demonstrated that interferon- $\beta$ 1a's mechanism of action is rather complex: it reduces the expression of the major histocompatibility complex class II on the antigen presenting cells, it decreases the peripheral activation of the T lymphocytes, it reduces the circulation of the inflammatory cells alongside blood brain barrier, it shifts the immune response towards a Th2-derived by increasing the levels of anti-inflammatory cytokines and decreasing the levels of pro-inflammatory cytokines, and also reduces the expression of matrix metalloproteinases [33, 40]. In spite of numerous studies on this aspect, the mechanism of action of interferon- $\beta$  is not fully recognized. The interferon- $\beta$  was the first disease modifying therapies used for multiple sclerosis' treatment, and even though the last years brought upon the world of immunomodulators an important number of molecules, it is still the highest used first

line disease modifying therapies. Due to the longevity of this agent, the characteristics regarding the tolerability and safety profile are very well known, with a stable equilibrium between efficiency and safety [8, 41].

The objectives for the present study are: 1. Assessment of the serum levels of pro and anti-inflammatory cytokines in naïve recurrent-relapsing multiple sclerosis patients and after one year of interferon- $\beta$ 1a treatment; 2. Identification of the cytokine profile in recurrent-relapsing multiple sclerosis patients in order to better apprehend the mechanism of action of this disease modifying therapies; 3. Evaluation of the correlations between the cytokine serum levels and the functional disability assessed by EDSS, relapses, duration of the disease and of the treatment; 4. Identification of predictive biomarkers for treatment's response in patients treated with interferon- $\beta$ 1a.

## Materials and Methods

### *Patients*

We performed a prospective study on 37 recurrent-relapsing multiple sclerosis patients, between January 2017 and June 2018, naïve to any disease modifying therapies, and 37 healthy controls (healthy controls), age and sex-matched. The patients are being followed in the Regional Multiple Sclerosis Center of Târgu Mures, Romania. The inclusion criteria were: 1. recurrent-relapsing multiple sclerosis diagnosis according to McDonalds Criteria 2010 [46]; 2. Over 18 years old; 3. Signed the informed consent form; 4. Naïve to any kind of disease modifying therapies. The exclusion criteria were: 1. Treatment with corticosteroids 30 days prior to the study inclusion; 2. Pregnancy or the wish to remain pregnant in the next 12 months (for female patients); 3. Contraindications for interferon- $\beta$ 1a treatment; 4. The patient interrupted interferon- $\beta$ 1a treatment in less than 12 months from initiation for various reasons, both personal or medical.

At the start of the study, all the study participants were evaluated based on their demographical (age, sex) and clinical data (onset of the disease, the total number of relapses, number of relapses in the past year). A full neurological work-up was administered to all patients, based on the EDSS score. In order to facilitate the statistical analysis, the initial EDSS score was defined EDSS\_0. All the patients underwent serum collection for immune analysis and the treatment with interferon- $\beta$ 1a with intramuscular administration once a week was administered. The healthy controls were assessed based on their demographical data. Serum samples were obtained from all the included subjects. At the end of the study, new serum samples were obtained from all the recurrent-relapsing multiple sclerosis patients, a new full neurological work-up was conducted, noted as EDSS\_1. The number of new relapses was also noted.

The study was conducted according to the Declaration of Helsinki and all the patients and the healthy controls signed the informed consent form.

#### *Serum sampling and Multiplex analysis*

For the cytokine evaluation, 20 mL of venous blood samples were collected. The samples were kept at room temperature for 20 minutes, afterwards, they were centrifuged at 3500 rpm. The obtained serum was aliquoted and kept in the freezer at  $-70^{\circ}\text{C}$  until analysed.

In order to determine the serum levels of the pro and anti-inflammatory cytokines, we used the xMAP technique, which allows the simultaneous assessment of numerous parameters from a small serum quantity. We evaluated 15 cytokines (IL-1 $\beta$ , TNF- $\alpha$ , IL-4, sCD40L, IL-6, IL-10, IL-33, IL-25, IL-17A, IL-17F, IL-21, IFN- $\gamma$ , IL-22, IL-23, IL-31) using the Bio-Plex Pro Human Th17 Cytokine Panel (Bio-Rad Laboratories, Inc. USA) with Flexmap 3D analyser (Luminex Corp, Austin Texas, USA). The values obtained at the beginning of the study for recurrent-relapsing multiple sclerosis patients were noted as: IL-1 $\beta$ \_0, TNF- $\alpha$ \_0, IL-4\_0, sCD40L\_0, IL-6\_0, IL-10\_0, IL-33\_0, IL-25\_0, IL-17A\_0, IL-17F\_0, IL-21\_0, IFN- $\gamma$ \_0, IL-22\_0, IL-23\_0, IL-31\_0 and the values obtained at the end of the study were noted as: IL-1 $\beta$ \_1, TNF- $\alpha$ \_1, IL-4\_1, sCD40L\_1, IL-6\_1, IL-10\_1, IL-33\_1, IL-25\_1, IL-17A\_1, IL-17F\_1, IL-21\_1, IFN- $\gamma$ \_1, IL-22\_1, IL-23\_1, IL-31\_1. The same cytokines were assessed for the healthy controls at the beginning of the study. Supplementary, we calculated the sum of all cytokines in the beginning of the study, both for recurrent-relapsing multiple sclerosis patients and

healthy controls, and at the end of the study for the recurrent-relapsing multiple sclerosis patients, noted as the “cytokine signature”, cytokine signature\_0 respectively cytokine signature\_1.

#### *Statistical analysis*

The statistical analysis was performed by GraphPad Prism 5.0. Parametric data were assessed by the mean and standard deviation (SD) and by range with minimum and maximum when evaluating non-parametric data. Normality was assessed using Kolmogorov-Smirnov and Shapiro-Wilk normality tests. The quantitative variables were assessed using Student's T-test and Mann-Whitney Test, respectively ANOVA or Kruskal-Wallis. The correlations were performed based on data distribution, either Spearman or Pearson. The statistical significance was set for  $p < 0.05$ .

## Results and Discussion

In both groups, we included 13 males and 24 females. The mean age of recurrent-relapsing multiple sclerosis patients at the beginning of the study was  $30.55 \pm 6.69$  years, while in the healthy controls group it was  $33.27 \pm 6.43$  years. The mean disease duration was  $60.63 \pm 49.18$  months, the mean study duration was  $13.36 \pm 1.91$  months. The mean number of relapses in the year prior to the study was  $1.09 \pm 0.53$ , the mean number of relapses during the study was  $0.45 \pm 0.93$ . The clinical neurological evaluation showed a slight increase between EDSS\_0 ( $1.3 \pm 1.23$ ) and EDSS\_1 ( $1.7 \pm 1.4$ ), but the difference was not statistically significant ( $p = 0.187$ ). The clinical, general and sociodemographic data is structured in Table I.

**Table I**

Clinical and sociodemographical characteristics of the recurrent-relapsing multiple sclerosis patients and healthy controls

	Multiple sclerosis patients (n = 37)	Healthy subjects (n = 37)
Age at the study onset	$30.55 \pm 6.69$	$33.27 \pm 6.43$
Male/Female	13/24	13/24
Multiple sclerosis duration (months)	$60.63 \pm 49.18$	
Treatment duration (months)	$13.36 \pm 1.91$	
Duration of the study (months)	$13.36 \pm 1.91$	
Relapses in the previous year	$1.09 \pm 0.53$	
Relapses during the study	$0.45 \pm 0.93$	
EDSS at study onset	$1.3 \pm 1.23$	
EDSS at the end of the study	$1.7 \pm 1.4$	

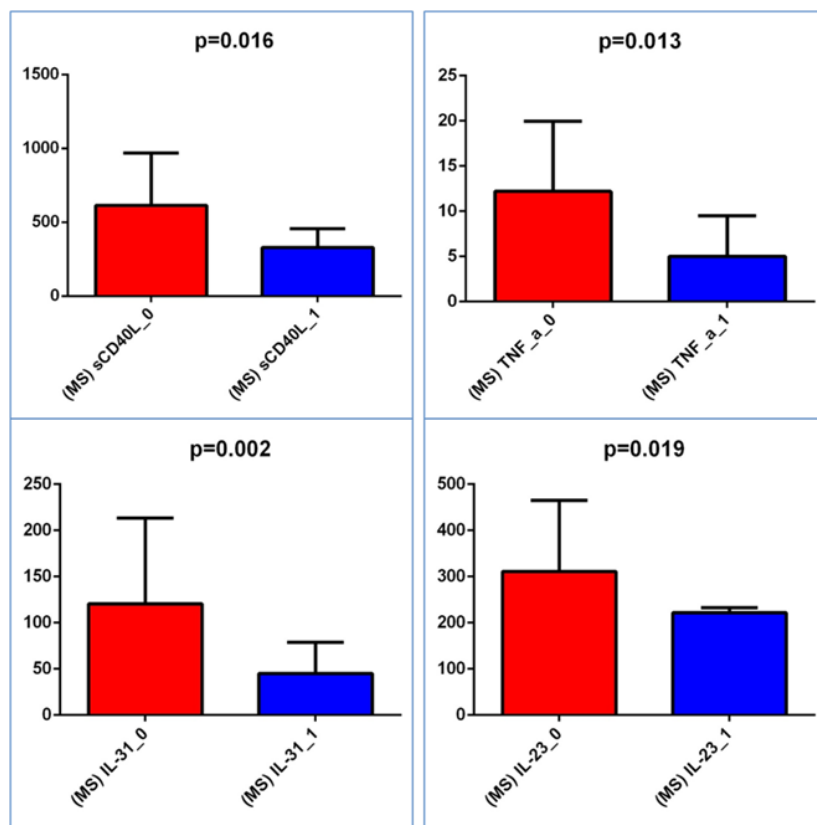
We obtained measurable serum values for 9 cytokines: IL-21, sCD40L, IL-23, IL-33, IL-17F, TNF- $\alpha$ , IL-10, IL-1 $\beta$  and IL-31. The results for the patients with recurrent-relapsing multiple sclerosis at the beginning and at the end of the study, respectively for the healthy controls are noted in Table II.

We compared the serum levels of the 9 cytokines at the beginning and the end of the study. A significant statistical difference was noted when comparing the serum levels of IL-23 ( $p = 0.019$ ), IL-31 ( $p = 0.002$ ), sCD40L ( $p = 0.016$ ) and TNF- $\alpha$  ( $p = 0.013$ ) (Figure 1).

**Table II**

Descriptive statistics of the serum values of cytokines of recurrent-relapsing multiple sclerosis patients and healthy controls and statistical analysis (comparison of serum values of cytokines at the beginning and end of the study in patients with recurrent-relapsing multiple sclerosis and comparison of cytokine values between recurrent-relapsing multiple sclerosis patients and healthy controls)

	IL-1 $\beta$	IL-10	IL-17F	IL-21	IL-23	IL-31	IL-33	sCD40L	TNF- $\alpha$	cytokine signature
MS_0 (pg/mL) Median Min:Max	0.36 (0.24:4.06)	4.97 (1.52:9.01)	36 (12:364)	200 (200:220)	232 (212:640)	92.26 (24.67:288.1)	191 (183:299)	576 (216:1488)	15.39 (3.31:21.81)	1317 (897:2698)
MS_1 (pg/mL) Median Min:Max	0.36 (0.26:0.61)	4.13 (0.58:7.84)	32 (20:36)	204 (200:212)	220 (200:236)	40.22 (2.97:118.5)	190 (186:200)	332 (136:596)	3.41 (1.89:17.49)	1037 (782:1328)
P value	0.625	0.105	0.064	0.08	<b>0.019</b>	<b>0.002</b>	0.23	<b>0.016</b>	<b>0.013</b>	<b>0.002</b>
HC (pg/mL) Median Min:Max	0.38 (0.26:0.46)	2.41 (0.58:8.24)	28 (20:40)	204 (200:212)	220 (212:232)	26.97 (0.79:56.92)	188 (184:208)	252 (128:388)	3.41 (2.84:9.84)	932 (790:1109)
p value	0.93	<b>0.036</b>	<b>0.02</b>	<b>0.001</b>	<b>0.05</b>	<b>0.002</b>	0.42	<b>0.002</b>	<b>0.006</b>	<b>0.002</b>

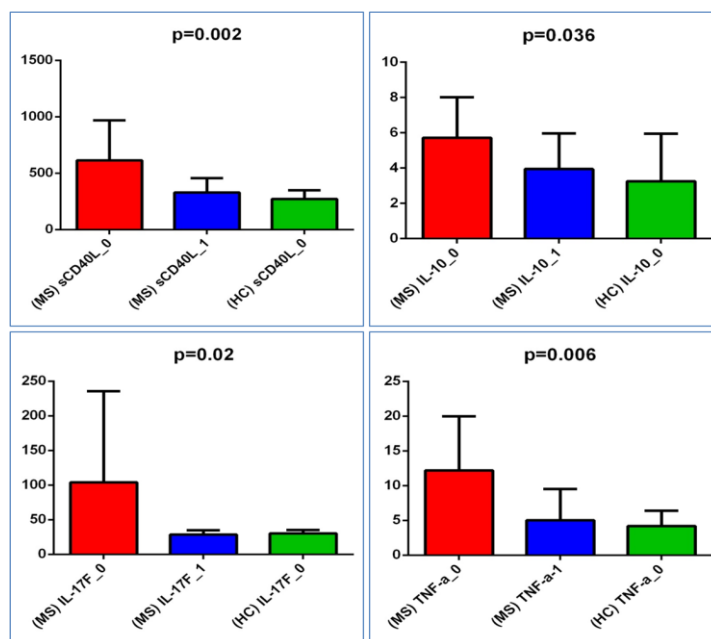


**Figure 1.**

Comparison between the serum levels of the sCD40L, TNF- $\alpha$ , IL-31, IL-23 at the beginning and at the end of the study

Furthermore, the serum levels of the selected cytokines were compared against the healthy controls. The recurrent-relapsing multiple sclerosis patients had significantly higher statistical values than the healthy controls, in both the beginning and at the end of the study for: IL-10 ( $p = 0.036$ ), IL-17F ( $p = 0.02$ ), IL-31 ( $p = 0.002$ ) and sCD40L ( $p = 0.006$ ). IL\_21\_0 was statistically significantly lower in recurrent-relapsing

multiple sclerosis than healthy controls ( $p = 0.001$ ), but at the end of the study they reached similar levels. IL-23\_0 and TNF- $\alpha$ \_0 were statistically significantly higher in recurrent-relapsing multiple sclerosis than in healthy controls ( $p = 0.05$ ,  $p = 0.006$ ), but the values decreased to the same levels as the healthy controls at the end of the study (Figure 2).

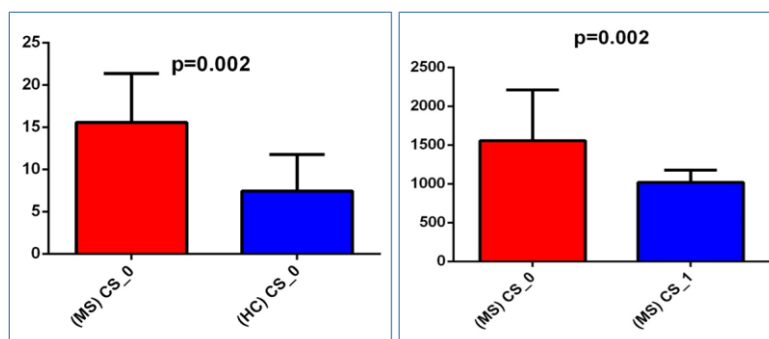


**Figure 2.**

Comparison between the serum levels of the cytokines in the recurrent-relapsing multiple sclerosis patients at the beginning and at the end of the study, and healthy controls

Regarding the cytokine signature, significantly higher values were found in the recurrent-relapsing multiple sclerosis patients at the beginning of the study, cytokine signature<sub>0</sub>, than at the end of the study

cytokine signature<sub>1</sub> ( $p = 0.002$ ). The recurrent-relapsing multiple sclerosis patients had significantly higher cytokine signature<sub>0</sub> values compared to healthy controls ( $p = 0.002$ ). (Figure 3).



**Figure 3.**

Comparison between cytokine signature in recurrent-relapsing multiple sclerosis patients at the beginning and end of the study; comparison between cytokine signature in recurrent-relapsing multiple sclerosis patients at the beginning of the study and healthy controls

Multiple correlations were assessed between the levels of the cytokines of the recurrent-relapsing multiple sclerosis patients (both at the beginning and the end of the study) and the clinical characteristics of the patients: age, treatment and disease duration, relapses<sub>0</sub> and relapses<sub>1</sub>, EDSS<sub>0</sub> and EDSS<sub>1</sub>. The correlations were performed by univariate analysis, using the Spearman's test for non-parametrical data. We noted the following negative statistically significant correlations between: disease duration and: IL-21<sub>0</sub> ( $r = -0.255$ ,  $p < 0.0001$ ), IL-23<sub>0</sub> ( $r = -0.605$ ,  $p = 0.05$ ), IL-33 ( $r = -0.678$ ,  $p = 0.023$ ), IL-17<sub>0</sub> ( $r = -0.796$ ,  $p = 0.003$ ),

IL-1 $\beta$ <sub>0</sub> ( $r = -0.636$ ,  $p = 0.04$ ), IL-31<sub>0</sub> ( $r = -0.736$ ,  $p = 0.012$ ); recurrences during the study and: IL-33<sub>1</sub> ( $r = -0.503$ ,  $p < 0.0001$ ), TNF- $\alpha$ <sub>0</sub> ( $r = -0.458$ ,  $p = 0.02$ ); EDSS<sub>0</sub> and TNF- $\alpha$ <sub>0</sub> ( $r = -0.748$ ,  $p = 0.005$ ); EDSS<sub>1</sub> and TNF- $\alpha$ <sub>0</sub> ( $r = -0.763$ ,  $p = 0.006$ ).

We noted the following positive statistically significant correlations between: EDSS<sub>0</sub> and IL-21<sub>0</sub> ( $r = 0.259$ ,  $p < 0.0001$ ); EDSS<sub>1</sub> and IL-21<sub>0</sub> ( $r = 0.409$ ,  $p < 0.0001$ ); Recurrences in the previous year and TNF- $\alpha$ <sub>0</sub> ( $r = 0.594$ ,  $p = 0.05$ ). All the data can be found structured in Table III.

**Table III**

Correlations between the serum values of cytokines and clinical and sociodemographical characteristics of the recurrent-relapsing multiple sclerosis patients

		Age	Treatment duration	Disease duration	Relapses in the previous year	Relapses during the study	EDSS_0	EDSS_1
(MS) IL-21_0	correl coef	0.502	<b>-0.255</b>	<b>-0.255</b>	0.318	0.638	<b>0.259</b>	<b>0.409</b>
	p value	0.181	<b>&lt; 0.001</b>	<b>&lt; 0.0001</b>	0.181	0.181	<b>&lt; 0.0001</b>	<b>&lt; 0.0001</b>
(MS) IL-21_1	correl coef	0.368	-0.392	-0.392	0.227	-0.060	-0.167	-0.201
	p value	0.267	0.161	0.161	0.515	0.097	0.349	0.295
(MS) sCD40L_0	correl coef	-0.013	-0.009	-0.590	-0.289	-0.075	-0.506	-0.436
	p value	0.961	0.927	0.060	0.287	0.327	0.111	0.179
(MS) sCD40L_1	correl coef	0.030	-0.111	-0.209	0.318	-0.017	0.084	0.111
	p value	0.930	0.691	0.539	0.338	0.424	0.805	0.743
(MS) IL-23_0	correl coef	-0.068	<b>0.636</b>	<b>-0.605</b>	-0.314	-0.255	-0.174	-0.270
	p value	0.824	<b>0.039</b>	<b>0.050</b>	0.258	0.117	0.521	0.377
(MS) IL-23_1	correl coef	0.422	-0.257	0.078	0.225	-0.238	-0.268	-0.316
	p value	0.194	0.341	0.820	0.503	0.145	0.327	0.302
(MS) IL-33_0	correl coef	0.009	0.370	<b>-0.678</b>	-0.428	-0.216	-0.508	0.469
	p value	0.983	0.260	<b>0.023</b>	0.101	0.105	0.073	0.114
(MS) IL-33_1	correl coef	-0.144	-0.173	-0.071	-0.203	<b>-0.503</b>	-0.217	-0.330
	p value	0.525	0.432	0.715	0.393	<b>&lt; 0.0001</b>	0.365	0.203
(MS) IL-17F_0	correl coef	0.095	0.548	<b>-0.796</b>	-0.457	0.153	0.095	0.194
	p value	0.781	0.084	<b>0.003</b>	0.119	0.650	0.781	0.563
(MS) IL-17F_1	correl coef	0.346	-0.0294	0.160	0.313	0.117	-0.185	-0.205
	p value	0.291	0.269	0.634	0.351	0.796	0.406	0.450
(MS) TNF- $\alpha$ _0	correl coef	0.004	0.116	-0.163	-0.169	<b>0.458</b>	<b>0.748</b>	<b>0.763</b>
	p value	0.994	0.732	0.633	0.491	<b>0.020</b>	<b>0.005</b>	<b>0.006</b>
(MS) TNF- $\alpha$ -1	correl coef	0.346	-0.292	-0.073	<b>0.594</b>	0.332	0.134	0.161
	p value	0.291	0.332	0.821	<b>0.050</b>	0.331	0.690	0.632
(MS) IL-10_0	correl coef	-0.239	<b>0.670</b>	-0.482	-0.219	-0.275	-0.007	-0.036
	p value	0.478	<b>0.024</b>	0.122	0.419	0.080	0.982	0.915
(MS) IL-10_1	correl coef	0.147	-0.245	-0.083	-0.064	0.503	-0.352	-0.042
	p value	0.661	0.378	0.743	0.763	0.129	0.205	0.748
(MS) IL-1 $\beta$ _0	correl coef	-0.086	0.297	<b>-0.636</b>	-0.420	-0.214	-0.466	-0.404
	p value	0.790	0.371	<b>0.040</b>	0.134	0.153	0.117	0.188
(MS) IL-1 $\beta$ _1	correl coef	0.531	-0.100	0.569	0.366	-0.080	0.038	-0.047
	p value	0.094	0.695	0.071	0.267	0.311	0.910	0.752
(MS) IL-31_0	correl coef	-0.123	0.186	<b>-0.736</b>	-0.328	-0.040	-0.381	-0.260
	p value	0.707	0.581	<b>0.012</b>	0.235	0.387	0.199	0.392
(MS) IL-31_1	correl coef	-0.278	0.252	-0.490	0.062	0.0	0.116	0.053
	p value	0.395	0.454	0.129	0.857	0.460	0.733	0.875

The multifactorial aetiology of multiple sclerosis is not fully cleared yet. The complex immunopathogenic processes are of active interest. The cytokines and their respective receptors play an important role in the development, maintenance and evolution of multiple sclerosis lesions, as well as in disease activity.

In the absence of an etiological factor to specifically target as a multiple sclerosis treatment, defining the molecular mechanisms that build the base of the inflammation, demyelination and neuronal toxicity processes, led to the development of numerous molecules that slow down disease progression, reduce the number of relapses and the imagistic activity. The development of disease modifying therapies significantly improved quality of life and the prognosis in an important number of patients [2].

Interferon- $\beta$ 1a is a first line disease modifying therapies for the treatment of recurrent-relapsing multiple sclerosis. Although it has been used for

almost 20 years, the mechanism of action is not fully understood. Assessment of serum cytokines in recurrent-relapsing multiple sclerosis patients treated with Interferon- $\beta$ 1a is important in order to understand the physio-pathological mechanism of multiple sclerosis together with the mechanism of action of interferon- $\beta$ 1a. Numerous hypotheses were raised: it increases the IL-10, IL-4 and IL-12 (which suppress the differentiation of Th17 lymphocytes), it decreases TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ , IL-23 and transforming growth factor  $\beta$  (TGF- $\beta$ ) (which induce the differentiation of Th17 lymphocytes). Two unknown variables compose this puzzle: one is the mechanism of action of Interferon- $\beta$ 1a and the other one is the immunopathogenesis of multiple sclerosis [3, 11, 35, 50].

IL-17 is produced by the Th17 lymphocytes and plays an essential role in the pathogenesis of multiple sclerosis. The naïve CD4 cells, stimulated by TGF- $\beta$ , IL-6 and IL-21 differentiate in Th17 cells, and IL-23

is required for maintaining and expanding this cell line. IL-17 has a strong pro-inflammatory effect by inducing the expression of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in the epithelial and endothelial cells, it attracts the neutrophils and stimulates dendritic cells maturation. Experimental studies on murine models demonstrated that, in IL-17 deficient mice, a reduction in experimental autoimmune encephalomyelitis activity was found. Various clinical studies revealed that a significant number of Th17 cells were found at the site of active lesions compared to inactive lesions, and also that the serum levels of IL-17 is higher in patients with an active multiple sclerosis. The endothelial cells of multiple sclerosis patients carry a higher number of IL-17 receptors and appear to be more permeable when faced with this cytokine. The Th17 cells pass through the blood brain barrier, thus initiating inflammation [2, 5, 6, 20, 25, 26, 53, 58].

In our study, the recurrent-relapsing multiple sclerosis patients had significantly higher IL-17 levels compared to healthy controls. The Interferon- $\beta$ 1a treatment for more than 1 year led to a reduction in IL-17 serum levels, but with no significant impact. The levels of IL-17 negatively correlated with the duration of the disease, in accordance with international data, suggesting that the inflammatory processes are more accentuated in the early stages, while the latter are marked by neurodegeneration and axonal loss.

IL-10 is a strong anti-inflammatory cytokine with effects upon both the innate and the acquired immunity. It is usually secreted by the monocytes, Th2 cells, regulatory T cells, mastocytes and eosinophilic cells, but in the certain conditions, it can be also produced by the B cells, macrophages, dendritic cells and other T cells. The secretion of IL-10 is dependent on the presence of exo- and endotoxins, its expression being minimal in unstimulated tissues. IL-10 is essential for the up-regulation of the immune response, carrying an important immunosuppressive role in the autoimmune processes by inhibiting the secretion of pro-inflammatory cytokines (IL-17 and IL-6), chemokines, adhesion molecules, neutrophils and by reducing class II major histocompatibility complex. It inhibits the Th1 response by reducing the antigen presentation to the antigen presenting cells, but can also act directly upon the T cells, reducing their proliferation and cytokine secretion. Recent studies showed that, in order to limit the Th1 response after infections, by reducing the lithogenic capacity of Th1 and Th17 cells, the Th1 cells also secrete IL-10, under the influence of IL-27 and TGF- $\beta$  [4, 10, 27, 29, 42, 44].

In multiple sclerosis patients, the serum levels of IL-10 are increased in the remission phase. Genetic studies demonstrated that depletion of IL-10 is associated with an increase in experimental autoimmune encephalomyelitis severity, and corollary,

high expression of IL-10 has a neuroprotective role [56].

In our study, the multiple sclerosis patients had significantly higher IL-10 levels compared to healthy controls. All the patients were in the remission stage of the disease, at least 30 days after the last relapse, and high serum levels were kept until the end of the study.

In multiple sclerosis, interferon  $\beta$  treatment inhibits the differentiation of the Th17 cells by reducing the activity of IL-1 $\beta$ , IL-23 and TGF- $\beta$ , cytokines actively involved in Th17 differentiation and increases the levels of IL-10, IL-27, IL-12 and IL-4, with suppressive properties upon the Th17 lineage [7, 19, 36].

Zhang *et al.* demonstrated that interferon- $\beta$  decreases the number of IL-17 secreting cells and increases the IL-10 secreting cells, and also dual IL-10 and IL-17 secreting cells. They hypothesized that fully differentiated Th17 cells produce IL-10, stimulated by interferon- $\beta$ , contributing to the treatment efficiency [59].

IL-23 is a heterodimer from the IL-12 family, actively involved in Th1 differentiation. It contains the same p40 subunit as IL-12, together with the unique p19 unit. IL-23 plays a crucial role in Th17 differentiation from naïve CD4 T cells. Studies upon IL-23 deficient mice revealed that experimental autoimmune encephalomyelitis couldn't be induced and also, disease resistance was correlated with IL-17 deficiency [1, 17].

In our study, the recurrent-relapsing multiple sclerosis patient had at the beginning statistically higher IL-23 levels compared to healthy controls. After one year of treatment, the serum levels dropped to the same levels as healthy controls, sustaining the evidence according to which, one of the interferon- $\beta$  effects is represented by a decrease in IL-23 levels. The serum levels of IL-23 negatively correlated with the disease duration.

IL-21 belongs to class I cytokines (including IL-2, IL-4, IL-7, IL-9 and IL-15) and is produced by activated T lymphocytes, natural killer T cells, Th follicular cells and Th17. IL-21 carries an important, dual role in the pathogenesis of multiple sclerosis. It inhibits the activation and the maturation of dendritic cells, mediated by granulocyte-macrophage colony-stimulating factor and by inhibiting the granulocyte-macrophage colony-stimulating factor it down-regulates the secretion of IL-6, IL-12, IL-1 $\beta$  and TNF- $\alpha$  by dendritic cells. IL-21 stimulates the differentiation of Th17 cells, even in the absence of IL-6, thus shifting the balance between Th17 and regulatory T cells towards the Th17 and stimulates the proliferation of the lymphoid cells. Together with IL-15, it increases T CD8 and natural killer cytotoxicity. Various clinical studies demonstrated that patients with a secondary progressive form of multiple sclerosis have higher IL-21 levels compared to healthy controls, and that IL-21 levels correlate with disease activity and severity in secondary

progressive multiple sclerosis patients [22, 23, 47]. Wang *et al.* found no statistically significant differences between IL-21 serum levels in multiple sclerosis patients with relapses and healthy controls [55]. Our data reveals that the lot of patients had slightly lower or comparable levels with the healthy controls, this being explained by the fact that all our patients were recurrent-relapsing multiple sclerosis. In our study, an increase in IL-21 levels at the beginning of the study correlated with disability, as per EDSS.

TNF- $\alpha$  is mainly secreted by the macrophages and the monocytes, but also by T and B lymphocytes. TNF- $\alpha$  has a dual role, dependent on the serum levels. A low TNF- $\alpha$  expression carries immune regulation effects, by inhibiting tumoral growth and protecting against various pathogens. In the central nervous system, it mediates the remyelination. High levels of TNF- $\alpha$  induce a pro-inflammatory response [14, 24, 38, 39]. The contrasting effects in the etiopathogenesis of multiple sclerosis shaped this cytokine as a double-edged sword. Directly inhibiting TNF- $\alpha$  in multiple sclerosis patients resulted in a paroxysmal augmentation of disease activity [52, 54]. Beck *et al.* demonstrated that TNF- $\alpha$  levels positively correlated with disease activity in multiple sclerosis patients and also that relapses were preceded by an increase of TNF- $\alpha$  production [9].

In our study, multiple sclerosis patients had significantly higher levels of TNF- $\alpha$  at the beginning, compared to healthy controls. Interferon- $\beta$ 1a treatment lead to a significant decrease in TNF- $\alpha$  levels, defining yet another one mechanism of action. High TNF- $\alpha$  levels at the study initiation correlated with a higher relapse rate during the study, thus supporting Beck *et al.* hypothesis.

IL-33, a member of the IL-1 family, has a high expression inside the central nervous system with an important immunomodulatory role. High levels of IL-33 have been reported in the serum and the cerebrospinal fluid of multiple sclerosis patients. Jiang *et al.* studied the effects of this cytokine on murine models treated with IL-33 and demonstrated the protective role in experimental autoimmune encephalomyelitis. IL-33 supposedly switches the immune response from a Th1 and Th17 derived to a Th2, a protective immune response, with IL-5 and IL-13 augmentation [13, 30].

In our study we did not find any statistically significant differences between IL-33 levels in recurrent-relapsing multiple sclerosis nor healthy controls. High serum levels at the end of the study were correlated with a reduced number of relapses, suggesting a protective role in multiple sclerosis.

sCD40L, the soluble ligand of CD40L, connects to CD40 receptor and has cytokine-like properties with a dual role in multiple sclerosis. Drescher *et al.* demonstrated that CD40L has a protective role

against demyelination and stimulates the remyelination in a mouse model of Theiler's murine encephalomyelitis virus [18]. Other studies demonstrated that sCD40L stimulates the production of proinflammatory cytokines and chemokines (by augmenting IL-6 expression), matrix metalloproteinases and increases the expression of cell adhesion molecules [12, 21, 28]. Zong *et al.* identified significantly increased serum levels of CD40L in multiple sclerosis patients compared to healthy controls [60]. Guerrero- García *et al.* performed a study in order to bring light upon this dual role of sCD40L, and noted that multiple sclerosis patients have higher serum levels of sCD40L in the first years of the disease, with peak values at 5 years and a slow decrease thereafter. They assessed serum values of sCD40L in patients treated with interferon  $\beta$  or glatiramer acetate and noted that interferon  $\beta$  treated group had significantly lower levels compared to glatiramer acetate – treated group [16].

In our study, recurrent-relapsing multiple sclerosis patients had significantly lower sCD40L values compared to healthy controls, our patients having a mean duration of the disease of 5 years. We noted that a short duration of the disease tends to associate with high sCD40L values, but with no statistical impact. One-year interferon- $\beta$  1a treatment leads to a significant decrease in sCD40L serum levels, therefore we can raise the hypothesis, according to which, this might be one of the mechanisms of action of Interferon- $\beta$ 1a in recurrent-relapsing multiple sclerosis.

The results of our study demonstrate that the serum levels of the proinflammatory cytokines, also called cytokine signature are higher in recurrent-relapsing multiple sclerosis patients compared to healthy controls, and that after one year of Interferon- $\beta$ 1a treatment, this inflammatory profile significantly decreases. Similar results were reported by O'Connell *et al.* [43].

## Conclusions

A fair understanding in the immunopathogenesis of multiple sclerosis, together with the mechanism of action of Interferon- $\beta$ 1a is necessary in order to be able to personalize the disease modifying therapies choice. After approximately one year of Interferon- $\beta$ 1a treatment in naïve multiple sclerosis patients, the following pro-inflammatory cytokine levels decreased: IL-23, IL-31, sCD40L, TNF- $\alpha$  and “cytokine signature”.

## Conflict of interest

The authors declare no conflict of interest.

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