

Interleukin-17A Levels Vary in Relapsing-Remitting Multiple Sclerosis Patients in Association with Their Age, Treatment and the Time of Evolution of the Disease

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Key Words

Glatiramer acetate · IFN- β · IFN- γ · IL-10 · IL-17A · Multiple sclerosis

Abstract

Objective: The present study was specifically designed to discern the possible existence of subgroups of patients with the relapsing-remitting form of multiple sclerosis (RRMS) depending on their gender, age, disease stage (relapsing or remitting), time of disease evolution and response to different treatments. **Methods:** We analyzed samples from patients with RRMS (50 females and 32 males) and healthy individuals (25 matched for age and gender) and determined serum concentrations of IFN- γ , IL-10 and IL-17A. We stratified patients by gender, age, treatment and disease evolution time, and subsequently correlated these independent variables with the concentrations of the previously mentioned cytokines. **Results:** We provided initial evidence that treatment

exerted possible differential effects depending on the time of disease duration. Results evidence the existence of different subgroups of patients with MS, who can be classified as follows: (a) male or female under or over 40 years of age; (b) disease duration according to treatment (under or over 8 years of disease); (c) classification according to fluctuating levels of IFN- γ , IL-10 and IL-17A in the following three stages of disease evolution: <5 years, between 5 and 10 years, and >10 years. **Conclusion:** These subgroups must be taken into account for the clinical follow-up of patients with MS in order to provide them with a better and more personalized treatment, and also for a deep and detailed analysis of progressive disease, in an attempt to comprehend fluctuations and clinical variability by means of a better understanding of intrinsically physiological variables of the disease.

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Introduction

Multiple sclerosis (MS) is a demyelinating disease that mainly affects the central nervous system (CNS) and is immune mediated [1–3]. The progression of MS is characterized by very different evolution patterns and variable rates of disability accumulation. There is a female preponderance in MS [4] and a defining feature comprises its fluctuating disease activity, with the occurrence of clinical relapses separated by periods of clinical stability (remissions) [5]. These latter stages can be prolonged for years, and gender appears to play a critical role in MS development, progression and response to treatment [6]. More than 80% of patients express a relapsing-remitting form of MS (RRMS), characterized by exacerbations of partially or nearly completely reversible neurological disability.

MS can be considered as a disease with different clinical phenotypes rather than an entity encompassing several distinct diseases [7]. RRMS is characterized by T-cell-mediated demyelination [8] involving T-cell subtypes, such as Th1 [9], T_{reg} [10] and Th17 [9, 11], which characteristically produce interferon (IFN)- γ , interleukin (IL)-10 and IL-17A, respectively. Consequently, these cytokines can be used as markers of the differential activity of the disease during different disease stages and also to monitor the response of the immune system to treatment. Treatment of MS consists of immunomodulatory drugs, such as glatiramer acetate (GA) and IFN- β , which affect and modulate the autoreactive immune response through cytokine pathways [12] and which comprise the first US Federal Drug Administration-approved treatment for MS [13]. However, the efficiency of these treatments varies widely among different MS patients, and unfortunately there is no cure of MS.

One of the main problems is related to the differential genetic background and also the disparity of epigenetic influences that affect the disease progression. Given that immunological events along relapsing-remitting periods are not well defined, more and novel analyses must be performed to better understand MS pathogenesis and to better comprehend the disparity of response to different treatment approaches by identifying subsets of patients (responders/nonresponders), with better/poor prognosis, depending on treatment, age, gender and disease evolution time. In the present work, we analyzed samples from 82 RRMS patients (50 females and 32 males) and determined serum concentrations of IFN- γ , IL-10 and IL-17A. We stratified patients by gender, age, treatment and disease evolution time, and sub-

Table 1. Demographic and clinical data of samples from RRMS patients

	Female	Male	Total
Patients, n	50	32	82
%	61	39	100
Age, years	34 \pm 8.5	32 \pm 9.9	33 \pm 9.0
Disease duration, years	8.6 \pm 5.2	6.9 \pm 5.3	7.9 \pm 5.3
EDSS (n = 50)	2.3	2.7	2.5
MSSS (n = 48)	3.1	4	3.4

Data on age and disease duration are expressed as means \pm SD.

sequently correlated these independent variables with the concentrations of the previously mentioned cytokines, in an attempt to ascertain the existence of different subgroups.

Patients and Methods

Patients

A total of 82 patients (50 females and 32 males) diagnosed with RRMS (41 in clinical relapse and 41 in clinical remission), who had received IFN- β (n = 29) and GA (n = 53), were recruited from the Neurology Service of the Western National Medical Center's Specialty Hospital of the Mexican Social Security Mexican Institute (IMSS) and the Neurology Service of Guadalajara Civil Hospital in Jalisco, a province of Mexico. Study patients with RRMS fulfilled the following criteria: (1) diagnosed with RRMS according to the revised McDonald diagnostic criteria (2005) [14]; (2) age between 20 and 60 years, and (3) RRMS treated with IFN- β or GA for at least 3 months. Clinical disability was evaluated using Kurtzke's [15] Expanded Disability Status Scale (EDSS), and disease severity was evaluated utilizing the MS Severity Score (MSSS) [16]. The clinical form of RRMS was determined according to the classification of Lublin and Reingold [17] published in 1996 and its revision in 2013 [18]. RRMS patients in clinical relapse and those in clinical remission were included. Clinical relapse is defined as an episode of acute worsening of neurologic functions and clinical remission as the period of variable degree of recovery with no relapse episodes occurring within the last 3 months prior to the time of enrollment in this study. Untreated RRMS patients were not included. Patients with the following conditions were excluded: (1) a diagnosis of secondary progressive MS or primary progressive MS; (2) treatment with corticosteroids in the previous 3 months; (3) a clinical history of autoimmune or inflammatory disease, and/or (4) a diagnosis of any other chronic-degenerative disease of the CNS. All patients gave their written informed consent for study inclusion. The control group consisted of 25 age- and gender-matched healthy individuals (15 females and 10 males) that were selected among the general population in the same geographical areas as the patients. The samples were collected at the same time in order to diminish bias associated with the circadian cycle. This study was conducted in accordance with the ethical guidelines of

Table 2. Serum cytokine levels (pg/ml) of RRMS patients treated with IFN-β and GA and grouped by different criteria

Subgroups	Cytokine levels					
	IFN-γ	p value	IL-10	p value	IL-17A	p value
Treatment						
IFN-β	28.71±3.86	0.06 ^a	6.31±2.35	0.20 ^a	1.87±0.32	0.22 ^a
GA	43.35±6.58		9.48±2.42		2.09±0.23	
Phase						
Relapsing	30.76±3.87	0.04 ^a	5.08±0.84	0.05 ^a	1.89±0.25	0.15 ^a
Remitting	45.81±8.03		9.30±2.41		2.14±0.28	
Gender						
Male	30.60±3.61	0.09 ^a	7.72±2.28	0.37 ^a	1.51±0.24	0.02 ^a
Female	43.02±6.98		6.84±1.55		2.34±0.26	
Disease duration <8 years						
IFN-β	26.64±4.20	0.03 ^a	6.99±3.20	0.47 ^a	1.69±0.34	0.04 ^a
GA	47.83±9.15		6.73±1.27		2.53±0.31	
Disease duration ≥8 years						
IFN-β	33.20±9.06	0.49 ^b	3.43±0.76	0.24 ^b	2.93±0.93	0.22 ^b
GA	39.61±9.46		8.10±2.70		1.61±0.31	
Age <40 years						
Male	31.94±4.17	0.14 ^a	7.90±2.67	0.48 ^a	1.62±0.27	0.08 ^a
Female	42.70±7.98		7.73±2.10		2.20±0.30	
Age ≥40 years						
Male	23.35±4.30	0.44 ^b	6.74±2.58	0.14 ^b	0.91±0.40	0.03 ^b
Female	43.83±14.66		4.57±1.05		2.70±0.56	
Severity (MSSS)/disability (EDSS)						
High	43.71±9.87	0.21 ^b	7.68±2.49	0.21 ^b	1.96±0.30	0.21 ^b
Low	42.64±9.18		9.43±3.76		2.57±0.51	
Control values	16.8±2.42		2.23±0.29		7.30±0.51	

Means ± SEM. Significant p values are italicized. ^a Student's t test. ^b Mann-Whitney U test.

the 2013 Declaration of Helsinki and was approved by the Ethical Committee of the IMSS in Mexico.

Blood Sample Collection

Peripheral blood was collected in BD Vacutainer® tubes without anticoagulant to obtain serum. Serum was obtained by centrifugation and aliquots were immediately stored at -80°C until assayed.

Cytokine Measurements

Serum cytokine levels (IFN-γ, IL-10 and IL-17A) were measured with a sandwich enzyme-linked immunosorbent assay using a commercial immunoassay (BioLegend, San Diego, Calif., USA) according to the manufacturer's instructions. Results were expressed in pictograms per milliliter. The specificity and the sensitivity for the cytokines was defined according to the manufacturer's instructions (specificity: except in IL-17A cytokine measurements in which cross-reactivity with human IL-17F was negligible, non-cross-reactivity was observed when those kits were used to analyze multiple human and mouse recombinant cytokines/chemokines at up to 50 ng/ml; sensitivity: the minimum detectable concentrations were 4 pg/ml for IFN-γ, 2 pg/ml for IL-10 and 1 pg/ml for IL-17A).

Statistical Analysis

A database was created using the Microsoft Office Excel program and statistical analysis was performed using GraphPad Prism version 5.0. Data analyses of cytokine levels were performed by calculating means, SD and SEM. The groups' continuous variables were compared using the Student t test, the Mann-Whitney U test, analysis of variance (ANOVA) or the Kruskal-Wallis test, when appropriate. Statistical significance was considered when $p < 0.05$.

Results

Demographic and clinical data of the samples from the patients with RRMS are presented in table 1. In addition, mean values obtained from the EDSS and MSSS tests are indicated; it is noteworthy that females exhibited slightly longer disease duration, but they were slightly less affected. Also, disease prevalence in our study population corresponds to previously published results, which show a nearly 3:1 female:male ratio [19]. Additionally, this find-

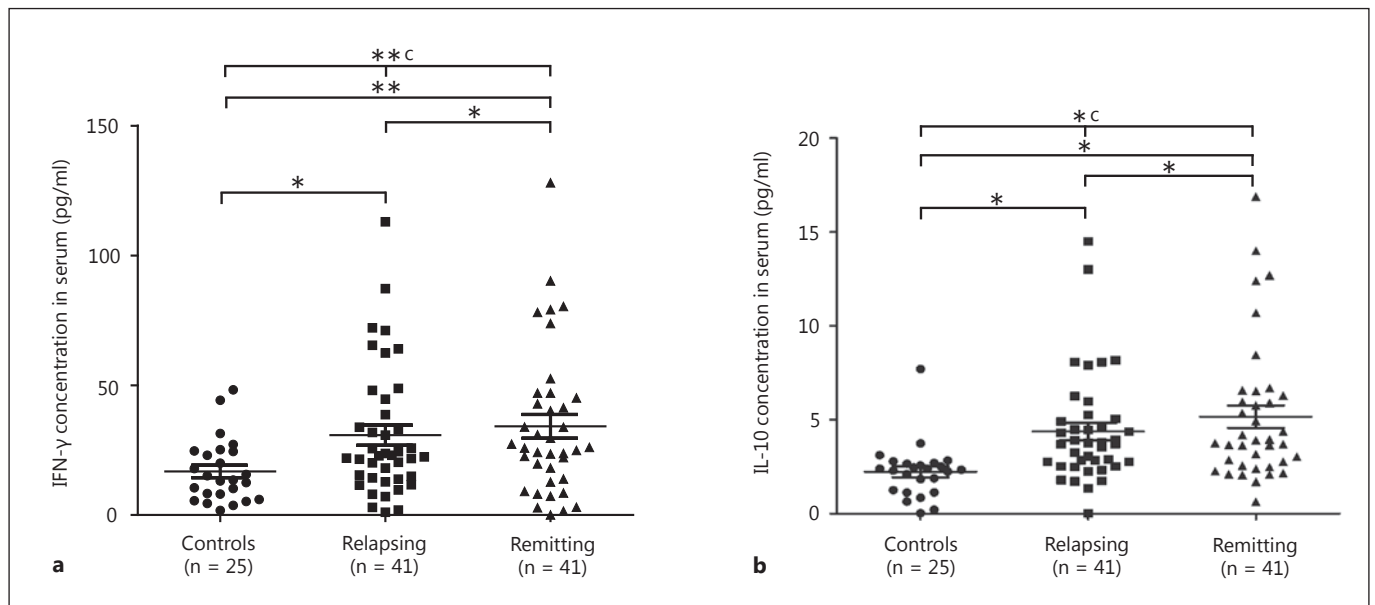


Fig. 1. IFN- γ (a) and IL-10 (b) serum levels of RRMS patients. IFN- γ and IL-10 tends to increase in remitting compared to relapsing RRMS patients and controls. The dot plots show the concentrations of IFN- γ and IL-10 in the serum of controls and RRMS. * $p < 0.05$, ** $p < 0.01$ (Student's t test); *c $p < 0.05$, **c $p < 0.01$ (ANOVA).

ing agreed with previous reports where women show less progression of disability than men during the disease course [20].

A summary of the results obtained for the concentrations of the three studied cytokines is shown in table 2. Additionally, table 2 includes the results obtained from the analysis of the different subgroups of patients. First, we divided our study group by treatment: IFN- β ($n = 29$) or GA ($n = 53$). Results showed that IFN- γ and IL-10 levels are higher in patients than in controls. Contrariwise, IL-17A levels were lower in patients treated with IFN- β or GA than in controls, although none of the three cytokines determined reached statistically significant differences among treatments. Second, we classified samples by disease phase: whether the patients were in the relapsing ($n = 41$) or remitting ($n = 41$) phase. According to this classification, it is important to mention that IFN- γ (fig. 1a) and IL-10 (fig. 1b) concentrations are increased in patients in the remitting disease phase ($p \leq 0.05$), whereas IL-17A presented no differences. Third, we grouped samples by gender: male ($n = 32$) and female ($n = 50$), and only IL-17A was increased in females versus males ($p < 0.05$; fig. 2a).

Based on this initial analysis and on the apparently differential distribution of some of the samples within each of these classifications, we decided to designate dif-

ferent subgroups. First, subgroups were distinguished when we classified samples by gender subdivided in two groups by age: one < 40 years and the other ≥ 40 years. Under these categories, IFN- γ and IL-10 concentrations did not vary, but again, IL-17A concentrations did ($p < 0.05$; fig. 2b), the latter being significantly higher in females than in males ≥ 40 years of age. In addition, these also tended to increase in females ≥ 40 years when compared with females < 40 years of age, but tended to decrease in males ≥ 40 years when compared with males < 40 years of age, although this did not reach statistical significance, probably due to the small number of patients aged ≥ 40 years.

Second, in relation to treatment, we subdivided the groups by disease duration and selected two groups: disease duration < 8 years ($n = 46$) and ≥ 8 years ($n = 36$). Under these classifications, IL-10 concentrations did not vary, but IFN- γ and IL-17A concentrations did ($p < 0.05$; fig. 3). It is relevant to note that samples from patients treated with IFN- β demonstrated an increase in IL-17A levels when these samples derived from patients with disease evolution ≥ 8 years; however, when patients were treated with GA, IL-17A levels decreased after 8 years of disease duration (fig. 3).

Third, we subclassified the samples by applying values assigned for disability and severity using the EDSS and

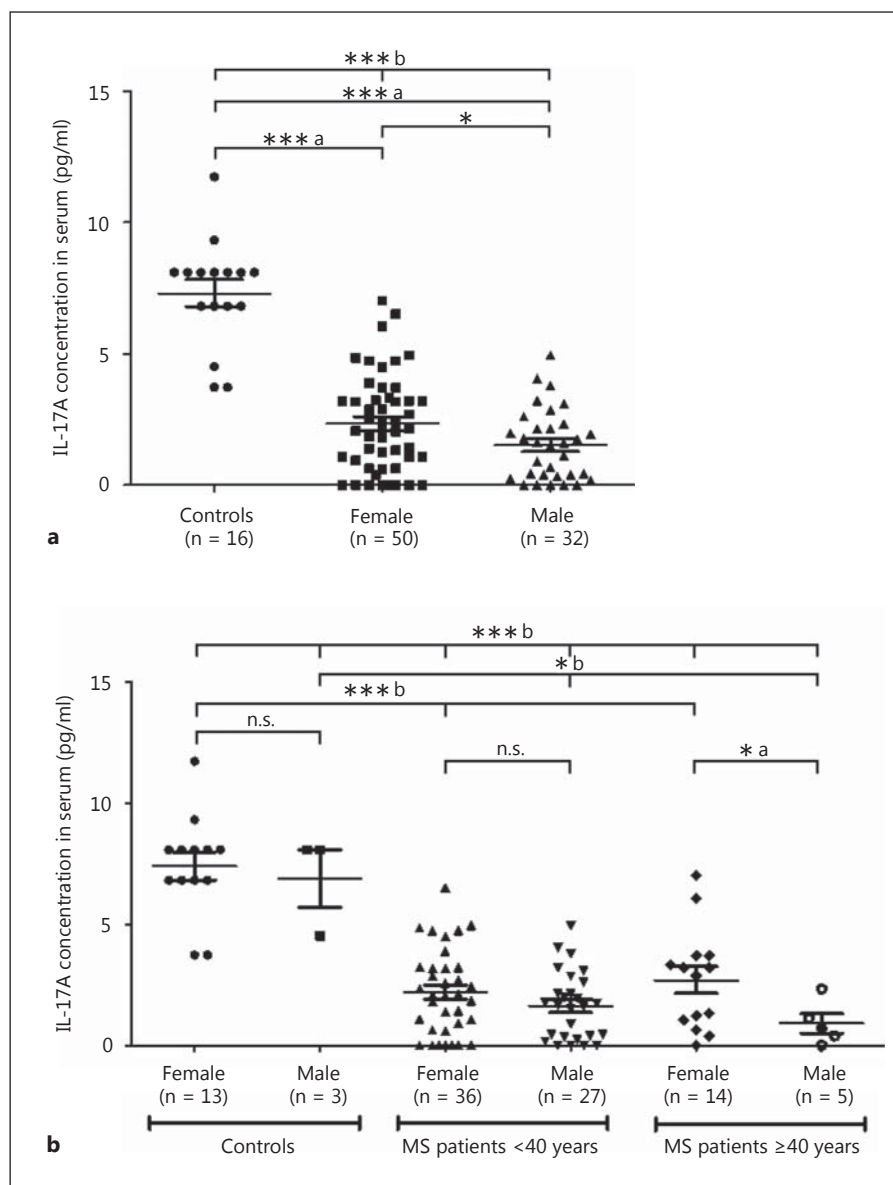


Fig. 2. IL-17A serum levels of RRMS patients classified by gender. **a** IL-17A concentration is reduced in MS patients compared to controls and tends to be higher in females versus males. **b** Subjects classified by age in two groups (<40 and ≥40 years). While IL-17A concentrations tend to decrease in aged males, it increased in aged females. n.s. = Nonsignificant; * $p < 0.05$ (Student's t test); *, ^a $p < 0.05$, ***, ^a $p < 0.0001$ (Mann-Whitney U test); *, ^b $p < 0.05$, ***, ^b $p < 0.0001$ (Kruskal-Wallis test).

the MSSS test, respectively, assigning patients into one of the following two groups: with high disability and severity, and with low disability and severity. However, none of the cytokines studied significantly varied among these subgroups.

Finally, given the data dispersion of the concentrations of the three cytokines studied, we decided to stratify the data by each year of disease evolution (fig. 4). Under this arrangement, it is noteworthy that the concentration of the three studied cytokines varied among different disease duration years, having a three-wave shape with maximal spikes at around 5 and 10 years (12

years in the case of IL-10) of disease evolution. Prior to and after these peaks, cytokine concentrations decreased significantly.

Discussion

The present study was specifically designed to discern the possible existence of subgroups of patients with RRMS depending on their gender, age, disease stage (relapsing or remitting), time of disease evolution and response to different treatments based on the serum levels of three

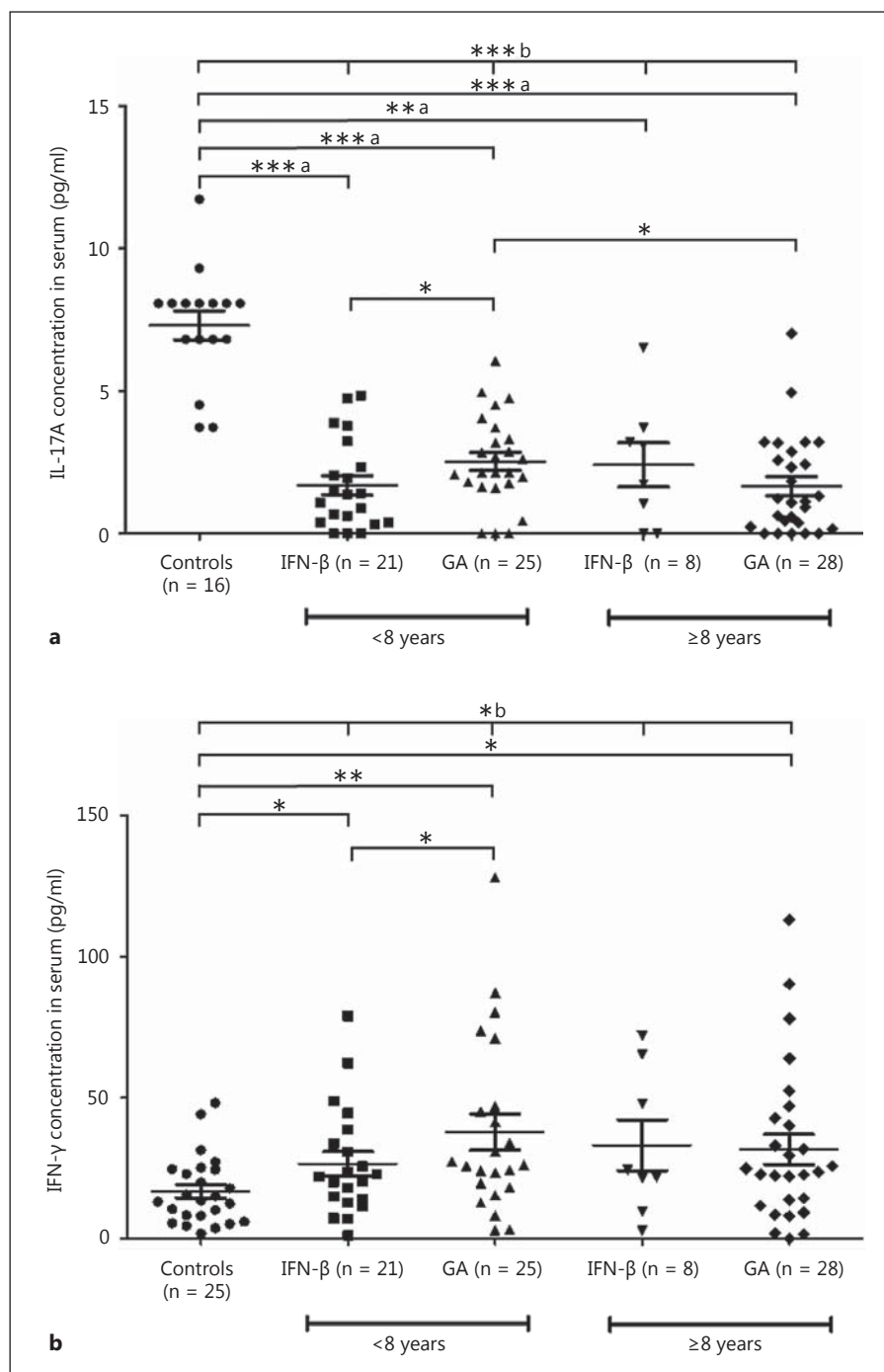


Fig. 3. IL-17A and IFN- γ serum levels in RRMS patients treated with IFN- β or GA and classified by disease duration. IL-17A and IFN- γ concentrations varied depending on the treatment but only when subjects are grouped by disease duration. **a** IL-17A: * $p < 0.05$ (Student's t test); **, ^a $p < 0.01$, ***, ^a $p < 0.0001$ (Mann-Whitney U test); ***, ^b $p < 0.0001$ (Kruskal-Wallis). **b** IFN- γ : * $p < 0.05$, ** $p < 0.01$ (Student's t test); *, ^b $p < 0.05$ (Kruskal-Wallis test).

cytokines, IFN- γ , IL-10 and IL-17A, which helped to monitor the activity of the main T-cell subpopulations involved in the pathophysiology of MS [11, 21, 22].

First, our study showed that IFN- γ and IL-10 levels tended to be higher in the remitting phase, which probably reflects an immunomodulatory effect that decreases

Th17 cell differentiation [23, 24]. The increase in IFN- γ mediates STAT1 (signal transducer activator of transcription 1) activation, which in turn enhances SOCS3 levels (a Th17 differentiation inhibitor); then, STAT3 phosphorylation is attenuated [25]. On the other hand, IL-10 levels were elevated in MS patients, being higher in

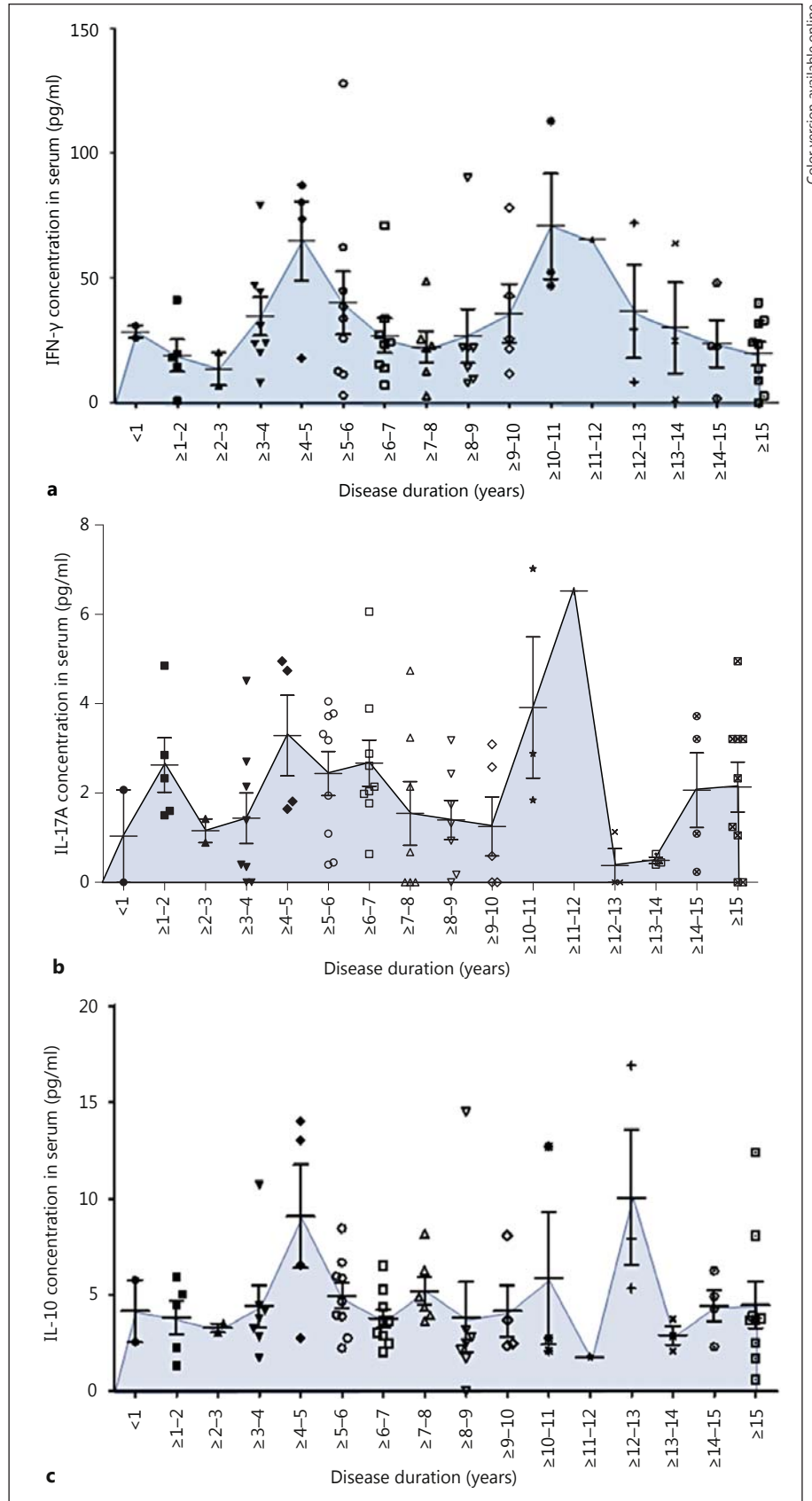
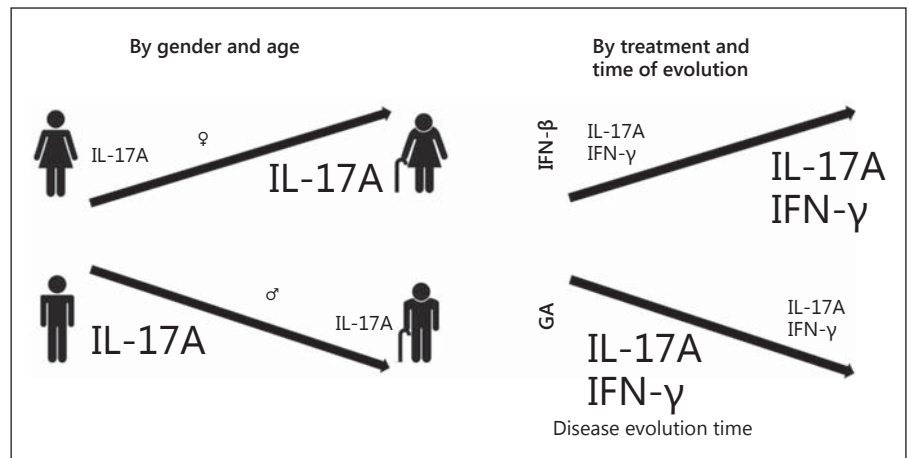


Fig. 4. IFN- γ (a), IL-17A (b) and IL-10 (c) serum levels in RRMS patients distributed by disease duration. Graphics show three-wave fluctuations with peaks at around 5 and 10 years of disease development, which tend to decrease after 15 years of evolution.

Fig. 5. Variations in IL-17A and IFN- γ in RRMS patients are depending on gender and age (a) and treatment and time of evolution of the disease (b).



those who are in remission. IL-10 is an immunomodulatory cytokine with predominantly suppressive actions that is produced by many cell types including T_{reg} cells [26]. Previous studies indicate that treatment with IFN- β or GA induces elevated IL-10 production [27–29], which enhances the immunomodulatory effect in the remission phase in our patients. Therefore, our study provided further support that IFN- γ and IL-10 may be involved in the pathophysiology of MS. Consequently, we must take into account whether the patients are in the relapsing or the remitting disease phase in order to interpret clinical analyses based on IFN- γ and IL-10 levels.

Second, of note, IL-17A significantly increased in females. Then, after separating samples from patients <40 years of age, the subgroup that maintained the statistical significance was the female group aged ≥ 40 years when compared with the group of males. In addition, we observed that in males, average serum levels of IL-17A tended to decrease in patients ≥ 40 years, while in females, average serum levels of IL-17A increased, which probably means that the levels of this cytokine are modified by a hormonal component that could lead to an increase in proinflammatory effects and subsequently to clinical exacerbations/manifestations of the disease. Hormonal influences appear to be involved in regulating the balance of cytokine secretion by Th1, Th2 and T_{reg} cells, as well as by antigen-presenting cells [30]. Additionally, some studies have been associated with a differential response of T cells to sex hormones in vitro and in vivo in patients with MS, revealing that progesterone and estradiol enhance T_{reg} function [31, 32]. Also, the late stages of pregnancy are associated with a decrease in clinical symptoms or relapse rates in MS patients and animal models of MS [33]; moreover, IL-17A serum levels are associated with estro-

gen deficiency in postmenopausal women [34]. Taken together, this evidence suggests that hormonal stage plays an important role in the secretion of the proinflammatory cytokine IL-17A. However, it is necessary to conduct future studies on the relationship between IL-17A serum levels and the effect of hormones on the pathogenesis of MS.

Third, but more relevant, we also provided initial evidence that treatment exerted possible differential effects depending on the time of disease duration, because serum levels of IL-17A differed between patients with disease duration <8 and ≥ 8 years, being lower in patients treated with IFN- β with disease duration <8 years; however, thereafter, IL-17A levels increased in patients treated with IFN- β , while they decreased in GA-treated patients. Among the proinflammatory cytokines involved in MS pathophysiology, IL-17A has gained attention in recent years owing to its ability to promote the transmigration of human CD4+ lymphocytes through the blood-brain barrier endothelial cells by disrupting tight junctions [11]. Furthermore, Th17 cells that release IL-17A might enhance glutamate excitotoxicity as an effector mechanism in MS pathogenesis [35]. Additionally, high levels of IL-17A are associated with several chronic inflammatory diseases, including rheumatoid arthritis, psoriasis and MS [36]; however, to the best of our knowledge, IL-17 fluctuations have not been reported previously. This could be relevant in terms of the different disease stages and the variability in disease extent.

In the same direction, our results showed lowest levels of IFN- γ in patients with <8 years of disease duration treated with IFN- β when compared with GA treatment. It has been documented that IFN- γ induces apoptosis in human oligodendrocytes contributing to MS pathophys-

iology by increasing the number of exacerbations [37, 38]. An earlier report documented that IFN- β therapy for 1 year diminished IFN- γ expression without significant changes in IL-17A [39]. In this sense, IFN- β seems to be more useful in the first years of disease treatment, but further studies in a larger patient cohort are needed in order to test this hypothesis.

In a recent study in 2015, Pasquali et al. [40] reported a negative correlation between EDSS and IL-17 serum levels, and a decrease in this cytokine when comparing patients who were 'non-active' (remitting) versus 'active' (relapsing). In contrast, we found higher levels of IL-17A in the relapsing versus the remitting phase, and we were unable to find any significant correlation between EDSS and IL-17A levels (nor between those of IL-10 or IFN- γ). This could be partially explained by the different inclusion criteria for both studies because, in our case, selected patients had been free of corticosteroid treatment for at least 3 months, while in the previously mentioned work, the authors selected patients with only 1 month without corticosteroid treatment; thus, it is possible that the system can continue to be influenced by the corticosteroid effect. Additionally, these authors included a small group of patients (9 males and 21 females), while our sample was larger (32 males and 50 females), that is more than three times the number of males and more than double the number of females. Despite these differences between studies, both coincide in the conclusion regarding the physiological and clinical relevance of the altered cytokine profiles in patients with MS. Therefore, further studies are necessary, including meta-analyses, in larger numbers of patients with more accurate clinical characterization and more precise categorization in subgroups, as we proposed, to avoid heterogeneous and conflicting results.

Finally, because we detected a wide range of the serum levels of the three cytokines studied, we performed an analysis by separating samples by each disease evolution

year (between 1 and 15 years) in an attempt to ascertain whether there was some correlation or fluctuating variations. As we expected, we found a three-wave profile for all three cytokines tested, which probably reflects the physiological cyclical variations between the relapsing and remitting stages of the disease and which could help to improve monitoring of the disease progression.

In conclusion, our study provides compelling evidence of the existence of different subgroups of patients with MS, who can be classified as follows: (a) age and gender, male or female <40 or \geq 40 years of age; (b) disease duration according to treatment in both groups: under or over 8 years of disease evolution; (c) disease duration according to fluctuating levels of at least the three cytokines reported here (IFN- γ , IL-10 and IL-17A) in three stages: <5 years; between 5 and 10 years, and >10 years (summarized in fig. 5). These subgroups must be taken into consideration for the clinical follow-up of patients with MS in order to provide them with improved and more personalized treatment, and also for a deep and detailed analysis of disease progression in an attempt to understand the fluctuations in the disease and clinical variability through a better understanding of its intrinsically physiological variables.

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Disclosure Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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