Original article

IL-18 PROMOTER POLYMORPHISM AND IL-18 SERUM LEVELS IN ASSOCIATION WITH RELAPSING-REMITTING MULTIPLE SCLEROSIS

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ABSTRACT

Purpose: Multiple sclerosis is the immune-mediated disorder whose etiology is not completely understood. The present study aimed to survey association between the promoter polymorphism IL18 -607C/A (rs1946518), the serum concentration of IL-18 and susceptibility to relapsing-remitting multiple sclerosis (RRMS) in Bulgarian patients

Material and Methods: This case-control study includes 159 RRMS patients with disease-modifying therapy (DMT) and 162 age-sex-matched healthy volunteers. All included subjects were genotyped by ARMS-PCR while serum levels were measured by enzyme-linked immunosorbent assay (ELISA).

Results: Our results revealed significant differences in the serum levels of IL-18 according to the gender, the onset of disease and the type of disease-modifying therapy. The serum levels of IL-18 are significantly higher in RRMS men compared to RRMS women and the RRMS men with late-onset of the disease (above 30 years) also demonstrated significantly increased serum levels than the women with late-onset of disease and even with healthy men. The RRMS patients treated with interferon-beta showed significantly increased IL-18 serum level than those treated with glatiramer acetate.

Conclusions: Our study shows that the promoter polymorphism IL18 -607C/A is not associated with susceptibility to RRMS in Bulgarian patients as well as the serum level of the cytokine. The observed differences in the serum level of IL-18 in RRMS patients according to gender and the response to therapy could be used as a biomarker to the course of the disease.

Keywords: relapsing-remitting multiple sclerosis, cytokine, rs1946518, SNP,

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system leading to neurological dysfunctions. It is known that MS is the most common immune-mediated disorder that has an influence on the central nervous system and affects twice as often women than men [1]. One of the types of MS - Relapsing-remitting MS is described by unpredictable relapses followed by periods of months to years of remission without a new symptom of disease activity. This type of disease is an initial course in 80% of individuals with MS [2]. Although there are many studies on the pathogenesis of the MS, the detailed mechanism has not yet been clarified. However, it is assumed that immunological, environmental, infectious and genetic factors have been implicated in the pathogenesis of MS [3].

The homeostasis of the immune system is maintained by the balance between pro-inflammatory and anti-inflammatory cytokines. Interleukin 18 is a pro-inflammatory cytokine that belongs to the IL-1 superfamily, also known as interferon-gamma inducing factor (IGIF). In the presence of IL-12 or IL-15, IL-18 participates in the Th1 response inducing the production of IFNγ. IFNγ has a prominent and stage-specific role in the pathogenesis of MS [4]. IL-18 is synthesized as an inactive precursor that is activated by proteolytic cleavage of caspase-1 in the NLRP3 inflammasome, producing the active mature molecule [5]. IL18 is produced mainly by macrophages and dendritic cells but also IL-18 mRNA has been reported in many other cell types as astrocytes, osteoblasts, keratinocytes, and epithelial cells [6]. The gene of IL-18 has been mapped to chromosome 11(11q22.2–22.3) and in its sequence are described some single nucleotide polymorphisms in both of the promoter and the coding region. The polymorphisms -137C/G and -607C/A in the promoter region of the IL-18 are extensively investigated for an association whit the risk of various immune-mediated diseases as type-1 diabetes, asthma, multiple sclerosis, et al. [7-10]. The increase of IL-18 expression and serum level have been associated with different types of cancers, inflammatory and some autoimmune diseases including MS [8, 9].

In this study, we focused on to promoter polymorphism in IL18 gene at position -607C/A (rs1946518), its significant role for the serum concentration of IL-18 and their association to the susceptibility of relapsing-remitting multiple sclerosis (RRMS) in Bulgarian patients

MATERIALS AND METHODS
Patients and controls

In this study were included 159 RRMS patients (114 women and 45 men), recruited from the Department of Neurology, Medical University-Plovdiv. The disease was diagnosed as relapsing-remitting multiple sclerosis according to the McDonald criteria [11]. The severity of the disease was estimated using the Expanded Disability Status Scale (EDSS) [12]. Age of onset of the disease was included in the analyses and the patients' group was divided into two subgroups: early-onset - under 30 years and late-onset - above 30 years. The criteria for the patients were as follows: age between 18 and 60 years; period of remission of the disease (defined as improvement period or a clinically stable condition for at least 3 months); under disease-modifying therapy with interferon – beta (INF - β) and glatiramer acetate (GA) for at least 6 months. A total of 162 unrelated healthy controls (120 females and 42 males) were involved for genotyping analyses of IL-18 -607C/A. For serological analyses in the study was enrolled 79 healthy volunteers (39 females and 40 males), as a control group matched with cases for gender and age. The characteristics of the two studied groups are shown in Table 1. All included participants were Caucasian. The informed consent was obtained from the patients and healthy controls according to the ethical guidelines of the Helsinki Declaration.

Table 1. Characteristics of the studied groups

Characteristics of groups	Healthy controls (n=162)	RRMS patients (n=159)	p-value
Gender, n %			
Female	120 (74.1%)	114 (71.7%)	0.632
Male	42 (25.9%)	45 (28.3%)	
Age (mean ± SD) in years	39.36±9.97	40.08±8.48	0.492
Age of the disease onset (mean±SD) in years	-	29.45±7.84	
Early-onset n (%) (< 30 years)		82 (51.6%)	
Late-onset (≥ 30 years)		77 (48.4%)	
Duration of the disease (mean±SD) in years	-	10.60±5.70	
Disease-modifying treatment n, (%)	-		
Glatiramer acetate		22 (13.8%)	
Interferon-beta		137 (86.2%)	
Duration of the disease-modifying treatment (mean±SD) in months	-	59.24±33.89	
EDSS (mean±SD)		1.89±0.71	

Genotyping of -607C/A (rs1946518) polymorphism in IL18

DNA was extracted from peripheral blood samples using the Gene Matrix Purification Kit (EURx, Poland) according to the manufacturer's protocol and stored at -70°C. The genotyping of the promoter polymorphism -607C/A in IL18 was performed by amplification refractory mutation system (ARMS)-PCR. The primer sequences that were used are: C allele: 5'-GTTGCAGAAAGTGTAAAAA TTATTAC-3', A allele: 5'-GTTGCAGAAAGTGTAAAAA TTATTAA-3' and the common primer: 5'-TAACCTCATTCAGGACTTCC-3'. The PCR amplification was performed in a total volume of 20 μl containing 10x PCR buffer, 0.7 U Taq polymerase, 2.5 mmol/l MgCl^2, 0.2 mmol/l of each dNTP, 0.3μmol/l of each primer. The conditions of the PCR reaction were as follow: initial denaturation in 95°C for 5min, followed by 35 cycles at 94°C for 30 sec, at 56°C for 30sec and 45sec at 72°C and completed by final extension step at 72°C for 5min. The PCR products were detected on agarose gel electrophoresis. The PCR reactions were conducted with GeneAmp PCR System 9700 (Applied Biosystems, USA) and reagents delivered by Metabion GmbH (Germany) and Thermo Scientific (USA).

Quantification of IL-18 serum levels

The blood samples for cytokine measurements were collected from 150 patients with RRMS and 79 age-sex-matched healthy volunteers. The serum was isolated after centrifugation at 2000rpm for 15min and the aliquots stored in a -70°C freezer until used. The serum levels of IL-18 were measured by sandwich Enzyme-linked immunosorbent assay (ELISA) method according to manufacturers' instructions (Human IL-18 ELISA kit, Medical & Biological Laboratories CO., LTD.). The optical density (O.D.) was measured by ELISA reader Thermo Scientific Multiscan EX. IL-18 serum levels were expressed in pg/ml. The sensitivity of the kit was 12.5 pg/ml.

Statistical analyses

All statistical analysis was performed with StatSoft software, version 12.0. The test for the compare of genotype distribution and allele frequencies between cases and controls were analyzed using the chi-square test (α^2 test). The StatPages.net website (http://statpages.org/index.html) was used to assess the odds ratios (ODs) with 95% confi-

dence intervals (95% CI) for a relation to the IL-18 -607C/A polymorphism and susceptibility of the disease. The nonparametric Mann-Whitney U test was used to compare the serum IL-18 levels among the groups and the data were expressed as a median ± interquartile range (IQR 25-75%). The results were considered for significant at p<0.05.

RESULTS
Genotype and allelic distribution of promoter polymorphism -607C/A in IL-18 (rs1946518)

In this study of the distribution of the IL-18 -607C/A polymorphism were included a group of 159 patients with RRMS and 162 healthy controls. The allele and genotype frequencies of IL-18 -607C/A polymorphism among RRMS patients and healthy controls are presented in Table 2. The genotype distribution was similar between cases and controls and showed no significant difference. The homozygous genotype of the variant allele-A in IL-18 polymorphism was not detected in the patients' group in contrast to controls. Accordingly, no significant differences in allelic frequencies were observed between studied groups (p=0.503). The frequency of CC-genotype and C-allele is higher in females with early onset of the disease than females with late-onset with bordering significance (p=0.084).

**Table 2.** Genotype and allele frequencies among RRMS patients and control group

IL-18 -607 (rs1946518)	MS, n (%)	Controls, n (%)	OR(95% CI)	p value
Total N	159	162		
CC	57 (36)	54 (33)	1	
AC	102 (64)	104 (64)	0.929 (0.585¸1.474)	0.755
AC+AA	102	108 (67)	0.895 (0.565¸1.418)	0.636
CC vs. AC+AA			1.118 (0.705¸1.771)	0.636
C allele	216 (68)	212 (65)	Reference	
A allele	102 (32)	112 (35)	0.894 (0.644¸1.241)	0.503
IL18 -607 (rs1946518)	Early onset, n (%)	Late onset, n (%)	OR(95% CI)	p value
Total RRMS 159	82	77		
CC	33 (40)	24 (31)	1	
AC+AA	49 (60)	53 (69)	0.672 (0.350¸1.293)	0.233
C allele	115 (70)	101 (66)	1	
A allele	49 (30)	53 (34)	0.812 (0.507¸1.301)	0.386
Female 114	53 (100)	61 (100)		
CC	22 (42)	16 (26)	1	
AC+AA	31 (58)	45 (74)	0.501 (0.227¸1.104)	0.084
C allele	75 (71)	77 (63)	1	
A allele	31 (29)	45 (37)	0.707 (0.405¸1.235)	0.222

Serum levels of IL-18

For the measurements of serum IL-18 levels, 150 patients with RRMS and 79 age-gender matched healthy volunteers were enrolled. Our analysis shows that the subdivision of both groups according to the gender revealed significantly higher serum levels of IL-18 in RRMS men compared to RRMS women (243.1 pg/ml, IQR 84.2-413.9 vs. 113.3 pg/ml, IQR 47.04-273.09; p=0.006). In a group of healthy controls, there was no significant difference in cytokine serum levels between males and females also between total studied groups (Fig. 1).

Fig. 1.
Serum levels of IL-18 in RRMS patients and controls subdivided to gender. The results are presented as a median with interquartile range and non-outliers ranges, as well as outliers and extremes. *p < 0.05 – Mann-Whitney test was used for comparison between groups.
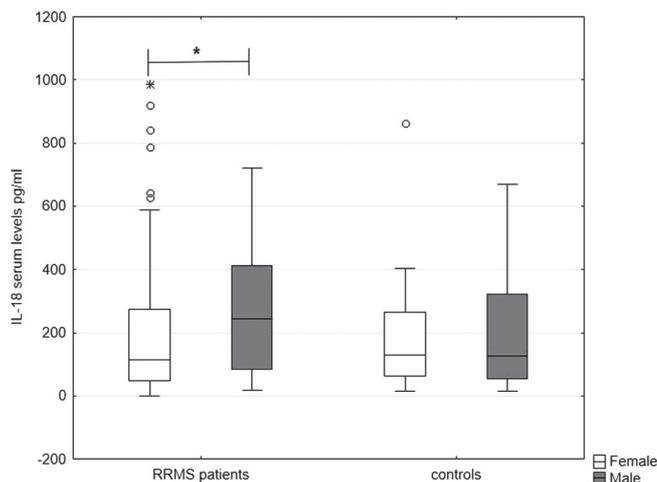
In the same line serum level of IL-18 in RRMS men with late-onset of the disease (above 30 years) was significantly increased compared to women with late-onset (274.9 pg/ml, IQR 121.3-442.1 vs. 129.99 pg/ml IQR 39.61-304.89; p=0.018). Also, a tendency for the elevation of the level of IL-18 was retained in men with late-onset of the disease than to healthy men (274.9 pg/ml, IQR 121.3-442.1 vs. 126.8pg/ml, IQR 54.55-321.68; p=0.048) (Fig. 2).

Fig. 2. The serum concentration of IL-18 in patients with early and late-onset of the disease, and healthy controls (HC), subdivided by gender. The results are presented as a median with interquartile range and non-outliers ranges, as well as outliers and extremes. *p < 0.05 – Mann-Whitney test was used for comparison between groups.
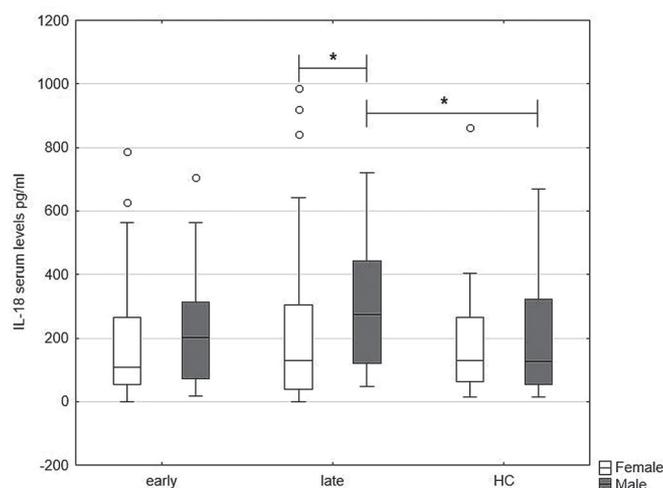
With regards to the type of treatment, RRMS patients treated with Interferon-beta (INF-β-n=129) showed a significantly higher serum concentration of IL-18 in comparison with those treated with glatiramer acetate (GA-n=21) (139.9 pg/ml, IQR 65.61-313.97 vs. 48.3 pg/ml, IQR 17.33-210.39; p=0.007). Cytokine serum levels of healthy controls were similar to RRMS patients with IFN-beta therapy and were significantly higher compared to those treated with GA (128.7 pg/ml IQR 61.9-298.9 vs. 48.3 pg/ml IQR 17.33-210.39; p=0.015) (Fig. 3).

Fig. 3. Serum levels of IL-18 of RRMS patients treated with Interferon-beta (INF-β), patients treated with Glatiramer acetate (GA) and healthy controls (HC). The results are presented as a median with interquartile range and non-outliers ranges, as well outliers and extremes. *p < 0.05; **p < 0.01 – Mann-Whitney test was used for comparison between groups.
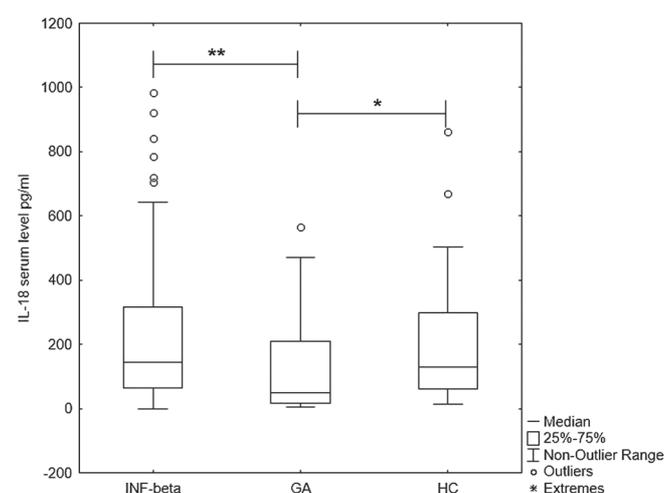
Serum levels of IL-18 in regards to the genotype of promoter polymorphism -607C/A in the control group are higher to compare with RRMS patients for the CC and CA genotypes. However, the presented data in table 3 did not show any significant differences.

Table 3. IL-18 serum levels of RRMS patients and healthy controls depending on genotype

IL-18 -607 (rs1946518)	RRMS patients	Healthy controls	p-value
	Median (IQR 25-75%) pg/ml		
CC	155.8 (61.90-304.89)	184.5 (72.4-296.84)	0.81
CA	117.6 (49.52-328.51)	141.52 (50.76-315.79)	0.84
CA+AA	117.6 (49.52-328.51)	115.62 (48.6-313.68)	0.95

DISCUSSION

Multiple sclerosis is an immune-mediated disorder that affects a significant number of people in their active working age. In this relation, it is important to note the necessity of appropriate biomarkers that can be used for prediction of disease onset, course of the disease, and treatment response. There are a limited number of studies that investigate the relationship between IL-18 -607C/A polymorphism and MS development. Even fewer are those who studied the effect of the polymorphism on serum levels of the cytokine and its association with MS development, as well as its potential role as a biomarker.

Our results from the current study carry out in 159 RRMS patients and 162 healthy volunteers reveal a lack of significant differences in the distribution of genotype in the IL-18 -607C/A in both groups. Similar results have been reported by Giedraitis and co-workers after analyzing the same polymorphism in 208 multiple sclerosis patients and 139 healthy controls in the Swedish population [10]. In contrast to this finding are the data obtained from a study of 113 predominantly RRMS patients and 135 healthy controls in the Turkish population. Even more, the

authors conclude that the IL-18 "607AA genotype indicated 6 times higher risk for the development of RRMS [8]. And although our patient group is more numerous and ethnically alike to the studied Turkish cohort, it should be noted as a limitation that there is none registered IL-18 -607AA genotype in our RRMS group. However, when we stratified our patients by gender a protective effect of genotypes containing A allele with OR= 0.501 was calculated. Another article also presents contradictory data about the risk of the disease and the studied polymorphism (IL-18 "607C/A) and showed the type of population as essential to the distribution of the genotypes. Yuanyuan et al. conducted a meta-analysis showing that one and the same IL-18 -607 C/A polymorphism decreased the risk of prostate cancer in the Asian population but increased risk in the Caucasian population [7].

The analyzed genotypes CC and CA in the IL-18 -607C/A polymorphism did not show a significant correlation whit the serum cytokine level measured in the RRMS patients and the control group as well as compared in between. The akin result has been obtained by Orhan et al. which indicated no statistically significant effect of the IL-18 "137C/G and "607C/A gene promoter polymorphisms on the levels of serum IL-18, although there is data indicated that common IL-18 promoter polymorphisms influence the expression of IL-18 [8,10]. Based on our results, obviously only the promoter polymorphism at positions -607C/A in the IL-18 gene is not determinative of its expression in our studied groups.

Similar to the results from Chen et al., our RRMS group also shows a higher level of serum IL18 in compared to the control group but without statistical significance [14]. As the other autoimmune diseases, MS is more common in females than in males. However, the available data are contradictory which of the two genders among the MS patients exhibits higher serum levels of pro-inflammatory cytokines, and the reason for this remains unclear [14]. We observed in our study significantly higher levels of serum IL-18 in RRMS male patients than RRMS female patients and with bordering significance compared to the healthy males. The comparable result has been reported by Chen et al. in their study conducted in Chinese patients whit MS [14]. These differences become more remarkable after analyzing the serum levels of IL-18 in the patient group according to the gender and onset of disease.

In general, men and women with late-onset of the disease show elevated cytokine serum levels than the patients with early-onset of the disease. The observed significant difference in the level of IL-18 in males and females is maintained but only for the patients with late-onset of disease, with the level of serum IL-18 in the male being significantly higher even than in the male of the control group. Our results indicate that the expression and secretion of IL-18 in patients' serum depend on the gender and age of onset of the RRMS.

All of our RRMS patients were under disease-modifying therapy with INF-β and GA for at least 6 months and during the measurement of cytokine were in a period of a clinically stable phase of the disease. Glatiramer acetate (GA) and INF-b are immunomodulatory drugs approved as efficient in the treatment of multiple sclerosis. Several studies have documented the role of GA and IFN-b in MS, as modulate the course of MS by a probably different mechanism but it is still not completely understood [15]. Constantly, different biomarkers are analyzed to be used for evaluation of the effectiveness and response to the therapy. IL-18 turns out to be a convenient biomarker in the blood serum to evaluate the response to therapy and has been studied for this purpose. Moreover, Robert et al. show elevated levels of proteins that promote the inflammasome dependent inflammatory response in MS and suggested caspase-1, the apoptosis-associated speck-like protein containing a caspase recruitment domain, and IL-18 as appropriate biomarkers that can be used in the course of MS [16]. Regarding disease-modifying therapy, our results showed a clear association between DMT and serum concentrations of IL-18. RRMS patients treated with GA show significantly lower serum levels of the cytokine than the patient's treatment whit IFN-beta and even healthy individuals. These data are in agreement with the other published results, which also suggest IL-18 as a potential biomarker for response to treatment with GA in MS patients [17].

In **conclusion**, our present study indicates significant differences in the serum level of IL-18 in RRMS patients depends on their gender and therapy which could be used as a potential biomarker for more precise therapeutic strategies.

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