



Gene expression of chemokines CX3CL1 and CXCL16 and their receptors, CX3CR1 and CXCR6, in peripheral blood mononuclear cells of patients with relapsing-remitting multiple sclerosis – A pilot study

Ekspresija gena za hemokine CX3CL1 i CXCL16 i njihove receptore, CX3CR1 i CXCR6, u mononuklearnim leukocitima periferne krvi bolesnika sa relapsno-remitentnom multiplom sklerozom – pilot studija

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Abstract

Background/Aim. *In vitro* and *in vivo* studies show that CX3CL1 and CXCL16 chemokines and their specific receptors, CX3CR1 and CXCR6, respectively, mediate mechanism of neuroinflammation during the pathogenesis of multiple sclerosis (MS). The aim of this study was to investigate relative messenger ribonucleic acid (mRNA) levels of CX3CL1, CXCL16, CX3CR1 and CXCR6 in peripheral blood mononuclear cells, as potential molecular markers of relapsing-remitting (RR) MS. **Methods.** The study included 43 unrelated RR MS patients, 20 of them with clinically active disease (relapse) and 23 with clinically stable disease (remission), and 28 unrelated healthy subjects as controls. Real-time polymerase chain reactions (PCR) were performed using TaqMan® gene expression assays. Relative expression (mRNA) level of each target gene in each sample of peripheral blood mononuclear cells was calculated as the mean normalized expression. **Results.** The levels of CX3CR1 mRNA were significantly higher in clinically active

RR MS patients compared to controls [fold change = 1.38, *p* (Mann-Whitney *U* test) = 0.009], and significantly lower in clinically stable *vs* active RR MS patients [fold change = 1.43, *p* (*t*-test) = 0.03]. Stable RR MS patients had significantly higher CXCL16 mRNA levels than controls [fold change = 1.33, *p* (Mann-Whitney *U* test) = 0.006]. A trend of increased CXCR6 gene expression was found in active RR MS patients compared to controls [fold change = 1.23, *p* (Mann-Whitney *U* test) = 0.08]. In either active or stable RR MS patients there were no significant correlations of the clinical parameters with expression levels of the target genes. **Conclusion.** The current results show that increased CX3CR1 mRNA levels in peripheral blood mononuclear cells could represent a proinflammatory molecular marker of clinically active RR MS.

Key words:

blood; chemokines; disease progression; gene expression; leukocytes; multiple sclerosis; rna, messenger.

Apstrakt

Uvod/Cilj. Studije *in vitro* i *in vivo* pokazuju da hemokini CX3CL1 i CXCL16 i njihovi specifični receptori, CX3CR1 i CXCR6, posreduju u mehanizmu neuroinflamacije tokom patogeneze multiple skleroze (MS). Cilj studije bio je ispitivanje relativnih nivoa informacione ribonukleinske kiseline (iRNK) za CX3CL1, CXCL16, CX3CR1 i CXCR6 u mononuklearnim leukocitima periferne krvi, kao potencijal-

nim molekularnim markerima relapsno-remitentne (RR) MS. **Metode.** Studijom su bila obuhvćena 43 bolesnika sa RR MS, koji nisu bili u srodstvu, od kojih je 20 bilo u klinički aktivnoj fazi bolesti (relaps), a 23 u klinički stabilnoj fazi bolesti (remisija), dok su 28 zdravih ispitanika, koji nisu bili u srodstvu, bili kontrola. Za izvođenje lančanih reakcija polimeraze u realnom vremenu korišćeni su genski ekspresioni esejci TaqMan®. Relativni nivo ekspresije svakog ciljnog gena (iRNK) u svakom uzorku mononuklearnih leukocita

periferne krvi bio je računat kao srednja normalizovana ekspresija. **Rezultati.** Nivoi CX3CR1 iRNK bili su značajno viši kod bolesnika u fazi relapsa u poređenju sa kontrolama [“fold change” = 1,38, *p* (Mann-Whitney *U* test) = 0,009] i značajno niži kod bolesnika u fazi remisije u poređenju sa bolesnicima u relapsu [“fold change” = -1,43, *p* (*t*-test) = 0,03]. Bolesnici u remisiji su imali značajno više nivoe CXCL16 iRNK nego kontrole [“fold change” = 1,33, *p* (Mann-Whitney *U* test) = 0,006]. Trend povećanja nivoa ekspresije CXCR6 gena je bio nađen kod bolesnika u relapsu u poređenju sa kontrolama [“fold change” = 1,23, *p* (Mann-Whitney *U* test)

= 0,08]. Ni kod jednog bolesnika, ni u fazi relapsa ni u fazi remisije, nije bilo značajnih korelacija između vrednosti kliničkih parametara i nivoa ekspresije ciljnih gena. **Zaključak.** Rezultati pokazuju da povećanje nivoa CX3CR1 iRNK u mononuklearnim leukocitima periferne krvi može predstavljati proinflamatorni molekularni marker relapsa, tj. klinički aktivne faze relapsno-remitentne MS.

Ključne reči:

krv; hemokini; bolest, progresija; geni, ekspresija; leukociti; multipla skleroza; rnk, informativna.

Introduction

Chemokines are a family of cytokines, representing small soluble proteins that have an essential role in the stimulation of cell migration and intercellular communication¹. Changes in expression of certain chemokines and chemokine receptors in the central nervous system (CNS) can be associated with the pathogenesis of chronic neuroinflammatory and autoimmune diseases, such as multiple sclerosis (MS)².

Both CX3CL1 (fractalkine) and CXCL16 chemokines are expressed in vascular endothelial cells³, while CXCL16 is also produced by monocytes/macrophages⁴, B cells⁵ and T cells⁶. Specific receptors for these two chemokines, CX3CR1 and CXCR6, respectively, are expressed on the surface of leukocytes, T cells^{7, 8}, monocytes/macrophages^{9, 10} and non-killer (NK) cells^{7, 8}. CX3CL1 and CXCL16 represent structurally and functionally unique chemokines. Each can exist as a soluble or a membrane-bound molecule and so can act as either a soluble chemoattractant or a membrane adhesion molecule, regulating both leukocyte migration and leukocyte adhesion to the vascular wall, which are key events in the inflammatory process^{5, 11}. Conversion of the transmembrane into the soluble form of these two chemokines is achieved through regulated proteolysis of their transmembrane forms, by ADAM10 and ADAM17 extracellular metalloproteinases¹².

CX3CL1 and CX3CR1 are constitutively expressed in the CNS – CX3CL1 predominantly in neurons and CX3CR1 in microglia, so they are important for the formation of intercellular connections between neurons and microglial cells¹. Inducible CX3CL1 expression was detected in astrocytes of the CNS inflammatory lesions in experimental autoimmune encephalomyelitis (EAE)¹³, while elevated levels of soluble CX3CL1 were measured in the cerebrospinal fluid and serum of MS patients^{14, 15}. CX3CL1 significantly increased the gene expression of proinflammatory cytokines in CD4+ T cells derived from patients with relapsing-remitting (RR) MS¹⁴. In the inflammatory brain lesions of rats and mice with EAE, there was accumulation of microglial cells and peripheral leukocytes expressing CX3CR1 messenger ribonucleic acid (mRNA), and CX3CR1 was responsible for the selective recruitment of NK cells into the CNS of these animals^{13, 16}. A significantly higher percentage of CD4+ CX3CR1+ T cells was detected in blood of RR MS patients

compared to healthy controls¹⁴, and CX3CR1 mediated the recruitment of cytotoxic T cells into the brain tissue of patients with MS¹⁷. In normal CNS tissue, CXCL16 expression is low and mostly restricted to endothelial cells¹⁸. However, production of CXCL16 in the CNS has been increased during both preclinical and acute EAE¹⁹. It was found that CXCL16 could act as a proinflammatory chemokine in the pathogenesis of MS because, in animals with EAE, application of CXCL16 monoclonal antibodies resulted in: reduced disease incidence, decreased infiltration of mononuclear leukocytes into the CNS, decreased level of serum interferon gamma (IFN γ) and decreased production of myelin-specific T cells²⁰. The levels of soluble CXCL16 in the human serum and cerebrospinal fluid were significantly increased in MS and other neuroinflammatory autoimmune diseases²¹. Expression of CXCR6 was typically detected in myelin-reactive IFN γ -producing CD4+ Th1 cells of EAE mice²⁰. Also, CXCR6 was upregulated in neutrophils, which accumulated in the brain prior to and during the acute EAE attacks¹⁹.

In vitro and *in vivo* studies indicate that CX3CL1 and CXCL16 chemokines and their specific receptors, CX3CR1 and CXCR6, respectively, are involved in the mechanism of neuroinflammation during the pathogenesis of MS. We had previously shown association of the single nucleotide variants in CXCL16 and CX3CR1 genes with susceptibility and progression of MS^{22, 23}. The aim of the current study was to investigate changes in relative gene expression of CX3CL1 and CXCL16 chemokines and their receptors at mRNA levels in peripheral blood mononuclear cells (PBMC), as potential molecular markers of RR MS.

Methods

Subjects

The study included 43 unrelated patients with MS, from the Clinic for Neurology of the Military Medical Academy (MMA), Belgrade, Serbia. All patients were diagnosed with clinically definite MS²⁴ and the clinical course of the disease was defined²⁵. For estimation of the disease severity, the Expanded Disability Status Scale²⁶ and the Multiple Sclerosis Severity Score²⁷ parameters were calculated, according to clinical data obtained at the time when blood specimens for genetic analysis were collected. Of 43 patients, 20 had clinically active RR MS (relapse) and 23 had clinically stable

RR MS (remission). Patients with stable RR MS were treated with 0.25 mg of interferon beta-1b (Betaferon[®], Bayer Pharma AG), every other day, over a period of at least 12 months. The control group consisted of 28 unrelated healthy volunteers of the Military MMA personnel. Both controls and patients were of Serbian ethnic origin. The Ethics Committee of the MMA approved the study, and each participant gave his/her written informed consent to participate in the study.

Real-time reverse transcription-quantitative polymerase chain reaction (PCR) and calculation of relative gene expression (mRNA) levels of CX3CL1, CXCL16, CX3CR1 and CXCR6

Fresh blood samples (3 mL) were used for separation of PBMC, with lymphocyte separation medium (PAA, GE Healthcare), and extraction of PBMC total RNA, with TRI Reagent (Ambion, Life Technologies). The quality and quantity of total RNA were assessed using RNA 6,000 Nano Kit, on 2,100 Bioanalyzer (Agilent, US).

Each PBMC sample total RNA (500 ng) was treated with DNaseI (Fermentas, Thermo Fisher Scientific) and the reverse transcription was done using First strand cDNA synthesis kit, with oligo-dT18 and random hexamer primers (Fermentas, Thermo Fisher Scientific), in a reaction volume of 20 µL. Real-time polymerase chain reactions (PCR) were performed on Applied Biosystems 7500 Real-Time PCR system, by use of the following TaqMan[®] gene expression assays: Hs00171086_m1 (for CX3CL1), Hs01055223_g1 (for CXCL16), Hs01922583_s1 (for CX3CR1), Hs01890898_s1 (for CXCR6), Hs99999905_m1 (for GAPDH), Hs99999904_m1 (for PPIA) and Hs99999901_s1 (for 18S rRNA). Each real-time PCR reaction contained 1 µL of the reverse transcription product, in a total reaction volume of 13 µL. All samples were run in duplicates.

NormFinder algorithm²⁸ was used for identification of the optimal endogenous control among the candidate genes (GAPDH, peptidylprolylcis-trans-isomerase A PPIA and 18S rRNA), according to their expression stability in a given sample group and a given study design. Based on the input data, representing Ct values were transformed to linear scale expression quantities by delta-Ct method. NormFinder calcu-

lated the stability value for each candidate gene, which was a direct measure of the estimated gene expression variation.

The relative expression level of each target gene in each PBMC sample was calculated as the mean normalized expression (MNE), according to the following formula²⁹: $MNE = (E_{reference})^{Ct_{reference, mean}} / (E_{target})^{Ct_{target, mean}}$, where E represented PCR amplification efficiency for the reference (endogenous control) gene ($E_{reference}$) and the target gene (E_{target}), and Ct represented an average cycle threshold value from the two replicates, for the reference gene (Ct reference, mean) and for the target gene (Ct target, mean). TaqMan[®] gene expression assays provide the amplification efficiency of 100%, meaning that $E_{reference} = E_{target} = 2$, so the above formula for calculating the relative expression level of each target gene in each sample has become: $MNE = 2^{-(Ct_{reference, mean} - Ct_{target, mean})}$, $MNE = 2^{-Ct_{target, mean} + Ct_{reference, mean}} = 2^{-dCt}$. For verifying the relative gene expression results, relative expression software tool REST 2009 was used³⁰.

Statistical analysis

The statistical analysis was performed using Statistica 8.0 software package (StatSoft, Inc. 1984–2007). Comparisons of continuous variables between the tested sample groups were done by *t*-test and Analysis of Variance or by Mann-Whitney *U* test and Kruskal-Wallis test, depending on whether variable values had a normal or a non-normal distribution. Correlations between continuous variables were tested by product-moment and partial correlations. In all statistical tests, *p* < 0.05 values were considered statistically significant. Graphs were designed using GraphPad Prism 5.00 (GraphPad Software, San Diego California USA).

Results

Controls and RR MS patients

Characteristics of controls and RR MS patients who participated in the study are shown in Table 1. There was no significant difference in age between controls, patients with clinically active and patients with clinically stable RR MS [Table 1; *p* (Kruskal-Wallis test) = 0.72]. In each of the three groups, female-to-male ratio was > 1 (Table 1).

Table 1

Characteristics of the study participants

Parameter	Controls (n = 28)	Active RR MS patients (n = 20)	Stable RR MS patients (n = 23)
Age (years)	33.0 (25.0–64.0) [#]	32.5 ± 8.2	35.2 ± 7.5
Gender (female/male)	21/7	16/4	12/11
Disease onset age (years)	–	29.0 ± 7.9	27.5 ± 8.6
Duration of disease (years)	–	2.0 (1.0–15.0) [#]	6.0 (1.0–31.0) [#]
Expanded disability status scale	–	1.5 (1.0–3.5) [#]	2.0 (1.0–4.5) [#]
MS severity score	–	3.5 ± 1.9	4.0 ± 1.6

Values of continual parameters with a normal distribution are presented as mean ± standard deviation; [#]values of continual parameters with a non-normal distribution are presented as median (minimum–maximum). MS – multiple sclerosis; RR – relapsing-remitting.

Relative expression (mRNA) levels of CX3CL1, CX3CR1, CXCL16 and CXCR6 genes in PBMC of controls and patients with RR MS

In all tested PBMC samples, we detected the amplification of each target gene's mRNA (complementary DNA), except of CX3CL1 mRNA. By comparing the values of statistical parameter that represents the expression stability for each of the three tested endogenous control genes and by comparing the amplification profiles of endogenous control genes with amplification profiles of target genes, PPIA was found to be the optimal endogenous control for normalizing the results of expression of target genes in the analyzed PBMC samples. The statistical analysis of relative expression levels of the target genes in PBMC of controls and MS patients is presented in Figure 1 and Table 2. The levels of CX3CR1 mRNA were significantly higher in clinically active RR MS patients compared to controls [fold change = 1.38, p (Mann-Whitney U test) = 0.009], and significantly lower in clinically stable vs active RR MS patients [fold change = -1.43, p (t -test) = 0.03] (Figure 1 A, Table 2). Stable RR MS patients had significantly higher CXCL16 mRNA levels than controls [fold change = 1.33, p (Mann-Whitney U test) = 0.006] (Figure 1 B, Table 2). A trend of increased CXCR6 gene expression was found in active RR MS patients compared to controls [fold change = 1.23, p (Mann-Whitney U test) = 0.08] (Figure 1 C, Table 2).

Table 2
Statistical analysis of CX3CR1, CXCL16 and CXCR6 relative gene expression (mRNA) levels in peripheral blood mononuclear cells

Parameter	Fold change	p
CX3CR1 relative gene expression		
active RR MS patients vs controls	1.38	0.009 **
stable vs active RR MS patients	-1.43	0.03 *
stable RR MS patients vs controls	-1.03	0.66
CXCL16 relative gene expression		
active RR MS patients vs controls	1.20	0.39
stable vs active RR MS patients	1.11	0.20
stable RR MS patients vs controls	1.33	0.006 **
CXCR6 relative gene expression		
active RR MS patients vs controls	1.23	0.08
stable vs active RR MS patients	-1.22	0.26
stable RR MS patients vs controls	1.01	0.97

RR MS – relapsing-remitting multiple sclerosis.

Fold change – mean relative gene expression level (mean 2^{-dCt}) of the target sample group to mean relative gene expression level of the reference sample group ratio (* – statistically significant difference when $p < 0.05$; ** – statistically significant difference when $p < 0.01$).

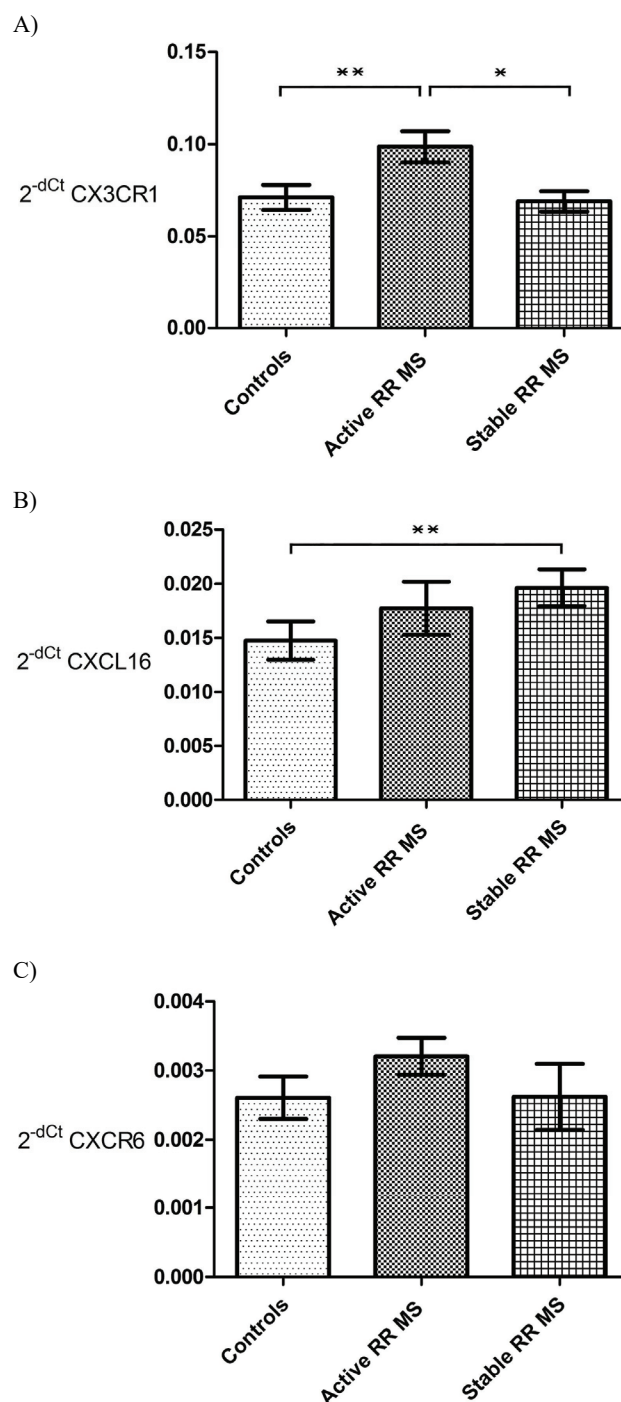


Fig. 1 – Relative expression (mRNA) levels of the target genes in peripheral blood mononuclear cells.

A) Relative expression of CX3CR1 gene; B) Relative expression of CXCL16 gene; C) Relative expression of CXCR6 gene. The analyzed groups are controls ($n = 28$), active relapsing-remitting multiple sclerosis (RR MS) patients ($n = 20$) and stable RR MS patients ($n = 23$). For each target gene, the expression levels are normalized to endogenous control gene peptidylprolyl cis-trans-isomerase A (PPIA) and shown for each analyzed sample group (graph bars) as mean relative gene expression level (mean 2^{-dCt}) with its standard error (* – statistically significant difference when $p < 0.05$; ** – statistically significant difference when $p < 0.01$).

Correlations between levels of the target mRNAs in controls and patients with RR MS

No significant correlations were established between CXCL16 and CXCR6 mRNA levels or between CX3CR1 and CXCR6 mRNA levels, within each analyzed sample group – controls, active RR MS patients or stable RR MS patients (product-moment and partial correlations, $p > 0.05$).

Correlations of clinical parameters with levels of the target mRNAs in patients with RR MS

In either active or stable RR MS patients there were no significant correlations between values of clinical parameters (Expanded Disability Status Scale, Multiple Sclerosis Severity Score) and mRNA levels of CX3CR1, CXCL16 or CXCR6 in PBMC (product-moment and partial correlations, $p > 0.05$).

Discussion

Important purpose of research regarding the molecular basis of MS is to identify gene expression changes in the immune cells of peripheral blood, which may reflect the pathological changes in the target CNS tissue. The aim of the present study was to analyze the relative gene expression of CX3CL1 and CXCL16 chemokines and their specific receptors, CX3CR1 and CXCR6, respectively, at the mRNA levels in PBMC samples of patients with RR MS and healthy control subjects. We found that changes in PBMC CX3CR1 mRNA levels may represent a molecular marker of RR MS.

A proposed proinflammatory role of CX3CL1 in the pathogenesis of MS is supported by the fact that CX3CL1 significantly increased the expression of IFN γ gene in CD4+ T cells and secretion of IFN γ from these cells, derived from RR MS patients but not healthy individuals¹⁴. In rheumatoid arthritis, representing a T cell-mediated inflammatory and autoimmune disease, such as MS, CX3CL1 was expressed in T cells of patients. Yet, there was a low overall proportion of CX3CL1-expressing peripheral T cells in both rheumatoid arthritis patients and controls³¹. Likewise, in the current study, expression of CX3CL1 mRNA was not detectable in PBMC of either MS patients or controls. On the other hand, inducible expression of CX3CL1 in endothelial cells, as well as CX3CL1 proteolytic cleavage from the surface of these cells, was demonstrated *in vitro*, in response to inflammatory mediators³². This finding³², along with ours, proposes that the inducible endothelial expression of CX3CL1 followed by its inducible proteolytic cleavage from the endothelial surface represents the main cause of a significant increase in circulating soluble CX3CL1 levels, having been shown in patients with RR MS^{14, 15}.

We detected significantly higher levels of PBMC CX3CR1 mRNA in patients with clinically active RR MS compared to both controls and patients with clinically stable RR MS. The study of relative gene expression, analyzed by real-time PCR, in PBMC samples of 28 healthy controls and 25 patients with RR MS showed that the levels of CX3CR1

mRNA, as well as percentage of CX3CR1+ cells, were significantly lower in patients compared to controls³³. This decreased CX3CR1 gene expression in patients may be explained by the fact that most of them were clinically stable³³. Likewise, we found a significant decrease in CX3CR1 mRNA levels in stable RR MS patients compared to the active ones. Furthermore, in microarray analysis by Infante-Duarte et al.³³, the only patient who suffered a relapse 14 days after venipuncture had an increased CX3CR1 gene expression in comparison to healthy individuals, which is consistent with our finding. Previous research indicated the proinflammatory properties of peripheral CX3CR1+ CD4+ T cells and CX3CR1+ NK cells, by which these cells should contribute to the process of neuroinflammation in RR MS, especially during relapse^{14, 17, 33}. Accordingly, our finding suggests that the increase in PBMC CX3CR1 mRNA levels represents a potential proinflammatory molecular marker of active RR MS.

Both CXCL16 chemokine and its receptor, CXCR6, are widely expressed in PBMC^{4-6, 8, 20}. CXCL16 from monocytes infiltrated in the CNS of EAE mice was suggested to induce chemotaxis and accumulation of activated myelin-specific CXCR6+ CD4+ Th1 cells in the CNS tissue, indicating a proinflammatory action of CXCL16 in the pathogenesis of EAE²⁰. Production of CXCL16 in the CNS was increased during the acute EAE¹⁹, and severity of EAE positively correlated with CNS mRNA and protein levels of CXCL16 and CXCR6²⁰. The analysis of brain tissue from MS patients demonstrated an increase in CXCL16 expression by foamy macrophages in the rims of chronic active brain lesions³⁴. Considering the foregoing results obtained *in vivo* and *in vitro*, we expected to find out elevated CXCL16 and CXCR6 gene expression levels in PBMC of the patients. In clinically active RR MS patients compared to controls, we detected no significant differences in either CXCL16 or CXCR6 mRNA levels, although there was a trend of increased CXCR6 gene expression. CXCR6 was an indicator of IFN γ production, since the intracellular synthesis of IFN γ significantly positively correlated with expression of CXCR6 on the surface of MBP-reactive CD4+ Th1 cells³⁵. The trend that we found, along with this finding of Calabresi et al.³⁵, suggests further investigation in order to evaluate the hypothesised role of CXCR6 as a proinflammatory marker of RR MS, typically of the active phase of the disease. In the present analysis of PBMC CXCL16 gene expression, we detected significantly higher mRNA levels only in patients with stable RR MS in comparison to controls. These patients were receiving interferon-beta treatment, which is known to suppress the secretion of proinflammatory cytokines³⁶. An *in vitro* study showed no correlation between CXCL16 expression and interferon-beta treatment³⁷. Considering the hypothesised proinflammatory action of CXCL16 in the pathogenesis of MS²⁰, the largest increase in CXCL16 gene expression is expected to be found in clinically active RR MS patients. As the intensity of inflammation and autoimmune response decreases during the stable phase of the disease, CXCL16 expression should also decrease. Yet, we did not find its decrease in

stable RR MS. Therefore, the increased PBMC CXCL16 mRNA levels in patients with clinically stable disease in the current study could be due to some pleiotropic effect(s) of CXCL16 on pathogenesis of RR MS, which is(are) not directly related to inflammation.

We aware that our study has limitations. One is the restricted inclusion of patients with clinically stable disease who are treatment-naïve. Thus, to create a homogenous group of clinically stable patients, we deliberately selected only those receiving interferon-beta treatments. We did not show which subpopulation(s) of PBMC underlay the detected significant changes in gene expression, and this represents another limitation of the study. Still, the expression changes were detected in total PBMC, which represent a valid source of potential MS biomarkers quantified at the level of mRNA and/or protein³⁸⁻⁴⁰.

Conclusion

The current study demonstrates that the increased CX3CR1 mRNA expression in PBMC could represent a pro-inflammatory molecular marker of clinically active RR MS. The results should be verified in future studies with a larger number of samples. In addition, more functional research is needed to fully clarify the roles of CX3CL1, CXCL16, CX3CR1 and CXCR6 in the pathogenesis of MS. Despite limitations, the present study adds value to investigation of CX3CL1, CXCL16, CX3CR1 and CXCR6 gene expression in the immune cells of RR MS patients, with respect to the disease activity.

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