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EKSPRESIJA GENA ZA HEMOKINE CX3CL1 I CXCL16 I NJIHOVE RECEPTORE, CX3CR1 I CXCR6, U MONONUKLEARNIM LEUKOCITIMA PERIFERNE KRVI PACIJENATA SA RELAPSNOSTO-REMITENTNOM MULTIPLOM SKLEROZOM - PILOT STUDIJA

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Abstract

**Background/Aim.** The *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 objective of this study was to investigate the relative messenger ribonucleic acid (mRNA) levels of CX3CL1, CXCL16, CX3CR1 and CXCR6 in the 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 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:** mRNA; CX3CL1; CXCL16; CX3CR1; CXCR6; multiple sclerosis.

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 ove studije bio je ispitivanje relativnih nivoa informacione ribonukleinske kiseline (iRNK) za CX3CL1, CXCL16, CX3CR1 i CXCR6 u
mononuklearnim leukocitima periferne krvi, kao potencijalnih molekularnih markera relapsno-remitentne MS. **Metode.** Studija je obuhvatila 43 pacijenta sa relapsno-remitentnom MS koji nisu u srodstvu, njih 20 u klinički aktivnoj fazi bolesti (relaps) i 23 u klinički stabilnoj fazi bolesti (remisija), i 28 zdravih ispitanika koji nisu u srodstvu, kao kontrola. Za izvođenje lančanih reakcija polimeraze u realnom vremenu korišćeni su genski ekspresioni eseji TaqMan®. Relativni nivo ekspresije svakog ciljnog gena (iRNK) u svakom uzorku mononuklearnih leukocita periferne krvi izračunat je kao srednja normalizovana ekspresija. **Rezultati.** Nivoi CX3CR1 iRNK su bili značajno viši kod pacijenata u fazi relapsa u poređenju sa kontrolama ("fold change" = 1.38, p (Mann-Whitney U test) = 0.009) i značajno niži kod pacijenata u fazi remisije u poređenju sa pacijentima u relapsu ("fold change" = -1.43, p (T-test) = 0.03). Pacijenti 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 nađen kod pacijenata u relapsu u poređenju sa kontrolama ("fold change" = 1.23, p (Mann-Whitney U test) = 0.08). Kod pacijenata, ni u fazi relapsa ni u fazi remisije, nije bilo značajnih korelacija između vrednosti kliničkih parametara i nivooa 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:** iRNK; CX3CL1; CXCL16; CX3CR1; CXCR6; multipla skleroza.

**Introduction**

Chemokines are a family of cytokines, representing small soluble proteins that have an essential role in the stimulation of cell migration and intercellular communication 1. 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 cells 3, while CXCL16 is also produced by monocytes/macrophages 4, B cells 5 and T cells 6. 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 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 metalloproteinases\(^12\).

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 cells\(^1\). 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) MS\(^14\). 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 controls\(^14\), and CX3CR1 mediated the recruitment of cytotoxic T cells into the brain tissue of patients with MS\(^17\). In normal CNS tissue, CXCL16 expression is low and mostly restricted to endothelial cells\(^18\). However, production of CXCL16 in the CNS has been increased during both preclinical and acute EAE\(^19\). 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 antibody resulted in: reduced disease incidence, decreased infiltration of mononuclear leukocytes into the CNS, decreased level of serum interferon gamma (IFN\(\gamma\)) and decreased production of myelin-specific T cells\(^20\). The levels of soluble CXCL16 in human serum and cerebrospinal fluid were significantly increased in MS and other neuroinflammatory autoimmune diseases\(^21\). Expression of CXCR6 was typically detected in myelin-reactive
IFNγ-producing CD4+ Th1 cells of EAE mice. Also, CXCR6 was upregulated in neutrophils, which accumulated in brain prior to and during the acute EAE attacks.

*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. The aim of the current study was to investigate changes in relative gene expression of CX3CL1 and CXCL16 chemokines and their receptors, at the mRNA level, in peripheral blood mononuclear cells (PBMC), as potential molecular markers of RR MS.

**Methods**

**Subjects**

The study included 43 unrelated patients with MS, from Clinic for Neurology of Military Medical Academy, Belgrade, Serbia. All patients were diagnosed with clinically definite MS and the clinical course of disease was defined. For estimation of disease severity the expanded disability status scale and the MS severity score 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 Betaferon® (Bayer Pharma AG) - interferon beta-1b, every other day, over a period of at least 12 months. The control group consisted of 28 unrelated healthy volunteers of Military Medical Academy personnel. Both controls and patients were of Serbian ethnic origin. The Ethical Committee of MMA approved the study, and each participant gave their 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 6000 Nano Kit, on 2100 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 microliters. Real-time PCR reactions 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 microliter of the reverse transcription product, in a total reaction volume of 13 microliters. All samples were run in duplicates.

NormFinder algorithm \(^ {28}\) was used for identification of the optimal endogenous control among the candidate genes (GAPDH, 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 transformed to linear scale expression quantities by delta-Ct method, NormFinder calculated 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 formula \(^ {29}\): 

\[
\text{MNE} = \left( \frac{E_{\text{reference}}^{Ct \text{ reference}, \text{ mean}}}{E_{\text{target}}^{Ct \text{ target}, \text{ mean}}} \right)
\]

where \(E\) represented PCR amplification efficiency for the reference (endogenous control) gene \((E_{\text{reference}})\) and the target gene \((E_{\text{target}})\), and \(Ct\) represented an average cycle threshold value from the two replicates, for the reference gene \((Ct \text{ reference}, \text{ mean})\) and for the target gene \((Ct \text{ target}, \text{ mean})\). TaqMan® gene expression assays provide the amplification efficiency of 100%, meaning that \(E_{\text{reference}} = E_{\text{target}} = 2\), so the above formula for calculating the relative expression level of each target gene in each sample has become: 

\[
\text{MNE} = 2^{\text{dCt}}.
\]

For verifying the relative gene expression results, relative expression software tool REST 2009 was used \(^ {30}\).

**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 the variable values had a normal or a non-normal distribution. Correlations between the 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).

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 analysed 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).

**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 analysed sample group – controls, active RR MS patients or stable RR MS patients (Product-moment and partial correlations, p > 0.05) (results not shown).

**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, MS severity score) and mRNA levels of CX3CR1, CXCL16 or CXCR6, in PBMC (Product-moment and partial correlations, p > 0.05) (results not shown).

**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 analyse the relative gene expression of CX3CL1 and CXCL16 chemokines and their specific receptors, CX3CR1 and CXCR6 respectively, at the mRNA level, in PBMC samples of patients with RR MS and healthy control subjects. We found that change 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 IFNγ gene in CD4+ T cells and secretion of IFNγ from these cells, derived from RR MS patients but not healthy individuals. 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 controls. 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 mediators. This finding, 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.

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, analysed 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 controls. This decreased CX3CR1 gene expression in patients may be explained by the fact that most of them were clinically stable. Likewise, we found a significant decrease in CX3CR1 mRNA levels in stable RR MS patients compared to the active. Furthermore, in microarray analysis by Infante-Duarte et al., 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. 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. 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 EAE. Production of CXCL16 in the CNS was increased during the acute EAE, and severity of EAE positively correlated with CNS mRNA and protein levels of CXCL16 and CXCR6. The analysis of brain tissue from MS patients demonstrated an increase in CXCL16 expression by foamy macrophages in the rims of chronic active brain lesions. 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 IFNγ production, since the intracellular synthesis of IFNγ significantly positively correlated with expression of CXCR6 on the surface of MBP-reactive CD4+ Th1 cells. The trend that we found, along with this finding of Calabresi et al., 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 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 cytokines. An in vitro study showed no correlation between CXCL16 expression and interferon beta treatment. Considering the hypothesised proinflammatory action of CXCL16 in the pathogenesis of MS, 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 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 protein.

**Conclusion**

The current study demonstrates that the increased CX3CR1 mRNA expression in PBMC could represent a proinflammatory 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 disease activity.

Acknowledgements

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References

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28. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes


Table 1.
Characteristics of the study participants.

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

N - number of controls/patients; 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).
Table 2.
Statistical analysis of CX3CR1, CXCL16 and CXCR6 relative gene expression (mRNA) levels in PBMC.

<table>
<thead>
<tr>
<th></th>
<th>Fold change</th>
<th>p</th>
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<tr>
<td>CX3CR1 relative gene expression</td>
<td></td>
<td></td>
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<tr>
<td>Active RR MS patients vs Controls</td>
<td>1.38</td>
<td>0.009 **</td>
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<tr>
<td>Stable vs Active RR MS patients</td>
<td>-1.43</td>
<td>0.03 *</td>
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<td>Stable RR MS patients vs Controls</td>
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<td>0.66</td>
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<th>CXCL16 relative gene expression</th>
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<td>Active RR MS patients vs Controls</td>
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<tr>
<td>Stable vs Active RR MS patients</td>
<td>1.11</td>
<td>0.20</td>
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<tr>
<td>Stable RR MS patients vs Controls</td>
<td>1.33</td>
<td>0.006 **</td>
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<table>
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<th>CXCR6 relative gene expression</th>
<th>Fold change</th>
<th>p</th>
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<tbody>
<tr>
<td>Active RR MS patients vs Controls</td>
<td>1.23</td>
<td>0.08</td>
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<tr>
<td>Stable vs Active RR MS patients</td>
<td>-1.22</td>
<td>0.26</td>
</tr>
<tr>
<td>Stable RR MS patients vs Controls</td>
<td>1.01</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Fold change - mean relative gene expression level (mean $2^{\Delta Ct}$) of the target sample group to mean relative gene expression level of the reference sample group ratio; asterisk (*) - statistical significance when $p < 0.05$, double asterisk (**) - statistical significance when $p < 0.01$. 
Figure 1. Relative expression (mRNA) levels of the target genes in PBMC.

A) Relative expression of CX3CR1 gene. B) Relative expression of CXCL16 gene. C) Relative expression of CXCR6 gene. The analysed groups are controls (N = 28), active 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 (PPIA) and shown for each analysed sample group (graph bars) as mean relative gene expression level (mean $2^{-\Delta Ct}$) with its standard error. Asterisk (*) - statistical significance when p < 0.05, double asterisk (***) - statistical significance when p < 0.01.
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