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Immune-cell BDNF expression in treatment-naïve relapsing-remitting multiple sclerosis patients and following one year of immunomodulation therapy

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ABSTRACT

Although neurons are the main source of neurotrophins in the healthy brain, neurotrophins can also be expressed in the immune system. We have previously shown that in relapsing-remitting multiple sclerosis (RRMS) lower immune-cell neurotrophin levels are associated with brain atrophy and cognitive impairment. The aim of the present study was to assess if immune-cell neurotrophin expression is impaired in MS as compared with the healthy controls, and to describe if these levels change in treatment-naïve RRMS patients, following one year of immunomodulation.

Fifty treatment-naïve RRMS patients were assessed at baseline and after one year of immunomodulation (beta-interferons/glatiramer acetate). The control group included 39 healthy subjects matched according to age and gender. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood using Ficoll-Histopaque gradient. The levels of brain-derived-neurotrophic-factor (BDNF), beta-nerve-growth-factor (beta-NGF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) were measured in PBMC lysates with ELISA.

BDNF levels were significantly lower in MS than in the healthy controls (median 613 vs. 1657 pg/mg protein, $p < 0.001$). After one year of immunomodulation, BDNF expression did not change significantly ($p = 0.06$) on the group level. In 70% of patients there was no increase in BDNF level, and in 30% it increased. We observed no differences between treatment groups. Other neurotrophins were detected in a minority of MS samples (as opposed to the controls).

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To conclude, we have shown that immune-cell production of neurotrophins is impaired in MS patients. In our MS cohort standard immunomodulation failed to restore normal BDNF levels in PBMCs within one year of therapy.

1. Introduction

Multiple sclerosis (MS) is an autoimmune disorder of the central nervous system (CNS), in which both, neuroinflammatory and neurodegenerative components, are responsible for endpoint disability in patients. So far it has not been established to which degree neurodegeneration can be independent of inflammation in MS. The interrelationship between the two components is complex. Although in MS inflammation drives neurodegeneration by means of oxidative stress and mitochondrial dysfunction [1], it can also exert neuroprotective effects, such as provided by immune-cell production of neurotrophic factors [2].

Neurotrophic factors are a family of polypeptides involved in neuronal development [3], survival [4] and synaptic plasticity [5]. They include neurotrophins, namely brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5). Neurons are the main source of neurotrophins in the healthy brain. However, when faced with MS-related damage, the additional supply of neurotrophins by peripheral blood mononuclear cells (PBMCs), which enter the brain via disrupted blood-brain barrier (BBB), could be essential in neuroprotection, especially in the periplaque area. It was previously shown, both in vivo and in vitro, that oligodendrocytes and astroglial cells serve as cellular targets for neurotrophins via trk receptors [6]. Neurotrophins, especially NT-3, were shown to regulate oligodendrocyte differentiation, which is the key process in remyelination [7]. Therefore, it may be hypothesized that in the periplaque area, where neuroinflammatory activity is the highest and so is the need for oligodendrocyte-mediated remyelination, immune cell-derived neurotrophic factors are key mediators of neuroregeneration and neuroprotection.

Following the concept of neuroprotective autoimmunity, we have previously shown that in relapsing-remitting MS (RRMS) patients neurotrophin levels are associated with general measures of brain atrophy, including brain parenchymal fraction (BPF) and corpus callosum cross-sectional area [8], and cognitive impairment [9]. We concluded that among RRMS patients, impaired immune-cell production of neurotrophins could be reflected by worse clinical outcome, as measured with brain atrophy and cognitive dysfunction parameters. Both these studies were of cross-sectional design and did not relate PBMC expression of neurotrophins in MS with the one in the healthy individuals. Therefore, one could not have assumed that all MS patients had impaired immune-cell production of neurotrophins. So far, only few studies measured neurotrophin expression within PBMCs in MS patients, mostly with regards to BDNF, while there is a number of papers assessing serum levels of neurotrophic factors. Serum BDNF levels, however, have not been considered reliable correlates of disease activity [10], mostly because the majority of serum BDNF stems from platelets, and not from immune cells [11]. As for immune cell source of neurotrophins, in one study BDNF was shown to be produced at lower levels than in PBMCs of the healthy controls [12]. In another study BDNF production by PBMCs was found to be increased during relapse phase, as compared with the remission and secondary progression [13]. On the contrary, Gielen et al. showed that BDNF expression (here assessed by mRNA levels, and not protein expression) was increased in MS as opposed to healthy controls and other neurological diseases [14].

The aim of the present study was to establish whether immune-cell production of neurotrophins is indeed impaired in treatment-naïve RRMS patients. Also, we wanted to assess if standard immunomodulatory treatment of MS could influence neurotrophin expression.

2. Methods

2.1. *Patient population*

Fifty-four patients diagnosed with relapsing-remitting multiple sclerosis according to the 2010 revised McDonald criteria [15] were screened and fifty were included in the study. The flow diagram of the study progress is presented in Fig. 1. All patients were treatment-naïve at the time of inclusion into the study. They were recruited consecutively in the Department of Neurology at the time of initiation of their immunomodulatory treatment, within 12 months of recruitment period. The study protocol was approved by the Internal Review Board at the Poznan University of Medical Sciences. All patients consented to the study in writing.

The study group consisted of 30 females and 20 males, with the mean age of 37 ± 9 years (min 18, max 64), median disease duration of 0.58 years (min 0.08, max 12.42 years) and median Expanded Disability Status Score (EDSS) of 2.0 (min 0.0, max 4.0). Clinical examination and blood sampling were performed at baseline, which was before therapy initiation, and after one year from treatment onset. All patients received standard first-line immunomodulatory drugs, namely beta-interferons (in 38 subjects) or glatiramer acetate (in 12 subjects). At follow up all patients were assessed clinically, including calculation of the Rio Score [16] and the complete set of study variables was assessed in 35 subjects.

The control group consisted of 39 healthy control subjects matched according to age and gender to MS study group. Written informed consent was obtained from control subjects, as well. Asymptomatic CNS pathology, especially radiologically isolated syndrome (RIS), was excluded in the healthy

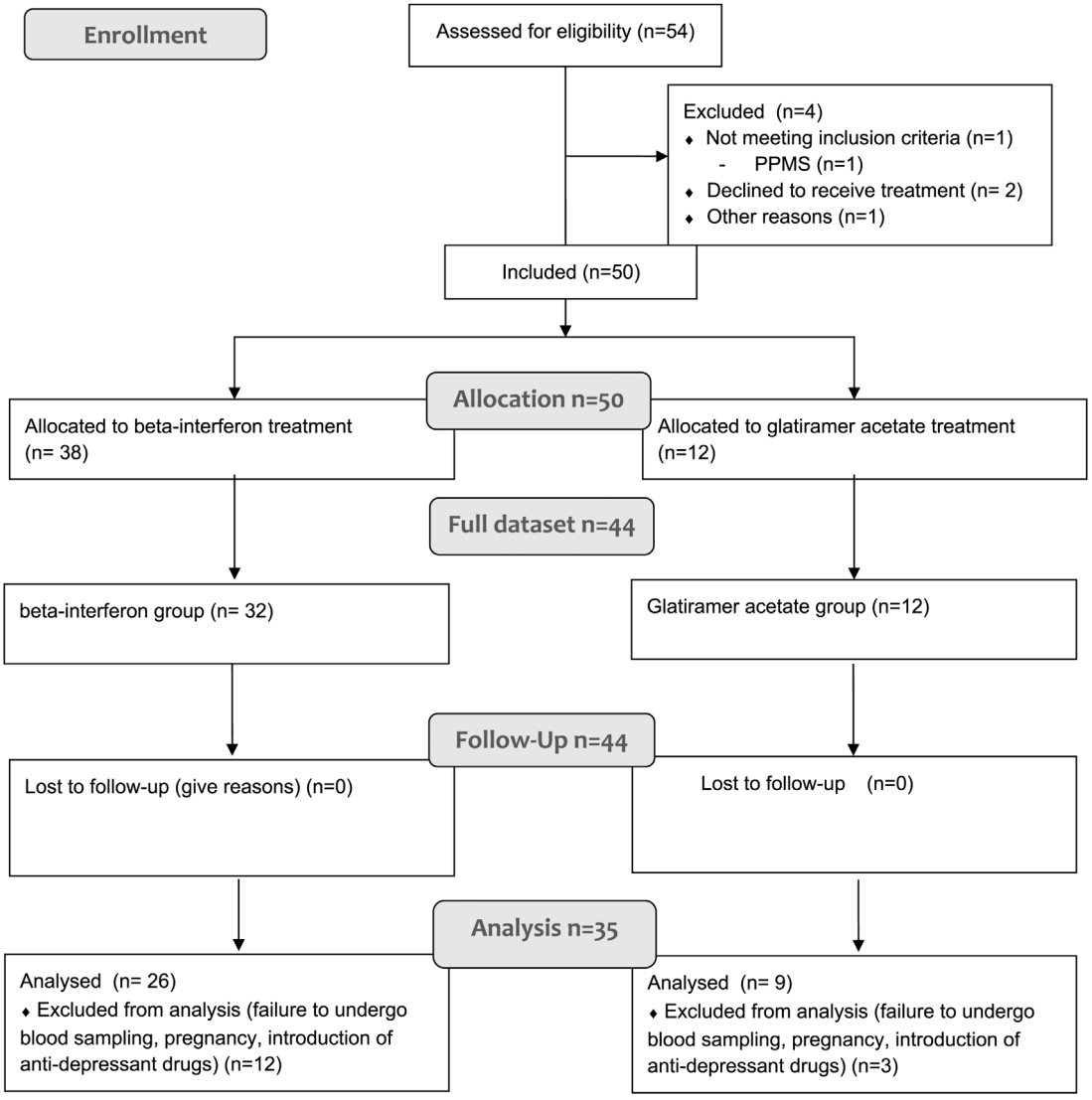

Fig. 1 – Flow diagram of the study progress. *Abbreviations*: MS – multiple sclerosis, PPMS – primary progressive MS.

controls with the use of standard magnetic resonance imaging.

Exclusion criteria were as follows: any history of psychiatric disorders, the use of antidepressants or other psychotropic drugs, alcohol abuse, malignancies, previous immunomodulatory treatment in MS subjects, intravenous steroid treatment within 2 months from blood sampling, other chronic diseases.

2.2. *Laboratory protocol*

Peripheral blood mononuclear cells (PBMCs) were isolated from patients' heparinized blood using Ficoll-Histopaque (Sigma–Aldrich) gradient method. Samples were kept frozen at −80 °C until further analysis. Before measurement of neurotrophin concentrations, PBMCs were resuspended in a lysis buffer (NaCl, Tris–HCl, EDTA) with Triton X-100 (Sigma–Aldrich) and a proteinase inhibitor cocktail (Sigma–Aldrich). The protein content was determined using the Lowry method [17]. BDNF, beta-NGF, NT-3 and NT-4/5 levels were measured in PBMC lysates with the use of ELISA method, according to manufacturer's instructions (MultiNeurotrophin Rapid Screening ELISA Kit: Human, Biosensis Pty Ltd, Australia). Neurotrophin concentrations were expressed as relevant weight units per one milligram of the protein.

2.3. *Statistical analysis*

Statistical analysis was performed with the use of MedCalc Statistical Software version 15.8 [MedCalc Software bvba, MedCalc statistical software, Ostend, Belgium (2015) https://www.medcalc.org]. Distribution of variables was evaluated using d'Agostino-Pearson normality test. The values were expressed as the mean and standard deviation (SD) for normally distributed variables and median and interquartile range (IQR) for parameters without normal distribution.

To compare the values between groups, *t*-test was used for variables with normal distribution and the nonparametric Mann–Whitney *U* test was used for variables with non-normal distribution. The *p*-value ≤0.05 was considered statistically significant.

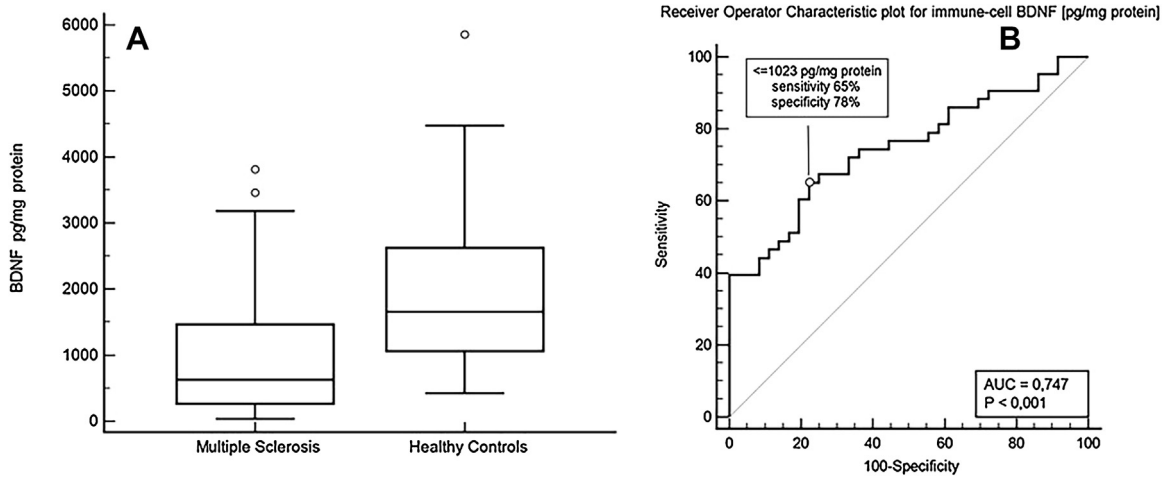

Fig. 2 – Comparison of immune cell BDNF levels in relapsing-remitting multiple sclerosis patients and in the healthy controls. (A) Box and whiskers plots for PBMC BDNF levels. (B) ROC curve analysis of discrimination between RRMS patients and healthy controls based on BDNF level in PBMCs. *Abbreviations*: RRMS – relapsing-remitting multiple sclerosis, PBMC – peripheral blood mononuclear cells, AUC – area under the curve, BDNF – brain-derived neurotrophic factor.

Receiver operating characteristic (ROC) curve analysis was applied to discriminate between MS and healthy controls, using neurotrophin concentrations. Calculated sensitivity and specificity pairs for each concentration threshold were used to create the ROC curve. The area under the curve (AUC) was calculated. Sensitivity, specificity and positive and negative predictive values were calculated for maximum accuracy threshold.

In order to assess the change in neurotrophin levels after one year of immunomodulation, the Wilcoxon signed-rank test was used.

3. Results

3.1. *Comparison of PBMCs neurotrophin expression in MS and in the healthy controls*

BDNF expression was detected in all MS and healthy controls PBMCs samples.

BDNF levels were significantly lower in MS than in the healthy control group (median 628 pg/mg protein, min 34, max 3815 vs. 1657 pg/mg protein, min 417, max 5858 pg/mg protein, $p = 0.0002$), see Fig. 2A.

NGF and NT-4/5 were detected in significantly fewer MS PBMCs samples (33% for NGF and 35% for NT-4/5) than in the healthy controls (92% for NGF and 81% for NT-4/5).

NT-3 was detected in 37% of MS samples and in 30% of healthy subjects PBMCs samples.

The detection pattern of different neurotrophins in MS, including baseline and follow-up data, and in the healthy controls cohorts is presented in Table 1.

We used ROC curve analysis to establish optimal accuracy of our cutoff BDNF level value to discriminate between MS patients and healthy controls, which was 1023 pg/mg protein (sensitivity 0.65, specificity 0.78, area under the curve, AUC 0.75, $p < 0.001$), see Fig. 2B.

3.2. *PBMCs neurotrophin expression in MS patients before and after one year of immunomodulation*

After one year of immunomodulation (beta-interferons or glatiramer acetate) BDNF expression did not change significantly (median 627.92 vs. 268.14 pg/mg protein, $p = 0.06$) on the group level. On the individual level, in 70% of patients there was no increase in BDNF level, and in 30% the level of BDNF increased. In comparison to the healthy control group, it was still significantly lower ($p < 0.0001$).

Table 1 – Detection level of different neurotrophins in multiple sclerosis and healthy controls study cohorts.

	BDNF		NGF		NT-3		NT-4/5	
	Baseline	After 1 year of therapy	Baseline	After 1 year of therapy	Baseline	After 1 year of therapy	Baseline	After 1 year of therapy
MS	100%	100%	33%	29%	37%	33%	35%	45%
HC	100%	n/a	92%	n/a	30%	n/a	81%	n/a

Abbreviations: MS – multiple sclerosis, HC – healthy controls, BDNF – brain-derived neurotrophic factor, NGF – nerve growth factor (NGF), NT-3 – neurotrophin 3, NT-4/5 – neurotrophin 4/5.

We observed no differences with regards to BDNF change in different treatment group ($p = 0.12$ for glatiramer acetate and $p = 0.57$ for beta-interferons).

Also, no changes were observed in the detection of other neurotrophins (after one year NGF was detected in 29% of samples, $p = 0.29$; NT-3 in 33% of samples, $p = 0.96$, and NT-4/5 in 45% of samples, $p = 0.77$).

3.3. *Correlation analysis for PBMC neurotrophin expression and clinical parameters in MS patients*

Correlation analysis was conducted only when neurotrophin detection was higher than 75%, which was the case for BDNF expression only.

We found that BDNF PBMC concentration at baseline did not correlate with any of the clinical parameters, including EDSS (Spearman $r = -0.11$, $p = 0.47$), disease duration (Spearman $r = -0.05$, $p = 0.73$), or age (Spearman $r = -0.09$, $p = 0.57$). It did not correlate with annualized relapse rate after one year of immunomodulation (Spearman $r = -0.17$, $p = 0.27$), or with Rio Score calculated after one year of therapy (Spearman $r = -0.28$, $p = 0.1$).

3.4. *Comparison of clinical outcomes in subgroups of patients with BDNF increase and non-increase after one year of immunomodulation*

We found that annualized relapse rate and Rio Score were higher in BDNF non-increase subgroup, however, these findings failed to reach statistical significance (median Rio Score 0, min 0, max 1 for BDNF increase vs. median Rio Score 1, min 0, max 2 for BDNF non-increase subgroup, $p = 0.07$).

4. Discussion

In the present study we confirmed our hypothesis, based on our previous findings [8,9] that immune-cell production of neurotrophins is impaired in MS patients. We found that BDNF was expressed in all samples of the study and control cohorts, however, its level was significantly lower in MS than in the healthy controls. While the detection of NGF and NT-4/5 was significantly lower in MS patients than in the controls, NT-3 was poorly expressed in both, MS and healthy subjects samples. Such discrepancy between NT-3 and other neurotrophins might be related to sample size, which was relatively small. However, it may also be due to different biology of NT-3, which is produced by T and B cells, but seems absent in macrophages [18], while BDNF, beta-NGF and NT-4/5 are secreted by all immune cells [19].

In our MS cohort standard immunomodulation failed to restore normal BDNF levels in PBMCs within one year of therapy. Importantly, immunomodulation did not affect PBMC ability to produce neurotrophins, either. While it would be encouraging to see that immune-cell BDNF production is enhanced by standard immunomodulatory treatment, one has to appreciate that it does not impair BDNF expression any further. By definition, immune-directed therapies are designed to restore the natural balance between pro- and anti-inflammatory components of the immune response in MS. Such mode of action could theoretically decrease the protective potential of autoimmune reaction, as well. However, our results deny this hypothesis.

In our study we analyzed patients receiving standard injectable immunomodulation, namely beta-interferons and glatiramer acetate. There is a large body of evidence that glatiramer acetate exerts a neuroprotective effect both, in vitro and in vivo. It was previously shown that glatiramer acetate could lead to enhanced BDNF expression in animal models [20,21]. Several studies provided conflicting results with regards to glatiramer acetate impact on BDNF expression in MS [22,23]. Whether this effect is clinically relevant in humans remains to be established. In our cohort there were only 12 patients treated with glatiramer acetate, which is too small a sample to draw definite conclusions. As for beta-interferons, they were also shown to influence BDNF levels in vivo. Mehrpour et al. found increased serum BDNF levels in MS patients on beta-interferons, as opposed to patients on mitoxantrone or receiving no immunotherapy [24]. Moreover, they found that higher BDNF levels were associated with smaller disability in their MS cohort. Another group found that T-cell BDNF production and trkB expression was enhanced in MS patients receiving beta-interferons, in comparison with the untreated group [25].

Similar findings were observed in other studies [26,27]. Our results do not confirm positive influence of first-line therapies on BDNF levels, however, they need to be interpreted with caution, as the sample size is relatively small. It is worth emphasizing that among clinical studies on MS patients, and not animal models, our study is one of the very few that analyze the whole spectrum of neurotrophins, including NGF (only 2 studies published with regards to beta-interferons [28,29] and none with regards to glatiramer acetate), NT-3 (one study with regards to glatiramer acetate [21], none with beta-interferons) and NT-4/5 (two studies for interferons [28,30], and one with glatiramer acetate [21]). Although we present detection data for NGF, NT-3 and NT-4/5, and direct level measurements for BDNF only, our study manages to present novel findings with regards to immune-cell neurotrophin expression in RRMS patients.

The limitations that we need to recognize are a relatively small sample size, which did not allow adequate comparison of different immunomodulatory therapies, and a relatively short follow-up period (12 months). This could be the reason for the fact that we did not observe any correlations between BDNF expression and clinical parameters in our MS group. Also, it would be interesting to supplement our study with assessment of BDNF polymorphisms in the studied subjects.

Undoubtedly, the advantages of our study include its prospective design, availability of longitudinal data, and treatment-naïve cohort that represents the early phase of MS course (median disease duration is 0.58 years).

Our data, suggesting that BDNF production is indeed impaired in MS patients, are especially relevant in the context of BDNF being a potential regulator of the number of oligodendrocyte progenitor cells, thus influencing the response to demyelination at acute lesion site [31,32]. If this was the case, immune-cell production of BDNF could be a compensatory response to white matter injury resulting from acute demyelination. As we have suggested previously,

heterogeneity among MS patients in the ability to upregulate immune neurotrophin production, could correspond with heterogeneity in disease severity, with high neurotrophin producers being protected from periplaque damage in the long term. Moreover, it has been indicated that BDNF molecular target can be influenced by small molecules that improve remyelination in injury models [33]. Such approach could be potentially useful in treatment of MS relapses.

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Conflict of interest

None declared.

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