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Interferon-beta, but not Glatiramer Acetate treatment induces gender-specific increase in BDNF serum levels in relapsing-remitting multiple sclerosis female patients

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Abstract

Neuroprotection is considered a major therapeutic target in Multiple Sclerosis (MS), a chronic inflammatory demyelinating disease of the central nervous system (CNS). Accordingly, current disease modifying drugs (DMDs) for MS have been reevaluated for their ability to stimulate production of neurotrophic factors that may provide support to neural cells against neurodegeneration. Brain-derived neurotrophic factor (BDNF) is considered the best candidate for such neuroprotective effects in MS. In the present study, we explored a potential effects of Glatiramer Acetate (GA), and Interferon-beta (IFN- β), the first two available treatments of MS, on circulating levels of BDNF considering the different BDNF forms. Using ELISA assays, we quantified mature and total BDNF in the serum of 20 relapsing-remitting MS (RR-MS) patients treated with GA and 31 with INF-beta compared with 20 age-matched RR-MS patients without any DMD. Mature BDNF but not total BDNF was significantly increased by treatment with IFN- β in female patients only, while GA had no effect. These results support a gender-specific role of BDNF in the treatment of RR-MS with INF- β . Of note, the present study was carried out with standardized ELISA assays which are commercially available and, prospectively, might be routinely used in the common clinical practice to monitor the individual response to the therapy with IFN- β .

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Introduction

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) with a high prevalence in young adults [1]. Even if MS etiology and pathogenesis are still incompletely understood, it is well known that inflammation with infiltration in the CNS by cellular and soluble mediators of the immune system plays a predominant role [2-4]. Accordingly, current therapeutic approaches are focused on trying to reduce the inflammatory processes by treatment with disease modifying drugs (DMDs) and the first available treatments to target inflammation were interferon-beta (IFN- β) and glatiramer acetate (GA). In the last years the increasing availability of both oral and injectable new medications has changed the clinical approach towards a personalized MS therapy [5]. However, the long-term efficacy of these immunomodulatory treatments is still debated and therefore, research has been recently oriented to neuroprotection as an alternative or complementary strategy for MS treatment [6]. Accordingly, new therapeutic strategies to provide neuroprotection of injured neurons are actively sought.

A potential therapeutic target is represented by the neurotrophin brain-derived neurotrophic factor (BDNF) which is one of the most potent neurotrophic factors supporting neuronal survival, and regulating neurotransmitters release and neuronal growth [7]. BDNF is released by neurons and activated astrocytes and in vivo application of BDNF prevents neuronal degeneration after experimental

axotomy and other forms of neuronal injury [8-11]. Not only neurons can produce BDNF but also immune cells like T cells, B cells and monocytes, especially after activation [7,12,13]. Indeed, enhanced BDNF levels were found in Th2 and Th1 cells following stimulation by GA [14,15] and in T cells from patients treated with IFN- β or the antibody alemtuzumab [16,17]. Considering that the receptor of BDNF, TrkB was found to be highly expressed in neurons and astrocytes within MS brain lesions [18], these findings fit very well into the concept of neuroprotective autoimmunity. This theory postulates that infiltration of CNS antigen-specific T cells or other immune cells can reduce neuronal degeneration especially because T cells secretion of BDNF and anti-inflammatory cytokines is enhanced by GA treatment [12,19,20]. However, more recent studies using experimental autoimmune encephalomyelitis (EAE) in conditional BDNF knockout mice, showed that, when BDNF is selectively ablated in astrocytes or in the whole CNS, animals showed a more severe course of the disease and increased axonal damage. Conversely, when BDNF was deleted in T cells or cells of the myeloid lineage alone no worsening of the course of EAE could be observed [21,22]. Given these contrasting results, it remains to be determined if the best neuroprotectant effect can be achieved by increasing expression of BDNF in neuronal or in immune cells.

In a previous study, we investigated the different BDNF forms produced in MS. BDNF is synthesized as a precursor protein of 32 KDa (proBDNF) which is cleaved by SKI-1 protease generating a large proteolytic product of 27 KDa called truncated BDNF, or by convertases

such as furin or MMP-7 producing a smaller protein of 14KDa, the mature BDNF. Notably, proBDNF and mature BDNF have opposite biological effects, while the biological function of truncated BDNF is still unknown [23]. In the serum of drug-naïve MS patients, we found a reduction in the serum levels of pro-apoptotic proBDNF and pro-survival mature BDNF while truncated BDNF was increased and thus, resulted to be the most abundant BDNF form in MS sera [24]. The increase in serum levels of truncated BDNF in MS patients appears to be specific because in sera from patients affected by schizophrenia we found a significant decrease of this BDNF proteolytic form [25].

In conclusion, therapeutic strategies to obtain neuroprotection by enhancing endogenous BDNF production in neurons, astrocytes or T-cells require a better understanding of the BDNF forms produced in the various phases of the disease and in response to MS disease-modifying drugs. First of all, because pro and mature BDNF have, respectively, pro- or anti-apoptotic effects and therefore, can affect neurons and immune cells in opposite ways. Secondly, because the functional role of truncated BDNF and its modulation during the course of MS are still unknown.

On the basis of the above mentioned issues, we have undertaken a study to determine how GA or IFN-b, the more common treatments for MS, affect the synthesis of total and mature BDNF. To this aim, we used two ELISA kits that in a previous study [26] we demonstrated to have the best performance above six commercial assays in measuring specifically total BDNF or mature BDNF in human serum. Information on this issue, will be instrumental for a better understanding of the effects of these treatments and for the design of future screening campaigns to identify drugs able to stimulate selective production of BDNF variants.

Results

Patient's characteristics

Clinical and paraclinical characteristics of the enrolled patients are reported in Table 1. All the considered variables were well-balanced in the groups; in particular, there were no significant differences of disease duration, annual relapse rate (ARR), type of disease onset, CSF profile between the groups (Chi-square test or Kruskal-Wallis test). A high percentage of patients both in the G1 and the G2 group underwent previous treatment before the current one; the reasons of the switch were the evidence of lack of efficacy, presence of neutralizing antibodies (NAbs), or adverse events (e.g. flu-like syndrome). We observed, as expected, a clear reduction in terms of ARR after last treatment onset, compared with the previous disease history in both G1 and G2 groups.

Total and Mature BDNF serum level measurements

Serum BDNF is considered a potential prognostic marker in monitoring the outcome of different therapies in RR-MS patients. Using western-blot technique, we previously showed that RR-MS patients are characterized by an altered balance between proteolytic forms of BDNF in the serum [24]. However, in a typical clinical setting, it is necessary to use simpler and more standardized techniques with respect to western-blot. Therefore, we used two commercial ELISA kits that we previously demonstrated [26] to be able to reliably measure total BDNF (Biosensis) and mature BDNF (Aviscera) in human serum. Since no commercial kit is available for truncated BDNF, this proteolytic form of BDNF was not measured in this study. We first measured circulating levels of total BDNF in the serum of RR-MS patients treated with interferon (G1) or glatiramer acetate (G2) with respect to age-matched untreated RR-MS subjects (G3). We

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observed no differences in total BDNF levels between all RR-MS patients groups, irrespectively of the administered therapy (Median G1=37.79 ng/ml, Q1-Q3=29.42-

43.18, Median G2=36.76 ng/ml, Q1-Q3=30.79-43.80, Median G3=42.88 ng/ml, Q1-Q3=35.02-52.26; p=0.22; see Figure 1A).

Table 1: Clinical and paraclinical characteristics of the enrolled patients. Mean \pm standard deviation or median (range); * Chi-square test or Kruskal-Wallis test, where appropriate. ARR: annual relapse rate; EDSS: Expanded Disability Status Scale; DMD: disease modifying drug; OCBs: oligoclonal bands; na: not available; ns: not significant.

Patient's characteristic	G1 Interferon (n=31)	G2 GA (n=20)	G3 no therapy (n=20)	p*
Gender M	9 (29%)	8 (40%)	3 (15%)	0.211
Gender F	22 (71%)	12 (60%)	17 (85%)	
Age (years)	41.8 \pm 9.7	40.5 \pm 11.6	44.3 \pm 10.2	0.753
Disease duration (months)	95 (23-346)	137 (5-305)	131 (6-353)	0.776
Total number of relapses	2 (1-12)	2 (1-28)	2 (1-12)	0.920
Annual Relapse Rates (ARR)	0.3 (0-1.3)	0.3 (0.0-2.4)	0.3 (0.1-3.4)	0.920
Expanded Disability Status Scale (EDSS)	1 (0-3)	1 (0-5.5)	1.3 (0-3.5)	0.828
Family history of MS	2 (6.5%)	3 (15%)	2 (10%)	0.606
No Family history of MS	29 (93.5%)	17 (85%)	18 (90%)	
Family history of autoimmune diseases	4 (12.9%)	5 (25%)	1 (5%)	0.186
No Family history of autoimmune diseases	27 (87.1%)	15 (75%)	19 (85%)	
Symptom at onset				
Optic pathways	7 (22.6%)	3 (15%)	5 (25%)	0.715
Supratentorial	6 (19.4%)	2 (10%)	2 (10%)	0.532
Brainstem/cerebellum	10 (32.2%)	6 (30%)	4 (20%)	0.622
Spinal cord	7 (22.6%)	8 (40%)	8 (40%)	0.298
Polyregional	1 (3.2%)	1 (5%)	1 (5%)	0.934
Complete Recovery from 1st relapse	27 (87.1%)	16 (80%)	17 (85%)	0.789
Incomplete Recovery from 1st relapse	4 (12.9%)	4 (20%)	3 (15%)	
CSF OCBs	17 (54.8%)	11 (55%)	13 (65%)	0.741
Absent CSF OCBs	5 (16.1%)	6 (30%)	2 (10%)	0.241
Not available CSF OCBs	9 (29%)	3 (15%)	5 (25%)	0.514
Time to 2nd relapse (months)	22 (1-162)	36 (2-157)	24 (2-278)	0.966
Time from last relapse (months)	51 (1-329)	42 (5-271)	24 (2-251)	0.264
Number of steroid treatment	1 (0-8)	1 (0-27)	1 (0-20)	0.831
Time from last steroid treatment (months)	45.5 (1-29)	45 (5-203)	27 (2-231)	0.630
Previous DMD	7 (22.6%)	8 (40%)	10 (50%)	0.117
No Previous DMD	24 (77.4%)	12 (60%)	10 (50%)	
Numbers of previous DMD	1 (1-2)	1 (1-3)	1 (1-3)	0.571
Duration of previous DMD (months)	34 \pm 31.2	58.5 \pm 33.7	77.3 \pm 36.9	0.056
Time from last DMD (months)	na	na	31.5 (9-51)	0.062
Current DMD duration	46 (9-175)	34 (2-146)	na	0.120
Number of relapses before last (ongoing) treatment	2 (1-10)	2 (1-26)	na	0.785
ARR before last (ongoing) treatment	0.9 (0.1-9.0)	0.5 (0.1-4.1)	na	0.359

Number of relapses during ongoing treatment	0 (0-3)	0 (0-2)	na	0.210
ARR during ongoing treatment	0.0 (0.0-1.2)	0.0 (0.0-0.3)	na	0.103
ARR worsening during ongoing treatment	2 (6.5%)	0 (0%)	na	0.247
EDSS before last (ongoing) treatment	1.5 (0-3)	1.5 (0-6)	na	0.364
EDSS worsening (≥ 1) during ongoing treatment	3 (9.7%)	3 (15%)	na	0.565
EDSS or ARR worsening during ongoing treatment	5 (16.1%)	3 (15%)	na	0.914
EDSS improving (≥ 1) during ongoing treatment	2 (6.5%)	3 (15%)	na	0.139

In a second set of experiments, we measured serum levels of mature BDNF. We previously demonstrated that RR-MS patients differ from healthy subjects for an imbalance in BDNF processing, leading to increased truncated BDNF and reduced mature BDNF serum levels [24]. Since mature BDNF is the form which promotes neuroprotection, we evaluated if treatment with interferon or glatiramer acetate may improve circulating mature BDNF levels as compared to untreated patients. Circulating

mature BDNF was measured in all RR-MS patient groups using an ELISA kit (Aviscera) which predominantly measures this form [26]. We found that none of the therapies was able to increase the serum levels of mature BDNF (Median G1=12.14 ng/ml, Q1-Q3= 9.52-16.40, Median G2=13.82 ng/ml, Q1-Q3= 10.37-18.58, Median G3=13.35 ng/ml, Q1-Q3= 11.50-15.48; $p=0.66$; see Figure 1B).

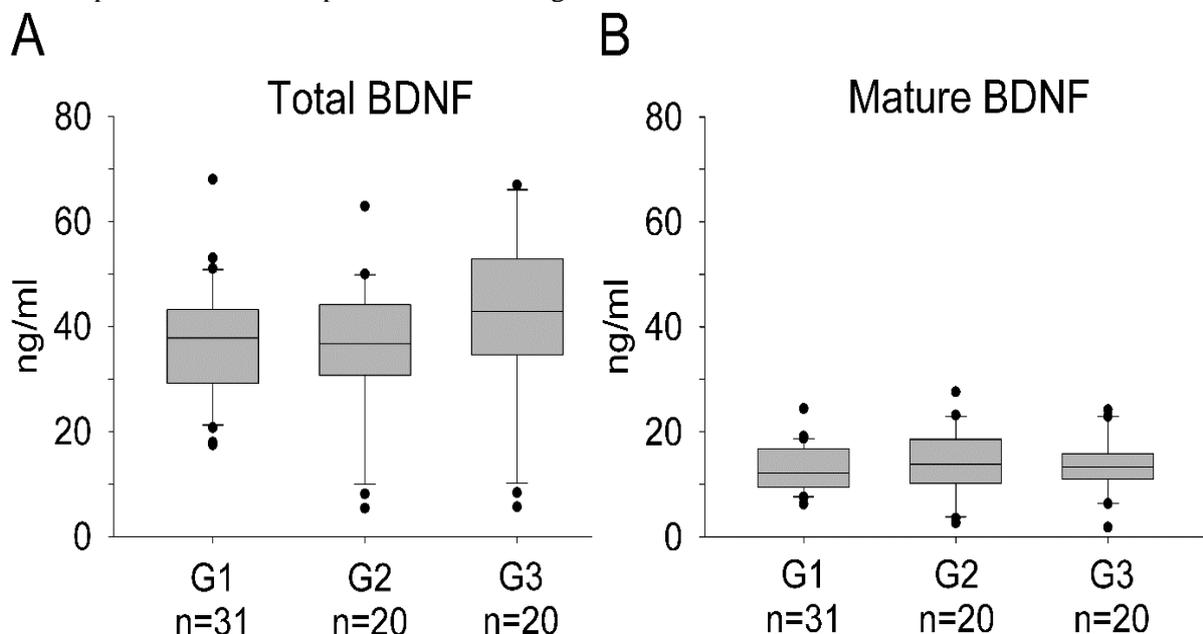


Figure 1: Total BDNF serum level measured by ELISA assay.

Box plot of total BDNF serum concentration (ng/ml) in healthy controls and RR-MS patients with different therapy: Group 1 (G1) interferon, Group 2 (G2) glatiramer acetate and Group 3 (G3) no-therapy groups. The upper line of the box marks the 75th percentile, the middle line is the median value and the lower line specifies the 25th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles, respectively. Dots indicate the outlier values within each group. B) Box plot of mature BDNF serum concentration (ng/ml), as described in panel A. n = number of patients in each group. Statistical analysis was done with Kruskal-Wallis One Way Analysis of Variance on Ranks test followed by correction for multiple comparison using the Dunn's method.

Total and mature BDNF serum level measurements in female subgroups.

Autoimmune diseases are known to show higher prevalence among females. To

investigate the possible gender effect, we analysed circulating levels of total and mature BDNF in male and female patient sera, separately. While we found no statistical difference between groups in male patients, we observed a statistical difference in female patients of the interferon group with respect to the glatiramer acetate and no-therapy groups ($P < 0.05$; G1 vs G2 and G1 vs G3; see figure 2A). In particular, we found a significant increase in circulating levels of total BDNF in female subjects treated with interferon (Median G1=38.28 ng/ml, Q1-Q3=29.13-43.63, Median G2=17.73 ng/ml, Q1-Q3=14.36-22.98, Median G3=21.32 ng/ml, Q1-Q3= 17.72-24.41; $P < 0.05$). Despite that, irrespectively of the administered therapy, in all RR-MS female subgroups, we observed no difference in mature BDNF levels (Median G1=12.95 ng/ml, Q1-Q3=8.98-16.80, Median G2=13.50 ng/ml, Q1-Q3=9.77-19.21, Median G3=12.78 ng/ml, Q1-Q3= 10.39-14.55; see Figure 2B).

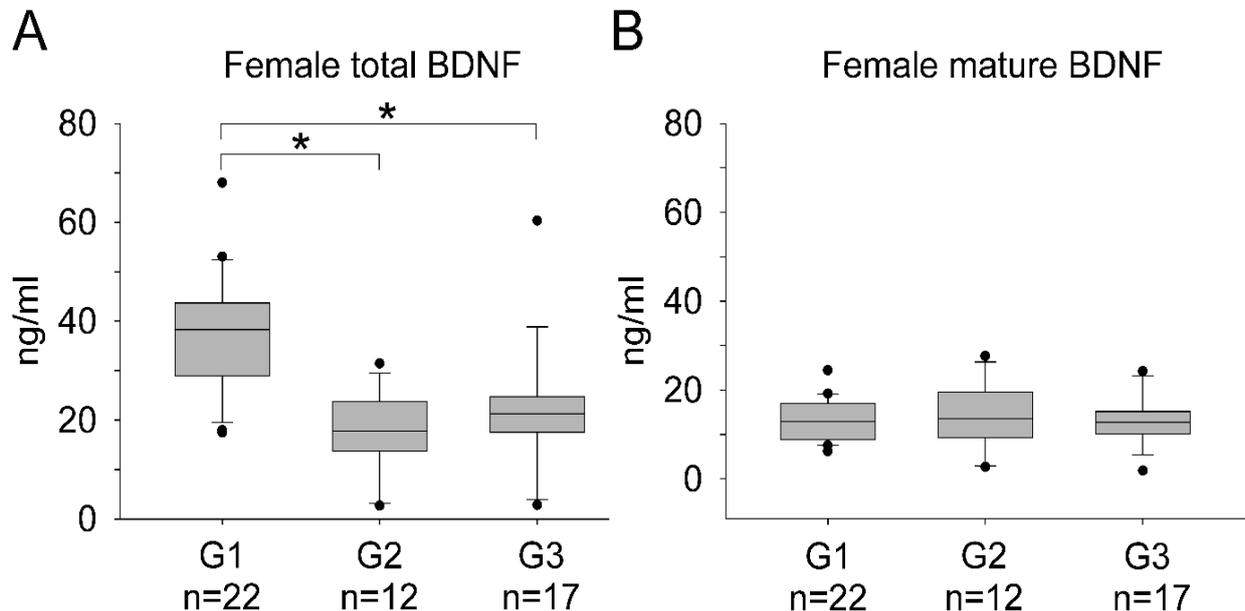


Figure 2: Genders-specific BDNF serum levels measured by ELISA assay in female patients.

A) Box plot of total BDNF serum concentration (ng/ml) in healthy controls and RR-MS female patients with different therapy: Group 1 (G1) interferon, Group 2 (G2) glatiramer acetate and Group 3 (G3) no-therapy groups. Box plot are defined as in figure 1. * = $P < 0.05$ G1 vs G3 and G1 vs G2 (Kruskal-Wallis One Way Analysis of Variance on Ranks test followed by correction for multiple comparison using the Dunn's method). B) Box plot of mature BDNF serum concentration (ng/ml) in female patients as described in panel A.

Discussion

In this study, we provide evidence that chronic treatment with interferon-beta significantly increases the levels of circulating BDNF in relapsing-remitting female patients, as compared to a population of RR-MS patients who refused any treatment. This effect was seen for total BDNF but not for mature-BDNF and was not detected in male patients or when the entire population of RR-MS patients was analysed (males + females). In addition, we found no effect of glatiramer acetate chronic treatment on serum BDNF levels, irrespective of the BDNF form or the sample population considered. The possibility to include a subgroup (G3) of rare RR-MS patients who refused any treatment for at least 6 months, although forcedly reduced the size of the study groups, made this study one the very few in the field of Multiple Sclerosis who investigated BDNF in these patients. An added value of the present study is that results were obtained with well standardized ELISA kits which are commercially available. In perspective these BDNF assays, might be used in a common clinical practice to monitor the efficacy of therapies and, in particular, the individual response to interferon-beta.

A major finding of this study is that INF-beta, but not GA, can restore the normal levels of BDNF in female RR-MS patients. This finding imposes three types of considerations, first

regarding the significance of the increased levels of total BDNF in response to INF-beta, second regarding the controversial literature on the effects of GA on BDNF, and lastly on the observed gender effect.

Our results are in agreement with a recent study showing that INF-beta can increase serum BDNF levels [27] and with a previous study demonstrating an increased production of BDNF by peripheral blood mononuclear cells following stimulation with INF-beta [28]. Serum levels of BDNF varies during to the different phases of RR-MS. Together with some pro-inflammatory cytokines, such as TNF-alpha and INF-gamma, BDNF is considerably increased in the acute phase and in case of complete or partial recovery from new symptoms [29,30] or after relapse [31]. In contrast, lower BDNF values were found in supernatants of peripheral blood mononuclear and in the CSF of patients with secondary-progressive (SP) MS in comparison with controls [29] while other studies found no difference in plasma BDNF levels between naïve MS patients and healthy controls [32,33]. However, other laboratories including ours have found that serum BDNF levels are reduced in RR-MS patients during remission in comparison to control patients [24,34]. It is worth to mention also the current debate regarding the significance of serum BDNF levels in terms of biomarker of disease progression. Mehrpour et al. [27], proposed serum BDNF with levels above a cut-off of 190 pg/ml as a biomarker of disease severity, while in another study, serum BDNF levels did not seem to correlate with the extent of neurodegeneration [33]. Besides the current difficulties in dissecting out the exact correlation between BDNF levels and phase or severity of the disease, our findings are in support of a possible neurotrophic effect of INF-beta in SM.

A second aspect of complexity is given by the controversial data on the effects of GA on BDNF levels. Although initial studies reported increased serum levels [34,35], more recent ones conducted on MS patients in different countries have consistently reported no effect of GA on serum levels of BDNF even after one year of treatment [36,37]. Such discrepancies might be due, as explained above to the fluctuating serum levels of BDNF in the different phases of MS. In addition, as we recently demonstrated, the use of different ELISA kits to detect serum BDNF may produce inconsistencies among studies due to different ability to detect BDNF or inter-assay variability of the kit used [26] (see discussion on the technical issue, below). On the other hand, various studies reported that when stimulated with GA, peripheral blood monocytes show increased release of BDNF in vitro, but only in a subpopulation of GA-responding cells and patients [38-41].

Third, in our hands INF-beta induces upregulation of serum BDNF in females, but not in males. The gender bias is particularly relevant in autoimmune diseases and MS is known to be more prevalent in females than in males. Of note, gender represents also an important factor with respect to the progression and aggressiveness of MS and is postulated to play a role also in remyelination and neuronal damage [42,43]. Interestingly, in experimental autoimmune encephalomyelitis (EAE) a gender difference in expression of molecules potentially related to myelin damage and repair has been described [44]. Besides these considerations, we are the first to observe a gender specific effect of INF-beta on serum BDNF levels. Additional studies are necessary to determine the mechanism of such gender difference in drug responsiveness.

The data on serum BDNF in MS however, impose caution regarding their interpretation, also in consideration of the biological complexity of BDNF. Indeed, as already

mentioned in the introduction, BDNF is produced as a pro-BDNF precursor, which undergoes proteolytic processing to generate the truncated or the mature BDNF forms. Pro and mature BDNF forms have antagonistic pro-apoptotic and anti-apoptotic effects, respectively; while the biological function of truncated BDNF is unknown [24,23]. We previously showed that in drug naïve MS patients, truncated BDNF was increased and therefore resulted to be the predominant form, because pro- and mature BDNF were significantly reduced with respect to the sera of healthy controls, thus leading to an altered balance between serum BDNF forms [24]. In this study, we found that INF-beta promotes an increase in serum BDNF levels in female MS patients, and that pro-BDNF, or truncated BDNF variants, or both contributes to this increase, because mature BDNF was not affected. Information regarding the capacity of conventional immunomodulatory drugs to restore the balance of BDNF forms observed in the serum of MS patients, is considered useful to understand the potential for neuroprotective effects of the different BDNF forms. The concept that neuroprotective autoimmunity induced by T-cells and other immune cells is partially mediated by BDNF has obvious implications for the therapy of MS and is the underlying theoretical framework of this study. Activated human T cells, B cells, and monocytes secrete BDNF in vitro. In T helper (Th)1- and Th2-type CD4(+) T cell lines specific for myelin autoantigens such as myelin basic protein or myelin oligodendrocyte glycoprotein, BDNF production is increased upon antigen stimulation [7,38-41]. However, there is no information on the type of BDNF variant secreted by immune cells. Pro and mature BDNF can exert opposite effects on cell survival by activating p75 or TrkB receptors, respectively. The BDNF receptor TrkB has been found to be upregulated in T cells from MS patients with respect to healthy controls suggesting that BDNF may promote also increased survival and proliferation of the

autoreactive T cells and hence further promote the disease [45]. The p75 receptor has been detected in B cells which are involved in forms of MS with GM involvement. Mice deficient for p75 receptor display a more severe EAE with reduced infiltrated B-cells, monocytes, macrophages and polymorphonucleate cells and almost doubled infiltrated T-cells [46].

There is an important technical issue with BDNF measurement that should be considered here. In a previous study, we investigated the type of BDNF produced in MS using a western-blot analysis with antibodies that allow to visualize all three BDNF forms, namely pro-BDNF, truncated BDNF and mature BDNF [24]. However, semi-quantitative western-blot analysis is a technique which is prone to some artefacts, due to poor sensitivity and the intrinsic variability in each experimental passage such as PAGE-electrophoresis, blot transfer, immune-detection and quantification. For these reasons, in the present study we aimed at quantifying BDNF forms using high-sensitive and well standardized methods consisting in two ELISA assays, one for total BDNF (Biosensis) and one for mature BDNF (Aviscera). We recently characterized these ELISA assays by comparing their performance amongst a pool of six different commercial assays and they resulted to be the most reliable ones in terms of sensitivity and inter-assay variations [26]. In addition, we showed that the Biosensis kit recognizes both pro- and mature BDNF, while the Aviscera kit is specific for mature BDNF only [26]. We inferred that Biosensis assay should also recognize the truncated BDNF form, and therefore it is suitable to measure total BDNF. Since INF- β induced an increase in total but not mature BDNF, the increase observed here is most likely accounted by pro-BDNF or truncated BDNF, or both in agreement with our previous study in untreated SM patients in which truncated and pro-BDNF forms showed the highest levels. These results indicate the absolute need of standardized new ELISA

assays able to discriminate the different BDNF forms.

This study presents some limitations, regarding the sample population, methodology and possible interpretation of the results. First, given the relatively small population investigated, a replica in a larger sample would be desirable. Second, as stated above, the ELISA assays available at the moment do not allow to quantify truncated BDNF and since we showed that this is the predominantly affected BDNF form in MS, it would be important to determine if drugs have an effect on its serum levels. Third, although it is known that most circulating BDNF is stored in platelets and is fully released after blood clotting [47], the source and biological relevance of circulating BDNF forms is still unclear.

Conclusions

This study was aimed at determining how GA and IFN- β , the two principle available treatments for MS, affect the synthesis of BDNF proteolytic forms. These two treatments are only partially functional in blocking MS inflammatory activity; therefore, in order to slow the disease progression, new therapeutical approaches towards a neuroprotective strategy are required. Finally, given the fact that GA and IFN- β mechanism of action is still not entirely understood, we propose that the strong relations that their pharmacological pathways share with neurotrophins production and regulation may possibly represent an important key factor to understand and possibly overcome their strength-limited efficacy. The results of this study highlights the modulation of BDNF forms in response to current MS treatments and suggest that this modulation can be easily detected using commercial ELISA kits.

Methods

Subject inclusion/exclusion criteria and sample collection

Blood samples have been routinely collected and stored during the every-day clinical practice in the Neurology Clinic of the Trieste University Hospital. All patients signed written informed consent to store the blood and participate to the study, and the project was conducted according to the Ethic Guidelines of the institute and the recommendations of the declaration of Helsinki and the Italian DL n° 675 of the 31-12-1996. We analysed the sera of seventy-one Caucasian RR-MS patients who accessed our clinic between April 2013 and February 2015. We grouped the patients as follows: thirty-one on interferon-beta treatment (G1), twenty on glatiramer acetate (G2), and twenty without any disease modifying drug (DMD) treatment (G3). The latter represents a subgroup of RR-MS patients who suspended (at least 6 months before blood collection) and/or refused all available treatment options, despite medical recommendation. Inclusion criteria were: diagnosis of RR-MS [48,49], DMD treatment for at least 2 months (for G1 and G2) and DMD-free condition for at least 6 months (for G3). For all groups, exclusion criteria were: relapse and/or corticosteroid therapy in the last month, concomitant treatment with antidepressants and/or mood stabilizers, antibiotics, antihypertensives, and gastroprotective agents. We retrospectively collected all the demographic, clinical (including EDSS score [49]), and paraclinical data of the patients, with particular attention to the therapeutic details.

Serum samples were prepared as previously described [26]. In brief, blood samples were collected from all subjects between 9:00 to 12:30 in fasting condition, and allowed to clot for 1 hour at room temperature, followed by 1 hour at 4°C. Serum was then separated by centrifugation at Relative Centrifugal Force

(RCF) 2000 g for 10 minutes at 4°C, aliquoted and stored at -80°C until use in 0.2 ml tubes strips (Sarstedt, Multiply® µStripPro).

BDNF measurements

Serum levels of total BDNF were measured by using BDNF *Rapid*TM ELISA Kit (Human, Mouse, Rat, 2 Plates; Cat #: BEK-2211-2P, Biosensis Pty Ltd., SA, Australia) ELISA kit, while mature BDNF serum levels were measured by human BDNF ELISA Kit (Cat #: SK00752-01, Aviscera-Bioscience, Santa Clara, CA, USA). All samples were assayed in duplicate. Protocols were performed according to the manufacturer's instructions. The optical density of each well was measured using an automated microplate reader (GloMax®-Multi Microplate Reader, Promega).

Statistical analysis

Clinical characteristics of the enrolled patients were analysed by chi-square test or Kruskal-Wallis test, where appropriate. BDNF values were not normally distributed and data are presented as median, 25th-75th percentile and range (see figures). Accordingly, differences in BDNF concentration among groups were assessed using Kruskal-Wallis One Way Analysis of Variance on Ranks test followed by correction for multiple comparison using the Dunn's method. Values of $p < 0.05$ were considered statistically significant. All statistical analysis and graphs were performed using SigmaPlot 11.0 (Systat Software, Inc.).

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Author contribution statement

P.M., A.B. and A.S. visited the patients. A.S., G.M., A.P. and A.B. collected and analysed the data. A.S., G.M., A.B., P.M., E.T revised the data and wrote the paper. E.T planned and supervised the project.

References

1. Mahurkar S, Suppiah V, O'Doherty C. 2014. Pharmacogenomics of interferon beta and glatiramer acetate response: a review of the literature. *Autoimmunity reviews*. 13: 178-186. Ref.: <https://bit.ly/2IJvRtm>
2. Correale J, Gaitán MI, Ysraelit MC, et al. 2017. Progressive multiple sclerosis: from pathogenic mechanisms to treatment. *Brain*. 140: 527-546. Ref.: <https://bit.ly/2IGytbr>
3. Schiess N, Calabresi PA. 2016. Multiple Sclerosis. *Seminars in Neurology*. 36: 350-356. Ref.: <https://bit.ly/2kDCIEo>
4. Yadav SK, Mindur JE, Ito K, et al. 2015. Advances in the immunopathogenesis of multiple sclerosis. *Current Opinion in Neurology*. 28: 206-219. Ref.: <https://bit.ly/2mcsmftr>
5. Feinstein A, Freeman J, Lo AC. 2015. Treatment of progressive multiple sclerosis: what works, what does not, and what is needed. *Lancet Neurology*. 14: 194-207. Ref.: <https://bit.ly/2kGpXJd>
6. Huang Y, Dreyfus CF. 2016. The role of growth factors as a therapeutic approach to demyelinating disease. *Experimental neurology*. Ref.: <https://bit.ly/2keDzve>
7. Kerschensteiner M, Eike Gallmeier, Lüder Behrens, et al. 1999. Activated human T cells, B cells, and monocytes produce brain-derived neurotrophic factor in vitro and in inflammatory brain lesions: a neuroprotective role of inflammation? *The Journal of experimental medicine*. 189: 865-870. Ref.: <https://bit.ly/2kGjyOa>
8. Sendtner M, Holtmann B, Kolbeck R, et al. 1992. Brain-derived neurotrophic factor prevents the death of motoneurons in newborn rats after nerve section. *Nature*. 360: 757-759. Ref.: <https://go.nature.com/2kGjIVM>
9. Weibel D, Kreutzberg GW, Schwab ME. 1995. Brain-derived neurotrophic factor (BDNF) prevents lesion-induced axonal die-back in young rat optic nerve. *Brain research*. 679: 249-254. Ref.: <https://bit.ly/2IIjpu0>
10. Gravel C, Gotz R, Lorrain A, et al. 1997. Adenoviral gene transfer of ciliary neurotrophic factor and brain-derived neurotrophic factor leads to long-term survival of axotomized motor neurons. *Nature medicine*. 3: 765-770. Ref.: <https://go.nature.com/2m3zIBA>
11. Kobayashi NR, et al. 1997. BDNF and NT-4/5 prevent atrophy of rat rubrospinal neurons after cervical axotomy, stimulate GAP-43 and Talphal-tubulin mRNA expression, and promote axonal regeneration. *The Journal of neuroscience: the official journal of the Society for Neuroscience*. 17: 9583-9595. Ref.: <https://bit.ly/2kgmt04>
12. Hohlfeld R, Kerschensteiner M, Stadelmann C, et al. 2000. The neuroprotective effect of inflammation: implications for the therapy of multiple sclerosis. *Journal of neuroimmunology*. 107: 161-166. Ref.: <https://bit.ly/2kEuBri>
13. Moalem G, et al. 2000. Production of neurotrophins by activated T cells: implications for neuroprotective autoimmunity. *Journal of autoimmunity*. 15: 331-345. Ref.: <https://bit.ly/2IHHvFh>
14. Ziemssen T, Kumpfel T, Klinkert WE, et al. 2002. Glatiramer acetate-specific T-helper 1- and 2-type cell lines produce BDNF: implications for multiple sclerosis therapy. *Brain-derived neurotrophic factor. Brain: a journal of neurology*. 125: 2381-2391. Ref.: <https://bit.ly/2INJBDC>
15. Aharoni R, Kayhan B, Eilam R, et al. 2003. Glatiramer acetate-specific T cells in the brain express T helper 2/3 cytokines and brain-derived neurotrophic factor in situ. *Proceedings of the National Academy of Sciences of the United States of America*. 100: 14157-14162. Ref.: <https://bit.ly/2kvBtY7>

16. Hamamcioglu K, Reder AT. 2007. Interferon-beta regulates cytokines and BDNF: greater effect in relapsing than in progressive multiple sclerosis. *Multiple sclerosis*. 13: 459-470. Ref.: <https://bit.ly/2keFIMX>
17. Jones N. 2010. Multiple sclerosis: HLA B*44 is protective in multiple sclerosis. *Nature reviews. Neurology*. 6: 584. Ref.: <https://go.nature.com/2IJzmA0>
18. Stadelmann C, Martin Kerschensteiner, Thomas Misgeld, et al. 2002. BDNF and gp145trkB in multiple sclerosis brain lesions: neuroprotective interactions between immune and neuronal cells? *Brain: a journal of neurology*. 125: 75-85. Ref.: <https://bit.ly/2lPtnJP>
19. Moalem G, Raya Leibowitz–Amit, Eti Yoles, et al. 1999. Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. *Nature medicine*. 5: 49-55. Ref.: <https://go.nature.com/2m3CJSq>
20. Schwartz M, Kipnis J. 2001. Protective autoimmunity: regulation and prospects for vaccination after brain and spinal cord injuries. *Trends in molecular medicine*. 7: 252-258. Ref.: <https://bit.ly/2kbjqpl>
21. Lee DH, et al. 2012. Central nervous system rather than immune cell-derived BDNF mediates axonal protective effects early in autoimmune demyelination. *Acta neuropathologica*. 123: 247-258. Ref.: <https://bit.ly/2k6Vgg2>
22. Linker RA, et al. Functional role of brain-derived neurotrophic factor in neuroprotective autoimmunity: therapeutic implications in a model of multiple sclerosis. *Brain: a journal of neurology* 133: 2248-2263. Ref.: <https://bit.ly/2kqpj5e>
23. Carlino D, De Vanna M, Tongiorgi E. 2013. Is altered BDNF biosynthesis a general feature in patients with cognitive dysfunctions? *The Neuroscientist: a review journal bringing neurobiology, neurology and psychiatry*. 19: 345-353. Ref.: <https://bit.ly/2lPIcMs>
24. Tongiorgi E, Arianna Sartori, Gabriele Baj, et al. 2012. Altered serum content of brain-derived neurotrophic factor isoforms in multiple sclerosis. *Journal of the neurological sciences*. 320: 161-165. Ref.: <https://bit.ly/2kDKT3F>
25. Carlino D, Emiliano Leone, Francesco Di Cola, et al. 2011. Low serum truncated-BDNF isoform correlates with higher cognitive impairment in schizophrenia. *Journal of psychiatric research*. 45: 273-279. Ref.: <https://bit.ly/2maIy0C>
26. Polacchini A, Giuliana Metelli, Ruggiero Francavilla, et al. 2015. A method for reproducible measurements of serum BDNF: comparison of the performance of six commercial assays. *Scientific reports*. 5: 17989. Ref.: <https://go.nature.com/2kGmQku>
27. Mehrpour M, Akhoundi, Fahimeh H, Delgosha, Maryam, et al. 2015. Increased Serum Brain-derived Neurotrophic Factor in Multiple Sclerosis Patients on Interferon-beta and Its Impact on Functional Abilities. *The neurologist*. 20: 57-60. Ref.: <https://bit.ly/2lHJedJ>
28. Lalive PH, Kantengwa S, Benkhoucha M, et al. 2008. Interferon-beta induces brain-derived neurotrophic factor in peripheral blood mononuclear cells of multiple sclerosis patients. *Journal of neuroimmunology*. 197: 147-151. Ref.: <https://bit.ly/2lKLjpf>
29. Sarchielli P, Greco L, Stipa A, et al. 2002. Brain-derived neurotrophic factor in patients with multiple sclerosis. *Journal of neuroimmunology*. 132: 180-188. Ref.: <https://bit.ly/2m946KV>
30. Caggiula M, Batocchi AP, Frisullo G, et al. 2005. Neurotrophic factors and clinical recovery in relapsing-remitting multiple sclerosis. *Scandinavian journal of immunology*. 62: 176-182. Ref.: <https://bit.ly/2lHJCJd>
31. Frota ER, David Henrique Rodrigues, Eduardo Antonio Donadi, et al. 2009. Increased plasma levels of brain derived neurotrophic factor (BDNF) after multiple sclerosis relapse. *Neuroscience letters*. 460: 130-132. Ref.: <https://bit.ly/2k6WnMK>
32. Kochanowski J, Dorota Uchman 1, Anna Litwiniuk, et al. 2015. Assessment of plasma brain-derived neurotrophic factor (BDNF),

- activity-dependent neurotrophin protein (ADNP) and vasoactive intestinal peptide (VIP) concentrations in treatment-naïve humans with multiple sclerosis. *Neuro endocrinology letters*. 36: 148-152. Ref.: <https://bit.ly/2ku9DLQ>
33. Damasceno A, Benito Pereira Damasceno, Fernando Cendes, et al. 2015. Serum BDNF levels are not reliable correlates of neurodegeneration in MS patients. *Multiple sclerosis and related disorders*. 4: 65-66. Ref.: <https://bit.ly/2kfWhTg>
34. Azoulay D, Vachapova V, Shihman B, et al. 2005. Lower brain-derived neurotrophic factor in serum of relapsing remitting MS: reversal by glatiramer acetate. *Journal of neuroimmunology*. 167: 215-218. Ref.: <https://bit.ly/2mbzfgV>
35. Azoulay D, Urshansky N, Karni A. 2008. Low and dysregulated BDNF secretion from immune cells of MS patients is related to reduced neuroprotection. *Journal of neuroimmunology*. 195: 186-193. Ref.: <https://bit.ly/2IHKNS7>
36. Ehling R, Di Pauli F, Lackner P, et al. 2015. Impact of glatiramer acetate on paraclinical markers of neuroprotection in multiple sclerosis: A prospective observational clinical trial. *Journal of neuroimmunology*. 287: 98-105. Ref.: <https://bit.ly/2kDliGZ>
37. Vacaras V, Major ZZ, Muresanu DF, et al. 2014. Effect of glatiramer acetate on peripheral blood brain-derived neurotrophic factor and phosphorylated TrkB levels in relapsing-remitting multiple sclerosis. *CNS Neurological Disorders Drug Targets*. 13: 647-651. Ref.: <https://bit.ly/2maEAoH>
38. Blanco Y, Moral EA, Costa M, et al. 2006. Effect of glatiramer acetate (Copaxone) on the immunophenotypic and cytokine profile and BDNF production in multiple sclerosis: a longitudinal study. *Neurosci Letters*. 406: 270-275. Ref.: <https://bit.ly/2k6XOL8>
39. Ziemssen T, Kümpfel T, Klinkert WE, et al. 2002. Glatiramer acetate-specific T-helper 1- and 2-type cell lines produce BDNF: implications for multiple sclerosis therapy. *Brain-derived neurotrophic factor Brain*. 125: 2381-2391. Ref.: <https://bit.ly/2m5NpzX>
40. Ziemssen T, Kümpfel T, Schneider H, et al. 2005. Secretion of brain-derived neurotrophic factor by glatiramer acetate-reactive T-helper cell lines: Implications for multiple sclerosis therapy. *Journal of Neurological Sciences*. 233: 109-112. Ref.: <https://bit.ly/2kaN6TV>
41. Kalinowska-Łyszczarz A, Pawlak MA, Wyciszkievicz A, et al. 2018. Immune-cell BDNF expression in treatment-naïve relapsing-remitting multiple sclerosis patients and following one year of immunomodulation therapy. *Neurology Neurochirurgia Poland*. 52: 483-489. Ref.: <https://bit.ly/2IIovq8>
42. Kuhlmann T, Goldschmidt T, Antel J, et al. 2009. Gender differences in the histopathology of MS? *Journal of the neurological sciences*. 286: 86-91. Ref.: <https://bit.ly/2KEzMrE>
43. Greer JM, McCombe PA. 2011. Role of gender in multiple sclerosis: clinical effects and potential molecular mechanisms. *Journal of neuroimmunology*. 234: 7-18. Ref.: <https://bit.ly/2kbApbD>
44. Massella A, Giulia D'Intino, Mercedes Fernández, et al. 2012. Gender effect on neurodegeneration and myelin markers in an animal model for multiple sclerosis. *BMC neuroscience*. 13: 12. Ref.: <https://bit.ly/2kGq3Ay>
45. De Santi L, et al. 2009. Higher expression of BDNF receptor gp145trkB is associated with lower apoptosis intensity in T cell lines in multiple sclerosis. *Journal of the neurological sciences*. 277: 65-70. Ref.: <https://bit.ly/2kGdoh3>
46. Kust B, Mantingh-Otter I, Boddeke E, et al. Deficient p75 low-affinity neurotrophin receptor expression does alter the composition of cellular infiltrate in experimental autoimmune encephalomyelitis in C57BL/6 mice. *Journal of neuroimmunology*. 174: 92-100. Ref.: <https://bit.ly/2mcz5WJ>
47. Lommatzsch M, Doerte Zingler, Katharina Schuhbaeck, et al. 2005. The impact of age, weight and gender on BDNF levels in human



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platelets and plasma. Neurobiology of aging. 26: 115-123. Ref.: <https://bit.ly/2m4u2Hx>

48. Polman CH, et al. 2005. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". Annals of neurology. 58: 840-846. Ref.: <https://bit.ly/2maNvqe>
49. Kurtzke JF. 1983. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). Neurology. 33: 1444-1452. Ref.: <https://bit.ly/2ktZbnD>