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https://hdl.handle.net/2324/26026

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Running Title:

BDNF production by PBMCs in MS

Keywords

Brain-derived neurotrophic factor (BDNF), multiple sclerosis (MS), interferon-beta (IFNβ), T cells, age

Character count for the title: 110 characters (including spaces)

Word count for the abstract: 194 words

Word count for the text: 4,553 words

Number of references: 46

Number of figures: 8 (5 figures and 3 supplementary figures)

Abstract

Background   Within multiple sclerosis (MS) lesions, brain-derived neurotrophic factor (BDNF) is detected in neurons and immunocytes.

Objective   To clarify BDNF production by peripheral blood immunocytes and its relationship to clinical parameters in MS.

Methods   Serum BDNF levels were measured by conventional ELISA while BDNF production by immunocytes was determined by an ELISA in situ assay in 74 MS patients, 32 healthy controls (HCs), and 86 patients with other neurological diseases (OND). The TrkB expression level in peripheral blood mononuclear cells (PBMCs) was measured by real-time PCR.

Results   MS patients showed significantly higher serum BDNF levels than HCs and OND patients. MS patients with high BDNF levels were younger, and showed fewer relapse numbers than those with low BDNF levels. BDNF production by T cells increased with age in HCs, but not in MS patients. IFNβ induced a significant increase in serum BDNF levels. BDNF production from T cells and TrkB expression levels in PBMCs were significantly enhanced in IFNβ-treated MS patients compared with untreated ones.

Conclusions   A high BDNF level is related to early mild disease in young MS patients. IFNβ potentiates BDNF production and BDNF receptor expression in PBMCs, which may act beneficially.

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). It is characterized by episodes of acute neurological dysfunction during the relapsing–remitting (RR) phase, leading to partial or full recovery [1]. However, with time, recovery from each episode becomes incomplete and persistent symptoms accumulate [1]. Thus, it is critical to understand the neuroprotection and repair mechanisms operating in this condition.

Among neuroprotective factors, brain-derived neurotrophic factor (BDNF), a member of the neurotrophin (NT) family which includes nerve growth factor (NGF), NT-3, and NT-4/5 [2], has recently received a lot of attention in MS research. BDNF has been shown to promote neuronal survival after experimental axotomy [3] and to enhance oligodendrocyte proliferation and myelination [4]. Not only neurons but also immune cells, such as T cells, B cells, and monocytes, produce BDNF in vitro and in inflamed CNS lesions in MS patients [5]. Furthermore, tyrosine kinase receptor B (trkB), a receptor for BDNF, was detected in reactive astrocytes within MS plaques and neurons in the immediate vicinity of such lesions [6]. Thus, BDNF and trkB are supposed to be key players in neuroprotective immunity [7].

There is accumulating evidence for a functional role of BDNF in the periphery, because BDNF is detected in both human serum and plasma [8]. More than 90% of blood BDNF is stored in platelets and can be released in serum on activation or clotting, which explains the 50 to 200-fold higher levels of BDNF in serum than in plasma [8, 9]. Platelets and megakaryocytes have low levels of mRNA for BDNF [8, 10]; however,

platelets bind and internalize BDNF from other sources via the blood circulation, through high affinity receptors other than trkB [10]. Alternative sources of blood BDNF identified to date include lymphocytes, monocytes [5], eosinophils [11], vascular endothelial cells [12], and vascular smooth muscle [13]. Human platelets are known to circulate for about 10 days in peripheral blood [14], while BDNF protein circulates in plasma for less than an hour [15]. Thus, serum or platelets are more stable components of blood to measure BDNF levels than plasma. Since BDNF is known to cross the blood-brain barrier in both directions, a substantial portion of circulating BDNF might originate from neurons and glia cells of the CNS [16]. Karege et al [17] observed a positive correlation between serum and cortical levels of BDNF, indicating that cortical BDNF is a possible candidate source for circulating BDNF, and that peripheral measurement of BDNF could be used as a surrogate measure for BDNF levels in the central nervous system. Collectively, the presence of high levels of BDNF in platelets and serum suggests a role of circulating BDNF in neural repair at injured sites.

While no study reports platelet BDNF release in MS, there have been several studies describing circulating BDNF levels: serum BDNF levels are similar in RRMS patients in remission and controls [18]; they are lower in RRMS patients, either at relapse or in remission, than in healthy controls [19]; and they are higher in patients in relapse than in those in remission [20]. Concerning BDNF production by immune cells in MS, some studies have revealed that BDNF production by peripheral blood mononuclear cells (PBMCs) is higher during relapse and the recovery phase than during the stable phase in RRMS patients [18, 20-22], and that it is significantly associated

with contrast-enhanced lesion volumes [23]. BDNF production by PBMCs was also reported to be higher in RRMS patients than in secondary progressive (SP) or primary progressive MS patients [20, 24]. On the contrary, other studies have reported that BDNF production by PBMCs is lower in RRMS patients in remission than in controls [25].

Regarding the effects of immunomodulatory therapies, PBMCs from glatiramer acetate (GA)-treated RRMS patients produced higher amounts of BDNF as compared with PBMCs from untreated RRMS patients and controls [26]. GA also significantly increased the serum levels of BDNF in MS patients [19], especially in responders [27]. However, there is conflicting data on the effects of IFNβ treatment on BDNF production; enhanced production of BDNF by PBMCs from IFNβ-treated patients [18] and by *in vitro* IFNβ-treated PBMCs [24] were reported in some studies, while no effect of IFNβ on BDNF expression was observed in others [21, 26]. Thus, there is no consensus regarding serum BDNF levels and BDNF production by peripheral blood immune cells in MS patients, or their modification by IFNβ, and there have been no studies regarding BDNF levels and production in Asian MS patients in general. Therefore, in the present study, we aimed to measure serum BDNF levels, as well as BDNF production and TrkB expression by PBMCs, in Japanese MS patients with and without IFN-β treatment, and ascertain their relationships with clinical parameters. Our study is the first to measure BDNF production by separated T cells and monocytes from MS patients using an ELISA *in situ* assay, and the first to assess trkB expression in PBMCs from untreated MS patients.

Methods

Subjects

In the present study, patients with MS, who were diagnosed as clinically definite MS according to the criteria of McDonald [28], at the MS clinic in the Department of Neurology, Kyushu University Hospital between 1999 and 2008, were enrolled after informed consent was obtained. For conventional ELISA experiments to determine serum BDNF levels, 74 MS patients (19 males and 55 females), 32 age-matched healthy controls (HCs) (15 males and 17 females), 29 patients with amyotrophic lateral sclerosis (ALS) (13 males and 16 females), 28 with spinocerebellar degeneration (SCD) (14 males and 14 females), and 29 with human T cell leukemia virus type 1-associated myelopathy (HAM) (10 males and 19 females) were enrolled; the ages at examination (mean±SD) in years were 39.9±11.9, 34.4±10.2, 60.1±12.4, 59.9±14.0, and 53.0±13.2, respectively. Among the MS patients, 66 patients had relapsing-remitting MS (RRMS) and eight had secondary progressive MS (SPMS); no patients with primary progressive MS were recruited. In MS patients, disease duration was 126.6±115.4 months, Kurtzke's Expanded Disability Status Scale (EDSS) [29] score was 3.38±2.04, the number of relapses was 5.39±4.88, and annualized relapse rate was 0.94±1.02. MS patients were clinically classified into two subtypes, OSMS and CMS, as described previously [30]. There were 31 patients with OSMS (42%) and 43 patients with CMS (58%). The ages at examination were not significantly different between the two subtypes of patients (mean±SD in years; 42.6±14.3 in OSMS and 37.8±9.3 in CMS). Anti-aquaporin-4 (AQP4) antibody was tested in all MS patients as previously

described [31, 32], and only 12 were positive for the antibody (10 OSMS patients and two CMS patients); 62 were negative. Nine patients with anti-AQP4 antibody and three patients without anti-AQP4 antibody also fulfilled the criteria for neuromyelitis optica (NMO) [33]. Thirty-one samples were obtained from 28 MS patients in the relapse phase, and 85 samples were obtained from 57 MS patients in the remission phase. All of the recruited patients were untreated for at least 6 months before study entry. The disability status of the patients was scored by one of the authors (J.K.) throughout the study, according to the EDSS [29]. Relapse was defined by the appearance of new neurological symptoms lasting at least 48 h in a patient who had been neurologically stable or improving for the previous 30 days, accompanied by objective changes on neurological examination. Thus, in the present study, the relapse phase was regarded as within 1 month after the onset of acute exacerbation, while the remission phase was regarded as either the stable stage or more than 1 month after exacerbation. In addition, in 12 patients (nine with RRMS and three with SPMS; eleven with CMS, one with OSMS and none meeting the diagnostic criteria for NMO [33]), serum BDNF levels were measured sequentially before and after the introduction of IFNβ (IFNβ-1b was used in all and blood drawing was done 926±624 days after initiation of the drug for the serum BDNF assay). We classified MS patients under treatment as clinical responders and sub-optimal responders to IFNβ therapy, based on EDSS progression and the numbers of relapses; the occurrence of more than one relapse or an increase in EDSS score of at least 1 point during the one year of IFNβ treatment, according to Pozzilli et al [34].

For the assay of BDNF production from T cells and monocytes using an ELISA *in situ* assay, 17 untreated MS patients (five males and 12 females), eight IFNβ-treated MS patients (three males and five females, seven on IFNβ-1b and one on IFNβ-1a), and 18 HCs (eight males and 10 females) were enrolled; their ages at examination (mean±SD) were 50.8±15.5 years, 42.0±8.3 years, and 44.9±17.0 years, respectively. In untreated and IFNβ-treated MS patients, disease durations were 200.8±122.9 months and 91.9±68.9 months, respectively, EDSS scores were 3.26±2.69 and 2.5±2.2, respectively, numbers of relapses were 4.76±3.73 and 3.13±2.23, respectively, and annualized relapse rates were 0.31±0.19 and 0.54±0.38, respectively. Among the untreated MS patients, 14 had RRMS and three had SPMS; six of these were diagnosed with OSMS and 11 were diagnosed with CMS. Among the IFNβ-treated MS patients, six had RRMS and two had SPMS; eight of these were diagnosed with CMS, while none fulfilled the criteria for OSMS. Anti-AQP4 antibody was present in one of the untreated patients and none of the IFNβ-treated patients; however, no patient fulfilled the criteria for NMO [33]. The IFNβ-treated patients had received the drug for at least 6 months before the commencement of the study and none underwent any additional immunomodulatory therapy while they were on IFNβ.

For examination of TrkB expression in PBMCs, 15 untreated MS patients (one male and 14 females), 11 IFNβ-treated MS patients (three males and eight females, ten on IFNβ-1b and one on IFNβ-1a), and 21 HCs (ten males and 11 females) were enrolled; their ages at examination were 52.5±15.8 years, 37.2±12.6 years, and 50.4±15.2 years, respectively. In untreated and IFNβ-treated MS patients, disease

durations were 220.7±138 months and 106±68.7 months, respectively, EDSS scores

were 4.03±2.45 and 2.64±2.68, respectively, numbers of relapses were 6.87±6.5 and

7.82±8.22, respectively, and annualized relapse rates were 0.38±0.27 and 0.79±0.52,

respectively. Among the untreated MS patients, 13 had RRMS and two had SPMS; four

of these were diagnosed with OSMS and 11 were diagnosed with CMS. Among the

IFNβ-treated MS patients, ten had RRMS and one had SPMS; two of these had OSMS

and nine had CMS. No patients used for this assay had anti-AQP4 antibody or met the

diagnostic criteria for NMO [33].

Conventional ELISA for serum BDNF levels

Serum samples of MS patients and controls were stored at -80°C until use. Serum

BDNF levels were determined using sandwich ELISA kits for BDNF (Promega,

Madison, WI, USA) according to the procedure supplied by the manufacturer. In this

procedure, flat-bottomed 96-well plates were coated with anti-human BDNF

monoclonal antibody (mAb) to bind soluble BDNF, and the plates were incubated

overnight at 4°C. After washing with wash buffer (Tris-HCl, pH 7.6), and blocking for

nonspecific binding with Block & Sample Buffer, the plates were incubated at room

temperature for 1 h without shaking and washed once. Samples were diluted 80 fold

with a calibrator prior to the assay. One-hundred microlitres of samples and BDNF

standards, in duplicate, were added to the appropriate wells and the plates incubated for

2 h at room temperature. A second BDNF-specific polyclonal antibody was added and

the plates were incubated for 2 h at room temperature so that the captured BDNF could

bind the polyclonal antibody. After washing, the amount of specifically bound

polyclonal antibody was then detected using species-specific anti-IgY antibody

conjugated to horseradish peroxidase as a tertiary reactant. Unbound conjugate was

removed by washing, which was followed by incubation with a chromogenic substrate

and stopping the reaction with 1 N hydrochloric acid. The absorbencies were measured

at 450 nm using an automatic ELISA microplate reader (IMMUNO-MINI NJ-2300,

Tokyo, Japan). The sensitivity of the assay (expressed as the minimum amount of

BDNF that could be detected) was 15.6 pg/ml, and the intra- and inter-assay coefficients

of variation were 6.0% and 8.5%, respectively.

ELISA in situ for BDNF produced by peripheral blood immunocytes

PBMCs were isolated from venous blood containing 0.2% ethylenediaminetetraacetic

acid (EDTA), diluted in a 1:1 ratio with saline, and subjected to density gradient

centrifugation for 20 min at 2000 × g at 20°C, using lymphoprep tubes (Nycomed.

Pharma, Oslo, Norway). Dead cells were removed using the MACS Dead Cell Removal

Kit (Miltenyi Biotech, Bergisch Gladbach, Germany). Viable PBMCs were determined

by trypan blue exclusion. T cells and monocytes were purified from PBMCs by negative

immunoselection (Miltenyi Biotech). The purity of the isolated T cells and monocytes

was >90% as determined by flow cytometry using anti-CD3 IgG and anti-CD14 IgG

and FITC-conjugated antibodies. Isolated T cells and monocytes were washed twice and

resuspended in X-VIVO 15 (Takara, Tokyo, Japan) at a concentration of 5×10^6 cells

per milliliter. We prepared UV-sterilized 96-well ELISA plates precoated with

anti-BDNF mAb to measure the levels of released BDNF. Then, 200 μl of purified

peripheral blood T cells and monocytes (1×10^5 cells) was added to the 96-well ELISA

plates, which were incubated at 37°C in a 5% CO_2-humidified atmosphere for 48 h.

Forty-six hours after the start of the assay, BDNF samples used to generate the standard

curve were incubated in the same plate as the cells. At the end of the cell culture period,

plates were extensively washed to remove all cells and cell debris, and the anti-BDNF

polyclonal antibody was applied, followed by subsequent steps according to Promega's

ELISA protocol. All experiments were performed in duplicate.

Real-time PCR for TrkB expression

TrkB mRNA expression in PBMCs from MS patients and HCs was examined by

real-time PCR. First, approximately 5×10^6 to 1×10^7 PBMCs were isolated from

venous blood containing 0.2% EDTA by density gradient centrifugation for 10 min at

$2000 \times$ g at 20°C on Ficoll-Paque. mRNA was extracted from total cellular RNA using

a commercially available mRNA-isolation kit (RNeasy Mini Kit, Qiagen, Hilden,

Germany). Reverse transcription was performed using 750 ng of mRNA from each

sample in a 20-μl reaction for 10 min at 65°C followed by 30 min at 55°C according to

the manufacturer's instructions (Transcriptor First Strand cDNA Synthesis Kit ; Roche

Diagnostics, Mannheim, Germany). As a control, 750 ng of mRNA from each sample

was treated according to the same protocol with the addition of distilled water instead of

the Reverse Transcriptase. Real-time PCR was performed on the Light Cycler

Instrument (BIORAD Miniopticon™, Bio-Rad Laboratories, Hercules, CA, USA)

using the DNA-binding dye SYBR Green I (Takara SYBR Premix Ex Taq™ II, Takara,

Kyoto, Japan). Specific primers for PCR were designed against GAPDH (sense:

5'-GAGTCAACGGATTTGGTCGT-3'; antisense:

5'-TTGATTTTGGAGGGATCTCG-3'; expected product length: 238 bp), and TrkB

(sense: 5'-CGAGATTGGAGCCTAACAGT-3'; antisense:

5'-CACCAGGATCAGTTCAGACA-3': expected product length: 272 bp). After an

initial denaturation step at 95°C for 5 sec, the PCR reaction was performed with an

annealing temperature of 55°C for 10 sec, followed by an extension phase at 72°C for

15 sec. At the end of each extension phase, fluorescence was observed at 72°C. The

PCR reaction was completed after 45 cycles. Melting point analysis was performed by

heating the amplicon from 50 to 95°C and revealed the characteristic melting point for

each product. After cooling to 40°C, the product was extracted from the capillary. Ten

microlitres of each reaction was separated on a 1.5% agarose gel and visualized by

staining with ethidium bromide.

Statistical Analysis

Statistical analyses of numerical variables were initially performed using the

Kruskal-Wallis H test. When a significant difference was found, the Mann-Whitney U

test was used to determine the significance of differences between subgroups. K-means

cluster analyses were used to identify subgroups among the MS patients according to

serum BDNF levels. For comparisons among the subgroups, we performed logistic

regression analyses. All analyses were performed using JMP 6.0.3 (SAS Institute, Cary,

NC, USA). Changes in serum BDNF levels were compared using the Wilcoxon signed

rank test.

Results

Serum BDNF levels determined by conventional ELISA

MS patients showed significantly higher serum BDNF levels than HCs, and patients with ALS, SCD, and HAM ($P<0.0001$ in all, Figure 1A). Serum BDNF levels tended to be higher in the relapse phase than in the remission phase ($P=0.094$, Figure 1B) while they were not significantly different between males and females (supplementary Figure 1A). Serum BDNF levels in OSMS patients were significantly higher than those in CMS patients ($P=0.015$, Figure 1C), while they were not significantly different between anti-AQP4 antibody-positive and -negative patients, or between those who fulfilled the NMO criteria and those who did not (supplementary Figure 1B,C). Exclusion of anti-AQP4 antibody-positive patients and those who met the NMO criteria gave essentially the same results. Serum BDNF levels were not significantly different between RRMS (mean $\pm$ SD = 32 $\pm$ 14.9) and SPMS patients (mean $\pm$ SD = 24.9 $\pm$ 4.9), in part due to the small sample size of SPMS patients, while none of the SPMS patients had BDNF levels higher than 1 SD above the mean level in RRMS patients (supplementary Figure 1D).

Correlation of serum BDNF levels with clinical parameters

Serum BDNF level showed a significant positive correlation with age at examination in HCs ($P=0.042$, $r=0.36$), while in MS patients it tended to show a weak negative correlation ($P=0.059$, $r=-0.18$) (Figure 2A, B). Serum BDNF levels were significantly negatively correlated with age at examination in patients with OSMS ($P=0.02$, r=-0.32),

but not in those with CMS (Figure 2C, D). Serum BDNF level was negatively

correlated with number of relapses (P=0.023, r=-0.21) but not with disease duration,

EDSS scores, or annualized relapse rate (supplementary Figure 2A-C). There was no

significant correlation between serum BDNF levels and EDSS after correcting for age

and disease duration by multiple logistic regression analyses (data not shown). In

addition, there was no significant correlation of serum BDNF levels at relapse (31

samples) with EDSS scores either at the peak of relapse or at three months after the

relapse (supplementary Figure 2C, D).

K-means cluster analyses revealed three subgroups on the basis of serum

BDNF levels: high (58.1 to 85.2 ng/ml, n=10), medium (31.4 to 50.8 ng/ml, n=30), and

low (11.2 to 31.0 ng/ml, n=76). Logistic regression analyses of the subgroups identified

by K-means clustering disclosed that as compared with the BDNF-low and medium

groups, the BDNF-high group showed significantly younger age at examination

(P=0.0108, and P=0.0237, respectively) and fewer relapses (P=0.0123, and P=0.0184,

respectively) (supplementary Figure 3A,B). Disease duration had a tendency to be

shorter in the BDNF-high group than in the BDNF-low and medium groups (P=0.0644,

and P=0.0994, respectively), while neither annualized relapse rate nor EDSS scores

differed significantly among the three groups (supplementary Figure 3C-E).

Introduction of IFNβ induced a significant increase in serum BDNF levels in MS

patients (P=0.0079) (Figure 2F). Among the 12 IFNβ-treated patients, 10 were regarded

as responders (nine were CMS patients and one was an OSMS patient who neither had

anti-AQP4 antibody nor met the diagnostic criteria for NMO [34]) and two were

regarded as sub-optimal responders (both with CMS). The two sub-optimal responders

(both with CMS) had the lowest and the second lowest serum BDNF levels among the

IFNβ-treated MS patients after IFNβ administration. The only patient who showed a

decrease in serum BDNF levels after therapy was one of the two sub-optimal responders,

who had the lowest serum BDNF level after IFNβ administration.

BDNF production from peripheral blood immunocytes by ELISA in situ

Levels of BDNF production by T cells and monocytes were not significantly different

between untreated MS patients and HCs (Figure 3A, B). In HCs, BDNF production by T

cells demonstrated a significant positive correlation with age at examination (P=0.017,

r=0.554), whereas in MS patients, such a correlation was not seen (Figure 3C, D). By

contrast, BDNF production by monocytes had no correlation with age in either MS

patients or HCs (Figure 3E, F). BDNF production by either T cells or monocytes was

not correlated with disease duration, number of relapses, annualized relapse rate, or

EDSS scores (data not shown). Levels of BDNF production by T cells and monocytes

were not significantly different between RRMS and SPMS patients (data not shown).

BDNF production by T cells was significantly increased in IFNβ-treated MS patients

compared with untreated ones (P=0.0249) (Figure 4A), while BDNF production by

monocytes did not differ significantly with IFNβ treatment (Figure 4B). Among

IFNβ-treated MS patients, six responders and two sub-optimal responders showed

similar levels of BDNF production by either T cells or monocytes (data not shown).

Real- time PCR analysis of TrkB expression levels

TrkB expression levels in PBMCs were significantly higher in IFNβ-treated MS patients than in untreated ones (P=0.03), but were not significantly different either between untreated MS patients and HCs or between IFNβ-treated MS patients and HCs (Figure 5). TrkB expression levels were not correlated with age, disease duration, number of relapses, annualized relapse rate, or EDSS scores (data not shown). TrkB expression levels were not significantly different either between RRMS and SPMS patients or between the eight responders and the three sub-optimal responders (data not shown).

Discussion

In the present study, we found that, compared with controls, BDNF levels in the sera of RRMS patients were significantly greater, especially in younger patients with fewer relapses and mild disease. Interestingly, a significant positive correlation of BDNF levels with age was found in HCs, whereas a trend toward a negative correlation was observed in MS patients. Such a reverse trend was apparent in OSMS patients while it was not in CMS patients. However, even the lower levels in older MS patients were compatible with those in HCs at the same ages. Thus, it is suggested that serum BDNF levels are increased in younger MS patients with an early disease course, but decreased in older patients in the late stage of the disease to the levels seen in HCs of similar ages. Thus, the reverse trend of BDNF levels in relation to age appears to be explained by the existence of young MS patients with high BDNF levels, but without anti-AQP4 antibody.

Our results are in line with previous studies showing that the PBMCs of RRMS patients produce more BDNF than those of SPMS patients [20, 24]. Furthermore, BDNF levels in our series showed a tendency to be higher at relapse than in remission, which is in accordance with previous results demonstrating that BDNF production by PBMCs is higher in the active phase than in the stable phase in RRMS patients [18, 20-23]. Therefore, at least in young patients, acute inflammation appears to induce enhanced BDNF production in peripheral blood during early relapses in MS patients, which is consistent with the concept of neuroprotective immunity [7]. Because BDNF production appears to decline during later relapses, such neuroprotective immunity may

contribute to good recovery and tissue repair only in the early course of the disease. However, our results are somewhat discrepant from those studies reporting similar or lower serum BDNF levels in MS patients compared with controls [18, 19]. Differences in the proportion of younger patients or ethnic backgrounds may account for these discrepancies. In the present study, considerable numbers of our MS patients presented with an OSMS phenotype, and most of these were seronegative for anti-AQP4 antibody (68% were seronegative), as reported previously [31, 32]. Moreover, OSMS patients had significantly greater serum BDNF levels than CMS patients. It is the OSMS patients who show a significant negative correlation of BDNF level with age. Thus, young OSMS patients without anti-AQP4 antibody in the early course of disease appear to have high BDNF levels, which may relate to the rare occurrence of a progressive course reflecting neuroaxonal degeneration in this subgroup [35, 36].

We could not find any increase in BDNF production by peripheral blood T cells or monocytes by an ELISA *in situ* assay, whereas previous studies have shown increased BDNF production by PBMCs [18, 20-22]. Differences in assay methods, stimuli employed, and the number of patients studied may in part explain these discrepancies. Sarchielli et al [26] reported a decrease in BDNF production by unstimulated PBMCs in SPMS patients compared with HCs. Petereit et al [21] described an increase in BDNF production by PMA- and ionomycin-stimulated PBMCs in RRMS patients in remission compared with HCs. Caggiula et al [22] disclosed that BDNF production by unstimulated PBMCs in RRMS patients was higher in relapse than in remission. Lalive et al [23] revealed that BDNF levels in PBMC lysates were

higher in lysates of PBMCs from IFNβ-treated RRMS patients than in those from untreated ones. All of the above-mentioned studies were done with unseparated PBMCs using an ELISA assay of the culture supernatants or cell lysates, whereas we separated PBMCs into T cells and monocyes and employed an ELISA *in situ* assay. The only other study using separated T cells and monocytes, performed by Azoulay et al [26], examined only two MS patients, and found similar BDNF levels to those in controls, which is in accord with our results.

In our hands, BDNF production by only T cells significantly increased with age in HCs, which may relate to an age-associated increase in serum BDNF levels in HCs. However, such a trend was completely absent in MS patients. Therefore, BDNF production by T cells in aged MS patients may be dampened, and this may partly account for the age-associated decrease in serum BDNF levels in MS patients. It is also possible that BDNF production may be up-regulated in other cell populations, such as B cells, platelets and eosinophils, which are also known to produce it [11, 37, 38]. Further studies on these populations are required to clarify the source of elevated BDNF levels in sera from young MS patients. Alternatively, secreted BDNF may not be utilized efficiently in the periphery. However, because TrkB expression levels in PBMCs from untreated MS patients did not differ significantly from those in PBMCs obtained from HCs, a comparison that had not previously been made, this possibility seems less likely.

Several reports have indicated that serum BDNF levels are lower in drug-free subjects suffering from major depression than in healthy controls [39-42]. In our series, no MS patient had major depression at the time of examination. However, follow-up

studies may be necessary to clarify whether low BDNF patients experience future development of depression.

Finally, for the first time, we have shown that IFNβ treatment in MS patients potentiates BDNF production in T cells but not in monocytes, which may account for the increase in serum BDNF levels after introduction of IFNβ observed in the present study. These findings are in line with the results of previous studies showing that BDNF production by PBMCs from IFNβ-treated patients is enhanced [18], and that GA, another disease-modifying drug for MS, also potentiates BDNF production in T cells [43]. We also found that TrkB expression levels in PBMCs were significantly elevated in IFNβ-treated MS patients compared with untreated ones. Among PBMCs, TrkB expression has been reported in human T [44, 45] and B cells [46]. Although there is a need for further study to exactly identify the cell populations showing an enhancement of TrkB expression by IFNβ, IFNβ-induced enhanced BDNF production in T cells and increased levels of serum BDNF thus appear to at least partly contribute to its beneficial effects in MS. Therefore, enhancement of neuroprotective immunity, as seen in early MS patients, may be an important target for future disease-modifying therapy in MS.

Acknowledgments

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Figure legends

Figure 1   Serum BDNF levels determined by conventional ELISA. MS patients show significantly higher serum BDNF levels compared with HCs, and patients with ALS, SCD, and HAM (**A**). Serum BDNF levels tends to be higher in the relapse phase than in the remission phase (**B**). Serum BDNF levels are significantly higher in OSMS patients than in CMS patients, although both OSMS and CMS patients show significantly increased levels of BDNF compared with HCs (**C**). ALS, amyotrophic lateral sclerosis; BDNF, brain-derived neurotrophic factor; HAM, human T cell leukemia virus type 1-associated myelopathy; HCs, healthy controls; MS, multiple sclerosis; *NS*, not significant; SCD, spinocerebellar degeneration.

Figure 2   Relationship between serum BDNF levels and clinical parameters. Serum BDNF levels show a significant positive correlation with age at examination in HCs (**A**), whereas it tends to decrease with age in MS patients (**B**). Serum BDNF levels are significantly negatively correlated with age at examination in OSMS patients (**C**), but not in CMS patients (**D**). Serum BDNF level is also negatively correlated with the number of relapses (**E**). Serum BDNF levels are significantly higher after IFNβ treatment than before treatment (**F**). Closed circles represent responders, while open circles indicate sub-optimal responders to IFNβ. BDNF, brain-derived neurotrophic factor; EDSS, Expanded Disability Status Scale; HCs, healthy controls; MS, multiple sclerosis; *NS*, not significant.

Figure 3   ELISA *in situ* analysis of BDNF production by immunocytes. BDNF production by T cells (**A**) and monocytes (**B**) is not significantly different between untreated MS patients and HCs. In HCs, BDNF production by T cells shows a significant positive correlation with age at examination (**C**). In MS patients, such a correlation between BDNF production and age is not seen (**D**). BDNF production by monocytes has no correlation with age in either HCs (**E**) or MS patients (**F**). BDNF, brain-derived neurotrophic factor; HCs, healthy controls; MS, multiple sclerosis; *NS*, not significant.

Figure 4   Comparison of BDNF production by immunocytes between IFNβ-treated MS patients and untreated ones. BDNF production by T cells shows a significant increase in IFNβ-treated MS patients compared with untreated ones (**A**). On the other hand, BDNF production by monocytes is not significantly different between IFNβ-treated and untreated patients (**B**). BDNF, brain-derived neurotrophic factor; IFN, interferon; *NS*, not significant.

Figure 5   Real-time PCR analysis of TrkB expression levels. TrkB expression levels in PBMCs are significantly higher in IFNβ-treated MS patients than untreated ones, but are not significantly different either between untreated MS patients and HCs or between IFNβ-treated MS patients and HCs. BDNF, brain-derived neurotrophic factor; HCs, healthy controls; IFN, interferon; HCs, healthy controls; *NS*, not significant.

Supplementary Figure Legends

Supplementary Figure 1   Serum BDNF levels determined by conventional ELISA.

Serum BDNF levels are not significantly different between males and females (**A**). Both

AQP4 antibody-positive and -negative patients show significantly higher serum BDNF

levels than HCs, while there is no significant difference in BDNF levels between the

two groups of patients (**B**). Similarly, BDNF levels are not significantly different

between patients who fulfill the NMO criteria and those who do not, while both have

significantly higher levels of serum BDNF compared with HCs (**C**). Serum BDNF

levels do not differ significantly between RRMS and SPMS patients (**D**). AQP4 (+),

anti-AQP4 antibody-positive patients; AQP4 (-), anti-AQP4 antibody-negative patients;

BDNF, brain-derived neurotrophic factor; HCs, healthy controls; MS, multiple

sclerosis; NMO, neuromyelitis optica; *NS*, not significant (*P*>0.1).

Supplementary Figure 2   Serum BDNF level is not correlated with disease duration

(**A**), EDSS scores (**B**), or annualized relapse rate (**C**). There is no significant correlation

of serum BDNF levels at relapse (31 samples) with EDSS scores either at the peak of

relapse (**D**) or at three months after relapse (**E**). BDNF, brain-derived neurotrophic

factor; EDSS, Expanded Disability Status Scale; *NS*, not significant.

Supplementary Figure 3   Logistic regression analyses of the subgroups identified by

K-means clustering. The BDNF-high group shows significantly younger age at

examination (**A**) and fewer relapses (**B**) than the BDNF-low and medium groups. There

is no significant difference in EDSS scores (**C**), disease duration (**D**), or annualized

relapse rate (**E**) among the three subgroups. BDNF, brain-derived neurotrophic factor;

EDSS, Expanded Disability Status Scale; *NS*, not significant.

Figure 1

Figure 1

(C)

Figure 2

(A)

HCs

$P=0.042$
$r=0.36$

BDNF (ng/ml)

Age at examination (years)

(B)

MS

$P=0.0595$
$r=-0.18$

BDNF (ng/ml)

Age at examination (years)

(C)

OSMS

$P=0.02$
$r=-0.32$

BDNF (ng/ml)

Age at examination (years)

(D)

CMS

NS

BDNF (ng/ml)

Age at examination (years)
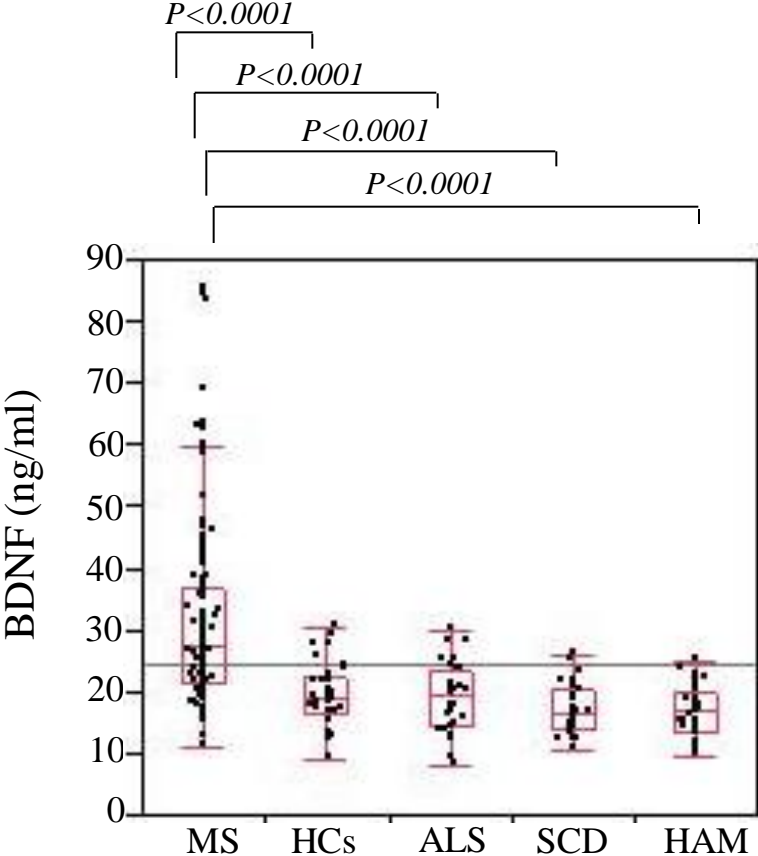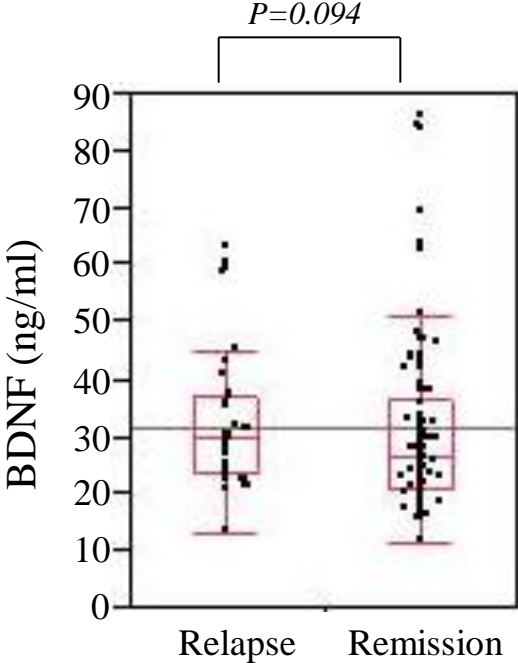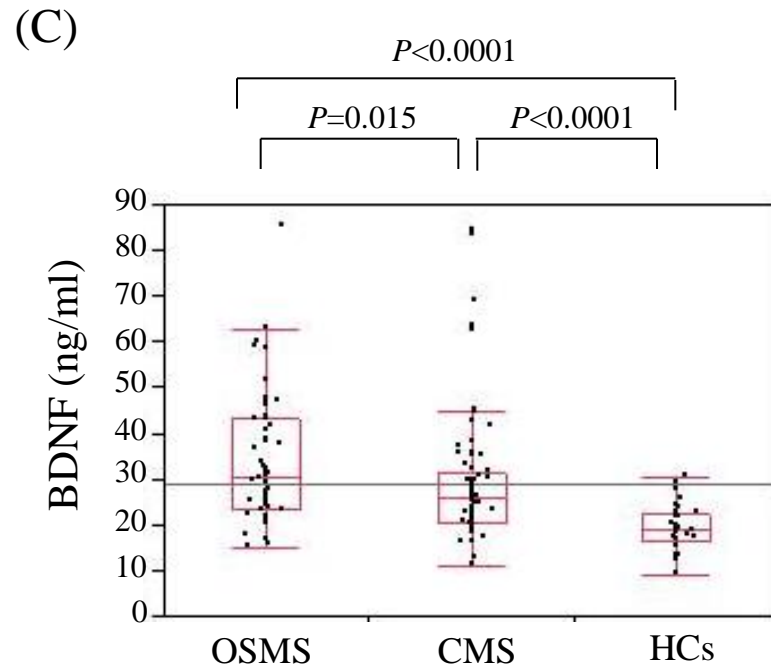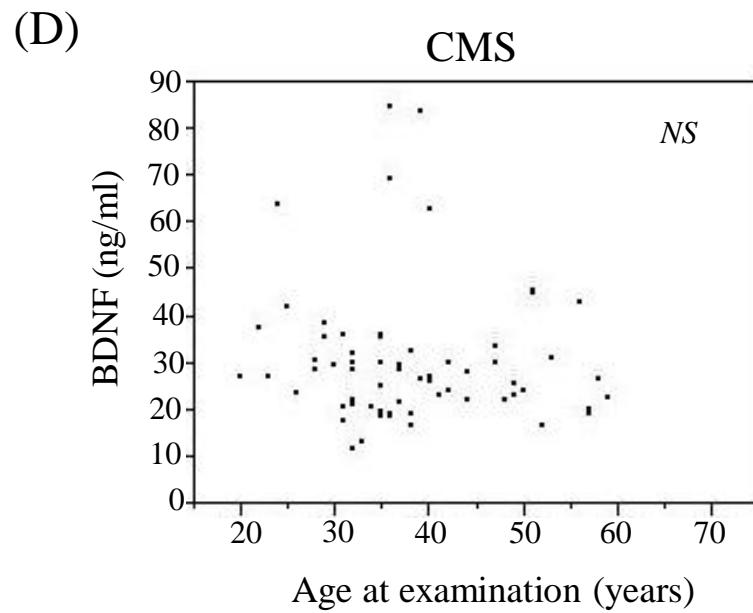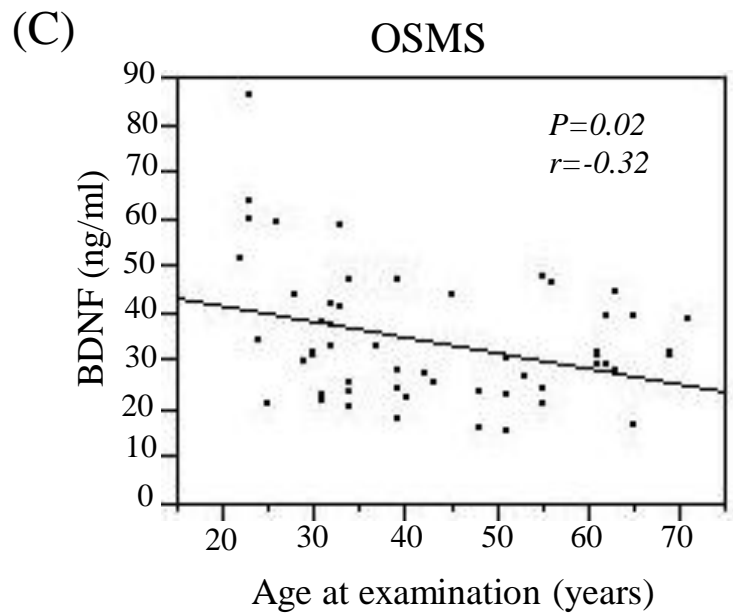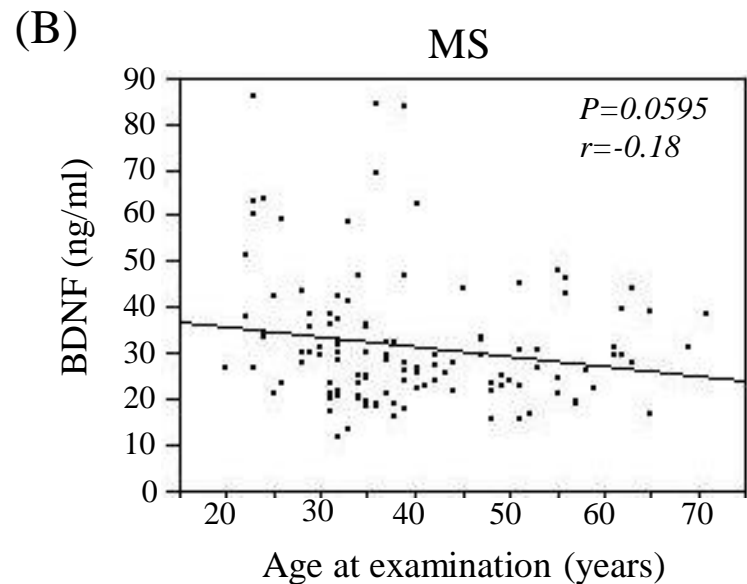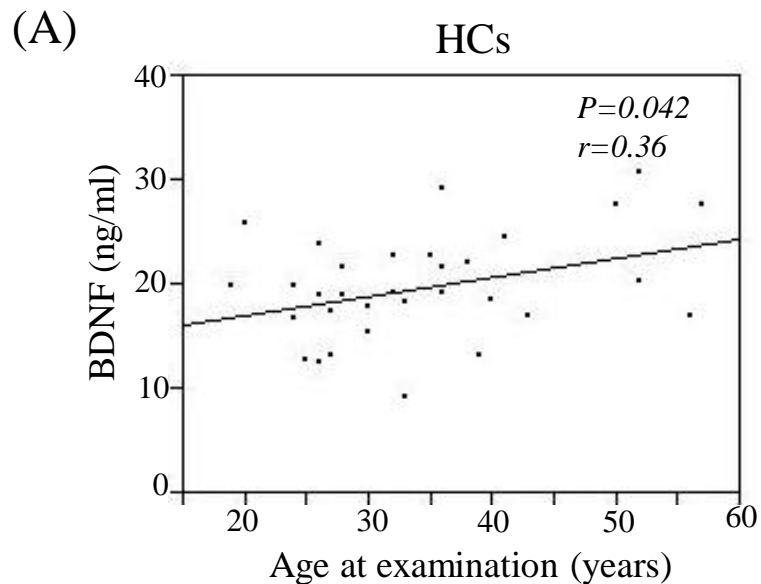
Figure 2

(E)

(F)

Figure 3

Figure 3

(E)

HCs

BDNF production by monocytes (pg/ml)

NS

Age at examination (years)

(F)

MS

BDNF production by monocytes (pg/ml)

NS

Age at examination (years)
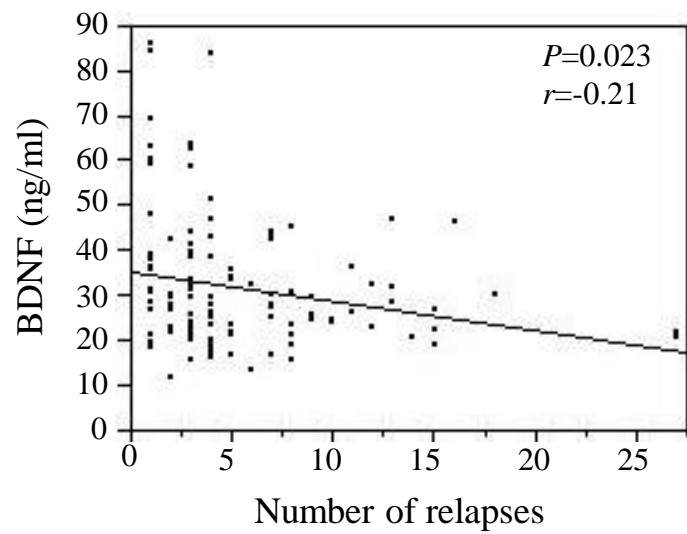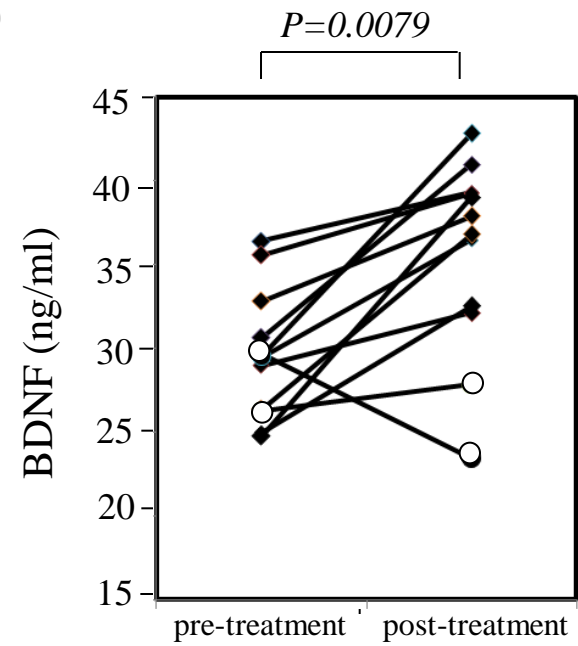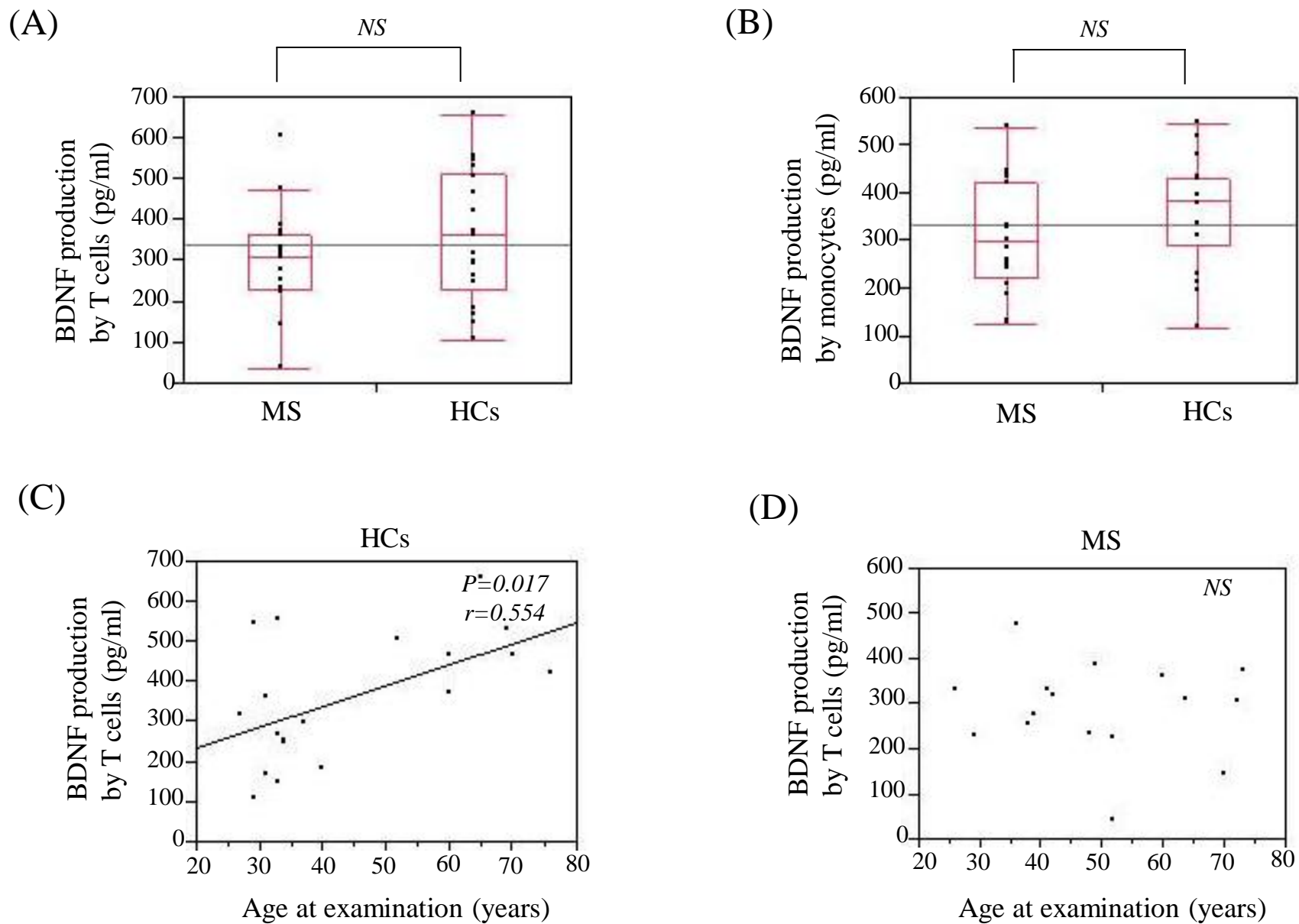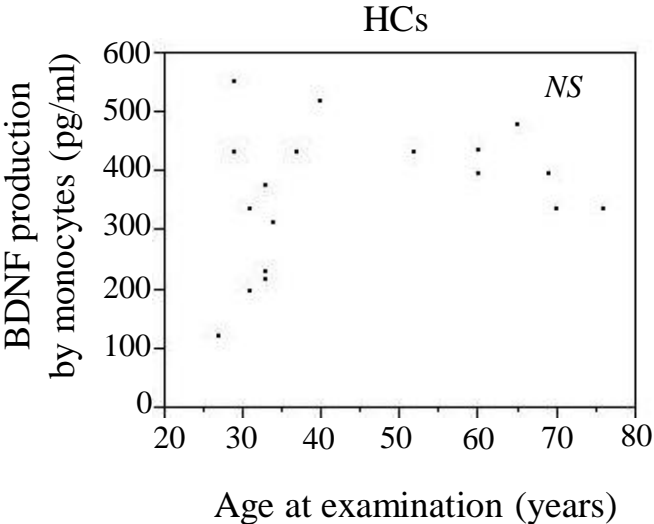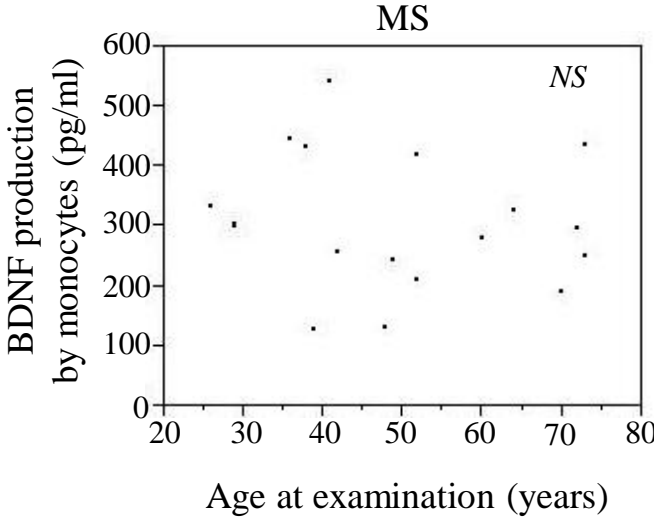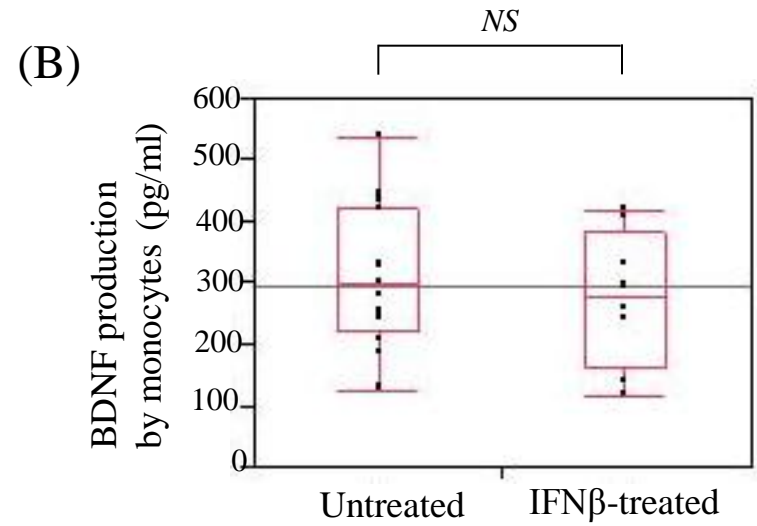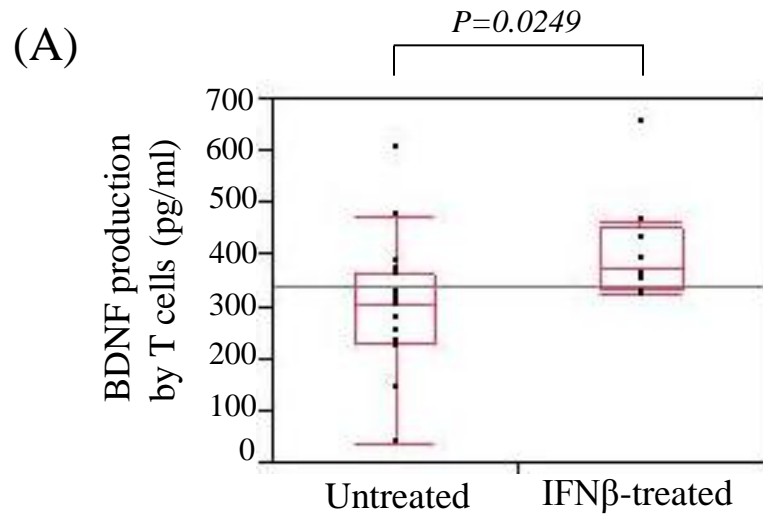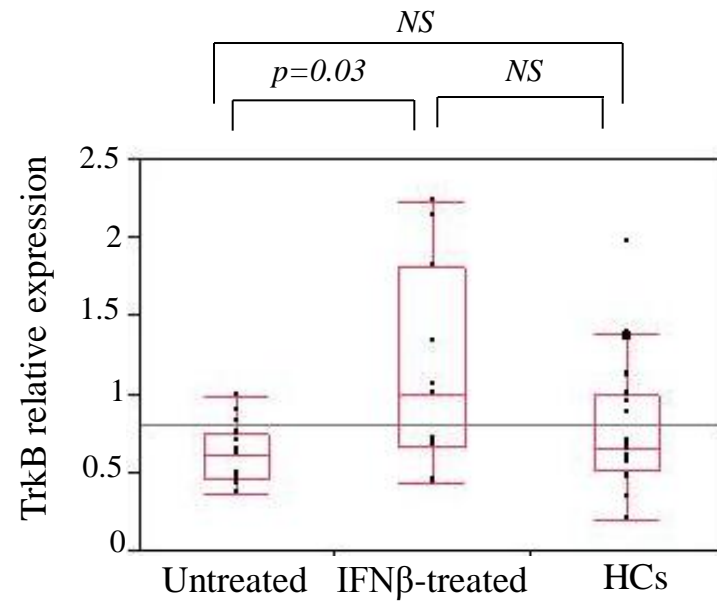
Figure 4

Figure 5

Supplementary Figure 1
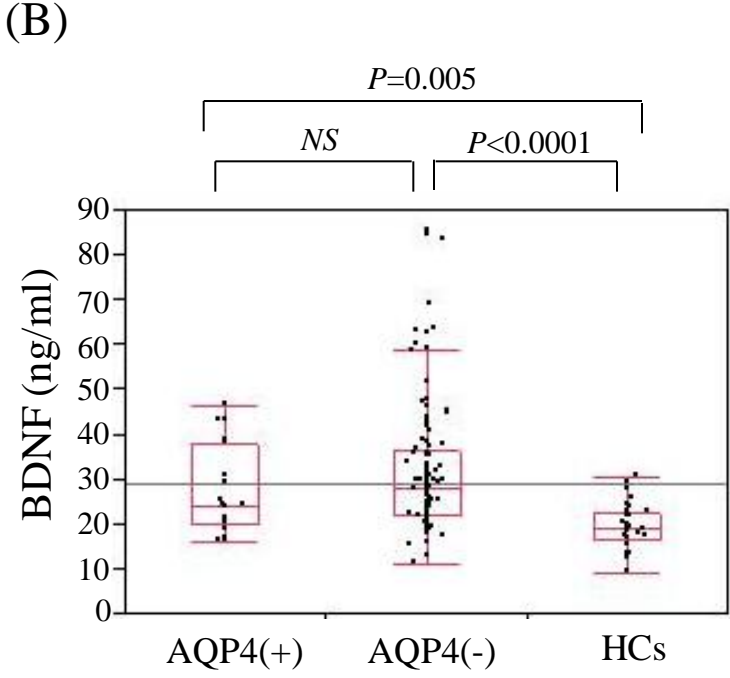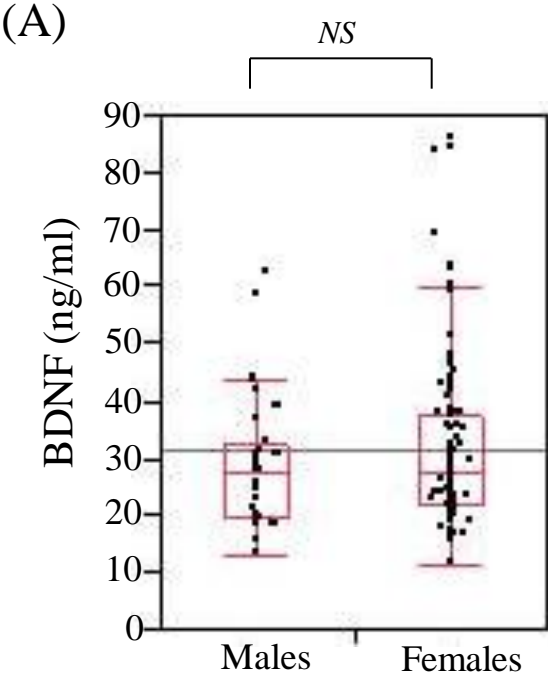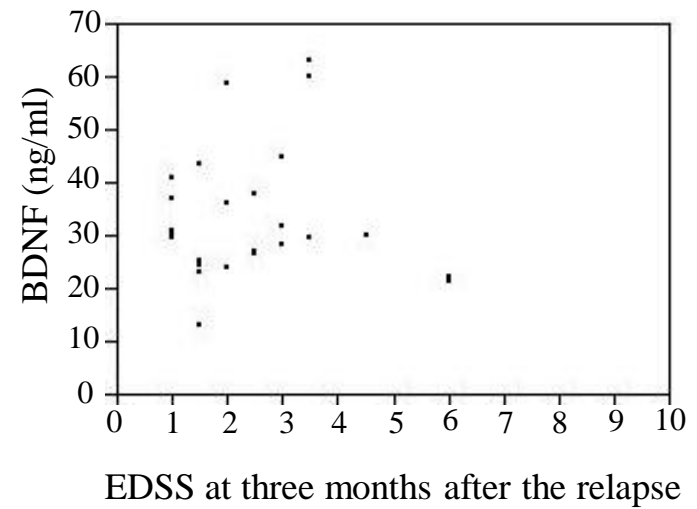
Supplementary Figure 1

(C)

(D)

Supplementary Figure 2

(A)

BDNF (ng/ml) vs Disease duration (months)
NS

(B)

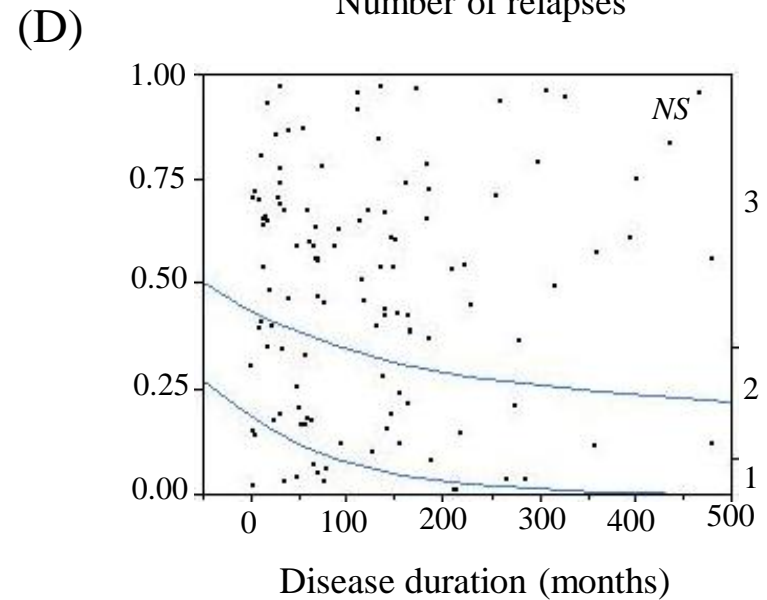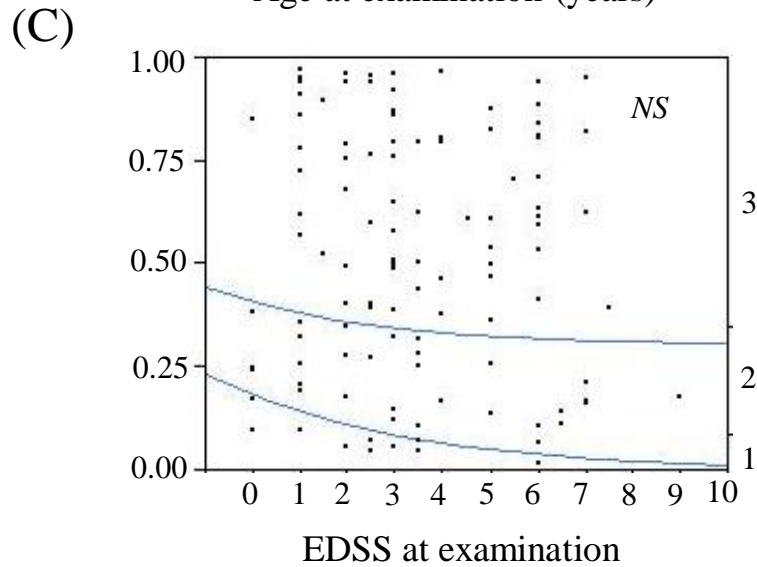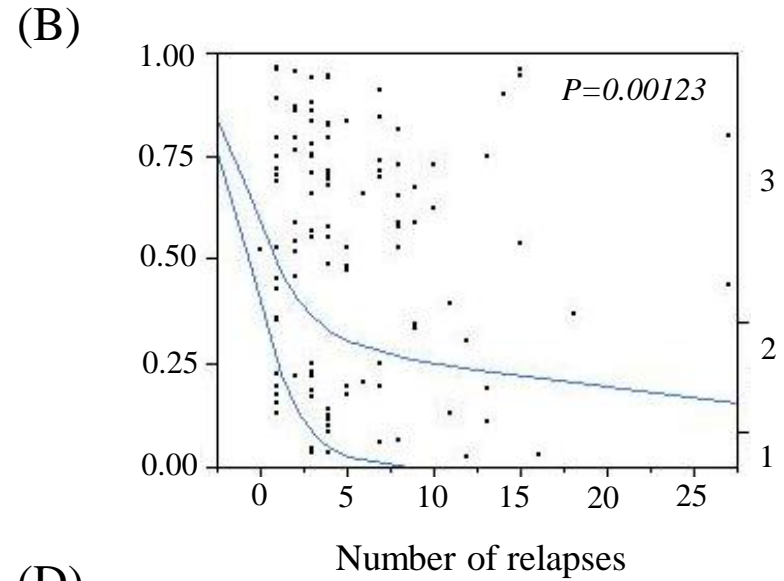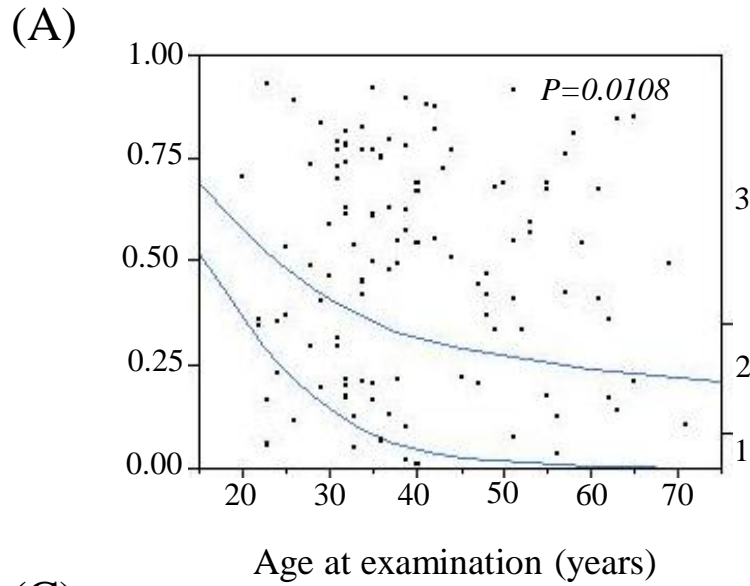BDNF (ng/ml) vs EDSS at examination
NS
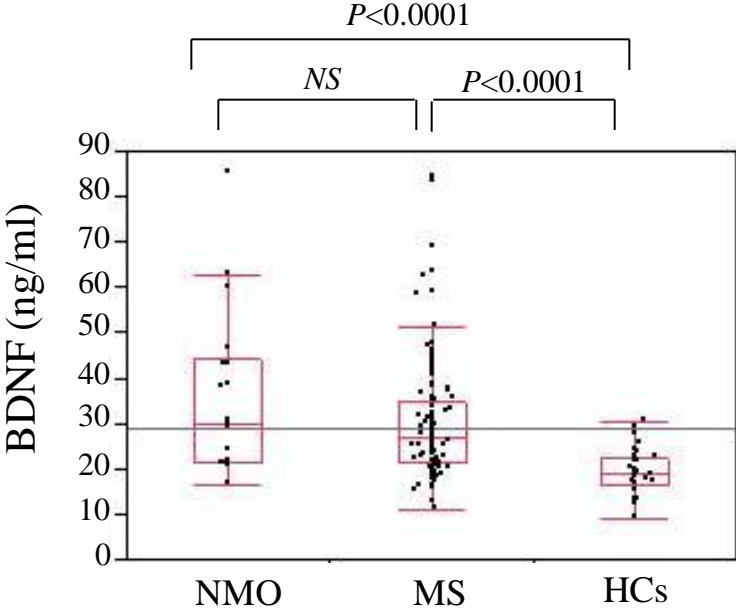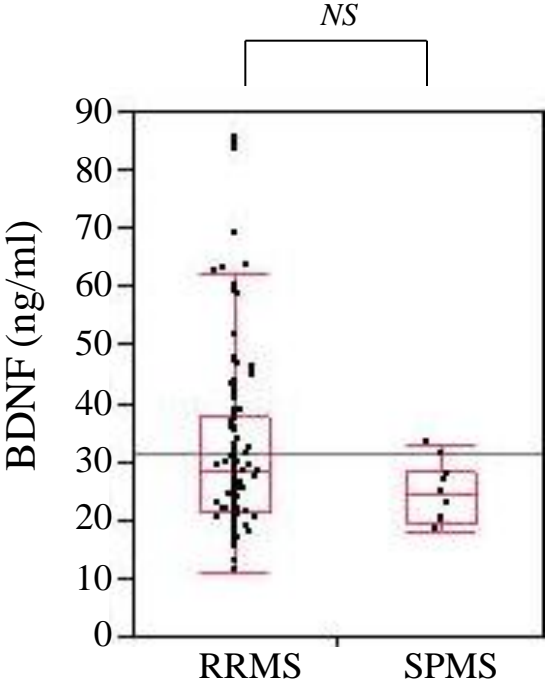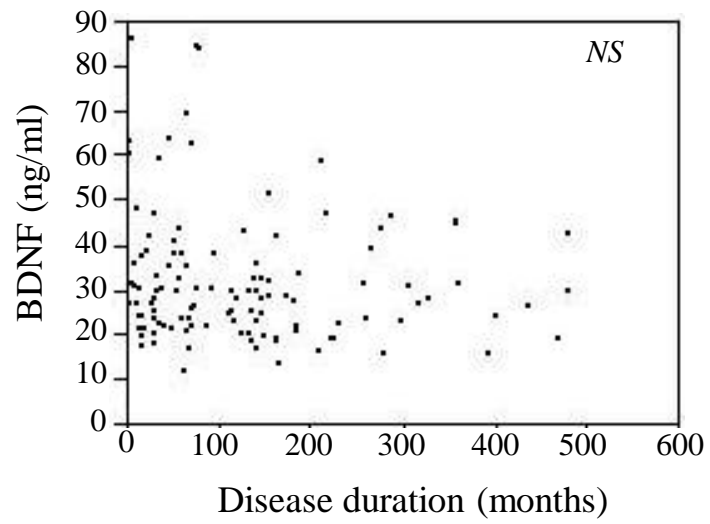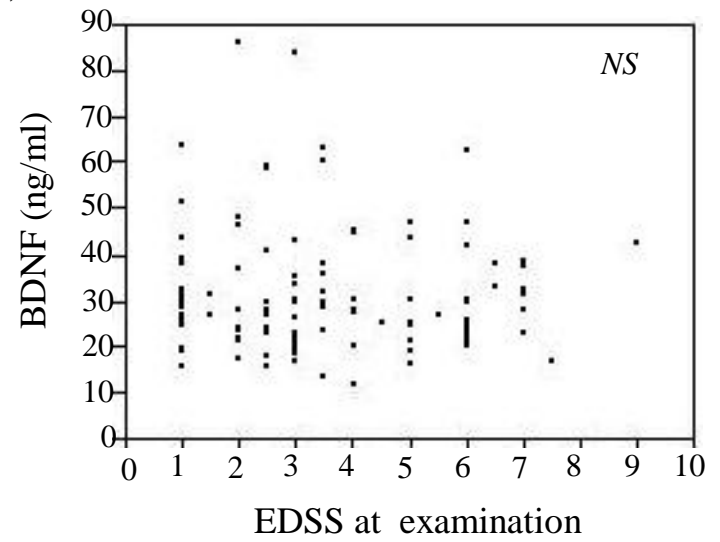
Supplementary Figure 2

(C)

Supplementary Figure 2

(D)

EDSS at the peak of relapse

(E)

EDSS at three months after the relapse

Supplementary Figure 3

(A) Age at examination (years)

(B) Number of relapses

(C) EDSS at examination

(D) Disease duration (months)

Supplementary Figure 3

(E)

Annualized relapse rate