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Immune-Inflammatory and Oxidative and Nitrosative Stress Biomarkers of Depression Symptoms in Subjects with Multiple Sclerosis: Increased Peripheral Inflammation but Less Acute Neuroinflammation

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Abstract There is evidence that activated immune-inflammatory and oxidative and nitrosative stress (IO&NS) pathways play a role in the pathophysiology of multiple sclerosis (MS) and depression. This study examines serum levels of interleukin (IL)-1β, IL-4, IL-6, and IL-10; peroxides (LOOH); nitric oxide metabolites (NOx); albumin; ferritin; C-reactive protein (CRP); and *tumor necrosis factor (TNF)-β NcoI* polymorphism (rs909253) and gadolinium-enhanced magnetic resonance imaging (MRI) scan in MS patients with (n=42) and without (n=108) depression and normal controls (n=249). Depression is scored using the depressive subscale of the Hospital Anxiety and Depression Scale (HADS). The extent of neurological disability is measured using the Expanded Disability Status Scale (EDSS) at the same time of the abovementioned measurements and 5 years earlier. Disease progression is assessed as actual EDSS—EDSS 5 years earlier. Three variables discriminate MS patients with depression from those without depression, i.e., increased IL-6 and lower IL-4 and albumin. Binary logistic regression showed that MS with depression (versus no depression) was characterized by more gastrointestinal symptoms and disease progression, higher serum IL-6, and lower albumin levels. In subjects with MS, the HADS score was significantly predicted by three EDSS symptoms, i.e., pyramidal, gastrointestinal, and visual symptoms. Fifty-eight percent of the variance in the HADS score was predicted by gastrointestinal symptoms, visual symptoms, the *TNFB1/B2* genotype, and contrast enhancement (both inversely associated). There were no significant associations between depression in MS and type of MS, duration of illness, age, sex, nicotine dependence, and body mass index. MS with depression is associated with signs of peripheral inflammation, more disability, disease progression, gastrointestinal and visual symptoms, but less contrast enhancement as compared to MS without depression. It is concluded that depression is part of the neurological symptoms of MS and that its expression is primed by peripheral inflammation while acute neuroinflammation and the *TNFB1/B2* genotype may be protective.

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Introduction

There is copious evidence that activated immune-inflammatory and oxidative and nitrosative stress (IO&NS) pathways play a role in the pathogenesis of multiple sclerosis (MS) [1–3]. Increased levels of IO&NS biomarkers are observed in the brain, cerebrospinal fluid (CSF), and blood of MS patients [4, 5]. For example, increased levels of the proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α), are observed in the plasma or CSF of patients with MS [6–11]. Increased concentrations of inflammatory mediators such as plasma TNF-α are often associated with the degree of disability as measured with the Expanded Disability Status Scale (EDSS) [12] and predict disease activity [13, 14]. Th1-like cytokines, such as IL-2, IL-12, and interferon (IFN)-γ, are often increased in active disease states [8–11], whereas Th2-like cytokines, including IL-4 and IL-10, may be increased during remission [15–18]. Nevertheless, concomitant increases in the expression of Th1- and Th2-like cytokines may be present during an acute attack [5, 19]. Recently, we observed that *TNF-β NcoI* polymorphism is significantly associated with MS, i.e., the *TNFB2/B2* genotype is associated with MS and with increased TNF-α levels [20].

The levels of oxidative stress biomarkers significantly increase during relapses but decrease during remission [21]. Oxidative stress levels in the plasma and CSF of MS patients are positively associated with the EDSS score [13, 14]. Increased inducible nitric oxide (NO) synthase (iNOS), NO production, and its metabolites nitrate and nitrite (NOx) are detected in the lesions, brain, and CSF of MS patients [22]. Increased NOx levels in the CSF, but not plasma, are correlated with disease progression [22]. Moreover, increased levels of oxidative stress are associated with the degree of gadolinium-enhanced brain lesions in magnetic resonance imaging (MRI) scan, an index of acute neuroinflammation and increased blood-brain barrier (BBB) hyperpermeability [14].

Clinical depression is the most common psychiatric manifestation of MS affecting more than 50 % of the patients [23, 24]. In MS, depression may precede the onset of neurological symptoms and is associated with a lowered quality of life and increased morbidity and mortality [24–26]. To complicate matters, treatment of MS with IFN-β may cause or exacerbate depression and a switch to treatment with glatiramer acetate is indicated when depression becomes an issue [27, 28]. The depression subscale of the Hospital Anxiety and Depression Scale (HADS) is a validated rating scale to measure depression in nonpsychiatric outpatients [29, 30].

Recently, we have reviewed that shared IO&NS pathways may underpin the comorbidity between MS and depression, including increased levels of monocytic and Th1-like cytokines, and increased reactive oxygen species (ROS), lipid peroxidation, and increased expression of iNOS [31]. During acute MS attacks, mRNA expression of TNF-α and IFN-γ is significantly correlated with severity of depression [32]. IFN-γ production by Th1 lymphocytes is significantly and positively correlated with depression in MS patients [33]. The initially increased production of IFN-γ by autoaggressive T cells in MS, which is associated with depression, is reduced during treatment with antidepressant drugs or psychotherapy [34].

The aims of the present study are to examine the differences in cytokine profile (IL-1β, IL-4, IL-6, and IL-10), inflammatory markers (ferritin, albumin, and C-reactive protein (CRP)), O&NS markers (peroxides and NOx), *TNF-β NcoI* polymorphism, and neuroinflammation (as measured with the gadolinium-enhanced MRI scan) in MS patients with and without depression and to examine the relationships between depression severity and IO&NS biomarkers, the extent of disability, and neurological symptoms.

Subjects and Methods

Subjects

We included 150 MS patients who consecutively attended the Neurology Outpatient Department of the Outpatient Clinical Hospital, State University of Londrina, Londrina, Brazil. The diagnosis of MS was made using the revised McDonald criteria [35, 36]. Patients were classified into relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), primary progressive MS (PPMS), and clinically isolated syndrome (CIS) [37]. All RRMS patients were in the remitting phase of illness, i.e., a period of recovery with no relapse episodes within the last 3 months prior to the time of enrollment in the study. Patients with neuromyelitis optica were excluded from this study.

We also included 249 healthy volunteers, i.e., healthy blood donors recruited at the Blood Bank of Londrina, Londrina, Brazil. We excluded controls with autoimmune disorders, blood-borne transmitted infection, and clinical symptoms of recent infections or inflammation. Written informed consent was obtained from all of patients and controls. The study protocol was approved by the Institutional Research Ethic Committee of the State University of Londrina, Londrina, Brazil.

Methods

A standard questionnaire and medical records were used to assess sociodemographic and clinical data. Self-reported ethnicity was registered as Caucasian, Asian, Black, or Mixed. Age at

onset of MS and duration of illness were assessed based on data from medical records [35]. The degree of disability of the patients was evaluated employing the EDSS [12], which scores severity of disability as mild (EDSS between 0.0 and 4.0), moderate (EDSS between 4.5 and 5.5), or severe (EDSS between 6.0 and 10.0). We also scored neurological symptoms as pyramidal (P), cerebellar (CII), brain stem (BS), sensory (S), bladder (Bb), gastrointestinal (Bi), visual (V), cerebral (Cb), and other (O) [12] symptoms. We measured the EDSS and the neurological symptoms prospectively in a subgroup of patients in 2006 and again in 2011. Disease progression was evaluated by computing the ΔEDSS, that is EDSS in 2011−EDSS in 2006. Severity of depression was assessed in 2011 using the depression subscale of the HADS [29]. In patients with MS, a cutoff score >8 on the HADS depression subscale can be used as a diagnostic criterion for major depression with a sensitivity of 90 % and a specificity of 87.3 % [38]. Consequently, we divided patients with MS into these with and without depression using the >8 threshold value.

The diagnosis of nicotine dependence was made using the criteria of the Diagnostic and Statistical Manual of Mental Disorders, Text Revision (DSM IV-TR). We also registered the use of medication, i.e., glucocorticoids, antidepressants (i.e., fluoxetine, nortryptiline, setraline, or amitryptiline), and disease-modifying drugs (DMDs) that slow down progression and attenuate flare-ups, i.e., IFN-β1a, natalizumab, or glatiramer acetate. We measured body weight using an electronic scale in individuals wearing light clothing, but no shoes, and height using a stadiometer. Consequently, body mass index (BMI) was calculated as weight (kg) divided by height (in meter) squared.

In 2011, MRI scans were performed in the brain, cervical, and spinal cord (T1, T2, and flair sequences) to detect lesion location and the presence of gadolinium contrast-enhancing lesions. Also, in 2011, blood samples were collected (with and without EDTA as anticoagulant), centrifuged at 3000 rpm for 15 min, and stored at −80 °C until thawed for assay of cytokines, albumin, ferritin, LOOH, and NOx. Samples were consecutively identified by number to guarantee the confidentiality. Serum levels of proinflammatory cytokines (IL-1β and IL-6) and Th2 cytokines (IL-4 and IL-10) were assayed using a sandwich enzyme-linked immunosorbent assay (ELISA, eBioscience, San Diego, CA, USA). The limits of detection were as follows: IL-1β 2.0 ng/mL, IL-6 1.0 ng/mL, IL-4 1.0 ng/mL, and IL-10 1.0 ng/mL. Albumin assays were performed using a biochemical autoanalyzer (Dimension™ Dade AR Dade Behring, Deerfield, IL, USA). Ferritin levels were determined by chemiluminescence microparticle immunoassay (Architech™, Abbott Laboratory, Abbott Park, IL, USA). High sensitivity C-reactive protein (hsCRP) was determined using nephelometry (NephelometerII™, DadeBehring-Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA) with an analytical sensitivity of 0.175 mg/L.

The stable NO metabolites, nitrate and nitrite (NOx), are used as an index of NO production and as a marker of NOS enzyme activity. NOx levels are assessed by determining plasma nitrite concentrations using an adaptation of the technique described by Navarro-Gonzálvez et al. [39]. The quantification of NOx is made in a microplate reader Asys Expert Plus, Biochrom ® (Holliston, MA, USA). Plasma levels of CL-LOOH were evaluated as described previously [40], and the results were expressed in counts per minute (cpm). TRAP was determined as reported by Reppeto et al. [41]. The system was calibrated with the vitamin E analog TROLOX, and the values of TRAP are expressed in equivalent of :M Trolox/mg uric acid.

Genomic DNA was extracted from the buffy coat using the reagent resin column (Biopur, Biometrix Diagnóstica, Curitiba, Brazil) according to the manufacturer's instructions. A 782-base-pair (bp) fragment of the *TNF-β* gene was amplified using polymerase chain reaction (PCR) as previously reported [42–44]. PCR was performed in a thermocycler (Applied Biosystems Veriti™ 96-Well Thermal Cycler, Life Technologies, Foster City, CA, USA). The PCR product was digested using *Nco*I restriction enzyme (Invitrogen Life Technologies, Carlsbad, CA, USA), and the genotypes were identified by the restriction fragment length polymorphism (RFLP) with electrophoresis on 3 % agarose gel (70 V, 70 min) after staining with ethidium bromide. The *TNFB1* allele includes a restriction site for *Nco*I, which results in 196- and 586-bp fragments after digestion, and the *TNFB2* allele (lacking the restriction site for *Nco*I) results a fragment with 782 bp. The heterozygous genotype *TNFB1/B2* results in three fragments (782, 586, and 196 bp).The PCR-RFLP profiles were captured and recorded by the photo documentation system L-PIX-HE (Loccus Biotecnologia, Cotia, SP, Brazil).

Statistics

We used analyses of contingency tables (χ^2 test) or Fisher's exact probability test to assess associations between diagnostic categories and clinical and sociodemographic data. Analyses of variance (ANOVAs) followed by Bonferroni-adjusted least significant difference to examine multiple comparisons among group means (only used when the overall ANOVA was significant) were employed to examine differences in clinical, sociodemographic, and IO&NS biomarker data between the diagnostic groups. Automatic stepwise multinomial logistic regression analyses with MS with and without depression and controls as dependent variables and clinical and IO&NS markers as explanatory variables were employed to delineate the significant explanatory variables that are associated with the diagnostic groups. Automatic binary logistic regression analyses with MS with depression as the dependent variable (and MS without depression as the reference group) were used to assess the significant clinical and IO&NS markers that predict the diagnostic groups. Multivariate general linear model

(GLM) analyses (followed by analyses of between-subject tests or univariate GLM analyses) were performed to delineate the relationships between clinical data (the EDSS, neurological symptoms, HADS, and disease progression) as dependent variables, and age, gender, nicotine dependence, medications, BMI, etc. as explanatory variables. Automatic linear (multiple) regression analyses with the HADS score as the dependent variable and the clinical variables and IO&NS biomarker data as explanatory variables were performed to delineate the significant predictors of severity of depression in patients with MS. Factor analysis, the principal component method, was used to interpret the associations between the HADS score and neurological symptoms using a quartimax rotation of the subtracted principal components. The Kolmogorov-Smirnov goodness-of-fit test was used to assess normality of distribution. Logarithmic (Ln) transformations of continuous data were used in the analyses when the variables were not normally distributed or when there was heterogeneity of variance (as assessed with the Levene test). We used the SPSS (Windows version 22), Statistica, and MaesStat to perform statistical analyses. All analyses were performed at $p=0.05$ (two-tailed).

Results

Demographic Data and Biomarkers

Table 1 shows that there were no significant differences in age, ethnicity, or BMI between controls and MS patients with or without depression. Using univariate analyses, there were no significant differences in hsCRP, IL-1, IL-6, and IL-10 between these three groups. IL-4 levels were significantly higher in patients with MS without depression versus controls. Ferritin and LOOH levels were significantly higher in MS patients than in controls. Albumin was significantly lower in patients with MS and depression than in controls. NOx levels were significantly lower in patients with MS with or without depression versus controls. TRAP levels were significantly lower in patients with MS without depression than in controls. The frequency of *TNFB2/B2* genotype was greater in MS patients without depression.

Effects of Possible Intervening Factors on the Biomarkers

In order to delineate the possible effects of intervening variables, including sex, age, nicotine dependence, ethnicity, and use of DMDs and antidepressants, we have carried out four different multivariate GLM analyses with the grouped biomarkers or clinical data as dependent variables. Multivariate GLM analysis with IL-1, IL-4, IL-6, and IL-10 as dependent variables showed that sex ($F=1.23$, $df=4/124$, $p=0.301$), age ($F=1.51$, $df=4/124$, $p=0.204$), nicotine dependence ($F=1.61$, $df=4/124$, $p=0.175$), ethnicity ($F=1.94$, $df=4/124$, $p=0.108$), DMDs (that is no drugs, IFN-β, or glatiramer; $F=1.20$, $df=4/124$, $p=0.315$), and antidepressants ($F=1.35$, $df=4/143$, $p=0.254$) were all not significant. Multivariate GLM analysis with LOOH, NO, and TRAP as dependent variables showed that sex was marginally significant ($F=3.85$, $df=3/101$, $p=0.012$) and that age ($F=1.51$, $df=3/101$, $p=0.217$), nicotine dependence ($F=1.26$, $df=3/101$, $p=0.293$), ethnicity ($F=0.39$, $df=3/101$, $p=0.764$), DMDs ($F=1.41$, $df=3/101$, $p=0.244$), and antidepressants ($F=0.33$, $df=3/109$, $p=0.809$) were not significant. Multivariate GLM analysis with ferritin, albumin, and hsCRP as dependent variables showed that sex had a significant effect ($F=15.56$, $df=3/136$, $p<0.001$; ferritin and albumin being higher in men), but that the other factors were not significant, that is age ($F=1.25$, $df=3/136$, $p=0.294$), nicotine dependence ($F=1.53$, $df=3/136$, $p=0.210$), ethnicity ($F=0.29$, $df=3/136$, $p=0.833$), DMDs ($F=2.01$, $df=3/136$, $p=0.115$), and antidepressants ($F=1.72$, $df=3/156$, $p=0.164$).

Multivariate GLM analysis with the EDSS score, ΔEDSS, and all neurological symptoms as dependent variables showed a significant effect of age (age being significantly and positively associated with all variables, except BS, Cb, and O symptoms and the ΔEDSS), while the other variables (including DMDs, antidepressants, and glucocorticoids) were not significant. We have also examined the effects of DMDs, coded as no use versus use of IFN-β, glatiramer, or natalizumab, on the EDSS variables and symptoms ($F=0.88$, $df=11/121$, $p=0.562$); the four cytokines ($F=1.73$, $df=4/142$, $p=0.146$); ferritin, albumin, and hsCRP ($F=1.37$, $df=3/154$, $p=0.253$); and LOOH, NOx, and TRAP ($F=1.13$, $df=3/108$, $p=0.339$) and could not find any effects. Multivariate GLM analyses showed that the use of glucocorticoids had no significant effect on the EDSS and symptoms ($F=0.81$, $df=11/120$, $p=0.999$); the four cytokines ($F=0.81$, $df=4/313$, $p=0.522$); ferritin, hsCRP, and albumin ($F=0.93$, $df=3/340$, $p=0.426$), but was significantly associated with LOOH, NOx, and TRAP ($F=3.32$, $df=3/260$, $p=0.020$). Analyses of between-subject tests showed that glucocorticoids significantly suppressed NOx ($F=4.19$, $df=1/262$, $p=0.018$) and TRAP ($F=5.16$, $df=1/262$, p=0.024). Positive contrast enhancement on the gadolinium-enhanced MRI scan was not significantly associated with DMDs ($\chi^2=1.32$, $df=2$, $p=0.518$) and the use of antidepressants (Fisher's exact test: $p=0.255$), but was positively associated with glucocorticoid use ($\chi^2=6.15$, $df=1$, $p=0.013$; odds ratio=3.00, lower and upper 95 % confidence of interval levels 1.22–7.36).

Using the HADS score as dependent variable and DMDs ($F=3.20$, $df=1/139$, $p=0.076$), glucocorticoids ($F=0.01$, $df=1/139$, $p=0.908$), and antidepressants ($F=0.29$, $df=1/139$, $p=0.112$) or any of their two-way interactions as explanatory variables did not show any effects of these drugs ($F=1.76$, $df=6/$

Table 1 Sociodemographic and biomarker data in normal controls (NC) and multiple sclerosis (MS) patients with (+MDD) and without (−MDD) an increased Hospital Anxiety and Depression Scale (HADS) score

Variables	NC (n=249)	MS−MDD (n=108)	MS+MDD (n=42)	F or χ^{2a}	df	p
Age (years)	36.7 (10.9)	40.1 (13.5)	44.8 (14.4)	9.16	2/396	0.001
Gender (female/male)	177/72	76/32	35/7	2.93	1	0.231
Non-Caucasian/Caucasian	50/199	18/90	10/31	1.22	2	0.542
BMI (kg/m^2)	25.1 (4.4)	25.2 (5.1)	25.4 (4.7)	0.07	2/320	0.929
IL-1 (ng/mL)[b]	9.2 (29.3)	5.0 (6.8)	4.0 (4.2)	1.38	2/307	0.252
IL-4 (ng/mL)[b]	1.4 (1.5)	4.6 (7.1)	3.2 (5.6)	15.44	2/310	<0.001
IL-6 (ng/mL)[b]	5.3 (14.7)	4.2 (6.3)	10.7 (32.7)	1.37	2/306	0.256
IL-10 (ng/mL)[b]	6.8 (8.8)	5.1 (3.3)	5.9 (3.2)	1.33	2/306	0.266
LOOH (mmol/L)[b]	16,770 (15,787)	24,675 (12,560)	24,092 (11,039)	27.65	2/279	<0.001
NOx (:M/L)[b]	46.0 (26.0)	19.7 (24.7)	26.3 (25.8)	73.55	2/247	<0.001
TRAP (:M Trolox)[b]	721.1 (144.6)	657.5 (141.0)	693.8 (106.6)	5.52	2/280	0.004
CRP (mg/L)[b]	2.95 (4.72)	2.53 (3.54)	3.24 (4.31)	1.21	2/360	0.300
Ferritin	94.6 (90.1)	214.9 (250.7)	213.8 (269.6)	19.02	2/336	<0.001
Albumin (mg/dL)	4.15 (0.31)	4.06 (0.40)	4.00 (0.41)	3.96	2/332	0.020
TNFB1B2 genotype N/Y	0.51	0.43	0.55	3.09	2	0.245
TNFB2B2 genotype N/Y	0.39	0.49	0.35	3.06	2	0.216

Results are shown as mean (±SD); or as frequencies

[a] All the results of analyses of variance (F values) or contingency analyses (O^2 tests)

[b] These variables are processed in Ln transformation

139, p=0.112). Bivariate logistic regression analysis showed that the use of DMDs, glucocorticoids, and antidepressants was not significantly associated with MS with depression (χ^2=6.68, df=4, p=0.154, Nagelkerke=0.065). In this analysis, the use of antidepressants (Wald=5.72, df=1, p=0.017), but not DMDs (Wald=0.89, df=2, p=0.640) or glucocorticoids (Wald=0.09, df=1, p=0.922), was positively associated with depression.

Immune-Inflammatory Biomarkers, MS, and Depression

Table 2 shows the results of automatic multinomial logistic regression analyses with MS with and without depression and the control group as the dependent variable and the IO&NS biomarkers as explanatory variables. We found that six variables were significant in explaining the diagnostic groups (χ^2=161.40, df=12, p<0.001; Nagelkerke=0.642; correctly classified subjects=82.9 %), i.e., IL-6 (χ^2=11.00, df=2, p=0.004), IL-4 (χ^2=20.00, df=2, p<0.001), LOOH (χ^2=21.03, df=2, p<0.001), NOx (χ^2=53.19, df=2, p<0.001), ferritin (χ^2=16.95, df=2, p<0.001), and albumin (χ^2=7.24, df=2, p=0.027). Increased IL-4, LOOH, and ferritin but lower NOx were associated with MS without depression versus controls. MS with depression was characterized by increased IL-6, LOOH, and ferritin and lowered albumin and NOx as compared to controls. Three explanatory variables discriminated MS patients with depression from those without depression, i.e., increased IL-6 and lowered IL-4 and albumin. Forced entry showed that nicotine dependence was not a significant explanatory variable (χ^2=4.45, df=2, p=0.349) and that IL-6 (Wald=5.02, df=1, p=0.025), IL-4 (Wald=5.74, df=1, p=0.017), and albumin (Wald=5.16, df=1, p=0.023) remained significant as explanatory variables separating MS with and without depression. Forced entry of DMDs (Wald=0.00, df=1, p=0.997) and the use of antidepressants (Wald=1.22, df=1, p=0.269) showed that these variables were not significant in discriminating MS with and without depression and that IL-6, IL-4, and albumin remained significant as explanatory variables. Forced entry of age, sex, and BMI showed that these variables were not significant.

Characteristics of MS with Depression

Table 3 shows the differences between individuals with and without depression. There were no significant differences between both groups with respect to positive gadolinium enhancement on MRI scan, the use of DMDs and glucocorticoids, and type of MS. Only two patients were treated with natalizumab, and therefore, we could not run separate analyses on this drug. The use of antidepressant drugs was higher in MS patients with depression than in those without depression. Patients with depression showed significantly higher scores on the total EDSS

Table 2 Results of multinomial logistic regression analysis with normal controls (NC) and patients with multiple sclerosis with (MS+MDD) and without (MS−MDD) depression as dependent variables and immune-inflammatory and oxidative and nitrosative stress biomarkers as explanatory variables

Contrasts	Explanatory variables	Wald	*df*	*p*	Odds ratio	95 % CI (lower–upper)
MS−MDD versus NC	IL-4	14.30	1	<0.001	3.57	1.85–6.90
	LOOH	12.87	1	<0.001	5.57	2.18–14.24
	NOx	31.54	1	<0.001	0.11	0.05–0.24
	Ferritin	9.41	1	0.002	1.01	1.00–1.01
MS+MDD versus NC	Albumin	4.63	1	0.031	0.17	0.03–10.84
	IL-6	6.40	1	0.011	2.01	1.17–3.46
	LOOH	13.10	1	<0.001	7.75	2.56–23.48
	NOx	18.07	1	<0.001	0.17	0.07–0.38
	Ferritin	10.23	1	<0.001	1.01	1.00–1.01
MS+MDD versus MS−MDD	Albumin	6.17	1	0.013	0.14	0.03–0.66
	IL-6	9.01	1	0.003	2.43	1.36–4.35
	IL-4	4.05	1	0.044	0.53	0.28–0.98

score (in 2011 but not in 2006), ΔEDSS, and P, S, Bb, Bi, V, Cb, and O symptoms in 2001 and Bi symptoms in 2006.

In order to delineate the best prediction of MS with versus depression, we performed a binary logistic regression analysis with MS and depression as the dependent variable and MS without depression as the reference group. Table 4 shows the results of three different automatic regression analyses: the first one used the EDSS 2011 data only and showed that Bi symptoms and ΔEDSS were significantly associated with MS with depression (χ^2=25.46, *df*=2, p<0.001; Nagelkerke=0.325). The second regression analysis was performed using all EDSS symptoms in 2006 and 2011 and showed that Bi symptoms in 2011 and V symptoms in 2006 were the most significant predictors of depression (χ^2=14.68, *df*=2, p<0.001; Nagelkerke=0.395).

The third binary logistic regression analysis was performed on the same clinical variables as shown in the first regression together with the IO&NS biomarkers. We found that four explanatory variables were significantly associated with depression, i.e., IL-6, Bi in 2011, and ΔEDSS (all positively) and albumin (negatively) (χ^2=34.15, *df*=4, p<0.001; Nagelkerke=0.572; 84.4 % of all cases were correctly classified with a sensitivity of 61.1 % and a specificity of 92.2 %).

Prediction of the HADS Score

Pearson correlation calculation showed that the HADS was significantly correlated with age (r=0.229, p=0.005, n=150), EDSS 2011 (r=0.365, p<0.001, n=146), and P (r=0.390, p<0.001, n=109), S (r=0.338, p<0.001, n=109), Bb (r=0.236, p=0.013, n=109), Bi (r=0.410, p<0.001, n=109), V (r=0.242, p=0.011, n=109), and C (r=0.252, p=0.008, n=109) symptoms in 2011, but not with duration of illness, BMI, or any of the IO&NS biomarkers.

Table 5 shows the outcome of two different automatic GLM analysis with the HADS score as the dependent variable and the EDSS and neurological symptoms in 2011 (regression 1) or these clinical variables and IO&NS biomarkers (regression 2) as explanatory variables. We found that the HADS score was significantly predicted by P, Bi, and V symptoms in 2011. Regression 2 shows that Bi and V in 2011 and V in 2011 were positively associated with the HADS, while the *TNFB1/B2* genotype and a positive gadolinium-enhanced MRI scan were inversely associated with the HADS score. Forced entry of the use of glucocorticoids (F=0.27, *df*=1/40, p=0.608), DMDs (F=0.82, *df*=2/39, p=0.449), and antidepressants (F=0.72, *df*=1/40, p=0.400) was not significant and did not change the abovementioned associations.

Results of Factor Analysis

Table 6 shows the results of a factor analysis with all neurological symptoms in 2011 and the HADS as variables. We found that without rotation, the HADS and the EDSS symptoms, except BS, were significantly loaded on the first PC explaining 27.5 % of the variance. Quartimax rotation showed that the HADS loaded together with P, S, Bb, Bi, V, and Cb symptoms on PC1. PC2 loaded highly on P, CII, and O, and PC3 loaded highly on BS, Bb, and V but negatively on Cb symptoms.

Discussion

The first major finding of this study is that depression in MS is accompanied by specific immune-inflammatory biomarkers. Thus, we found increased serum IL-6 but lower serum IL-4 and albumin levels in MS patients with depression as

Table 3 Clinical and biomarker data in patients with multiple sclerosis with (MS+MDD) and without (MS−MDD) depression, divided according to a cutoff score >8 on the Hospital Anxiety and Depression Scale

Variables	MS−MDD	MS+MDD	F/χ^{2a}	*df*	*p*
HADS	3.1 (2.4)	10.1 (1.8)	292.13	1/148	<0.001
EDSS in 2006	1.15 (1.68)	1.31 (1.41)	0.62	1/148	0.593
EDSS in 2011	2.40 (2.00)	3.62 (1.32)	14.28	1/144	<0.001
ΔEDSS	1.26 (1.47)	2.36 (1.65)	15.49	1/144	<0.001
Pyramidal functions 2006	1.86 (1.26)	1.64 (0.85)	0.57	1/76	0.451
Pyramidal functions 2011	1.91 (1.49)	3.25 (1.32	19.60	1/108	<0.001
Cerebellar functions 2006	0.38 (0.78)	0.59 (1.09)	0.96	1/76	0.331
Cerebellar functions 2011	0.92 (1.34)	1.25 (1.34)	1.41	1/108	0.237
Brain stem functions 2006	0.23 (0.54)	0.14 (0.47)	0.54	1/76	0.467
Brain stem functions 2011	0.09 (0.37)	0.06 (0.35)	0.13	1/108	0.722
Sensory functions 2006	0.48 (0.74)	0.23 (0.53)	2.17	1/76	0.144
Sensory functions 2011	0.73 (1.11)	1.34 (1.07)	7.05	1/108	0.009
Bladder functions 2006	0.32 (0.81)	0.73 (1.49)	2.39	1/76	0.126
Bladder functions 2011	0.85 (1.41)	1.59 (1.85)	5.31	1/108	0.023
Bowel functions 2006	1.02 (0.65)	1.41 (0.80)	5.06	1/76	0.027
Bowel functions 2011	1.04 (0.47)	1.97 (1.43)	26.56	1/108	<0.001
Visual functions 2006	0.51 (0.99)	0.68 (1.43)	0.36	1/76	0.558
Visual functions 2011	0.21 (0.59)	0.50 (0.92)	4.04	1/108	0.047
Cortical functions 2006	1.04 (0.66)	1.09 (0.43)	0.13	1/76	0.718
Cortical functions 2011	0.99 (0.25)	1.16 (0.45)	6.25	1/108	0.014
Other functions 2006	0.95 (0.30)	1.05 (0.21)	2.03	1/76	0.158
Other functions 2011	1.14 (0.53)	1.44 (0.50)	7.35	1/108	0.008
RR versus other (N/Y)	26/82	12/31	0.24	1	0.624
Gadolinium-enhanced MRI (N/Y)	75/24	28/7	0.26	1	0.610
No DMDs	17	7	1.19	2	0.551
IFN-γ	78	26			
Glatiramer	12	7			
No DMDs	17	7	0.06	1	0.814
Use of DMDs	90	33			
Glucocorticoids (N/Y)	94/14	38/5	0.05	1	0.823
Antidepressants (N/Y)	98/10	32/10	4.35	1	0.037

Results are shown as mean (± SD)

EDSS Expanded Disability Status Scale, *RR* relapsing-remitting versus the other MS subtypes, *DMDs* the disease-modifying drugs, IFN-γ or glatiramer

[a] All the results of analyses of variance (*F* values) or contingency analyses (χ^2 tests)

compared to those without depression. Increased IL-6 and lowered albumin are well-established inflammatory markers of depression [45–48]. Our data that IL-6 levels are increased in patients with MS and depression extend those of Koutsouraki et al. [49] showing that increased serum IL-6 levels were found in acute phase MS patients with depression

Table 4 Results of binary logistic regression analysis with multiple sclerosis with an increased HADS score (MS+ MDD) as the dependent variable and MS patients with a HADS score <8 as the reference group

Significant explanatory variables	Wald	*df*	*p*	Odds ratio	95 % CI (lower–upper)
Bowel functions 2011	11.37	1	0.001	3.48	1.69–7.18
ΔEDSS	8.64	1	0.003	1.64	1.18–2.28
Visual functions 2006	5.38	1	0.020	2.55	1.16–5.64
Bowel functions 2011	8.84	1	0.003	4.70	1.69–13.06
IL-6	6.57	1	0.010	2.91	1.29–6.59
Albumin	4.18	1	0.041	0.11	0.01–0.91
Bowel functions 2011	10.17	1	0.001	9.64	2.40–38.82
ΔEDSS	8.20	1	0.004	2.53	1.34–4.78

versus those without depression. Thus, not only depression in the remission phase (our study) but also depression in the acute state of MS is accompanied by increased IL-6 levels. The lowered IL-4 levels in our MS patients with depression may indicate a lowered negative immunoregulatory potential and thus an elevated capacity to immune-inflammatory responses. As such, depression in patients with MS is related to an increased peripheral immune-inflammatory potential.

There were, however, no significant differences between the O&NS biomarkers in MS patients with and without depression. Thus, LOOH and NOx were significantly higher and TRAP was significantly lower in MS with and without depression than in controls. In addition, we were unable to detect any significant correlations between the HADS score and the O&NS biomarkers. Major depression, on the other hand, is accompanied by increased levels of O&NS biomarkers and lowered antioxidant levels [31].

We also observed that the severity of depression in MS is inversely associated with the *TNFB1/B2* genotype, while the TNFB2 allele was associated with MS per se [20]. Thus, it may be that the *TNF-β NcoI* polymorphism is differently associated with MS per se versus depression during MS. The *TNFB2/B2* genotype is associated with increased TNF-α and decreased IL-4 and IL-10 levels, suggesting that this genotype is accompanied by increased inflammatory potential as compared to the other genotypes [20]. This may explain our findings that the *TNFB1/B2* genotype which shows lowered inflammatory potential (i.e., less TNF-α but increased IL-4 and IL-10) is protective against depression.

One limitation of this study is that we measured IL-6 but not the soluble IL-6 receptor (sIL-6R) levels and sgp130 levels. Classical IL-6 signaling proceeds when IL-6 binds to the IL-6R (or CD126) on leukocyte subsets, hepatocytes, megakaryocytes, and some neuronal cells and forms a complex with gp130 activating the Janus kinase/signal transducer and activator of transcription (JAK/STAT) and the mitogen-activated protein kinase (MAPK) pathways [50, 51]. However, many other cells, including some neuronal cells, without IL-6R expression can mount IL-6 signaling when serum IL-6 first binds to serum sIL-6Rs thereby forming a sIL-6R/IL-6 complex, which may drive IL-6 trans-signaling in cells expressing membrane gp130 [50, 51]. Interestingly, IL-6 has dual, Janus face-like effects: while classical IL-6 signaling has homeostatic effects, including neurotrophic, regenerative, and anti-inflammatory effects, IL-6 trans-signaling causes detrimental proinflammatory effects [50, 51]. Major depression and bipolar disorder are characterized by increased levels of serum IL-6 and sIL-6R levels without significant changes in sgp130 levels, suggesting that depression is accompanied by classical IL-6 and IL-6 trans-signaling [51]. Thus, our results do not allow to conclude whether the increased IL-6 levels in MS with depression indicate increased anti-inflammatory or proinflammatory effects. Interestingly, Koutsouraki et al. [49] found that serum IL-6 and sIL-6R levels were significantly higher in acute phase MS patients with depression than in the remission group.

The second major finding of this study is that severity of depression was inversely associated with contrast enhancement on the gadolinium MRI scan. In other studies, depression was associated with T1 lesions but not with bright T2 lesions or enhancement [52]. At first sight, our results may appear to contradict the findings of a greater peripheral immune-inflammatory response in MS subjects with depression. Indeed, contrast enhancement on gadolinium MRI scan is associated with increased BBB permeability and acute perivascular inflammation [53]. The gadolinium MRI scan enhancing active lesions differentiates between active versus chronic lesions [54, 55]. Thus, our results may indicate that the acute injury of BBB disruption and thus acute inflammation is associated with attenuated depressive symptoms. The acute inflammatory state is followed by a subacute and a late phase characterized by resorption of inflammatory edema, degeneration, and repair including remyelination [55–57]. Although this progressive state is generally not accompanied by gadolinium enhancement, inflammation and tissue injury may occur even in the absence of BBB breakdown [55]. Pathological studies show that in the chronic active or smoldering

Table 5 Results of two different automatic GLM analysis with the Hospital Anxiety and Depression Scale (HADS) score as the dependent variable and the Expanded Disability Status Scale (EDSS) and neurological symptoms (regression 1) or the combination of neurological symptoms and immune-inflammatory and oxidative and nitrosative stress biomarkers (regression 2) as explanatory variables

Variables	*B*	SE	*F*	*df*	*p*	Partial eta squared
Model regression 1	–	–	13.86	3/105	<0.001	0.28
Intercept	1.28	0.68	3.55	1/105	0.062	0.03
Pyramidal functions 2011	0.72	0.22	11.26	1/105	0.001	0.10
Bowel functions 2011	1.44	0.39	13.40	1/105	<0.001	0.11
Visual functions 2011	0.95	0.46	4.35	1/105	0.039	0.04
Model regression 2	–	–	11.27	5/41	<0.001	0.58
Intercept	3.17	0.85	0.05	1/41	0.831	0.00
Gadolinium-enhanced MRI scan	−4.00	1.09	13.48	1/41	0.001	0.25
TNFB1B2 genotype	−2.00	0.82	5.99	1/41	0.019	0.13
Bowel functions 2011	2.07	0.47	19.39	1/41	<0.001	0.32
Visual functions 2006	0.97	0.38	6.54	1/41	0.014	0.14
Visual functions 2011	1.80	0.61	8.65	1/41	0.005	0.17

state, macrophages are present at the plaque edges [55]. Moreover, the BBB dysfunction hypothesis considers that oxidative stress and neuroinflammation when co-occurring with neurovascular dysfunction may cause subtle increases in BBB permeability which are associated with depression [58]. Thus, it may be that a condition characterized by chronic low-grade inflammation, subtle increases in BBB permeability, degeneration, and repair, is more likely to be accompanied by elevated depressive symptoms than the acute inflammatory state. Nevertheless, as described in the "Introduction," during acute MS attacks, the increased production of IL-6, TNF-α, and IFN-γ is significantly associated with increased depression or severity of depression [32, 49]. Thus, it appears that depressive symptoms are related to cytokine production and immune-inflammatory markers not only during the acute phase of illness but also during the progressive course of illness, characterized by systemic inflammation, as shown in the present study, macrophage infiltration around the plaques, degenerative processes, and tissue repair. The continued immune-inflammatory disturbances in MS may explain that depression in MS has often a chronic course in contrast to an episodic course in the general population [59].

The third major finding of this study is that the total EDSS score, the changes in the EDSS score over the last 5 years, and neurological symptoms of MS were significantly and positively associated with depression and/or severity of depressive symptoms. Some authors [60, 61] were unable to find significant correlations between the EDSS score and depressive scores. Most studies, however, found that MS patients with higher EDSS scores had significantly higher depression scores, while significant positive correlations were found between the EDSS and depression scores [62–68]. This is important as MS functional disability and especially depression predict physical health and quality of life [69–71]. Our findings showing that depression is associated with progression of MS, as measured with the EDSS over a 5-year period, extend those of previous reports. Thus, higher depression scores were detected in MS patients with disability progression [72]. We observed significant associations between the severity of depression and neurological symptoms, especially with gastrointestinal and visual

Table 6 Results of factor analysis, principal component (PC) method, on the neurological symptoms of multiple sclerosis and the Hospital Anxiety and Depression Scale (HADS) with and without quartimax rotation

Variables	Unrotated PC1	Rotated PC1	Rotated PC2	Rotated PC3
Pyramidal	*0.702*	*0.511*	*0.479*	0.201
Cerebellar	*0.542*	0.107	*0.829*	0.042
Brain stem	0.063	−0.067	0.132	*0.641*
Sensory	*0.467*	*0.628*	−0.107	0.007
Bladder	*0.536*	*0.491*	0.171	*0.443*
Intestinal	*0.661*	*0.718*	0.140	−0.216
Visual	*0.309*	*0.404*	−0.128	*0.561*
Cerebral	*0.407*	*0.363*	0.265	*−0.556*
Other	*0.514*	0.119	*0.775*	−0.085
HADS	*0.698*	*0.744*	0.193	−0.008
Explained variance (%)	27.5 %	21.8 %	17.8 %	13.5 %

The significant loadings (>0.300) are in italics

symptoms, but also with pyramidal and sensory symptoms. Irritable bowel syndrome, obstipation or diarrhea, and fecal incontinence are common symptoms in MS that are more prevalent when depression is present [73]. In fact, gastrointestinal symptoms in MS may be explained by underlying neuronal (brain or spinal) pathways or depression-related factors [73]. Interestingly, depression is accompanied by increased IgM and IgA responses to commensal bacteria, indicating increased bacterial translocation, and the same findings were made in MS patients [74–77]. Our factor analysis showed that depression loaded on a first unrotated factor, which indicates severity of the illness, together with all neurological symptoms, excluding cerebellar symptoms. Our findings strongly suggest that depression is part of the other symptoms of MS and thus is a symptomatic manifestation of MS. Nevertheless, the best prediction of depression severity was obtained when gastrointestinal symptoms and disability progression were combined with IL-6 and albumin. This may suggest that depression in MS is at least in part related to IL-6-mediated mechanisms and the pathophysiological processes that underpin gastrointestinal symptoms and the chronic inflammatory and degenerative processes that characterize disability progression.

The findings should be interpreted in the context of its strengths and limitations. In this study, we have controlled for the putative effects of sex, age, nicotine dependence, BMI, and the drug state of the patients, i.e., the use of disease-modifying drugs, glucocorticoids, and antidepressants. Thus, although these drugs have immune-modifying effects, we may exclude the possible effects on the associations reported in this study. A first limitation of this study already discussed above is the measurement of IL-6 without concomitant assay of the sIL-6R. Future research should measure serum IL-6, sIL-6R, and sgp130 levels together with MAPK and JAK/STAT expression in patients with MS with and without depression. A second limitation is that contrast enhancement on gadolinium MRI scan persists for only 2 to 6 weeks [53], and therefore, repeated MRI scans offer more information. In addition, future research should examine the prevalence of depression in relationship to increased levels of IO&NS biomarkers in different phases and different subtypes of MS with and without depression.

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Authors' Contributions All authors contributed equally to this paper.

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