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**Endocrine and immune substrates of depressive symptoms and fatigue
in multiple sclerosis patients with comorbid major depression**

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ABSTRACT

Objective: Depression and fatigue are among the most common symptoms of multiple sclerosis (MS). These symptoms frequently co-occur and partially overlap in MS but their underlying biological substrates are unclear. In this study, we examined the relative role of cytokines and hypothalamic-pituitary-adrenal (HPA) axis activity for depression and fatigue in patients with relapsing-remitting MS (RRMS).

Methods: HPA axis function and frequency of stimulated cytokine (IFN γ and TNF α) producing T cells was measured cross-sectionally in 44 female patients with RRMS. All subjects completed a neurological exam, the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I) and self-report questionnaires.

Results: Ten patients met diagnostic criteria for MDD. MS patients with comorbid MDD showed normal morning but elevated evening salivary cortisol levels resulting in a flattened slope. While higher frequency of cytokine producing CD8⁺ T cells was also seen in MS patients with MDD, these markers were more closely associated with fatigue than depression.

Conclusions: This study supports a role of HPA axis hyperactivity for major depression in MS. In addition, inflammatory and neuroendocrine factors may differentially mediate fatigue and depressive symptoms.

Keywords: Inflammation – T cells – Cortisol – Multiple Sclerosis

INTRODUCTION

Depression and fatigue are among the most common symptoms of multiple

sclerosis (MS) (1). Depression has a point prevalence of 13-30% and a lifetime risk of 25-50% (2) with a significant impact on cognitive function (3), quality of life (4), work performance (5), and treatment compliance (6). It is also one of the strongest predictors of suicide (7). Fatigue is experienced by up to two thirds of MS patients and often perceived as the most debilitating symptom (8, 9). Importantly, fatigue represents the leading cause for absence from work (10). While this clearly illustrates the importance of depression and fatigue for the socioeconomic and psychosocial burden of MS, the underlying mechanisms are not well understood.

Earlier studies have not found evidence for an association of depression and fatigue in MS (11, 12), however, later reports have usually confirmed a moderate correlation (13-17). This suggests that while sometimes linked in MS, fatigue and depression may be mediated by at least partially independent pathological mechanisms. Given the high clinical relevance of depression and fatigue in MS, a better understanding of potentially underlying biological pathways is crucial for developing effective therapeutic strategies.

Hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis is among the most consistently reproduced biological findings in psychiatric patients with major depressive disorder (MDD) (18). A role for inflammation in the pathogenesis of depression has also been demonstrated (19). Fatigue and depressive symptoms often occur together as part of 'sickness behavior' and may share some biological correlates (20) but the differential role of inflammatory and neuroendocrine pathways remains to be elucidated.

Inflammation is the hallmark of MS and cytokines such as $\text{TNF}\alpha$, which have been implicated in major depression (21, 22), play a key role in MS pathology. Associations between cytokines such as $\text{TNF}\alpha$ and $\text{IFN}\gamma$ and fatigue have been described in MS (23,

24). In addition, a growing number of studies provide evidence for HPA axis hyperactivity in up to 50% of MS patients (25, 26) and gene variants involved in HPA axis regulation have recently been associated with MS (27). Clinical studies including both relapsing-remitting as well as progressive patients suggest that HPA axis hyperactivity in MS is associated with more progressed disease and global neurodegeneration (28-31). However, there are indications that during the earlier phases of MS, subtle HPA axis alterations may be a marker for depressive symptomatology (32).

In the current study, we examine the role of HPA axis activity and cytokines (IFN γ , TNF α) in T cell subpopulations for major depression in patients with relapsing-remitting MS. We hypothesized that patients with comorbid MDD would exhibit HPA axis hyperactivity and higher frequencies of cytokine producing T cells. Finally, we expected neuroendocrine and immune markers to be differentially associated with depression and fatigue, respectively.

METHODS

Participants and recruitment

Patients were recruited through the MS outpatient clinic at the University Hospital Hamburg Eppendorf. We contacted female patients from our database within the age range 20-50 who had been diagnosed with clinically definite relapsing-remitting MS (33). Patients were excluded if during an initial phone interview they met exclusion criteria such as a medical history of endocrine abnormalities (diabetes mellitus, uncontrolled thyroid disease), neoplasms, addiction or substance abuse, schizophrenia, or anorexia. Patients were excluded if they had a relapse or received steroid/immunosuppressive

treatment in the previous 4 weeks, or were currently taking benzodiazepines, neuroleptics or antidepressants. Patients were allowed to be on immunomodulatory therapies if the treatment had not been started or changed in the previous six months. In addition, patients were excluded if they were pregnant.

Recruitment of patients was completed over a time period of 14 months. Eligible patients were scheduled for an appointment at the MS outpatient clinic and underwent standard neurological exam to obtain clinical information and disability rating (Expanded Disability Status Scale, EDSS) (34). All procedures were approved by the Ethics committee of the Medical Board Hamburg and all subjects provided written informed consent prior to enrolment in the study.

Diagnosis of major depressive disorder (MDD) and clinical questionnaires

Diagnosis of current MDD was established by an experienced Ph.D. level clinical psychologist (K.J.Z.) using the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I). Depressive symptoms were quantified using the Hospital Anxiety and Depression Scale (35), a 14-item screening tool specifically developed to assess depression in medical and surgical settings, which excludes symptoms that might arise from somatic aspects of the illness such as fatigue. Fatigue due to MS was assessed using a recently developed and validated German version of the Modified Fatigue Impact Scale and the Fatigue Severity Scale (36).

HPA axis assessment

Salivary cortisol samples were collected using Salivette devices (Sarstedt AG, Nümbrecht, Germany). We obtained circadian profiles (awakening, 11 a.m., 3 p.m., 8 p.m., and 10 p.m.) on two consecutive days. On each day, we also collected samples for the cortisol awakening response (awakening, +15 min, +30 min, +45 min, +60).

In addition, a low dose (0.25 mg) oral dexamethasone suppression test was obtained as described (37). At 11 p.m. of day 2, subjects ingested a dexamethasone pill and salivary cortisol levels were measured the next morning. Cortisol levels were determined in duplicate by radioimmunoassay (DRG, Marburg, Germany).

Flow cytometry

Intracellular cytokines (IFN γ , TNF α) in CD4⁺ and CD8⁺ T cell populations were measured using commercially available flow cytometry kits (Becton Dickinson Fastimmune Cytokine Systems). Whole blood (500 μ l) was stimulated with 0.2 μ l/ml PHA and 5 μ l costimulatory antibodies (CD28/CD49d) in the presence of the 10 μ l secretion inhibitor Brefeldin A (BFA) at 37° C. After a stimulation period of 6 hours, 50 μ l EDTA were added to the sample in order to arrest activation and to remove adherent cells from the activation vessel. This step was followed by the simultaneous lysis of erythrocytes and fixation of leukocytes using 1 ml BD FACS Lysing Solution. Cells were then washed and permeabilized with BD FACS Permeabilizing Solution. After an additional wash, surface and intracellular staining antibodies were added in a single staining step. Finally, the cells were washed and fixed for flow cytometric analysis (FACS Calibur, Becton Dickinson). The method used a three-colour staining to identify CD4 T cell responses (anti-cytokine FITC, CD69 PE, CD4 PerCP-Cy5.5) and a four-

color staining system to identify CD8 T cell responses (anti-cytokine FITC, CD69 PE, CD8 PerCp-Cy5.5, CD3APC). CD69 staining was used to allow better clustering of cytokine positive cells and to ensure that cells defined as antigen-responsive have been sufficiently stimulated to express this activation marker. The Becton Dickinson Fastimmune CD8 Anti-HU-IFN γ detection kit includes CD3 APC to avoid misidentification of NK cell responses (CD8 dim) upon antigenic stimulus. Results are presented as % positive cells.

Statistical analysis

Corresponding cortisol values were averaged across the two days and log transformed. The circadian slope was computed using linear regression as described previously (32). As a measure of overall cortisol levels, we computed the area under the circadian profile (AUC) using trapezoidal estimation. Similarly, the area under the curve was computed for the cortisol awakening response (CAR).

Group differences between depressed and non-depressed RRMS patients in clinical characteristics, cortisol and endocrine markers were evaluated using independent samples *t* tests. If Levene's test indicated a violation of the equality of variance assumption, degrees of freedom were adjusted accordingly. When appropriate, group differences were also tested using univariate ANOVAs with disease modifying therapy (DMT) or EDSS entered as a covariate. Three group comparisons (untreated vs. interferon β vs. glatirame acetate) were conducted using univariate ANOVAs. Associations between endocrine and immune measures were tested using Pearson's correlation coefficients. Stepwise linear regression models were used to test the relative

contribution of cortisol and immune markers to depression and fatigue severity. Values of $p < .05$ were considered statistically significant. All statistical analyses were conducted using Predictive Analytics Software (PASW) Statistics 18.0 for Macintosh.

RESULTS

Forty-four female relapsing-remitting MS patients were enrolled in the study, 10 (23%) of whom met diagnostic criteria for current major depressive disorder (MDD). Patients with comorbid MDD and non-depressed MS patients were well matched (see Table 1). There were no differences in disease modifying therapies (DMTs) between the groups. As expected in this population, several patients were on symptomatic therapies including stimulants (modafinil n=3), pain/spasticity medication (ibuprofen n=2, gabapentin n=1), anti-cholinergic drugs for bladder symptoms (oxybutynin n=1, throsplumchlorid n=1), beta blocker eye drops (timolol n=1), thyroid hormones (L-thyroxin n=3), or homeopathic substances (n=3). While there was a slight difference in disability (EDSS), disease severity (as measured by EDSS disability increase per year since diagnosis) was not significantly different between the groups (see Table 1). Disease duration was not significantly different and was also not correlated with HADS depression scores ($r = -.01$, $p = .93$) or fatigue ($r = -.03$, $p = .88$).

As expected, patients with MDD had significantly higher levels of depressive symptoms as measured by the HADS depression subscale, as well as higher levels of anxiety and fatigue (see Table 1).

Table 1: Clinical characteristics of relapsing-remitting (RR) MS patients with or without comorbid Major Depressive Disorder (MDD).

	RRMS (n=34)	RRMS+MDD (n=10)	p
Age	35.8+0.7	37.2+2.2	.47
Education [years]	12.2+0.5	11.5+0.7	.47
Marital status (Single / Married / Rel / Div)	5 / 19 / 8 / 2	3 / 4 / 3 / 0	.49
Employment (FT / PT / Unemployed)	18 / 6 / 8	4 / 2 / 4	.62
BMI	25.2+1.1	24.6+1.8	.79
Day in menstrual cycle	11.5+1.5	9.3+3.5	.50
Smoking [pack years]	7.1+1.3	11.1+3.2	.17
Disease duration [years]	7.8+0.6	8.4+1.6	.66
Disability (EDSS)	2.2+0.2	3.3+0.3	.01
Progression Index	0.4+0.1	0.7+0.2	.10
DMT (none / IFN / GA)	13 / 9 / 12	5 / 4 / 1	.30
HADS Depression	2.3+0.5	11.7+1.1	<.001
HADS Anxiety	4.9+0.6	10.1+1.2	<.001
Fatigue	14.7+3.0	45.9+4.2	<.001

BMI: Body Mass Index; FT: Full time employment; PT: Part time employment; Rel: In a relationship; Div: Divorced or separated; EDSS: Expanded Disability Status Scale; DMT: Disease-modifying therapy; IFN: Interferon, GA: Glatiramer Acetate; HADS: Hospital Anxiety and Depression Scale; Progression Index: EDSS disability increase per year since diagnosis.

HPA axis activity

MS patients with co-morbid MDD showed hyperactivity of the HPA axis characterized by normal morning cortisol but elevated evening levels (Figure 1A), resulting in higher cortisol values across the day (area under the circadian curve, MS: 17.5 ± 1.1 ; MS+MDD: 22.0 ± 1.5 ; $p=.04$) and a significantly flatter slope ($p=.017$, Figure 1B). No significant differences were seen in the cortisol awakening response (CAR, $p=.88$, Figure 1C). In addition, there were no significant differences in cortisol patterns after overnight dexamethasone suppression ($p=.27$, Figure 1D). Cortisol slope was not significantly associated with CAR ($r=-.12$, $p=.43$) or post dexamethasone cortisol ($r=-.16$, $p=.30$), however, there was a significant association between CAR and post dexamethasone cortisol ($r=.45$, $p<.01$). The difference in cortisol slope between depressed and non-depressed patients remained significant after statistically controlling for EDSS ($p=.03$).

Some evidence suggests that prolonged treatment with interferon β may affect HPA axis activity in MS (38, 39), and interferon α administration has been associated with a flattening of cortisol profiles in other patient groups (40). Thus, we more closely examined the role of disease modifying therapies on HPA axis activity in our sample. Indeed, patients taking interferon β had flatter cortisol slopes that showed a statistical trend (see Supplementary Table 1, $p=.07$). However, the difference in cortisol slope between depressed and non-depressed patients remained significant after statistically controlling for disease modifying therapy ($p=.02$).

Supplementary Table 1: Association of disease modifying therapy with clinical, endocrine and immune characteristics.

	Untreated	Glatiramer Acetate	Interferon β	p
n	18	13	13	
HADS	4.6+1.3	2.5+1.1	6.1+1.4	.18
Fatigue	26.2+5.7	14.7+4.8	24.5+5.8	.32
Cortisol slope	-.07+0.01	-.08+0.01	-.05+0.01	.07
Cortisol CAR	58.1+4.0	51.9+6.3	54.3+4.9	.67
Post dex cortisol*	1.7+0.4	1.1+0.3	0.6+0.2	.11
IFN γ CD4+ [%]	14.7+1.7	12.3+0.9	9.2+1.1	.04
IFN γ CD8+ [%]	18.3+1.0	19.6+1.8	14.2+2.3	.07
TNF α CD4+ [%]	27.0+2.7	31.6+3.4	30.1+3.8	.57
TNF α CD8+ [%]	13.1+1.6	18.2+2.2	11.3+2.1	.06

CAR: cortisol awakening response, Post dex: Cortisol levels after overnight low dose (0.25mg) dexamethasone *Values are given as ng/ml, however, statistical tests were run using log-transformed cortisol values.

Immunological measures

MS patients with or without MDD did not differ in absolute numbers of lymphocyte subsets (Table 2). However, there were differences in T cell function as indicated by significantly higher proportion of TNF α producing CD8+ T cells after PHA stimulation ($p=.049$) and a trend towards higher frequency of stimulated IFN γ producing CD8+ T cells ($p=.08$) in MS patients with comorbid MDD (Figure 2). The differences in

cytokine producing CD8+ T cell populations between depressed and non-depressed patients were similar when statistically controlling for EDSS (TNF α p=.05; IFN γ p=.048).

Table 2: Absolute counts of lymphocyte subpopulations [cells/ μ l] in relapsing-remitting MS patients with or without comorbid Major Depressive Disorder (MDD).

	MS	MS+MDD	p
CD4+	812.2+54.0	889.4+84.1	.49
CD8+	350.7+19.3	387.2+53.5	.43
B cells	195.7+15.9	210.4+16.4	.64
NK cells	144.7+12.9	149.6+25.8	.86

Frequencies of TNF α and IFN γ producing CD8+ T cells were significantly correlated, however, no significant associations were found between immune and HPA axis measures (see Supplementary Table 2). IFN β therapy was associated with significantly lower levels of TNF α and IFN γ producing CD8+ T cells (see Supplementary Table 1), however, after controlling for DMTs, a statistical trend remained for both cytokines (IFN γ p=.053; TNF α p=.087).

Supplementary Table 2: Correlations between endocrine and immune markers.

	INF γ CD4+	IFN γ CD8+	TNF α CD4+	TNF α CD4+
INF γ CD4+	1.00			
IFN γ CD8+	.27	1.00		
TNF α CD4+	-.01	.39*	1.00	
TNF α CD8+	.20	.65**	.67**	1.00
Slope	-.07	-.20	-.24	.02
CAR	.21	-.25	-.10	-.18
Post dex cortisol	.26	-.08	-.16	-.01

CAR: cortisol awakening response, Post dex: Cortisol levels after overnight low dose (0.5mg) dexamethasone [log]. * $p < .05$; ** $p < .01$

Prediction of depression and fatigue severity

Using a linear regression model, cortisol slope was the only significant predictor of HADS depression severity ($R = .34$, Adjusted $R^2 = .09$, $p = .03$) while frequency of IFN γ producing CD8+ T cells was the only significant predictor of fatigue scores ($R = .44$, Adjusted $R^2 = .17$, $p = .01$).

DISCUSSION

In the present study we found that RRMS patients with comorbid MDD exhibit hyperactivity of the HPA axis as characterized by normal morning cortisol but elevated evening levels, resulting in a flattening of the circadian slope. MS patients with comorbid MDD also showed increases in cytokine-producing CD8+ T cells, however, these

immunological markers were more closely associated with measures of fatigue than with depression.

Using both a clinical diagnosis of MDD as well as a quantitative measure of depression severity that is not confounded by somatic aspects of the disease such as fatigue, our study indicates that increased evening cortisol may be clinically relevant and could represent a depression-specific biological correlate in MS.

In humans, the circadian trough of cortisol secretion is largely mediated via the high-affinity mineralocorticoid receptor (MR), while circadian peak levels are more strongly dependent on low-affinity glucocorticoid receptors (GR) (41). In line with this notion, we found no significant associations between cortisol slope and the dexamethasone suppression test, which reflects glucocorticoid receptor (GR) function. While post dexamethasone cortisol levels were associated with the awakening response, neither of these tests showed significant differences between depressed and non-depressed MS patients. Together, this suggests that major depression in MS may be mediated by MR-dependent HPA axis hyperactivity. In line with this hypothesis, elevated evening cortisol levels in an independent sample of depressed MS patients were recently linked to atrophy in the cornu ammonis 2-3 and dentate gyrus subfield of the hippocampus (32), where MR is highly expressed (42). Thus, novel strategies targeting MR signaling (43) may be promising treatment options for depression in MS.

Cytokines such as $\text{TNF}\alpha$ and $\text{IFN}\gamma$ have been implicated in the pathogenesis of ‘sickness behavior’ (19). Here, we provide evidence that the frequency of $\text{TNF}\alpha$ and $\text{IFN}\gamma$ producing CD8^+ T cells is upregulated in MS patients with comorbid MDD, but that these markers are more closely correlated with fatigue than depression. This is in line

with our previous results on associations between $\text{IFN}\gamma$ and $\text{TNF}\alpha$ with fatigue scores and daytime sleepiness in non-depressed MS patients (24). In psychiatric patients, cytokines are thought to be produced peripherally and affect CNS processes through several indirect pathways (22). MS presents a unique situation where cytokines are directly released in the CNS by invading peripheral immune cells. Although CD4^+ T cells appear to be most important for the initial steps in MS pathogenesis, the majority of T cells found in acute and chronic lesions of MS are actually CD8^+ T cells, outnumbering CD4^+ T cells 3- to 10-fold in chronically inflamed MS plaques (44). Thus, our finding of higher percentages of $\text{IFN}\gamma$ - and $\text{TNF}\alpha$ -producing peripheral CD8^+ T cells upon stimulation *ex vivo* may reflect similar responses of these CD8^+ T cells once they are restimulated in the CNS.

MS patients with MDD showed higher frequency of $\text{IFN}\gamma$ producing CD8^+ T cells despite elevated circulating cortisol levels. A similar lack of associations between increased evening cortisol, flattened cortisol curves and increases in cytokine levels have been recently shown in hepatitis C patients treated with interferon α (40). This argues for a decoupling of inflammatory mechanisms from HPA axis-mediated regulation. Interestingly, decreased sensitivity of immune cells to GC inhibition has been described both in MDD (45) and MS (46). Thus, an overshoot of inflammation despite HPA axis hyperactivity may represent a shared biological mechanism that could contribute to the high incidence of depression in MS.

Some limitations of this study need to be considered. First, our study did not include a healthy control group. Our main interest was to investigate biological substrates of depression within the MS population, i.e. what biological characteristics are seen in

MS patients who have high levels of depression compared to patients who do not. Pronounced HPA hyperactivity has been described in later stages of progressive MS, however, in the early stages of relapsing-remitting MS, HPA abnormalities are less obvious (25). In a recent study, with a sample of RRMS patients comparable to the current study, only patients with elevated depressive symptoms showed flattened cortisol profiles while non-depressed patients had circadian profiles indistinguishable from matched healthy control subjects (32). Thus, flattened cortisol slopes indeed seem to specifically identify depressed MS patients and are not characteristic for MS itself. This is corroborated and extended by our current data using a clinical diagnosis of MDD. Secondly, our data provide indirect evidence that depression in MS may be linked to MR, rather than GR, dysfunction. However, whether the observed HPA abnormalities are indeed linked to an imbalance in MR/GR needs to be further examined using selective agonists/antagonists *in vivo* or *in vitro*. Third, our study enrolled only female patients. This was done to eliminate the confounding impact of gender differences in endocrine regulation. While this approach increases the power of the study, especially in small samples, it also decreases generalizability of the findings. Thus, it remains to be determined if the same mechanisms can be observed in male patients. However, it should be noted that both RRMS as well as MDD have a female preponderance of 2:1 to 3:1 suggesting that our results may be relevant to the majority of patients. In addition, we have observed very similar HPA axis alterations in depressed RRMS patients in our previous study that enrolled both male and female patients (32). Forth, our sample of MS patients with comorbid MDD is comparatively small and these results need to be confirmed in larger studies. Lastly, due to the cross-sectional nature of this study, we

cannot infer a causal relationship between neuroendocrine and immunological abnormalities and depression or fatigue in MS. Further studies are needed to establish if changes in these systems are associated with changes in depressive symptoms or fatigue.

Taken together, our study suggests that although HPA axis hyperactivity and inflammation often coexist in depression, they may differentially mediate aspects of ‘sickness behavior’ such as mood alterations or fatigue. Our approach also indicates that using the unique pathogenetic situation of MS as a paradigm can help to further enhance our understanding of the clinical relevance of neuroendocrine and inflammatory processes with regard to medical depression and ‘sickness behavior’ in humans.

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FIGURE LEGENDS

Figure 1: Hypothalamic-pituitary-adrenal axis dysregulation in patients with comorbid multiple sclerosis (MD) and major depressive disorder (MDD). MS patients with MDD showed normal morning cortisol levels but elevated evening cortisol (**A**), resulting in a significantly flatter circadian slope (**B**). No significant differences were detected in the cortisol awakening response (CAR, **C**) or after overnight low dose dexamethasone suppression (**D**). Data are presented as mean±standard error of mean (SEM). All statistical analyses were run using log-transformed cortisol values.

Figure 2: Immunological alterations in patients with comorbid multiple sclerosis (MD) and major depressive disorder (MDD). A higher frequency of TNF α producing CD8 $^{+}$ T cells (PHA stimulated) was observed in MS patients with comorbid MDD. In addition, there was a trend towards higher percentage of IFN γ producing CD8 $^{+}$ T cells (PHA stimulated). However, no differences were found for IFN γ or TNF α producing CD4 $^{+}$ T cells. Data are presented as mean±standard error of mean (SEM).



