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### The stress-axis in multiple sclerosis: Clinical, cellular and molecular aspects

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## **HPA-axis activity in multiple sclerosis correlates with disease severity, lesion type and gene expression in normal-appearing white matter**

Jeroen Melief, Stella de Wit, Corbert van Eden, Charlotte Teunissen, Jörg Hamann, Bernard Uitdehaag, Dick Swaab, and Inge Huitinga

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## ABSTRACT

The hypothalamus-pituitary-adrenal (HPA)-axis is activated in most, but not all multiple sclerosis (MS) patients and is implicated in disease progression and comorbid mood disorders. In this post-mortem study, we investigated how HPA-axis activity in MS is related to disease severity, neurodegeneration, depression, lesion pathology and gene expression in normal-appearing white matter (NAWM). In 42 MS patients, HPA-axis activity was determined by measuring cortisol in cerebrospinal fluid (CSF) and counting hypothalamic corticotropin-releasing hormone (CRH) expressing neurons. Degree of neurodegeneration was based on levels of glutamate, tau and neurofilament in CSF. Duration of MS and time to EDSS 6 served as indicators of disease severity. Glutamate levels correlated with numbers of CRH expressing neurons, most prominently in primary progressive MS (PPMS) patients, suggesting that neurodegeneration is a strong determinant of HPA-axis activity. High cortisol levels were associated with slower disease progression, especially in females with secondary progressive MS. Patients with low cortisol levels had greater numbers of active lesions and tended towards having less remyelinated plaques than patients with high cortisol levels. Interestingly, NAWM of patients with high cortisol levels displayed elevated expression of glucocorticoid-responsive genes, such as CD163, and decreased expression of pro-inflammatory genes, such as tumor necrosis factor- $\alpha$ . Thus, HPA-axis hyperactivity in MS coincides with low inflammation and/or high neurodegeneration, and may impact on lesion pathology and molecular mechanisms in NAWM and thereby be of great importance for suppression of disease activity.

## INTRODUCTION

Multiple sclerosis (MS) is characterized by inflammatory demyelination and axonal damage in the central nervous system (CNS), leading to progressive neurological decline. Several lines of evidence implicate the hypothalamus-pituitary-adrenal (HPA)-axis in MS pathogenesis, disease progression and occurrence of comorbid mood disorders.<sup>25,89,90,128</sup> Cortisol, the end product of the HPA-axis, is immunosuppressive and routinely used in its synthetic form to treat MS relapses.<sup>39,92</sup> In the animal model for MS, experimental autoimmune encephalomyelitis (EAE), rats with low HPA-axis responsiveness indeed show higher disease susceptibility and reduced recovery rates.<sup>93,94</sup> However, clinical studies in MS patients demonstrated elevated basal cortisol plasma levels as well as marked HPA-axis hyperactivity in the combined dexamethasone-CRH test.<sup>95–99,128</sup> Post-mortem studies also showed chronic activation of the HPA-axis in MS, as indicated by enlarged adrenal glands, higher cortisol levels in the cerebrospinal fluid (CSF) and increased corticotropin-releasing hormone (CRH)-producing neurons co-expressing vasopressin (VP) in the hypothalamus.<sup>25,100,101</sup> Apparently, hyperactivity of the HPA-axis in MS is not sufficient to prevent disease. Interesting in this respect is that we and others identified MS patients with a hypoactive HPA-axis and a very severe clinical course of MS.<sup>25,95</sup> In addition, we found an inverse correlation between the number of CRH neurons and the number of active MS lesions in the hypothalamus.<sup>25</sup> In line with this, cortisol release in the combined dexamethasone-CRH test was reported to be negatively correlated to the presence and number of gadolinium-enhancing lesions in MS patients.<sup>89</sup> Thus, the HPA-axis is generally activated in MS, but patients with a hypoactive HPA-axis have particularly severe MS and more active lesions. Based on these findings we hypothesize that a hyporesponsive HPA-axis in MS is associated with increased susceptibility of normal appearing white matter (NAWM) to develop lesions, enhanced inflammatory demyelination, increased neurodegeneration and decreased remyelination in MS lesions, resulting in fast disease progression. In addition, mood disorders are strongly associated with MS as well as with abnormal functioning of the HPA-axis and were found to correlate to the extent of CNS inflammation in MS

Table 1 Overview of included subjects

NBB no.	Sex	Age	MS/C	PMD	pH	Onset	Duration	Time to EDSS6	Type	EDSS	Death cause
97-070	f	82	MS	4:30	6.38	59	23	10	PP	8-9	dehydration and kidney failure
98-176	m	83	MS	7:05	6.40	31	52	37	PP	8-9	basal pneumonia
99-056	m	72	MS	8:30	6.91	16	56	14	PP	9	respiratory insufficiency
00-024	f	52	MS	8:25	6.10	30	22	n/a	PP	9	pneumonia with respiratory insufficiency
00-124	m	64	MS	7:30	6.84	30	34	13	PP	9	cardiac arrest
01-003	m	69	MS	10:00	6.37	35	34	n/a	PP	9	pneumonia
02-025	m	77	MS	4:15	6.25	51	26	25	PP	8-9	cerebral vascular accident
02-055	f	48	MS	5:50	6.63	23	25	9	PP	8-9	legal euthanasia
02-063	f	72	MS	12:00	6.85	59	13	13	PP	8-9	pneumonia
03-020	f	68	MS	7:30	6.30	30	38	34	PP	8	aspiration pneumonia
93-136	f	39	MS	7:00	6.95	36	7	6	SP	4	legal euthanasia
94-122	m	47	MS	6:00	6.84	21	26	n/a	SP	9	legal euthanasia
95-057	m	46	MS	8:10	6.95	36	10	9	RR	6	cachexia
96-025	f	34	MS	6:50	6.46	24	10	6	SP	9	airway infection
96-026*	f	69	MS	9:15	6.40	44	25	16	SP	9	respiratory insufficiency
96-074*	f	40	MS	7:00	6.74	26	14	11	SP	8-9	dehydration
96-076*	f	81	MS	4:15	6.93	32	49	44	SP	6	cachexia
96-104	m	72	MS	4:45	6.92	50	22	22	SP	6	carcinoma of bladder and intestine
96-121*	f	53	MS	7:15	6.54	35	18	8	SP	9	pneumonia
97-006*	f	62	MS	6:45	6.49	33	29	22	SP	9	cardiac asthma
97-077	m	50	MS	5:40	6.20	33	17	10	SP	9	pneumonia
97-123	m	46	MS	3:45	6.55	23	23	6	SP	9	pneumonia
97-160*	f	40	MS	7:00	6.33	29	11	8	SP	9	aspiration pneumonia with cardiac decompensation
97-168	f	54	MS	7:00	6.70	17	37	22	SP	9	bronchopneumonia and urinary tract infection
98-009	f	70	MS	6:30	6.30	38	32	n/a	SP	9	cardiac arrest
98-158*	f	76	MS	14:15	5.93	23	53	24	SP	9	respiratory insufficiency
99-025*	f	64	MS	7:45	6.22	29	35	21	SP	9	pneumonia and dehydration
99-051	f	45	MS	10:55	6.62	31	14	n/a	SP	9	legal euthanasia
99-054*	f	58	MS	8:10	6.30	38	20	13	SP	9	legal euthanasia
99-073*	f	71	MS	8:00	6.80	47	24	30	SP	9	pneumonia
99-086*	f	71	MS	10:25	6.35	47	24	22	SP	9	respiratory insufficiency
99-109	m	70	MS	6:25	6.28	48	22	7	SP	9	aspiration pneumonia
99-119*	f	38	MS	5:15	6.55	28	10	n/a	RR	3	cardiac arrest
99-121	m	51	MS	7:50	6.36	22	29	9	SP	9	kidney failure and cachexia
00-120*	f	69	MS	13:20	6.12	43	26	10	SP	9	probable viral infection
01-018*	f	48	MS	8:10	6.55	40	8	7	SP	6.5	legal euthanasia
01-093*	f	66	MS	6:20	6.44	23	43	30	SP	9	liver failure due to cancer metastases
01-126*	f	80	MS	9:35	6.20	21	59	51	SP	9	acute leukemia
01-130	f	53	MS	10:45	6.86	26	27	16	SP	9	legal euthanasia
01-135	m	43	MS	8:30	6.48	26	17	10	SP	7.5	pneumonia
02-053*	f	48	MS	5:50	6.64	27	21	14	SP	8	heart failure
03-014	f	49	MS	5:45	6.43	29	30	23	RR	5	metastasized mamma carcinoma and dehydration
92-037*	f	61	C	7:15	7.18	-	-	-	-	-	cachexia
95-078*	f	74	C	6:40	6.70	-	-	-	-	-	cachexia
96-014*	f	54	C	8:00	6.45	-	-	-	-	-	acute renal failure
96-037*	f	80	C	6:15	6.96	-	-	-	-	-	dehydration
97-068*	f	32	C	30:00	n/a	-	-	-	-	-	bronchopneumonia
00-025*	f	68	C	5:45	6.97	-	-	-	-	-	euthanasia
01-069*	f	41	C	13:30	n/a	-	-	-	-	-	pulmonary artery hemorrhage

patients.<sup>128</sup> Therefore, we postulate that both hyper- and hypoactivity of the HPA-axis in MS may lead to an increased vulnerability for comorbid depression. To test this, 42 MS patients were studied for indicators of HPA-axis activity, disease severity (time to death and time to EDSS 6), occurrence of depressive episodes and markers for neurodegeneration in the CSF (tau, glutamate and neurofilament). Additionally, neuropathological characteristics of MS lesions were analyzed and NAWM was investigated for expression of glucocorticoid-responsive and immune-related genes.

## SUBJECTS & METHODS

### Human brain material and CSF

Hypothalami, NAWM and CSF of subjects (table 1) were provided by the Netherlands Brain Bank (NBB), Amsterdam, The Netherlands. Informed consent was obtained for brain autopsy and the use of tissue and clinical information for research purposes. Exclusion criteria were death due to sepsis and glucocorticoid treatment within 8 weeks prior to death. Clinical diagnoses of MS were confirmed by a neurologist (Prof. C.H. Polman, VUmc, Amsterdam or Dr. S. Luchetti, NIN, Amsterdam, The Netherlands). NAWM and hypothalami were dissected at autopsy and either snap-frozen or fixed in 4% w/v formaldehyde for 30 days. CSF was taken from the lateral ventricles, centrifuged to discard cells and frozen at -80°C.

Within the NBB framework, MS lesions were evaluated by a neuropathologist (Dr. W. Kamphorst, VUmc, Amsterdam, The Netherlands) for demyelination, microglia/macrophage activity and axonal damage using sections (immuno)stained with Luxol Fast Blue and Bodian, and for proteolipid protein (PLP) and human leukocyte antigen (HLA)-DR.<sup>129</sup> These detailed evaluations are documented in neuropatho-

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< **Table 3 text** NBB no.=Donor registration number of the Netherlands Brain Bank; PM-D=Post-mortem delay (h); pH=pH value of cerebrospinal fluid; Onset=Age of disease onset (years); Duration=Disease duration (years); Type=MS subtype; EDSS=Score at death on the Expanded Disability Status Scale; PP=Primary progressive MS; RR=Relapsing remitting MS; SP=Secondary progressive MS; n/a=not available; \*=Included for study of gene expression in normal appearing white matter.

logical reports, which were used to quantify different lesions types in tissue blocks dissected on macroscopic appearance of MS lesion pathology from periventricular and subcortical white matter of the cerebrum. Active lesions were defined as demyelination in the presence of lipid-laden (foamy) macrophages. Remyelination was identified by a relative decrease in myelin density throughout or around the lesion in the absence of foamy macrophages, whereas axonal damage was determined by the presence of transected axons.

Out of 7 patients that died by euthanasia, 6 cases were due to severe MS. Subjects studied for gene expression in NAWM are indicated in table 1. Further characteristics of these patients are depicted in supplementary table 1. Only the pH value of the CSF differed significantly between controls and MS patients with high ( $p=0.011$ ) and low ( $p=0.012$ ) cortisol.

### **Quantification of CRH-producing neurons and cortisol, glutamate, tau and neurofilament heavy chain in the CSF**

Numbers of CRH expressing neurons in the paraventricular nucleus (PVN) were quantified in fixed tissue as described in detail previously.<sup>25</sup> In short, serial 6  $\mu\text{m}$  frontal sections were cut on a microtome. Delineation of the PVN was determined in thionine-stained sections. During the CRH staining sessions, paired series of MS and control subjects were run together, and a section from a previous session was included to check consistency of the staining sensitivity.

Each 100th section through the PVN was stained for CRH. Neurons that showed a nucleolus and expressed CRH were counted blinded. The total number of CRH expressing neurons in the PVN was calculated on the basis of cell counts and the distance between the sections. Examples of PVN sections stained for CRH are shown in supplementary figure 1. Cortisol was measured by radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA). Enzyme-linked immunosorbent assay (ELISA) was used to measure total tau concentrations (INNOTEST® hTAUAgW; Innogenetics, Ghent, Belgium). Neurofilament heavy chain (NfH) levels were determined with an ELISA as described elsewhere.<sup>130</sup> and glutamate levels were measured with an enzymatic assay using spectrophotometry.<sup>131</sup>

**Analysis for life time occurrence of a mood disorders**

Post-hoc analysis for the lifetime occurrence of an mood disorder (i.e. depressive episode) was performed by a psychiatrist in-training (Dr. S.J. de Wit). Insufficient documentation was available for diagnosis according to standardized criteria. Therefore, the clinical files of the NBB were examined for depressive symptoms, the use of anti-depressants, diagnoses by mental healthcare professionals and reported suicide attempts in our study population. This information was used to determine an evidence score for the lifetime occurrence of an mood disorder as follows: 0=no depressive symptoms mentioned; 1=depressive symptoms mentioned, but not enough evidence for a mood disorder; 2=possible mood disorder (start anti-depressant medication around mentioned start or worsening of depressive symptoms, or suicide attempt); 3=probable mood disorder (diagnosis of depressive episode reported by a mental health care professional).

**RNA isolation, cDNA synthesis and quantitative real-time PCRs**

Series of 10 cryostat sections (20  $\mu\text{m}$  each) of subcortical NAWM were homogenized in Trizol. Sections preceding and following these series were stained for PLP (Serotec, Oxford, UK) and HLA-DP, -DQ, -DR (DakoCytomation, Glostrup, Denmark) to confirm the absence of MS lesion pathology. RNA isolation and assessment of its quality by RNA integrity number (RIN) was performed as described previously.<sup>132</sup> Synthesis of cDNA was done with the Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Quantitative real-time polymerase was performed and analyzed as described elsewhere, with minor adaptations.<sup>129</sup> The amount of cDNA used per reaction was based on an input of 5 ng original RNA in a final volume of 20  $\mu\text{l}$ . Primers used for qPCR are shown in supplementary table 1.

**Statistical analysis**

In all analyses, patients with secondary progressive MS (SPMS; n=30) and relapsing remitting (RR) MS (n=3) were considered as one group, as they represent phases of the same MS type.

Correlations were calculated using the Spearman's non-parametric correlation, except for those between glutamate, tau and NfH, which were corrected for possible confounding effects of post-mortem delay (PMD) by partial correlation analysis. For analysis of neuropathological characteristics and gene expression, Kruskal-Wallis tests were performed to compare more than 2 patient groups and Mann-Whitney tests were performed for pair-wise comparisons of patient groups. Evidence scores for lifetime occurrence of a mood disorder were analyzed using Fisher's exact test. Correlation coefficients were interpreted as follows:  $r < 0.1$ : no correlation;  $0.1 < r < 0.3$ : weak correlation;  $0.3 < r < 0.5$ : moderate correlation;  $r > 0.5$ : strong correlation. Inferential statistics are intended to be exploratory (hypotheses generating), not confirmatory, and are interpreted accordingly. The local significance level was set to 0.05. No adjustment for multiple testing was performed. Therefore, an overall significance level is not determined and cannot be calculated. The local significance level was set to 0.05. We used SPSS software version 24 (IBM, Armonk, NY, USA) for all analyses.

## RESULTS

Age was higher in primary progressive MS (PPMS) than in SPMS patients (median  $\pm$  interquartile range =  $70.7 \pm 17.3$  and  $53 \pm 23.8$  years, respectively;  $p = 0.013$ ). PMD correlated with levels of glutamate ( $r = 0.553$ ,  $p < 0.001$ ), tau ( $r = 0.384$ ,  $p = 0.017$ ) and NfH ( $r = 0.468$ ,  $p = 0.003$ ) in CSF, but not with any other parameter. Furthermore, pH did not correlate with any of the CSF parameters, including cortisol. Age correlated strongly with duration of MS ( $r = 0.645$ ,  $p < 0.001$ ), time to score 6 on the Expanded Disability Status Scale (EDSS;  $r = 0.675$ ,  $p < 0.001$ ) and moderately with cortisol levels ( $r = 0.391$ ,  $p = 0.010$ ).

### **Duration of MS strongly correlates to time to EDSS 6**

First, we investigated whether disease duration is a reliable indicator of MS severity. Duration of MS is defined as time between disease onset and death and showed a moderate negative correlation with age of disease onset ( $r = -0.340$ ,  $p = 0.028$ ). How-

ever, duration of MS correlated strongly with time to EDSS 6 ( $r=0.745$ ,  $p < 0.001$ ), which did not show a correlation with age of disease onset. This indicates that disease duration and time to EDSS 6 are reliable indicators of disease severity in this cohort. In all further analyses, total disease duration was used as the main parameter for disease severity, since data on time to EDSS 6 were not available for 14% of the patients.

**Table 2** Correlations between indicators of HPA-axis activity and severity of MS

Correlation	All subtypes	n	SPMS	n	PPMS	n
All subjects						
Cortisol – CRH	0.356 ( $p=0.021$ )	42	0.415 ( $p=0.018$ )	32	0.309 ( $p=0.385$ )	10
Cortisol – Duration	0.378 ( $p=0.014$ )	42	0.412 ( $p=0.019$ )	32	0.267 ( $p=0.455$ )	10
Cortisol – Time to EDSS 6	0.283 ( $p=0.095$ )	36	0.397 ( $p=0.036$ )	28	0.084 ( $p=0.844$ )	8
Females						
Cortisol – CRH	0.351 ( $p=0.062$ )	29	0.383 ( $p=0.065$ )	24	-0.100 ( $p=0.873$ )	5
Cortisol – Duration	0.507 ( $p=0.005$ )	29	0.556 ( $p=0.005$ )	24	0.200 ( $p=0.747$ )	5
Cortisol – Time to EDSS 6	0.477 ( $p=0.016$ )	25	0.512 ( $p=0.018$ )	21	0.400 ( $p=0.600$ )	4
Males						
Cortisol – CRH	0.280 ( $p=0.354$ )	13	0.405 ( $p=0.320$ )	8	0.600 ( $p=0.285$ )	5
Cortisol – Duration	0.025 ( $p=0.936$ )	13	-0.434 ( $p=0.283$ )	8	-0.564 ( $p=0.322$ )	5
Cortisol – Time to EDSS 6	-0.037 ( $p=0.915$ )	11	0.164 ( $p=0.726$ )	7	-0.800 ( $p=0.200$ )	4

Depicted are coefficients and p-values of Spearman's rank correlations and numbers of patients per group. Note the strong correlation of cortisol with disease duration and time to EDSS 6 in females with SPMS.

### **Cortisol levels in CSF correlate with numbers of CRH-producing neuron and indicators for disease severity**

Numbers of CRH expressing neurons correlated with cortisol levels ( $r=0.356$ ,  $p=0.021$ ; table 2). In addition, cortisol was correlated to disease severity, as we found that its levels in CSF correlated moderately with disease duration ( $r=0.378$ ,

$p=0.014$ ), which was most pronounced in female SPMS patients ( $r=0.556$ ,  $p=0.005$ ). Along with this, cortisol and time to EDSS 6 correlated most strongly in females with SPMS ( $r=0.512$ ,  $p=0.018$ ). This demonstrates that low cortisol levels are associated with fast disease progression that occurs in severe MS.

### **Glutamate in CSF correlates with numbers of CRH-producing neurons and tau levels**

To investigate whether HPA-axis activity in MS was related to neurodegeneration, markers of neuronal pathology in CSF were correlated to numbers of CRH expressing neurons in the PVN. Levels of glutamate correlated with numbers of CRH expressing neurons ( $r=0.331$ ,  $p=0.034$ ), which was most manifest in PPMS patients ( $r=0.745$ ,  $p=0.013$ ; table 3). Levels of glutamate and tau correlated moderately to each other ( $r=0.490$ ,  $p=0.002$ ), which was also most prominent in PPMS patients ( $r=0.876$ ,  $p=0.004$ ). No correlations were present between cortisol levels and markers for neurodegeneration in CSF.

### **Increase of active lesions and reduction of remyelinated lesions in patients with low cortisol and severe MS**

The median cortisol level (236 nmol/l) was used to objectively divide the patient population into two subgroups (figure 1A), allowing comparison of groups for indicators of neuronal pathology in CSF and MS neuropathology that might reveal associations with HPA-axis responsiveness. For analysis of lesion characteristics, 3 patients were excluded from analysis due to unavailability of required neuropathological information.

Proportions of SPMS and PPMS patients did not differ between the two groups according to Fisher's exact test ( $p=1.000$ ). No differences were present for levels of glutamate, tau and NfH between patients with high and low cortisol ( $p=0.197$ ,  $p=0.686$  and  $p=0.435$  respectively). Also, total numbers of MS lesions found in cerebral tissue blocks dissected for the appearance of macroscopically detectable MS lesions did not differ between the groups ( $p=0.444$ ; mean  $\pm$  SEM:  $19.3\pm 3.4$  in patients with low cortisol and  $13.9\pm 2.0$  in patients with high cortisol). Percentages of

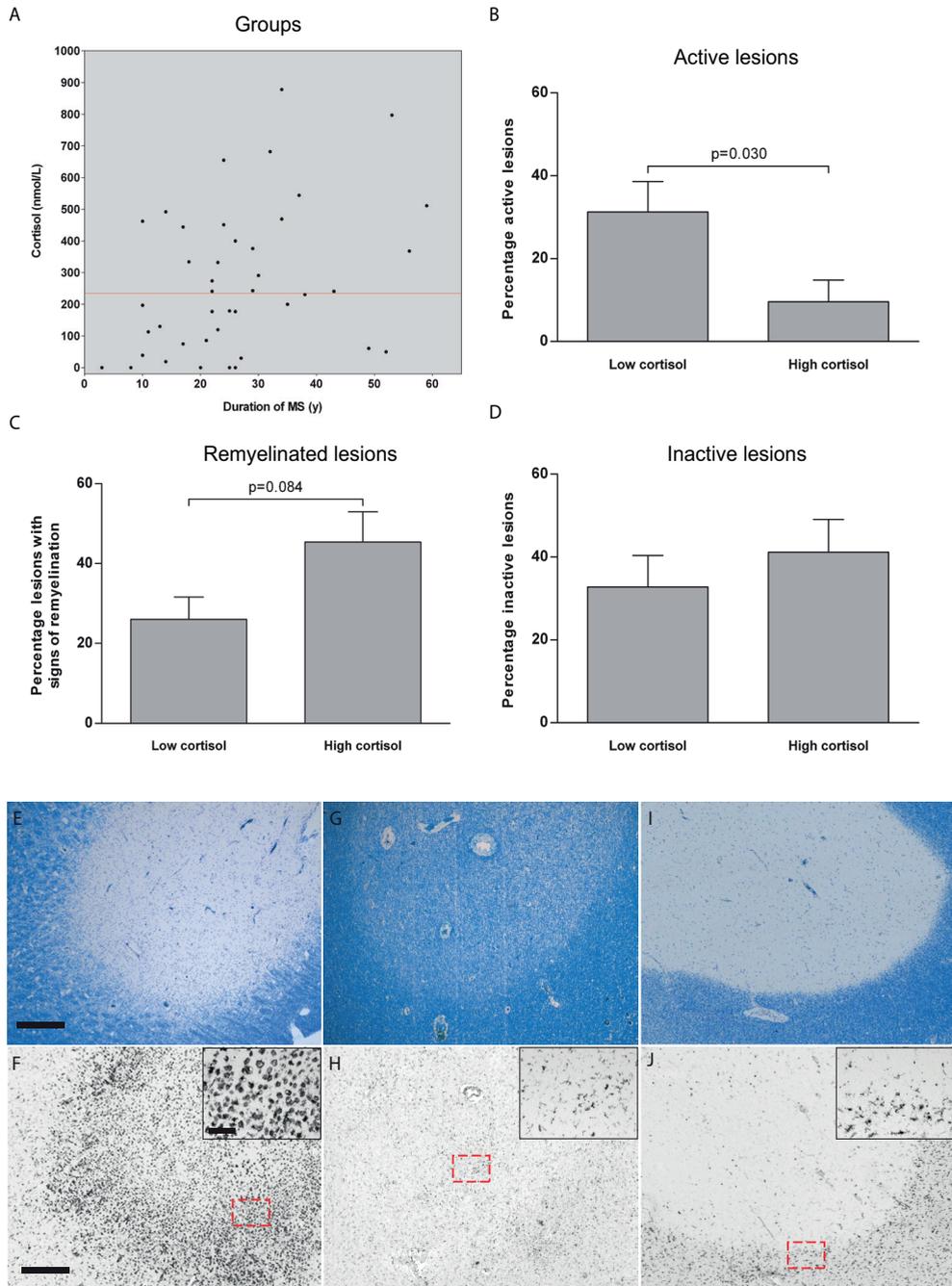
**Table 3** Correlations between parameters of neuronal pathology and HPA-axis activity

Correlation	All subtypes	n	SPMS	n	PPMS	n
<i>All subjects</i>						
Glutamate – CRH	0.331 (p=0.034)	41	0.277 (p=0.132)	31	0.745 (p=0.013)	10
Tau – glutamate	0.490 (p=0.002)	38	0.352 (p=0.066)	29	0.876 (p=0.004)	9
Tau – NfH	0.466 (p=0.004)	38	0.587 (p=0.001)	29	0.366 (p=0.372)	9
<i>Females</i>						
Glutamate – CRH	0.443 (p=0.018)	28	0.426 (p=0.043)	23	0.700 (p=0.188)	5
Tau – glutamate	0.568 (p=0.003)	26	0.320 (p=0.169)	21	0.967 (p=0.033)	5
Tau – NfH	0.347 (p=0.090)	26	0.621 (p=0.004)	21	0.474 (p=0.526)	5
<i>Males</i>						
Glutamate – CRH	-0.143 (p=0.642)	13	-0.524 (p=0.183)	8	0.600 (p=0.285)	5
Tau – glutamate	-0.235 (p=0.487)	12	-0.349 (p=0.443)	8	0.316 (p=0.796)	4
Tau – NfH	0.899 (p<0.001)	12	0.711 (p=0.073)	8	0.967 (p=0.163)	4

Depicted are coefficients and p-values of Spearman's rank correlations and numbers of patients per group. Note the strong correlation of glutamate with numbers of CRH expressing neurons and with tau in patients with PPMS.

active lesions were higher in patients with low cortisol (p=0.030; figure 1B), while in patients with high cortisol there was trend towards a significantly higher percentage of lesions with signs of remyelination in (p=0.084; figure 1C). No differences were present in percentages of inactive lesions (p=0.531; figure 1D) and lesions with axonal damage (p=0.967; data not shown). When analyzed for patients with relatively severe MS (i.e. a total disease duration shorter than the population median of 24.5 years), the patients with low cortisol had markedly higher percentages of active lesions (p=0.002) as well as strongly reduced percentages of lesions with signs of remyelination (p=0.001) compared to patients with high cortisol.

A moderate to strong negative correlation was present between percentages of active lesions and cortisol (r=-0.316, p=0.050), duration of MS (r=-0.414, p=0.009) and time to EDSS 6 (r=-0.521, p=0.002), further substantiating the latter two parameters as indicators for disease severity. Percentages of lesions with signs of remyelination correlated moderately with time to EDSS 6 (r=0.437, p=0.010) and



**Figure 1 Analysis of neuropathological evaluations of MS lesions. (A)** Scatter plot of MS duration and post-mortem cortisol levels in cerebrospinal fluid, depicting two groups defined by the median cortisol level. **(B-D)** Overview per subgroup of the percentages of macroscopically dissected cerebral lesions exhibiting various neuropathological features as described

negatively with percentages of lesions with axonal damage ( $r=-0.361$ ,  $p=0.024$ ). Examples of lesions as typically observed in patients with high and low CSF cortisol levels are depicted in figure 1E-J.

### **No association between HPA-axis activity and lifetime occurrence of mood disorders**

We also investigated our study population for lifetime occurrence of a mood disorder, which is a frequent co-morbidity in MS and associated with hyperactivity of the HPA-axis.<sup>128,133,134</sup> Again, the median cortisol level was used to objectively divide the study population into a group with low and high HPA-axis responsiveness. Though depressive symptoms were reported for 20 out of all 42 patients, no differences were found between the groups with high and low HPA-axis responsiveness for proportions of individuals with the respective evidence scores for lifetime occurrence of a mood disorder ( $p=0.416$ ; proportions in the group with low HPA-axis responsiveness were 5%, 14%, 24% and 57% for score 0 to 3 respectively; proportions in the group with high HPA-axis responsiveness were 24%, 14%, 14% and 48% for score 0 to 3 respectively).

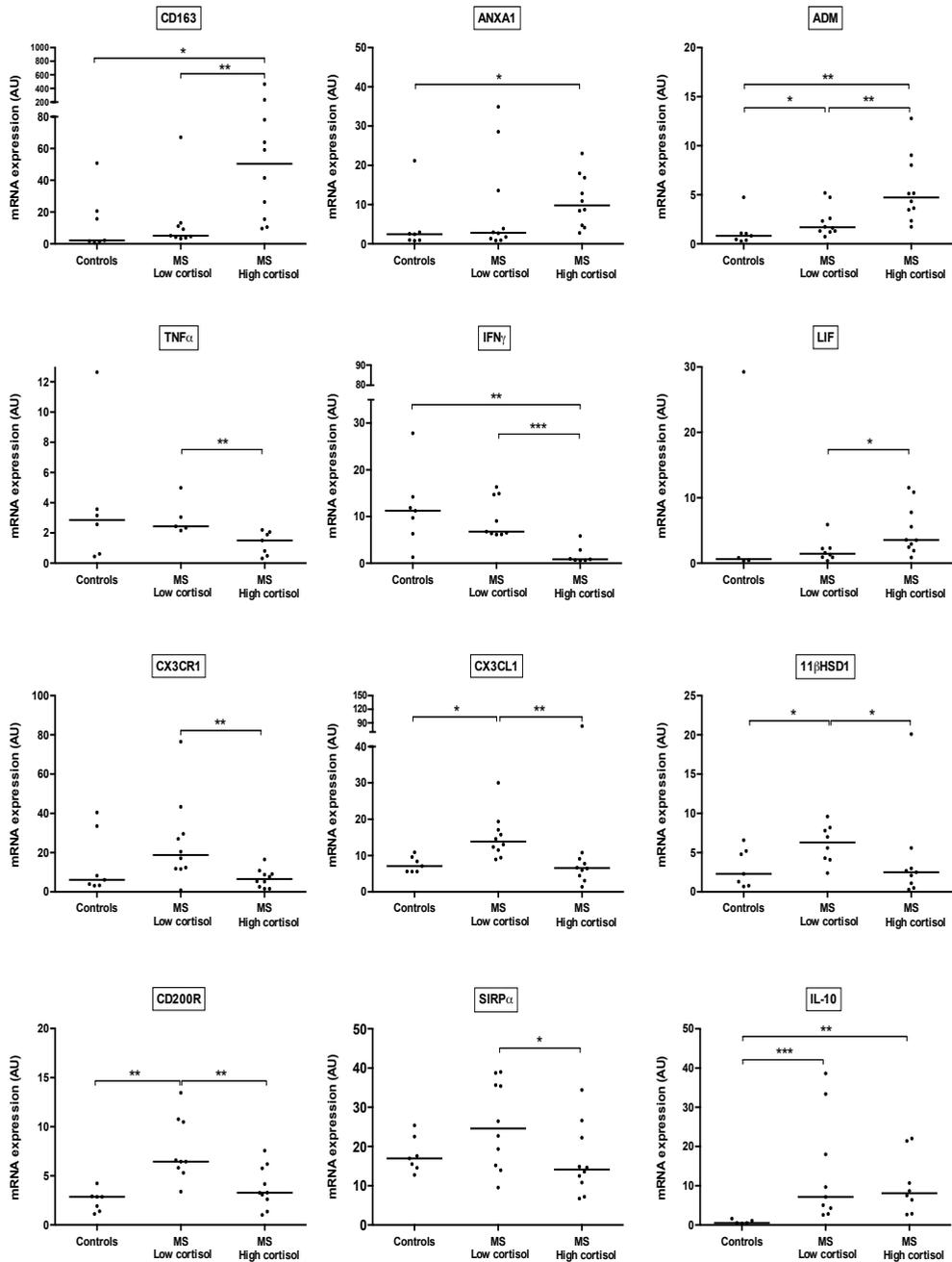
### **Glucocorticoid-associated gene expression profiles in NAWM of MS**

Finally, we studied gene expression in NAWM of female patients only, considering that cortisol and duration of MS correlated most strongly in females and thereby ensuring homogeneity of our sample. Subcortical NAWM was obtained for 20 female MS patients, which were separated into a high and a low cortisol group by the medi-

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#### **< Figure 1 legend continued**

in the pathological reports of the NBB ( $n=19$  for donors with low cortisol;  $n=20$  for donor with high cortisol).  $y$ =years. **(E–J)** Lesions as typically seen in patients in the studied population: **(E)** Klüver and **(F)** HLA-DP-DQ-DR staining of a chronic active lesion containing large amounts foamy macrophages representative for plaques most frequently observed in patients with low cortisol; **(G)** Klüver and **(H)** HLA-DP-DQ-DR staining of a remyelinated lesion as typically observed in patients with high cortisol. **(I)** Klüver and **(J)** HLA-DP-DQ-DR staining of a chronic inactive lesion more typically observed in patients with high cortisol. Scale bar represents 100  $\mu\text{m}$  for inset pictures and are demarcated by red dotted lines in pictures made at a lower magnification, in which scale bars represent 500  $\mu\text{m}$



**Figure 2 Cortisol-related gene expression in NAWM of MS patients.** Gene expression was determined by qPCR analysis. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001

an value for this parameter for objective group-wise comparison for cortisol-associated gene expression. No differences between the groups were present for RIN. Target genes were selected for glucocorticoid-responsiveness and/or a prominent role in CNS inflammation, immunoregulation or neuroprotection (see also supplementary table 2). Expression of the established glucocorticoid-responsive molecules CD163 and annexin A1 (ANXA1), as well as adrenomedullin (ADM), was indeed elevated in patients with high cortisol levels compared to those with low cortisol levels and controls (figure 2). In line with this, cortisol levels correlated with CD163 ( $r=0.584$ ,  $p=0.009$ ) and ADM ( $r=0.558$ ,  $p=0.011$ ) mRNA levels. In addition, MS patients in the high cortisol group had significantly decreased levels of the pro-inflammatory cytokines interferon-gamma (IFN $\gamma$ ) and TNF $\alpha$ , which both correlated negatively with cortisol (respectively  $r=-0.763$ ,  $p=0.001$ ;  $r=-0.699$ ,  $p=0.011$ ).

Expression of leukemia inhibitory factor (LIF), a molecule that promotes oligodendrocyte survival, was elevated in high cortisol MS patients, and correlated to cortisol ( $r=0.608$ ,  $p=0.007$ ). Expression of immunosuppressive molecules such as CX3CR1, CD200 receptor (CD200R) and signal-regulatory protein alpha (SIRP $\alpha$ ), as well as the cortisol producing enzyme 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ HSD1), was increased in patients with low cortisol levels, and correlated inversely with cortisol (respectively  $r=-0.664$ ,  $p=0.001$ ;  $r=-0.549$ ,  $p=0.015$ ;  $r=-0.516$ ,  $p=0.020$ ;  $r=-0.537$ ,  $p=0.020$ ). Negative correlations with disease duration were present for expression of IFN $\gamma$  ( $r=-0.694$ ,  $p=0.003$ ), CD200R ( $r=-0.555$ ,  $p=0.014$ ) and IL-1b ( $r=-0.526$ ,  $p=0.017$ ). Disease duration correlated positively with LIF ( $r=0.624$ ,  $p=0.006$ ) and, with a trend towards significance, ADM ( $r=0.398$ ,  $p=0.082$ ).

## DISCUSSION

In this post-mortem study we confirmed earlier findings that low HPA-axis activity in MS is associated with increased disease severity. In addition, this study shows that low cortisol levels are clearly associated more active MS lesions and tended to have less remyelination. Moreover, in patients with high HPA-axis activity, gene expres-

sion profiles in the NAWM were partially cortisol related and less inflammatory, more neuroprotective and permissive for remyelination. These data suggest that HPA-axis activation as generally observed in MS patients may directly impact on lesion activity and molecular mechanisms in NAWM, thereby reducing disease severity. Additionally, HPA-axis hyperactivity in MS may also coincide with high levels of neurodegeneration, whereas the opposite holds true for low HPA-axis activity. Interestingly, a recent study showed acute MS relapses to be associated with a decrease of cortisol levels in CSF, but not in serum, implying that cortisol levels in CSF are a more accurate indicator of actual concentrations in the brain parenchyma and directly relate to MS disease activity.<sup>126</sup>

For two reasons we analyzed NAWM rather than MS lesions for gene expression. Firstly, mRNA expression profiles in MS lesions likely reflect the severity of inflammation and neuronal damage and could therefore possibly overrule glucocorticoid-related effects. More importantly, assessing gene expression in NAWM would give us a first indication of the extent to which HPA-axis activity in MS may play a role in (preventing) the initiation of new MS lesions. Several of the genes that were up-regulated in NAWM in relation to cortisol contain a glucocorticoid response element (GRE), such as ADM and CD163. Production of ADM occurs in cerebral endothelial cells and astrocytes and through autocrine signaling at the endothelium decreases blood-brain-barrier permeability. Moreover, ADM has anti-apoptotic effects on neurons and was reported to inhibit the pro-inflammatory transcription factor NF- $\kappa$ B in macrophages.<sup>135–137</sup> Expression of CD163 is restricted to myeloid immune cells and is strongly induced by glucocorticoids in cultured macrophages and microglia and *in vivo* even in monocytes in human subjects.<sup>138–140</sup> The functional roles of CD163 are manifold. It is able to serve as a scavenger receptor for free hemoglobin, thereby preventing its toxic effects in tissue.<sup>141,142</sup> Moreover, it is thought to have immunomodulatory effects by down-stream signaling and as a soluble molecule after proteolytic cleavage.<sup>138,143,144</sup> In line with this, elevated soluble CD163 levels were reported in plasma of MS patients and were negatively correlated to cytokine levels.<sup>143</sup> Together, these data suggest that high cortisol production by the HPA-axis induces expression of molecules that may have beneficial effects on pathogenic events in MS, such as

macrophage and microglia activation, and compromised blood-brain barrier integrity.

The actual presence of enhanced glucocorticoid-induced signaling in NAWM of MS patients with high cortisol is indicated by increased expression of ANXA1, which is a key downstream mediator of anti-inflammatory effects of glucocorticoids.<sup>145</sup> Moreover, high cortisol levels in CSF were associated with decreased expression in NAWM of the pro-inflammatory cytokines IFN $\gamma$  and TNF $\alpha$ , which are both well-known targets of glucocorticoid-induced immunosuppression through NF- $\kappa$ B.<sup>146</sup> Expression levels of IFN $\gamma$  and TNF $\alpha$  in NAWM in control subjects were equal to those in MS patients with low cortisol, though the latter suffer from an inflammatory condition and may therefore be expected to display enhanced cytokine expression. One explanation for this finding is that no activate inflammation is present in NAWM. Moreover, our group showed in a previous study that expression profiles in control white matter of IFN $\gamma$ , TNF $\alpha$  and IL-1 $\beta$  were similar to those in NAWM of MS patients, even when measured directly adjacent to active and inactive MS lesions.<sup>132</sup>

The NAWM of MS patients with high cortisol levels also had decreased mRNA levels of the inflammatory chemokine CX3CL1 and its neuroprotective receptor CX-3CR1, which are both negatively regulated by glucocorticoids and play an important role in neuroinflammation by promoting leukocyte recruitment from the periphery and simultaneously suppressing microglial neurotoxicity.<sup>147,148</sup>

Interestingly, high cortisol levels and long disease duration were also associated with increased expression of LIF, which can be expressed by astrocytes and myelin-specific T cells and limits demyelination in EAE and MS by promoting oligodendrocyte survival and proliferation of oligodendrocyte progenitors, while inhibiting T helper 17 cell differentiation.<sup>149–153</sup> Moreover, LIF modulates HPA-axis responsiveness to inflammation, by reducing GR-mediated negative feedback in the hypothalamus and pituitary.<sup>154</sup>

A notable finding in NAWM of MS patients with low cortisol levels is the increase in expression of 11 $\beta$ HSD1, which enhances local glucocorticoid signaling by reduction of cortisone into cortisol. Therefore, increased expression of 11 $\beta$ HSD1 may reflect a compensatory mechanism for the limited cortisol availability in these subjects, in line with a study by Heidbrink et al.<sup>126</sup> Likewise, the NAWM of the same

patients displayed higher mRNA levels of the macrophage inhibitory molecules CD200R and SIRP $\alpha$ , possibly to compensate for inadequate glucocorticoid-mediated immunosuppression. Together, these data suggest that high cortisol levels in the CNS through various mechanisms renders NAWM less vulnerable for development of new MS lesions.

Analysis of MS neuropathology in macroscopically dissection tissue blocks indicated that HPA-axis hyperactivity also impact on lesion activity and remyelination, as high cortisol levels in CSF were associated with lower percentages of active lesions and increased amounts of remyelinated plaques. No differences were found for percentages of plaques with axonal damage, though it could have been expected that the presence of more active inflammation would be associated with axonal pathology. However, axonal injury in MS lesions was found to be independent of demyelinating activity and to be correlated primarily to numbers of macrophages and CD8<sup>+</sup> T cells.<sup>35</sup>

In PPMS patients, glutamate strongly correlated with HPA-axis activity. Indeed, glutamate, when present in the CSF or in glutamatergic projections, can activate the HPA-axis through direct binding to various glutamate receptors on CRH-producing neurons in hypothalamus.<sup>155–158</sup> A prominent positive correlation between tau and glutamate was present in patients with PPMS, which suggests that CSF glutamate is related to neurodegeneration and reflects widespread neuronal damage associated with this type of MS.<sup>159</sup> This may imply that cortisol induces neurodegeneration or, vice versa, that neurodegeneration drives HPA-axis activity. It has been described that cortisol may cause neuronal damage, but this has not unequivocally been proven in humans.<sup>160,161</sup> Conversely, high levels of neurodegeneration in MS were reported to be associated with the presence of gene alleles that lead to high glutamate levels.<sup>24</sup> Thus, glutamate levels in CSF more likely reflect MS pathology and would therefore be a major determinant of HPA-axis activity in PPMS rather than a consequence of cortisol-induced neurodegeneration.

In some MS patients in our study, low cortisol levels did not coincide with fast disease progression, whereas in others fast disease progression occurred in the presence of high cortisol levels (see also figure 1A). Changes in cortisol sensitivity

due to genetic polymorphisms in the GR might account for these observations. However, we did not find any differences in these patients for frequencies of five different GR haplotypes associated with altered glucocorticoid sensitivity (data not shown).<sup>162</sup> Apparently, multiple factors beside glucocorticoids determine MS severity.

In disagreement with previous studies, we did not find an association of cortisol levels with lifetime occurrence of a mood disorder.<sup>163</sup> However, this relation may be hard to find in our population due to the fact that patients with HPA-axis hyperresponsiveness were compared to a group of patients that besides extremely low cortisol levels had very severe disease, which probably made them more inclined to have depressive symptoms as well. Importantly, retrospective assessment of psychiatric symptoms in post-mortem studies was reported to be a reliable approach to determine lifetime occurrence of depression.<sup>164</sup> On the other hand, HPA-axis activity was found to normalize after remission of depression and the retrospective clinical data in the patient files concerning depression should be considered with caution, which may explain why post-mortem indicators of HPA-axis activity do not relate to lifetime occurrence of depression in our study population.<sup>165</sup>

Post-mortem cortisol levels in CSF are about 20-fold higher than those determined in living patients, which results from HPA-axis responsiveness to the physiological process of dying.<sup>101,166</sup> Importantly, the mild but significant correlation between cortisol levels and numbers of CRH positive neurons in the PVN clearly show that both parameters assessed post-mortem indeed reflect HPA-axis activity during life. This is also evident from the finding that post-mortem cortisol levels in CSF strongly correlate with ante-mortem levels in CSF and serum in control subjects and MS patients.<sup>101,166</sup> Also, PMD and pH value did not correlate with cortisol levels and had therefore no confounding effects.

The validity of disease duration as an indicator for severity of MS is confirmed by its strong correlation with time to EDSS 6, which is a widely accepted measure of MS severity.<sup>162,167</sup> We also studied the extensive clinical files of the brain donors for clinical symptoms in their final years. These files confirmed that many MS patients of our study group died due to causes directly related to severe MS, such as legal euthanasia and pneumonia, which makes it more likely that disease duration does

reflect MS severity. Moreover, duration of MS and time to EDSS 6 both correlated to percentages of active lesions, showing that both parameters are also associated with pathological correlates of MS disease severity.

Gender differences in MS are well recognized.<sup>168,169</sup> This study indicates that HPA-axis regulation in MS might also be gender-specific, as correlations between cortisol levels and indicators of disease severity were more prominent in female patients and absent in the male patient group. However, the fairly small sample size, especially of males, warrants verification of these findings in larger populations.

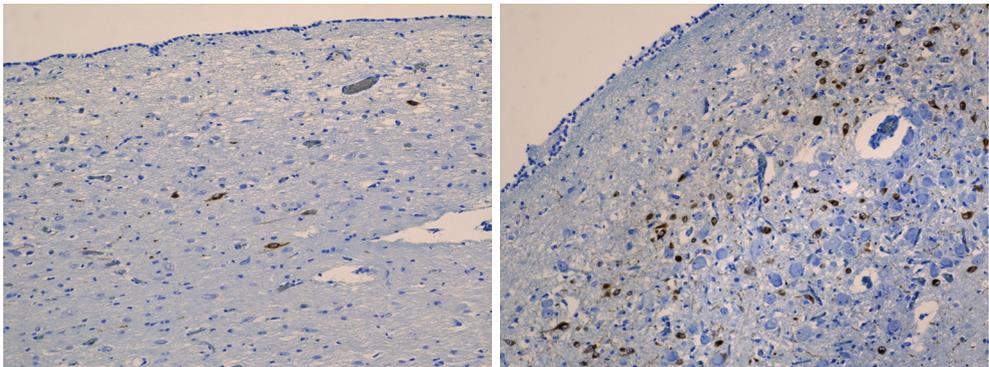
Several clinical studies reported an absent relation between HPA-axis activity and disease duration.<sup>89,96,99</sup> Since these observations were done in living patients, MS duration does not reflect the overall rate of disease progression in these studies. Importantly, patients with particularly severe MS may, due to their disability, be less inclined to participate in these studies and thus be underrepresented in these data. Our findings also seem to contradict reports that high HPA-axis responsiveness in MS patients is predictive for fast disease progression and associated with cognitive and neurologic disability.<sup>90,96</sup> However, observations made in those studies may very well reflect HPA-axis activation due to a high rate of neurodegeneration early in the disease course, which is associated with rapid neurological decline during follow-up.

The cause of low HPA-axis responsiveness in some MS patients is not clear. Experimental studies show that disease progression is known to be strongly associated with a specific desensitization of the HPA-axis to pro-inflammatory cytokines.<sup>94</sup> Many of the aforementioned studies applied the combined dexamethasone-CRH test to determine HPA-axis functioning in MS, which does not give information on responsiveness of CRH neurons to inflammatory mediators. Clearly, there are ethical and practical limitations to assessment of HPA-axis responsiveness to inflammation in MS patients. However, patients that fail to produce adequate cortisol levels in response to the inflammatory component of MS might be at particular high risk for rapid neurological decline.<sup>22</sup> Therefore, finding appropriate ways to evaluate MS patients for HPA-axis responsiveness to inflammation might be of considerable prognostic relevance.

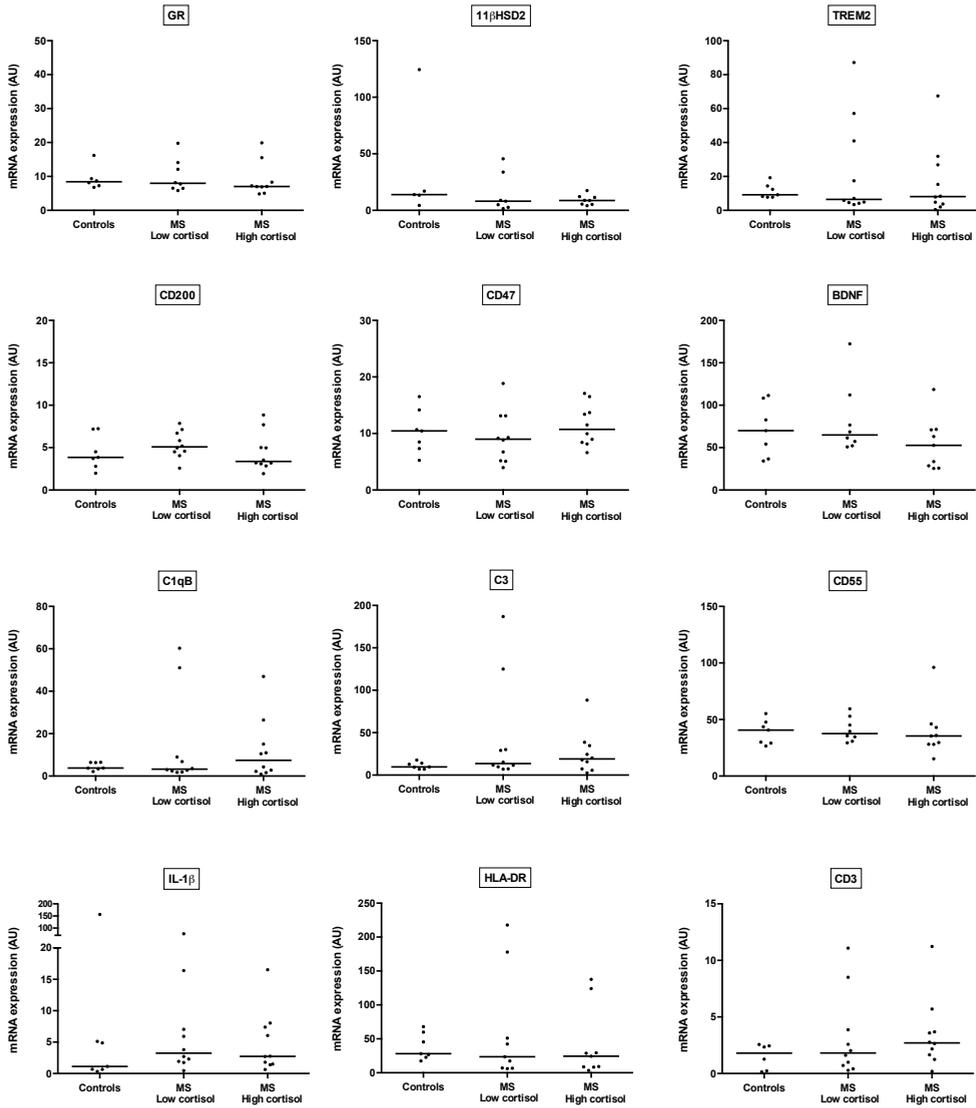
## ACKNOWLEDGMENTS

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## SUPPLEMENTAL DATA



**Supplementary figure 1 Representative immunostainings used for counting CRH producing neurons.** Immunostaining for CRH in the periventricular nucleus (PVN) of the hypothalamus in a subject with only low (left panel) and high (right panel) numbers of positive neurons. Sections are taken from identical levels in the PVN.



**Supplementary figure 2 Non-regulated genes in NAWM of MS patients.** Gene expression was determined by qPCR analysis. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

**Supplementary table 1** Characteristics of subjects studied for gene expression in NAWM

	Controls (n=7)		MS low cortisol (n=10)		MS high cortisol (n=10)	
	Median	IQR	Median	IQR	Median	IQR
Age (y)	61	41.0-71.0	55	46.0-69.0	70.5	59.8-77.0
MS type (SP/PP)	-	-	9/1	-	9/1	-
MS duration (y)	-	-	20.5	10.8-28.3	26.5	21.8-45.5
Cortisol (nmol/L)	-	-	100	29-178	472	310-662
PMD (h:m)	8:00	5:45-13:30	8:00	5:40-8:40	7:05	6:27-9:47
pH CSF	7	6.5-7.1	6.4	6.2-6.6	6.4	6.3-6.6
RIN	7.4	7.1-8.4	8	6.6-8.4	7.7	6.7-7.9

Characteristics of patients included for study of gene expression in normal-appearing white matter. RIN=RNA integrity number; IQR=interquartile range.

**Supplementary table 2** Sequences of primers used for qPCR

Gene	NCBI database no.	Primer sequence forward	Primer sequence reverse
<i>Related to inflammation</i>			
CD163	NM_004244.5	AAGACGCTGCAGTGAATTGCA	GGATCCCGACTGCAATAAAGGAT
IL10	NM_000572.2	TGCCTTCAGCAGAGTGAAGACTT	TCCTCCAGCAAGGACTCCTTTA
TNF	NM_000594	ATGTCCCAAGACAAGGGCAAGA	GGGCAGTCAATGGTCAGATATTGA
IL1B	NM_000576.2	CCAGCTACGAATCTCGGACCACC	TTAGGAAGACACAAATTCATGGTGAAGTC
IFNG	NM_000619	GCAAGATCCCATGGGTTGTGT	CTGGCTCAGATTGCAGGCATA
HLA-DR	NM_019111.4	CCCAGGGAAGACCACCTTT	CACCCTGCAGTCGTAAACGT
C1QB	NM_000491	CCGCTTCGACCACGTGAT	AGTAGAGACCGGGCACCTT
C3	NM_000064	CGGACGGTCATGGTCAACAT	ATGTCCCAAGACAAGGGCAAGA
CD55	NM_00574	GGGCAGTCAATGGTCAGATATTGA	GGCTGTTTGAGGGATGCAGAAT
CD3	NM_000733	AAGCAAACCAGAAGATGCGAACTT	CCAGTGATGCAGATGTCCACTATGA
<i>Related to neuroprotection</i>			
BDNF	NM_001143816.1	AAAAGGCATTGGAACCTCCAG	GCCAGCCAATCTCTTTTTGTC
LIF	NM_002309.4	ATGAACCAGATAGGAGCCAACT	CCACATAGCTTGCCAGGTTGTT
ADM	NM_001124.1	ATGAACCAGATCAGGAGCCAAC	CTGTGGACATCCCGCAGTT
<i>Related to glucocorticoid signaling</i>			
GR	NM_001018077	GGATCCCGACTGCAATAAAGGAT	CAACCGATCCACCTCACCTT
ANXA1	NM_000700.1	TGAAGTGCGCCACAAGCAA	GCCTTATGGCGAGTTCCAACA
HSD11B1	NM_005525.3	ATGGGTTCTTCTCCTCCATC	TATCCAGAAACTGCCTTCA
HSD11B2	NM_000196.3	CCAAACCAGGAGACATTAGCC	CAGCTCCGCATCAGCAACTA
<i>Related to immunoregulation</i>			
CX3CL1	NM_002996	CAGGCCACAAGACCCTTGT	GCCCAGCCTCAGGAAAGAAT
CX3CR1	NM_001337	TTGGCCTGGTGGGAAATTTGT	AGGAGGTAAATGTCGGTGACACT
CD200	NM_005944.5	CCAGGAAGCCCTCATTGTGA	TCTCGCTGAAGGTGACCATGT
CD200R	NM_138806	GAGCAATGGCACAGTGACTGTT	GTGGCAGGTACCGGTAGACA
CD47	NM_001777	ATGGAGCTCTAAACAAGTCCACTGT	TGTGAGACAGATCACTCTTATCCAT
SIRP $\alpha$	NM_080792	GTCTGGAGCAGGCACCTGA	GGACTCGCAGGTGAAGCT
TREM2	NM_018965.3	CCACCCACTTCCATCCTTCT	GTCCCTGGCTTCTGTCCAT