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

Melief, J.

Publication date
2014

[3]

Glucocorticoid receptor haplotypes conferring increased sensitivity (*BclI* and N363S) are associated with faster progression of multiple sclerosis

Jeroen Melief, Jan Willem Koper, Erik Endert, Holger Møller, Jörg Hamann, Bernard Uitdehaag, Dick Swaab, and Inge Huitinga

ABSTRACT

High cortisol production in multiple sclerosis (MS) is associated with suppressed disease activity. Cellular effects of cortisol are primarily mediated by the glucocorticoid (GC) receptor (GR). Therefore, GR polymorphisms associated with altered GC sensitivity may affect MS disease activity by changing inhibition of the hypothalamus–pituitary–adrenal (HPA)-axis and/or immunosuppression. GC-induced immunosuppression of microglia and macrophages coincides with increased expression of CD163, which is shed at elevated rates in MS. In this post mortem study, we investigated whether GR haplotypes are related to MS disease course and levels of cortisol and soluble CD163 (sCD163) in CSF. In total 137 MS brain donors were genotyped for five GR polymorphisms (9β, ER22/23EK, *Tth111*I, *Bcl*I and N363S). Cortisol and sCD163 levels in CSF were determined by radioimmunoassay and enzyme linked immunosorbent assay, respectively. A more aggressive disease course defined by disease duration and time to Expanded Disability Status Scale 6 (EDSS 6), was associated with GR haplotypes ($p=0.006$) that confer increased GC sensitivity. Cortisol and sCD163 levels in CSF correlated to each other ($r=0.494$, $p < 0.001$), but did not differ between GR haplotype carriers. From this, we conclude that GR haplotypes conferring high GC sensitivity coincide with more aggressive MS but do not affect cortisol secretion by the HPA-axis or CD163 shedding.

Introduction

Accumulating evidence indicates that the clinical course of multiple sclerosis (MS) is strongly affected by cortisol, the main endogenous glucocorticoid (GC) hormone, which is released by the adrenal glands as final product of hypothalamus–pituitary–adrenal (HPA)-axis activity.[89,170] While several studies show that the HPA-axis is generally activated in MS, we and others found that patients with a hypoactive HPA-axis have particularly severe MS and more active lesions.[25,89,91,95–97] In post-mortem cohorts, we identified a negative correlation between the number of corticotropin-releasing hormone (CRH)-expressing neurons and the number of active lesions in the hypothalamus of MS patients.[25] Moreover, high post-mortem levels of cortisol in cerebrospinal fluid (CSF) were associated with a more benign disease course, lower amounts of active lesions, and increased numbers of remyelinated plaques in the cerebrum of MS patients.[170] In addition, neurodegeneration was found to be a strong determinant of HPA-axis activity. Notably, the same study indicated that normal-appearing white matter (NAWM) of patients with high cortisol and slow disease progression displayed elevated expression of GC-responsive and protective genes, such as CD163, and decreased expression of pro-inflammatory genes, such as tumor necrosis factor-α (TNFα). Thus, cortisol hypersecretion by the HPA-axis in MS coincides with low inflammation and/or high neurodegeneration, while it may impact on lesion pathology as well as molecular mechanisms in NAWM and thereby suppress disease activity. Consequently, MS patients may benefit from genetic predispositions associated with enhanced GC sensitivity of the immune system and/or low GC-mediated inhibition of the HPA-axis.

The cellular effects of GC are mediated by the GC receptor (GR), which exerts it immunomodulatory effects through transcriptional regulation via protein–protein interactions with transcription factors, such as NF-κB, and by binding to conserved DNA motifs in gene promoter regions known as a GC-response elements.[171] The GR is encoded by a gene (NR3C1) on chromosome 5q31–33, and is a member of the nuclear receptor subfamily 3. Various genetic polymorphisms of the GR have been described: N363S (rs6195), ER22/23EK (rs6189 and rs6190),) *Bcl*I (rs41423247), 9β

(rs6198) and *Tth111*I (rs10052957). The N363S (rs6195) and ER22/23EK (rs6189 and rs6190) polymorphisms are located in the transactivation domain of the GR gene and are associated with, respectively, increased and reduced GC sensitivity *in vitro* and *in vivo*, as determined by the dexamethasone suppression test (DST).[172–175] It has been reported that the *Tth111*I polymorphism is not functional, as no differences were found in cortisol values at baseline or after DST, though another study found that homozygous carriers of the *Tth111*I minor allele showed higher total and evening cortisol values with a trend for elevated diurnal cortisol levels.[176,177] Carriers of the ER22/23K polymorphism by definition also had the minor *Tth111*I allele and showed less cortisol suppression after DST compared to the carriers of the *Tth111*I minor allele only.[178] The 9β polymorphism leads to more stable expression of the GRβ and may thereby decrease GC sensitivity.[179] The *Bcl*I polymorphism is associated with increased GC sensitivity, as elderly carriers of the minor allele have lower cortisol levels after DST.[180] Together, these polymorphism make up 6 haplotypes, some of which are associated with altered GC sensitivity.[174,177] MS patients carrying the *Tth111*I-ER22/23EK-9β haplotype, which is associated with decreased GC sensitivity, were found to be have a more aggressive disease course.[23] GR polymorphisms were also shown to be associated with disease severity and/or susceptibility in other autoimmune disorders, such as rheumatoid arthritis and Crohn's disease.[181,182]

In vitro, GC induce in microglia and macrophages an anti-inflammatory phenotype that coincides with upregulation of the scavenger receptor CD163, a molecule that is thought to have immunomodulatory effects.138,139,143,183 Moreover, increased membrane expression of CD163 and shedding of soluble CD163 (sCD163) is a common feature of macrophages in various chronic inflammatory disorders, including MS.184–187 As cortisol and GR polymorphisms have been implicated in progression of MS, we performed a post-mortem study in 137 MS brain donors of the Netherlands Brain Bank to investigate the association of known genetic polymorphisms in the GR with MS disease progression, cortisol production by the HPA-axis and sCD163 levels in the CSF. With this, we aimed to explore the prognostic relevance of assessing GR haplotypes and CSF levels of cortisol and sCD163 in MS patients.

SUBJECTS AND METHODS

Patients and controls

In total, 137 donors with MS were included for genetic analysis, while cortisol and sCD163 were measured in CSF of respectively 104 and 118 of these subjects. For an overview of the characteristics of the study population, see table 1. All human material was provided by the Netherlands Brain Bank (www.brainbank.nl). Informed consent was obtained for brain autopsy and the use of tissue and clinical information for research purposes. Clinical diagnoses of MS were confirmed by a neurologist (Prof. C.H. Polman, VU Medical Center, Amsterdam or Dr. S. Luchetti, NIN, Amsterdam, The Netherlands). DNA was isolated from post-mortem brain tissue dissected from the cerebellum or blood leukocytes, while CSF was taken from the lateral ventricles, centrifuged to discard cells and frozen at -80ºC. For analyses using disease duration as an indicator for MS severity, two donors were excluded because of premature death due to causes unrelated to MS (euthanasia due to cancer and sudden death). Excluded for analysis of cortisol or sCD163 levels in CSF were donors that died due to sepsis (n=17) or that had been treated with synthetic GC 2 months prior to dying (n=14), as these factors are known to impact on HPA-axis activity and endogenous GC levels.

GR genotyping

To compare our study cohort with a reference population of healthy controls, we used data obtained from a total of 317 healthy Caucasian blood donors, described previously.[23] DNA was extracted according to standard techniques. Briefly, 150 mg of brain tissue was homogenized and incubated with 20 mg/ml proteinase K for at least 4 h at 56ºC, followed by DNA extraction using phenol–chloroform–isoamyl alcohol. Subsequently, DNA was purified on a column using a DNA Midi kit (Qiagen, Hilden, Germany), precipitated with isopropanol, and diluted in Tris-EDTA buffer. DNA was genotyped by allelic discrimination using the TaqMan ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and TaqMan Universal PCR master mix (Applied Biosystems, Branchburg, NJ, USA). The poly-

Table 1 Characteristics of included subjects

Characteristic	Value
Total no. of subjects	137
No. of females (percentage)	91 (64%)
Median age (interquartile range)	66 yr (55–78)
Median age at onset (interquartile range)	32 yr (23–41)
Median duration (interquartile range)	26 yr (14–38)
Median time to EDSS6 (interquartile range)	15 yr (5–26)
No. of SPMS patients	77
No. of PPMS patients	34
No. of subtype undetermined	26

No.=number; EDSS=expanded disability status scale; SPMS=secondary progressive multiple sclerosis; PPMS=primary progressive multiple sclerosis

morphisms 9β (rs6198), *Tth111*I (rs10052957), ER22/23EK (rs6189 and rs6190, respectively), N363S (rs6195), and *Bcl*I (rs41423247) were determined as described previously.[178,188] In 137 patients, all five polymorphisms were determined, and six haplotypes were inferred, based on Bayesian linkage disequilibrium analyses using the Phase Reconstruction Method version 2.1, a statistical approach for haplotype reconstruction.[19]

Measurement of cortisol and sCD163 in CSF

sCD163 was measured using a non-commercial enzyme-linked immunosorbent assay (ELISA) essentially as described before.[190] Samples were initially diluted 1:4. A few samples were rerun in higher dilutions. Control samples and standards traceable to purified CD163 were co-analyzed in each run. Cortisol was measured by radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA).

Statistical analysis

We analyzed whether the distributions of genotypes deviated from Hardy–Weinberg equilibrium. Log-rank test was used to study carriers and non-carriers of the individual GR haplotypes for differences in disease severity, which was based either on total disease duration (i.e. time between disease onset and dying) or time to reach score

Table 2 Allele frequencies in the studied MS donor population compared to a general population of healthy blood donors

Allele	MS cohort (n=137)	Blood donors (n=317)
WT	44.1%	42.0%
*Bcl*I	19.8%	23.0%
*Tth111*I	15.3%	14.0%
9β	14.2%	14.0%
ER22/23EK	3.1%	2.5%
N363S	3.5%	4.5%

WT=wild type

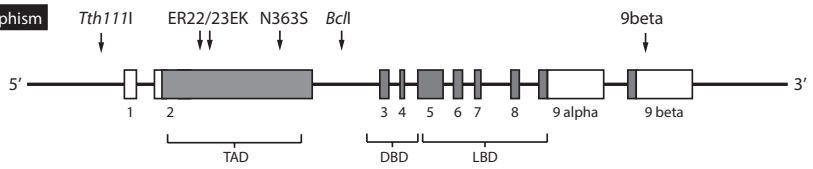
6 on the Kurtzke Expanded Disability Status Scale (EDSS 6). Correlations were calculated using the Spearman's non-parametric correlation test. Mann–Whitney U tests were used to make group-wise comparisons. Chi-square test was applied to compare clinical MS subtypes for proportions of subjects with an allelic makeup of the GR associated with normal, reduced or increased GC sensitivity. A p-value< 0.05 was considered significant in all tests.

RESULTS

Genotype distribution of polymorphisms and disease susceptibility

We studied six different GR haplotypes in a post-mortem cohort of 137 MS brain donors. An overview of their genetic makeup and relative GC sensitivity is depicted in figure 1A. To investigate whether the haplotypes are involved in susceptibility to MS, we compared their relative frequencies with those in a cohort of 317 healthy blood donors, which were genetically characterized for GR polymorphisms.[162] No differences were found in frequencies of any of the haplotypes in the studied MS brain donors as compared to frequencies in the general population (table 2). Moreover, no differences were found between carriers and non-carriers of the studied GR haplotypes for the age of onset or subtype of MS (data not shown).

A



Haplotype	GC-sensitivity	n
1. Wildtype	Normal	122
2. *BclI*	High	52
3. *Tth111I BclI*	Normal	44
4. *Tth111I* 9β	Low	39
5. N363S	High	9
6. *Tth111I* ER22/23EK 9β	Low	8

Group	Allelic makeup	n
GS-N	N/N	50
GS-H	H/H and H/N	44
GS-L	L/L and L/N	33
Excluded	H/L	10

B

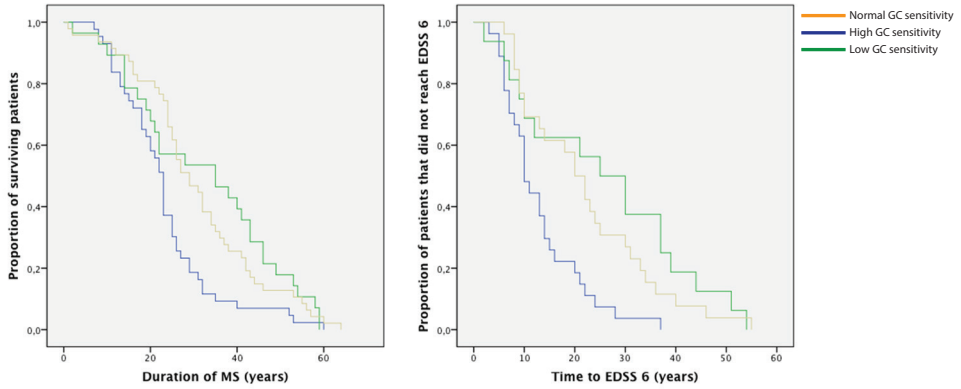


Figure 1 Duration of MS and time to EDSS 6 versus GR haplotypes associated with altered GC sensitivity. A. Schematic representation of the GR polymorphisms and haplotypes investigated in this study and their grouping based on GC sensitivity. TAD=transactivation domain; DBD=DNA binding domain; LBD=ligand binding domain; GS=GC sensitivity; N=normal (GC sensitivity); H=high (GC sensitivity); L=low (GC sensitivity). B. Kaplan–Meier curves for duration of MS and time to reach EDSS 6 for the patient groups with normal, high and low GC sensitivity, based on the grouping shown in panel A. For both duration of MS and time to EDSS 6 the curves differ significantly (respectively p=0.006 and p=0.001, log-rank test).

No association of GR haplotypes with MS subtype or gender

No differences were present between genders or clinical MS subtypes, for proportions of subjects with an allelic makeup of the GR associated with normal, reduced or increased GC sensitivity (data not shown).

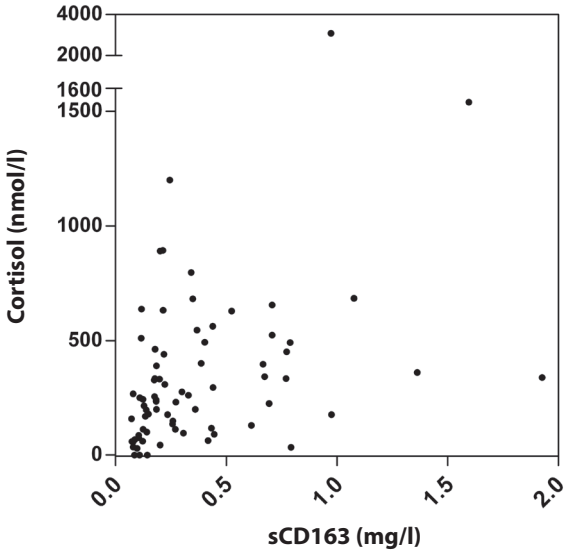


Figure 2 Scatterplot of sCD163 and cortisol levels in CSF. Levels of cortisol and sCD163 in CSF were correlated (r=0.494, p < 0.001).

More severe MS in patients with high GC sensitivity

Based on the haplotypes of both alleles, patients were divided into groups with normal (GS-N), high (GS-H) or low (GS-L) GC sensitivity (figure 1A). Figure 1B shows Kaplan–Meier survival curves for the three groups, which differed significantly from each other in relation to both total disease duration (p=0.006) and time to EDSS 6 (p=0.001). In relation to disease duration, pair-wise comparison of Kaplan–Meier survival curves indicated that MS patients in the GS-H group had a more aggressive disease than those in the GS-N (p=0.004) and GS-L (p=0.020) group, whereas no difference in survival was present between the GS-N and GS-L group (p=0.578). Similarly, pair-wise comparison of Kaplan–Meier survival curves in relation to time to EDSS 6 also revealed a more aggressive course of MS in the GS-H group compared to the GS-N (p=0.003) and GS-L (p=0.002) group, and no difference in survival between the GS-N and GS-L group (p=0.418). Analysis for the haplotypes separately indicated that carriers of both the *Bcl*I and N363S haplotype, both associated with

increased GC sensitivity, had a more aggressive course of MS, as indicated by a shorter disease duration compared to non carriers (p=0.007 in both cases; table 3). The same observation was made when using time to EDSS 6 as parameter for disease severity (p=0.002 for *Bcl*I; p=0.001 for N363S; data not shown). This analysis also indicated that MS progression was slower in carriers of the wild type (WT) haplotype compared to the WT non-carriers, possibly due to a relatively high percentage of MS patients with a *Bcl*I or N363S haplotype among the WT non-carriers (67%), in comparison to the WT carriers (22%). No differences were found between the GS-N, GS-H and GS-L groups in CSF levels of cortisol (p=0.837) or sCD163 (p=0.395).

Table 3 Comparison of survival between carriers and non-carriers of the studied GR haplotypes

Haplotype	Mean duration ± SEM	n	Mean duration ± SEM	n	Log Rank test
	Carriers		**Non-carriers**		
WT	30.1 ± 1.5	94	22.4 ± 2.1	43	p = 0.011
*Tth111*I - *Bcl*I	26.5 ± 2.6	37	28.0 ± 1.5	100	p = 0.755
*Bcl*I	23.5 ± 1.7	46	29.8 ± 1.7	91	p = 0.007
N363S	18.7 ± 2.6	9	28.3 ± 1.4	128	p = 0.007
*Tth111*I - 9β	27.4 ± 3.0	35	27.7 ± 1.4	102	p = 0.970
*Tth111*I - ER22/23EK - 9β	38.0 ± 4.2	8	26.9 ± 1.3	129	p = 0.201

SEM=standard error of the mean

Cortisol correlates to sCD163 levels in CSF

Neither pH, post-mortem delay (PMD) nor age correlated with cortisol and sCD163 levels in CSF. Cortisol levels in CSF correlated to those of sCD163 (n=76, r=0.494, $p < 0.001$; figure 2). The correlation between cortisol and sCD163 was strongest in the GS-L group (r=0.754, p=0.001) and less so in the GS-N (r=0.447, p=0.010) and GS-H (r=0.419, p=0.041) groups. No correlations were present between cortisol and disease duration (r=-0.094, p=0.673) or time to EDSS 6 (r=0.050, p=0.713). Similarly, sCD163 levels in CSF did not correlate with disease duration (r=-0.064, p= 0.562) or time to EDSS 6 (r=0.141, p=0.292).

DISCUSSION

This study investigated whether GR haplotypes associated with altered GC sensitivity are related to clinical severity of MS, and levels of cortisol and sCD163 in post-mortem CSF. We show that faster disease progression occurs in MS patients in the GS-H group, who have an allelic makeup of the GR that is associated with increased GC sensitivity due to the presence of the *Bcl*I or the N363S haplotype. The GS-N, GS-L and GS-H groups did not differ in their levels of cortisol and sCD163 in CSF. At the same time, levels of cortisol were correlated to those of sCD163. Together, these data suggest that the faster MS progression in the GS-H group is not related to a change in cortisol production by the HPA-axis or altered expression and/or shedding of CD163. However, sCD163 may be a good biomarker of cortisol-induced phenotypes in macrophages and microglia in MS patients.

Our data are in contrast with previous reports that studied the same GR haplotypes and did not find an association of the *Bcl*I and N363S haplotype with faster progression of MS.[23,191] In fact, these studies indicated that a more aggressive disease course occurs in MS patients carrying the *Tth111*I-ER22/23EK-9β haplotype, which is associated with a reduced GC sensitivity.[23,191]

One reason that we could not confirm this finding may be that our study population was relatively small and may thus lack the power to demonstrate the effect of the Tth111I-ER22/23EK-9β haplotype on MS disease course, especially since the carriers of this haplotype represent only an minor percentage of the population. Of note is that the studies investigated the effect of GR polymorphisms on MS disease course by comparing carriers and non-carriers. A consequence of this approach is that subjects having the Tth111I-ER22/23EK-9β haplotype are included in the group of non-carriers, thus decreasing survival in the group used as a reference when studying the effect of the BclI and N363S haplotype on the clinical course of MS. This may in part explain the discrepancy with the study by van Winsen et al. regarding the association of this polymorphism with more aggressive MS.[23,191] However, we also analyzed our data by comparing carriers and non-carriers of each haplotype and actually found an association between the BclI and N363S haplo-

type and a more severe MS disease course, which is still in sharp contrast with the previous studies.[23,191] A final, and more likely, explanation for these discrepancies is that about 50% of the patient population studied by van Winsen and co-workers had relapsing-remitting MS, whereas population consisted of already deceased patient of which the large majority had progressive MS.[162] Perhaps the effects of GR haplotypes on MS course are different depending on the clinical subtype or disease stage. New insights may thus be gained if data from the MS populations used in previous studies would be combined with data from our cohort and re-analyzed, also for the separate clinical subtypes and with different types of grouping of the GR haplotypes carriers (i.e. carriers versus non-carriers or grouped by GC sensitivity).

Given the fact that cortisol is protective in MS, it seems counterintuitive that increased GC sensitivity due to presence of the *Bcl*I or N363S GR haplotype leads to more aggressive MS, unless it would lead to inhibition of HPA-axis activity through increased negative feedback without potentiating effects of GC on immune cells. Indeed, *in vitro* experiments on leukocytes failed to demonstrate an association of the N363 and *Bcl*I minor allele with differences in affinity and concentration of the GR.[172,192] It has also been convincingly shown that the N363S and *Bcl*I haplotype are associated with a increased sensitivity of the HPA-axis to GR-mediated suppression, as carriers in a well-sized population of healthy elderly had lower cortisol levels after DST, assessed at two time points with different dosages of dexamethasone.[172,180] However, our study does not provide evidence for this, considering that CSF cortisol levels did not differ between the GS-N, GS-H and GS-L group nor between carriers and non-carriers of the *Bcl*I or N363 haplotype. It has been shown that post-mortem cortisol levels in the CSF reflect HPA-axis responsiveness to the process of dying, rather than basal activity. The physiological process of dying induces a strong HPA-axis response that leads to a rise in cortisol levels, which are well correlated with numbers of CRH positive hypothalamic neurons post-mortem and with ante-mortem cortisol levels in CSF and serum.[101,166,170] However, HPA-axis responsiveness to dying may not necessarily reflect GC-induced negative feedback, in contrast to well-controlled DST in living subjects. Thus, post-mortem cortisol may not (accurately) indicate variability in GR-mediated suppression of the HPA-axis.

The mechanisms underlying the association of the haplotype with an aggressive MS disease course therefore remain to be elucidated.

We did not find a correlation between levels of cortisol and indicators of MS disease severity, which is in contrast with earlier studies in our group.[25,170] Possibly, this is due the fact that these previous studies were performed on populations that displayed a much stronger variability in MS severity and HPA-axis activity and comprised a relatively large amount of patients with particularly severe MS and/or low activity of the HPA-axis.

Expression of CD163 within the CNS is found on macrophages and in the MS brain also on some microglia, and both cell types are known to strongly upregulate CD163 mRNA and protein levels under the influence of GCs.[143,193] Moreover, CD163 expression is also related to pathology-specific activation of macrophages, for example in rheumatoid arthritis and Gaucher's disease.[184–186] The latter also applies to MS, as CD163 expression is present on macrophages and microglia in demyelinating lesions and is increased on perivascular macrophages.[143] An important finding in this respect is that lesion-associated CD163 expression is particularly high on foamy macrophages and coincides with upregulation of 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1), the enzyme that increases local GC signaling by converting cortisone into cortisol.[126,143] Moreover, acute relapses were found to coincide with lowered cortisol levels in the CSF and not in serum. This local cortisol decrease was associated with poor local activation of cortisone via 11βHSD1 and/or cortisol inactivation via 11βHSD2. Accordingly, 11βHSD2 was found to be expressed on early activated macrophages within active plaques, whereas 11βHSD1 was expressed by foamy macrophages. Thus, endogenous regulation of cortisol in the CNS is reflected by its levels in the CSF and is strongly related to MS disease activity and CD163 expression by microglia and macrophage subpopulations within lesions. In line with this, no correlation was found between cortisol and sCD163 in serum of MS patients.[187] The correlation found in this study between cortisol and sCD163 levels in CSF may be of clinical relevance, as it suggests that the latter may indicate the cortisol-mediated cellular effects within the MS brain.

In conclusion, this study shows that more severe MS occurs in patients with

the *Bcl*I and N363S GR haplotype, which are associated with increased sensitivity to GC-induced suppression of the HPA-axis, without affecting immunomodulation of leukocytes. In addition, we show that sCD163 levels may serve as an indicator for GR-mediated immunomodulation within the MS brain. Therefore, further research is warranted to evaluate the prognostic value of assessing GR haplotypes and sCD163 levels in MS patients.

ACKNOWLEDGEMENTS

We thank the NBB team for their excellent work (www.brainbank.nl). This study was financed by MS Research, grant MS03-525ext.