Glucocorticoid sensitivity in multiple sclerosis; what makes the difference?

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Chapter 2

Glucocorticoid sensitivity in MS

Chapter 2.1

Sensitivity to glucocorticoids is decreased in relapsing remitting multiple sclerosis

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Abstract

Endogenous glucocorticoids (GC), which are under control of the hypothalamic-pituitary-adrenal (HPA) axis, play an important role in controlling chronic inflammatory demyelinating diseases, like MS. Increased HPA axis activity has been found in MS patients and appeared to be negatively associated with acute inflammation. Exogenous GCs are frequently used to treat relapses in MS, but the response to this treatment differs among patients, suggesting differences in sensitivity to GC. Previous, relatively small studies investigating GC sensitivity, have yielded conflicting results. In the present study, we have investigated GC sensitivity in peripheral blood cells of MS patients (n=117) and healthy controls (HC) (n= 45). GC sensitivity was measured by the in vitro suppressive effect of GC on LPS stimulated TNF-α production. Blood cells of MS patients, especially relapsing remitting (RR) MS patients, were less sensitive to GC compared to blood cells of healthy controls. This turned out to be unrelated to previous treatment with exogenous GC expressed as frequency of courses of intravenous steroids or interval since last course. The use of interferon β (IFNβ) was found to be associated with a lower GC sensitivity. However, also after correction for the use of IFNβ, RR MS patients remained less sensitive to GC.

Introduction

Exogenous as well as endogenous glucocortoids (GC) play an important role in controlling chronic inflammatory diseases, like multiple sclerosis (MS). Exogenous GCs are frequently used as therapy for MS. Treatment with GC reduces the severity of clinical deficits and shortens the relapse. However, response to GC differs among patients, suggesting differences in sensitivity to GC. Endogenous GC are considered to restrain the immune system in such a way that the probability of recovery from relapse is increased (35-37, 61). In MS sensitivity to GC may therefore be related to the clinical course or the susceptibility to the disease.

The production of cortisol is regulated by the hypothalamic-pituitary- adrenal (HPA) axis. An increased activity of the HPA axis leads to an increased production of cortisol. Cortisol exerts its action via interaction with GC receptors that are present in nearly every cell in the human body. Because of a negative feedback system mediated via the GC receptors elevated levels of cortisol will usually lead to a decrease in HPA axis activity.

In subgroups of MS patients this feedback system seems to be disturbed. Signs of increased basal secretion of cortisol have been found. In post-mortem studies in MS patients an increased size of adrenal glands was observed (62). Other post mortem studies revealed higher numbers (63) and activity of hypothalamic neurons producing corticotrophin releasing hormone (CRH) (9). The clinical relevance of an increased HPA axis activity is supported by the observation that this phenomenon is related to the clinical disease course (10). Moreover, a recent study revealed that the HPA axis may have a modulatory effect on inflammatory disease activity in MS. High HPA axis activity was associated with lower amount of Gadolinium (Gd) enhanced lesions on magnetic resonance imaging (MRI), suggesting a protective effect of increased HPA axis activity (11).

The mechanism behind increased HPA axis activity in MS is unknown, but it may be a result of a reduced negative feedback mechanism. Functional studies showed a decreased response to the dexamethasone suppression test (DST) (12), and increased cortisol levels after combined DST- CRH tests (10, 13-16). In depressive patients, increased HPA axis activity was associated with decreased peripheral GC sensitivity (17). Despite elevated cortisol levels clinical signs of hypercortisolism in MS are unusual. Taken together these observations suggest that increased HPA axis activity is accompanied by a reduction in the GC sensitivity (16). Some older studies show conflicting results with respect to GC sensitivity in MS (18-20). A small-scale study in 19 MS patients did not show differences in GC sensitivity between MS patients and healthy controls (HC) nor between patients with and without Gd enhancement on MRI (21). A recent study in 24 patients however showed a reduced GC sensitivity in relapsing-remitting MS patients (22).

To evaluate the hypothesis that peripheral GC sensitivity is decreased in MS compared to healthy controls we have set up a larger study. In addition we examined the relation between GC sensitivity and clinical disease characteristics. GC sensitivity was determined using the suppressive effect of GC on *in vitro* stimulated cytokine production. In our analyses the possible

effect of prior treatment with corticosteroids on GC sensitivity was taken into account, since it has been shown that binding of GC to the GR results in a significant down-regulation of the expression of the GR (23). Based on preliminary observations that interferon beta (IFNβ), a nowadays frequently used drug in MS, can influence GC sensitivity (24, 25), we were also interested in the effect of *in vivo* treatment with IFNβ on GC sensitivity.

Materials and Methods

Patients and controls

Whole blood was obtained from 126 MS patients and 51 healthy volunteers. All patients visited the outpatient clinic and met the Poser criteria for clinically definite MS (26). Patient's files were scrutinized to obtain data on all prior exposure to corticosteroids. The volunteers were recruited mainly from personnel working in the hospital or the laboratory. The study was approved by the ethics committee of the VU Medical Centre, Amsterdam and all patients and healthy volunteers gave informed consent.

Whole- blood cultures

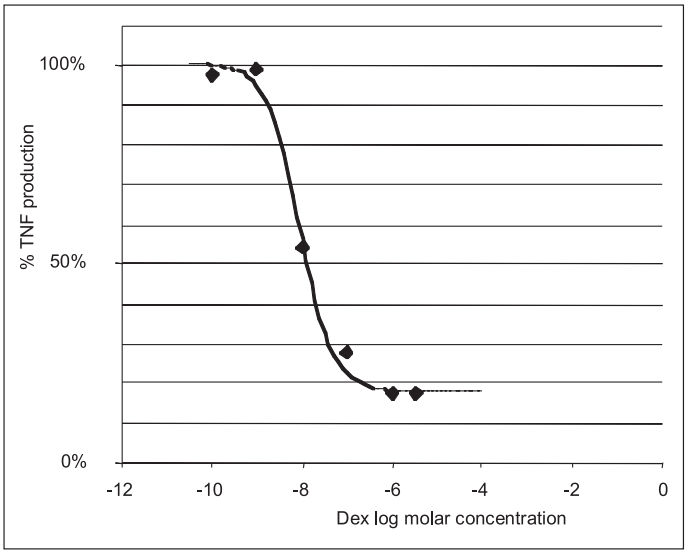
The whole blood stimulation procedure was based on a previously described method (27) with some minor modifications. Briefly, blood was collected by venapuncture and collected in chromogenix endotoxin free, heparinized tubes (CN Smith, Chromogeneix Endotube, Molndal, Sweden). To limit the influence of circadian variation in cortisol levels and cytokine production (28) blood was obtained between 9 and 11:00 am. The blood was diluted 1:1 in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 0.01 % L-Glutamine 200nM (GIBCO). All experiments were performed in 96- well tissue culture plates (Greiner, Frickenhausen, Germany). Cultures were set up in triplicate with each well containing 200 µL of diluted whole blood. During pipetting, the cell suspension was frequently vortexed to prevent sedimentation. To induce TNF-α production cells were stimulated with lipopolysaccharide (LPS); *Escherichia coli*; final concentration 100 ng / ml) except for a triple control for spontaneous TNF-α production. A dose-related response to dexamethasone (DEX; Sigma, St. Louis, MO) was studied by the addition of DEX in a final concentration ranging from 0, 10^{-10} M to 10^{-5} M. One stock solution of 10^{-2} M was prepared in ethanol and stored at -80 °C in 150 µL aliquots. The supernatants of the cultures were collected after four hours incubation at 37 °C and stored at -20 °C pending TNF-α measurements.

Cytokine measurements

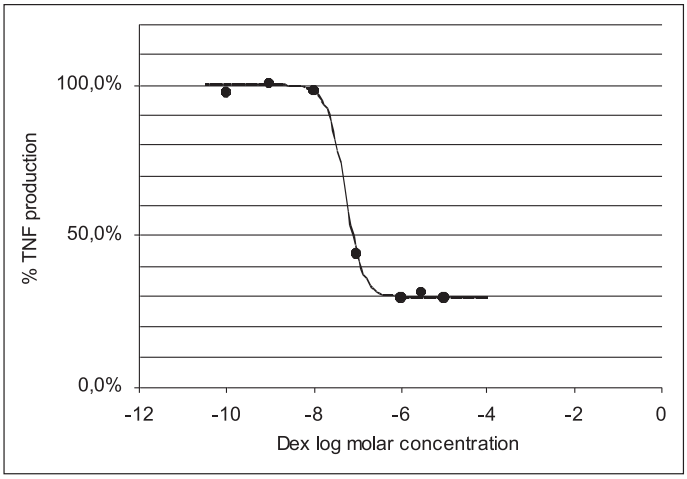
TNF-α in the cell culture supernatants was measured with a commercial ELISA (Biosource, Nivelles, Belgium) using maxisorp surface 96 wells plates (NUNC, Roskilde, Denmark). Recombinant TNF-α was diluted in culture medium and used as a standard. The standard curves ranged from 4 to 1000 pg/ml.

IC50 calculation

IC 50 was determined as log concentration DEX causing 50% of the maximum inhibitory effect. Inhibition of TNF-α by DEX was expressed as a percentage of LPS-induced TNF-α in the absence of DEX. To calculate the IC 50 for each subject, a sigmoid dose response curve for inhibition by DEX was fitted using a tangens inversus function (*TNF-α = a * TANH [b * log concentration (DEX –c)] +d)* (Figure 1.a – b). As a result of this calculation IC 50 takes into account the maximum TNF-α value (TNF max) after stimulation with LPS and the suppressive effect of different concentrations DEX ranging from very low concentrations to the maximum achievable suppression at high concentrations DEX (29).



1a: relative sensitive case. (IC 50 = 10-8.04 M/L = 9.1 nmol/L)



1b: relative insensitive case. (IC 50 = 10-7.28 M/L = 52.5 nmol/L)

Figure 1. Two examples of IC 50 curves. The points represent the mean of the 3 measured values. The lines represent the calculated curves based on the measured values.

Occasionally extremely low or high responders can be encountered (30). Since in our experience the corresponding IC50 values are not reliable, we decided a priory to exclude these cases from analyses. The threshold for that was arbitrarily set at TNF-α values outside the linear area of the standard curve.

Statistical analysis

Mean values of IC 50 between patients and HC were compared using independent Student's t-test, with a two-tailed significance at 0.05. MS subtypes and healthy controls were compared by analysis of variance (ANOVA) and Bonferroni's *post hoc* correction for multiple tests was used to compare MS subtypes and HC. In a subsequently performed ANOVA, the use of IFNβ was included as a covariate. To investigate the contribution of a predefined set of patient's and clinical disease characteristics on the IC 50, multiple linear regression analysis was undertaken. Cox regression analysis was performed to study the effect of the IC 50 on disease progression as defined by time to EDSS 6. The level of significance was set at 0.05. All statistical analyses were performed using SPSS for windows, version 11.0 (SPSS, Chicago, IL).

Results

Patient's characteristics

The group of MS patients consisted of 74 patients with relapsing remitting MS (RRMS), 35 patients with secondary progressive MS (SPMS) and 17 patients with primary progressive MS (PPMS) (31). Of the RR and SP MS patients 38 were on IFNβ treatment. 74 patients had previously been exposed to systemic corticosteroids. In all cases this systemic treatment of corticosteroids consisted of a course of high dose IVMP, either given in a dose of 1000 mg for 3 consecutive days, or as 500 mg for 5 consecutive days. The number of IVMP treatments varied from 1 to 15, the minimum interval between last exposure to corticosteroids and blood withdrawal was 30 days. In none of the subjects there was any detectable spontaneous secretion of TNF-α *i.e.* in the absence of LPS levels of TNF-α were below the level of assay sensitivity.

In 15 subjects TNF-α production after stimulation with LPS was outside the linear area of the standard curve and therefore these subjects, consisting of 6 HC (12 %) and 9 MS patients (7 %) were not included in the analysis. Characteristics of patients and HC that were included in the analysis are shown in table 1. Characteristics of RR and SP patients with and without IFNβ treatment are shown in table 2, and are comparable except with respect to previous exposure to IVMP; more patients on IFNβ treatment had been exposed to IVMP compared to patients without IFNβ treatment and the number of courses was higher as well ($p<0.01$). Among all patients treated with IVMP in the past the clinical effect of IVMP was not any different between those who were not treated versus those who were treated with IFNβ. If any, the treatment effect in the latter group was even better ($p=0.037$, not corrected for multiple

Table 1. Characteristics of healthy controls (HC) and MS patients, subdivided for MS subtypes having relapsing-remitting MS (RR MS) , secondary progressive MS (SP MS) and primary progressive (PP MS).

	HC N=45	MS N=117	RRMS N=69	SPMS N=32	PPMS N=16
Age[a]	35	42	38	46	52
Women n (%)	29 (64%)	75 (64%)	49 (71%)	17 (53%)	9 (53%)
EDSS[b]	n.a.	4.0 (2.5-6.0)	3.0 (2.0-4.0)	6.0 (4.0-6.5)	6.3 (4.8–6.9)
EDSS ≥ 6	n.a.	38	9 (13%)	19 (59%)	10 (59%)
Time to EDSS 6 (months)[b]	n.a.	72 (38-142)	53 (9-82)	117(62-213)	62 (26-81)
Disease duration (months)[a]	n.a.	116	88	176	112
IFNβ n (%)	n.a.	38 (33%)	38 (32%)	25(36%)	13 (41%)
IVMP in past n (%)[b]	n.a.	74 (64%)	38 (55%)	26 (81%)	10 (63%)
months since last iv-MP[b]	n.a.	8 (2-27)	11 (1-27)	13 (5-34)	5 (2-8)
Clinically active disease n (%)	n.a.	26 (22%)	13 (19%)	9 (28%)	4 (25%)

[a] Expressed as mean
[b] expressed as median (interquartile range)
n.a. = not applicable

Table 2. Characteristics of relapsing remitting (RRMS) and secondary progressive (SPMS) patients with and without IFNβ treatment.

RR/SP MS patients n= 101	no IFNβ n=63	IFNβ yes n=38
women n (%)	41 (65%)	25 (66%)
age[a]	41 (8)	39 (11)
IC 50 nmol/L[a]	34 (19)*	42 (21)*
RRMS n (%)	44 (70%)	25 (66%)
Age at onset[a]	32 (8)	28 (10)
Disease duration a (months)	111 (84)	126 (88)
EDSS[b]	3.5 (2.5-5.3)	4.0 (2-6.3)
EDSS 6 reached n (%)	16 (25%)	12 (32%)
Time to EDSS 6 (months)[b]	104 (5-276)	70 (0-353)
IVMP in past n (%)	33 (52%)*	31 (82%)*
number of IVMP courses[b]	1 (0 to 2)*	3 (1 to 5)*

RR = relapsing remitting MS, SP = secondary progressive MS and PP = primary progressive MS
* significant at the 0.05 level
[a] Expressed as mean and standard deviation
[b] expressed as median (interquartile range).

comparisons). In patients who were treated with IVMP both before and after onset of IFNβ treatment the clinical effects in these two periods were comparable.

Peripheral GC sensitivity in MS versus HC and MS subtypes

Blood cells of MS patients were less sensitive to GC compared to HC ($p < 0.001$). In HC the mean IC 50 was 23.7 nmol/L, in MS patients the mean IC 50 was 35.4 nmol/L (95%CI of the difference, 11.7, is 5.6 to 17.8 nnmol/L). ANOVA revealed a difference between clinical MS subtypes with respect to the IC 50 (figure 2). Bonferroni's *post hoc* test showed that this difference was due to a difference between HC and RRMS ($p = 0.001$) and HC versus SPMS

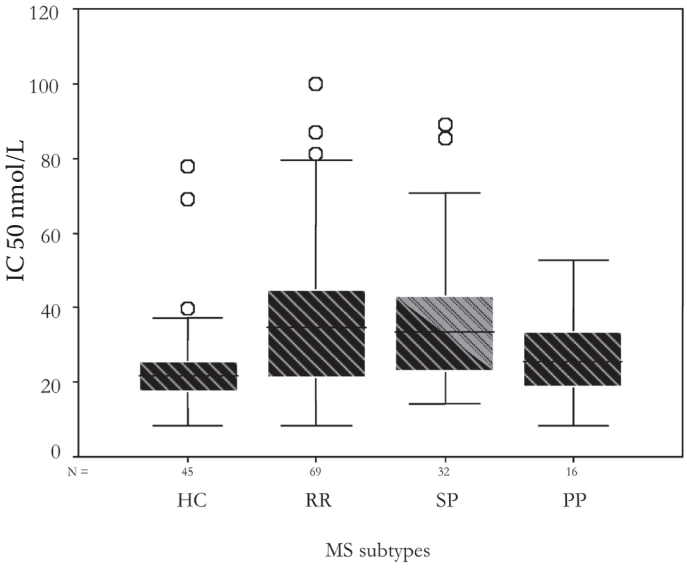


Figure 2. Box plots (each box shows the mean and inter-quartiles) and outliers (○) of IC 50 nmol/L of healthy controls (HC) and MS subtypes (ANOVA $p < 0.001$).
(Bonferroni's post hoc test: RRMS vs. PPMS p= 0.32, SPMS vs. PPMS p= 0.41 HC vs. RRMS p= 0.001, HC vs. SPMS p= 0.008 HC vs. PPMS p= 1.0)

RR = relapsing remitting MS, SP = secondary progressive MS and PP = primary progressive MS

(p= 0.008), whereas there was no difference between HC and PP MS. Within the MS patients the differences between the several subgroups were not significant (RR vs. PP p= 0.23, SP vs. PP p= 0.28 and RR vs. SP p= 1.0).

Peripheral GC sensitivity and disease parameters

In a multiple linear regression analysis, in which the IC 50 was used as the dependent variable and, as independent variables were entered: gender, age, MS subtype, EDSS score, disease duration, IFNβ treatment, number (no.) of IVMP treatments in the past, time in months since last IVMP treatment and whether patients were in an active or stable disease phase and season of blood sampling, only IFNβ treatment significantly contributed to the IC 50 (beta 0.254, p< 0.01). Patients on IFNβ treatment had a lower GC sensitivity. None of the other parameters were shown to have an effect on GC sensitivity.

After excluding patients on IFNβ treatment in the ANOVA analysis with Bonferroni correction, the difference in IC 50 between HC and MS patients remained significant (p=0.003), but only RR MS remained significantly less sensitive compared to HC (p= 0.021), as illustrated in figure 3.

A Cox regression analysis with time to EDSS 6 as dependent variable showed no significant impact of the IC 50, neither when entered as continuous variable, nor when entered as categorical variable after conversion based on percentile groups. Including disease type and the use of IFNβ as covariates did not affect the predictive value of IC 50.

Effects of exogenous glucocorticoids on IC 50

Mean IC 50 for patients with or without IVMP treatment in the past was respectively 36.2 nmol/L (standard deviation (SD) 19.7) and 34.1 nmol/L (SD 18.4). MS patients who had never

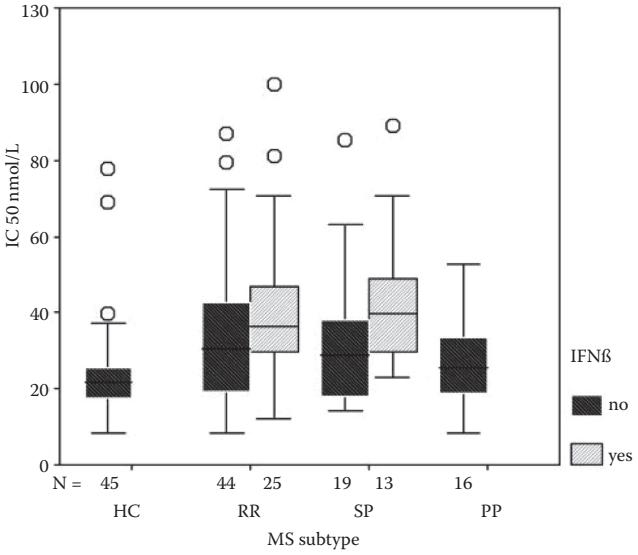


Figure 3. Box plots (each box shows the mean and interquartiles) and outliers (○) of IC 50 nmol/L per MS subtype subdivided for use of IFNβ. (for patients **without** IFNβ: ANOVA p= 0.022, Bonferroni's *post hoc* test: RRMS vs. PPMS p= 0.929, SPMS vs. PPMS p = 1.000 HC vs. RRMS p= 0.021, HC vs. SPMS p=0.301 HC vs. PPMS p = 1.000).

RR = relapsing remitting MS, SP = secondary progressive MS and PP = primary progressive MS

been treated with IVMP were less sensitive compared to HC 23.7 nmol/L (p= 0.003; 95%CI of the difference –17.1 to –3.6). Patient characteristics show that patients, who never had been treated with GC, were mainly RRMS patients, with lower EDSS scores, and who were less often on IFNβ therapy (table 3).

Time (in months) since last IVMP treatment or number of IVMP courses in history did not correlate with IC 50 in MS nor in the different subtypes of MS (data not shown). The observations of an increased IC 50 in RR MS patients remained after excluding either those patients that received IVMP in the three months prior to blood withdrawal or those that were ever treated with IVMP.

Discussion

In this study we show that blood cells of MS patients are less sensitive to GC compared to blood cells of healthy controls. Especially RR MS patients were found to have a reduced GC sensitivity. This observation can partly be explained by the use of IFNβ, which was related to a lower GC sensitivity. But also after correction for the use of IFNβ, RRMS patients remained less sensitive to GC.

In other inflammatory or autoimmune diseases such as rheumatoid arthritis (32), asthma (33), Crohn's disease (34) and ulcerative colitis (35, 36) evidence of decreased GC sensitivity of blood cells has been reported earlier. The underlying mechanism leading to this reduction in GC sensitivity is unknown. It has often been suggested that a possible explanation for this reduced GC sensitivity might be the frequent administration of exogenous GC, which may be the case in some of these diseases. However, in this study, we applied multiple methods to

Table 3. Disease characteristics of MS patients with and without prior exposure to GC treatment. RR = relapsing remitting MS, SP = secondary progressive MS and PP = primary progressive MS.

	MS never treated with IVMP(n= 43)	MS ever treated with IVMP (n= 74)	all MS patients (n= 117)
age	43 (11)	41 (10)	42 (11)
women n (%)	33 (74%)	42 (57%)	75 (63%)
disease duration[a] (mo)	104 (82)	124 (83)	116 (83)
RR no. (%)*	31 (72%)	38 (51%)	69 (59%)
SP no. (%)*	6 (14%)	26 (35%)	32 (27%)
PP no. (%)*	6 (14%)	10 (14%)	16 (14%)
onset age[a]	34 (9)	30 (10)	32 (10)
EDDS score[b] *	3.3 (2.0-4.0)	4.8 (3.0-6.5)	4.2 (2.5-6.0)
EDSS 6 reached n (%)[#]	7 (16%)	31 (42%)	38 (33%)
time to edds 6 (months)[b]	93 (72- 86)	71 (38-136)	72 (38-142)
IFNβ yes n (%)*	7 (16%)	31 (42%)	38 (33%)
IC 50 nmol/L	34 (18)	36 (20)	35 (19)

* pearson chi square p <0.05
[#] independent t-test p <0.05
[a] Expressed as mean and SD
[b] expressed as median (interquartile range).

analyse this possible correlation and were unable to identify any relation between decreased GC sensitivity and either number of courses of IVMP or interval since last course. In fact, even after excluding all patients who had ever been treated with exogenous GC, MS patients were still less sensitive to GC compared to controls.

Until now, studies evaluating peripheral GC sensitivity in MS patients showed conflicting results ((18-22). In most of these studies GC sensitivity was measured as suppressive effect of DEX on phytohemagglutinin (PHA) induced mitogenic response of isolated peripheral blood mononuclear cells (PBMCs). In our study GC sensitivity was measured as suppressive effect of DEX on LPS stimulated TNF-α production which is predominantly monocyte -driven (37). Using whole blood preserves the "natural environment" (including endogenous cortisol) of cytokine producing cells, which will at least partially be influenced by the HPA axis (3, 4). Our results are in line with a recent study in which the suppressive effect of DEX on interleukin-6 after whole blood LPS stimulation was evaluated (22). In this study however no clear distinction between PP and SP MS was made. Furthermore our study is based on, as of yet, the largest sample. Intra-individual variation of IC 50 has been investigated in lymphocytes in HC and was as great as between-individual variation (29). Other studies showed that GC sensitivity of lymphocytes in MS patients remained constant over time (18, 21). All these studies have been done in isolated PBMCs in which there is more intra-individual variability regarding cytokine production (28).

In MS, HPA axis dysregulation has been described, but the underlying mechanism is still unclear. HPA axis dysregulation was presumed to be due to diminished corticoid receptor function (16). Apart from being caused by a decreased peripheral GC sensitivity, a chronic activation of the HPA axis may result in a down regulation of the peripheral GC sensitivity to

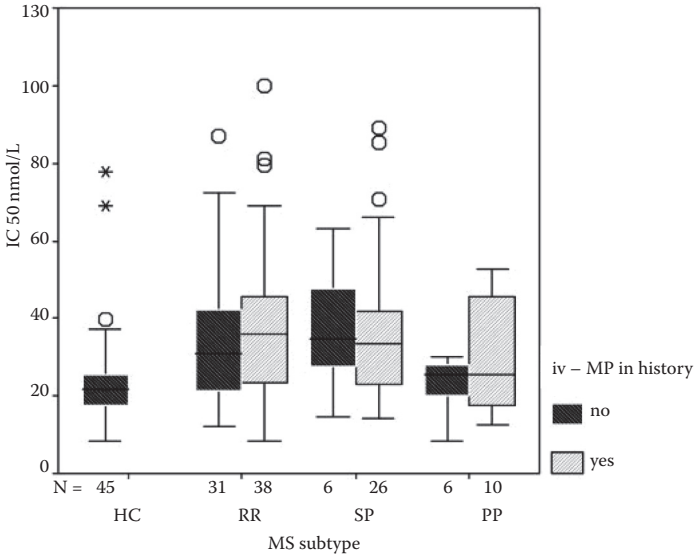


Figure 4. Box plots (each box shows the mean and interquartiles) and outliers (○) of IC 50 nmol/L per MS subtype subdivided for prior treatment with IVMP (*for patients never treated with IVMP* ANOVA p= 0.009, Bonferroni's *post hoc* test: RRMS vs. PPMS p= 0.519, SPMS vs. PPMS p = 0.904 HC vs. RRMS p= 0.011, HC vs. SPMS p= 0.393 HC vs. PPMS p = 1.000).

RR = relapsing remitting MS, SP = secondary progressive MS and PP = primary progressive MS, IVMP = methylprednisolone treatment

protect tissue from increased cortisol levels, as is the case in patients with the GC resistance syndrome and in endogenous Cushing' syndrome although this protecting mechanism seems to be insufficient in the latter patients because they do develop clinical signs of GC excess (38). Also in MS, an increased HPA-axis activity is likely to go along with a decreased peripheral GC sensitivity since clinical signs of hypercortisolism are unusual. However, HPA axis activity was not formally addressed in this study. Moreover the activity of the HPA-axis may be independent from the responsiveness to acutely exogenous administered GC (39).

There are several possible explanations for a change in GC sensitivity but these have thus far not been studied in MS. Different ratio's between the receptor isoform GR-α and GR-β, an alternatively spliced variant of GR-α which does not bind to GC but antagonizes the *trans*-activating activity of the classic GR-α receptor (40), have been described as possible explanations for differences in GC sensitivity in asthma (41) and ulcerative colitis (42). Decreased GC sensitivity may also be genetically determined. Several polymorphisms in the GR have been described. Some have been associated with altered GC sensitivity in the primary GC resistance syndrome, but also in HC (43). It will be interesting to investigate the role of these polymorphisms in MS.

In this study we evaluated the role of several parameters that could have confounded our observations. First, we looked at previous treatments with GC. In the MS patients studied here, prior iv-MP treatment, number of IVMP in history, nor months since last iv-MP therapy did not influence IC 50. Therefore, our finding that RRMS and SPMS patients have decreased GC sensitivity compared to HC and PPMS patients, can not be explained by previous iv-MP treatment. Even in analyses excluding all patients who had ever received exogenous GC RRMS

patients were still less sensitive to GC compared to HC. Second, we looked at disease activity because acute inflammation may influence IC 50. Unfortunately MRI data were not available for all patients at the time of data collection. Therefore we could only rely on clinical observations with respect to disease activity. We did not observe differences between patients in a clinically active phase of the disease versus those in a stable phase. This could be due to the relative small number of patients in active disease (n= 26), or it may suggest that GC sensitivity, as measured in this study, is a relative constant factor changing under influence of chronic inflammation rather than during acute inflammation. Thirdly, seasonal variation in glucocorticoid activity has been described (44) in healthy men, and in MRI and immune markers in MS patients (45). We compared IC50 in the four seasons determined as spring (March, April, and May), summer (June, July, and August), autumn (September, October and November) and winter (December, January and February). No significant differences were found.

Finally the use of IFNβ. IFNβ can influence the HPA axis, resulting in increased endogenous cortisol levels, as was shown in patients with cancer (46), chronic hepatitis (47) as well as in HC (48). One small study in 10 MS patients did not establish an effect of 4-6 months IFNβ treatment on HPA axis parameters (49). In our study IFNβ treatment was associated with lower GC sensitivity. IFNβ treatment is especially initiated in patients with clinical active disease, which is probably caused by an increased and ongoing inflammation in the central nervous system. This may be related to or - via a defective suppression by endogenous GC - even caused by a decreased GC sensitivity. The observed effect of IFNβ should perhaps not be contributed to IFNβ itself but rather explained by selection bias. When carefully comparing the disease characteristics of our RR and SP patients we observed a significant difference in exposure to iv-MP treatment thereby supporting the latter explanation.

The aim of our study was to gain more insight in the presence and role of peripheral GC sensitivity in MS compared to HC. MS patients, especially RR MS patients, were less sensitive to GC. It is tempting to speculate that this is related to a defective mechanism in controlling inflammation in MS patients. Further studies to elucidate the implications and causes of a reduced GC sensitivity in MS are warranted.

Acknowledgement

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