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Glucocorticoid sensitivity in multiple sclerosis; what makes the difference?

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2009

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citation for published version (APA)

van Winsen, L. M. L. (2009). *Glucocorticoid sensitivity in multiple sclerosis; what makes the difference?* [PhD-Thesis - Research and graduation internal, S.I.]. s.n.

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Chapter 1

General introduction and outline of this thesis







In this thesis different aspects of glucocorticoid (GC) sensitivity in patients with multiple sclerosis (MS) will be evaluated. Differences in peripheral GC sensitivity, single nucleotide polymorphisms (SNPs) in the glucocorticoid receptor (GR) gene leading to differences in GC sensitivity, and methods to measure different effects of treatment of GC therapy will be addressed.

In the introduction MS in general will be discussed, as well as the role of genetics in MS. GCs as treatment for exacerbations, and the role of endogenous GC will be assessed. Finally factors that influence GC sensitivity will be considered.

Multiple sclerosis

Multiple sclerosis (MS) is an inflammatory, demyelinating and neuro-degenerative disease, which occurs in genetically susceptible people. The pathological hallmarks are perivascular inflammation (T-helper cells, few B-cells and plasma cells and extensive macrophage and microglial activation), demyelination and axonal loss in the central nervous system (CNS) (white as well as grey matter). What drives the inflammatory response is still unclear. Autoimmune mechanisms and viral infections have been suggested (1-3). It has been thought that inflammation eventually leads to axonal degeneration. However, recently published papers based on large MS databases point towards the hypothesis that axonal degeneration in MS is an age-dependent process and, contrary to what has been thought before, independent of previous relapses (4). This is in line with the finding that also in very early stages of the disease, axonal degeneration has been found. The exact pathogenesis of (early) degeneration is yet unclear (2, 5).

History of MS

Tracing the historical roots of MS has proven to be difficult, given the lack of knowledge of clinical-anatomic localization in neurology prior to the late 19th century. Notable is the paucity of convincing MS-like illnesses in the historical record prior to this time. Two reports from the late 13th century described women afflicted with chronic, multifocal, and partially remitting neurological illnesses that conceivably might have been MS. These were the cases of a woman named Hall in the Icelandic Saga of St. Thorklar and of a Dutch woman named Lidwina van Schiedam. These uncertain cases excepted, MS makes its first appearance in 1822 in the diaries of Augustus D'Este, the illegitimate grandson of King George III (Firth 1948).

From: the Neurobiology of multiple sclerosis: Genes, inflammation, and neurodegeneration. Hauser 2006.



Epidemiology

The prevalence of MS varies considerably around the world. One of the salient features of MS epidemiology, is the observation between latitude gradient and prevalence of MS (6). The highest prevalence is found in Caucasian populations, especially, in the Northern Europe, South Australia and North America (Figure 1). An increasing incidence over the past century has been observed, partly due to improved diagnostic possibilities, but as most investigators believe, there is also a real increase, suggesting the presence of new environmental triggers (7). Persons, who migrate, before a certain age (around 15 years), from a high prevalence area to a low prevalence area, have lower risk for MS. Females have an increased risk of MS. These findings are fascinating, and have led to hypotheses and further research about environmental factors influencing susceptibility. One of these, the 'hygiene' hypothesis, has been evolved 30 years ago, from observations on sanitation and MS incidence in Israel (8). It is based on the theory that the immature immune system needs to be exposed to infectious agents early in life to develop normally. Indirect studies, for example whether the number of older siblings or any of the other sib ship characteristics studied, is associated with risk for multiple sclerosis, did not support the hygiene hypothesis (9). To address this issue quantitatively, the global prevalence of *Trichuris trichiura*, a relatively common human helminth, as a surrogate marker for infection with other parasites and low levels of sanitation, was used. It was found that

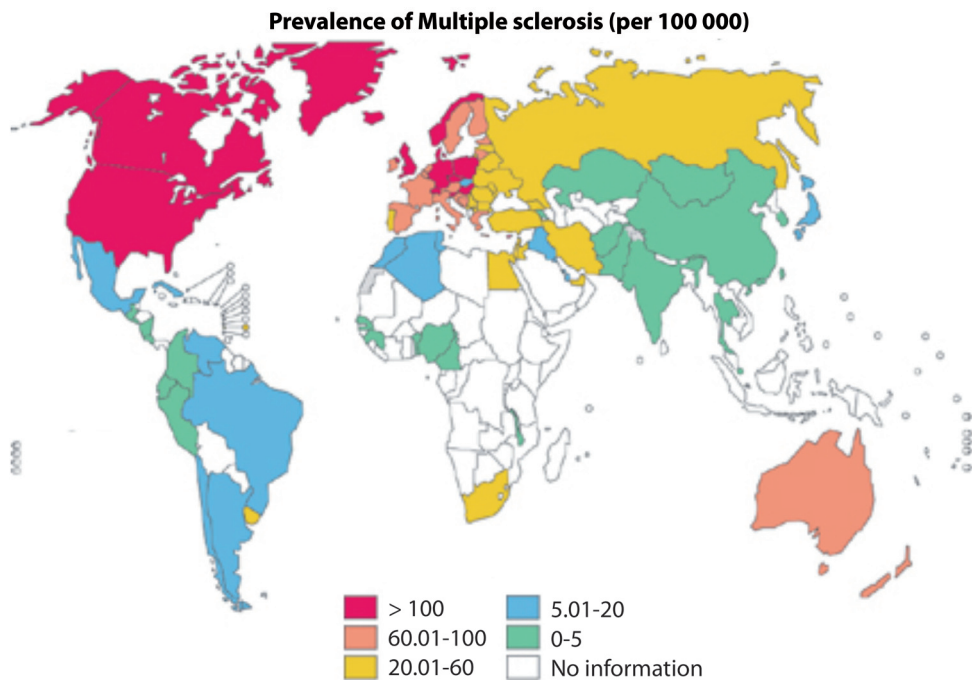
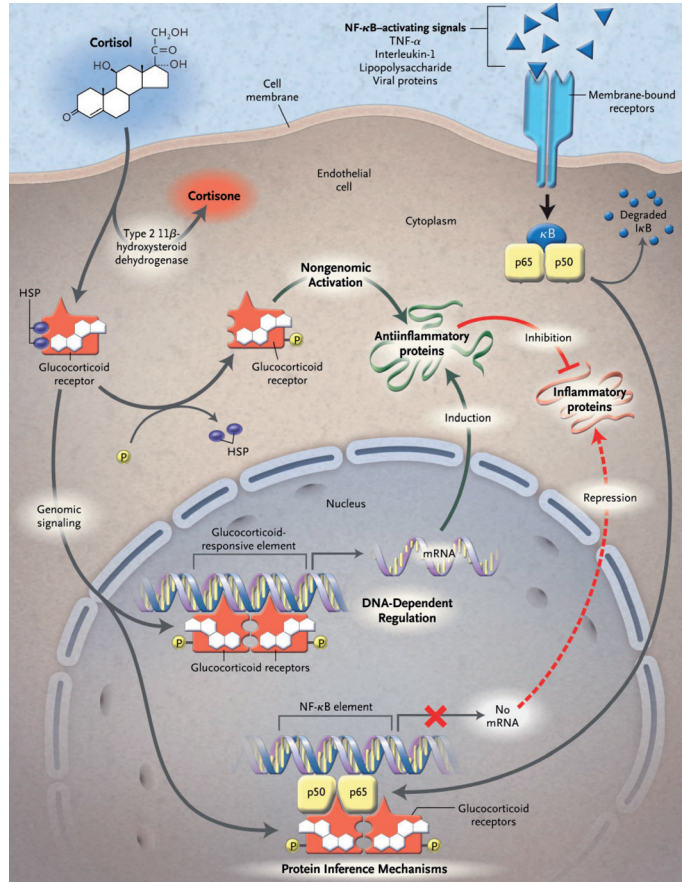


Figure 1. Prevalence of Multiple sclerosis. Adapted from WHO/MSIF.

Figure 2. Three general mechanisms of action of glucocorticoids and the glucocorticoid receptor in the inhibition of inflammation.

TNF-alpha denotes tumor necrosis factor alpha, HSP heat-shock protein, mRNA messenger RNA, and P phosphate. The three mechanisms are non-genomic activation, DNA-dependent regulation, and protein interference mechanisms (e.g. NF-kappa-B elements). Black arrows denote activation, the red line inhibition, the red dashed arrow repression, and the red X lack of product (i.e. no mRNA)

From: Rhen T, Cidlowski JA. Anti-inflammatory action of glucocorticoids-new mechanisms for old drugs. *N Engl J Med* 2005



the prevalence of MS falls steeply once a critical threshold of *T. trichiura* prevalence (about 10%) is exceeded (10). Paradoxical to this hygiene hypothesis, is the finding that persons who are seronegative for Epstein Barr virus (EBV), which suggests a more 'hygienic' upbringing compared to persons who are seropositive for EBV, have a higher risk for MS. Especially those who experienced or infectious mononucleosis clinical extremely low (6). In the same period as the hygiene hypothesis has been evolved, the hypothesis that increased exposure to sunlight reduced the risk for MS, started to be subject of research (11). As sunlight is for most people the main source of vitamin D, this led to associations between vitamin D and MS. Recently,

Human leucocyte antigen (HLA)-genes is the name for the Major histocompatibility complex (MHC) in humans, this is a gene dense region, related to the immune system, on chromosome 6p21, with extensive linkage disequilibrium and extreme levels of polymorphisms. DRB1 encodes the most prevalent beta subunit of HLA-DR.

there is an increase in interest in this subject (12) suggesting a beneficial effect of preventing hypo vitaminosis D on susceptibility of MS.

Genetics in MS

In Northern Europe, where MS has the highest the frequency, the prevalence is typically around 1 per 1000 (0, 1%) and 15 to 20% of the affected individuals have a family history of MS. The concordance rate in monozygotic twins is higher than in dizygotic twin, 31% versus 5%, which is the same as in first degree relatives of MS patients (13). It has been consistently shown that familial clustering results from shared genetic factors although the mode of inheritance is complex (14).

The association between one of the HLA-genes, the DRB1*1501 allele, and MS is best established. Populations with a high frequency of this allele, e.g. in Scotland, have the highest risk of MS (2). The exact mechanism(s) by which the DRB1 gene influences susceptibility for MS is not clear, but is likely related to the function of HLA molecules in immune responses.

Whole genome association studies, in which single nucleotides polymorphisms (SNP's) are used to divide the whole genome in tagged pieces, have further revealed an association between the IL-7 receptor gene and the IL-2 receptor gene (15) and susceptibility to MS. These two genes, however, display a low odds ratio to develop MS in the order of 1.3. Other genes with probably lower odds ratio's or occurrence in the population play also a role, however very large populations are needed to find these.

The severity of the disease or other aspects of the clinical phenotype of MS may also be genetically influenced (7). Candidate gene studies have pointed to an association between several genes and disease course (IL1 β –receptor gene, IL-1-receptor antagonist genes, immunoglobulin Fc receptor genes and the Apo-E gene) (2). Unfortunately, these findings could not be confirmed in later studies (16, 17).

Diagnosis in MS

Before the advent of MRI, the diagnosis was primarily based on clinical criteria, as described by Poser et al (18). The visualisation of cerebral MS lesions using MRI has greatly improved the sensitivity and specificity of the criteria for diagnosis of MS (19, 20), as described by McDonald et al (21), and more recently by Polman et al (22).

Clinical course

The clinical course is characterized by exacerbations and progression. Of approximately 85% of patients debuted with relapsing remitting MS (RRMS), this phase is characterized by exacerbations and remissions of neurological symptoms. In between these exacerbations there



is no progression of the disease, although residual symptoms can cause a new level of disability. RRMS often transforms to secondary progressive MS (SPMS) after a variable number of years. The course of MS in an individual patient is largely unpredictable. Ten percent of the patients do well for more than 20 years, and thus are considered to have benign MS. On the other hand, in rare cases, patients with a fulminate disease die within months after the onset of MS (2).

Neurological symptoms depend on the place of the lesions in the brain or spinal cord. Cognitive deficits are common, especially in advanced disease. Depression is experienced by around 60% of patients during the course of the illness (7).

Treatment in MS

At the moment all approved treatments in MS target inflammation and or adaptive immunity. Treatment response has been measured as reduction of relapses, with clinical outcome scales like the Expanded Disability Status Scale (EDSS) (23) and Multiple Sclerosis Functional Composite (MSFC) (24) and by measuring the reduction of T2 lesions and gadolinium (Gd) enhancing lesions on MRI. Over the years, several disease modifying therapies (DMTs) have been approved for the treatment of MS, including glatiramer acetate, IFN β , mitoxantrone, and a monoclonal antibody (MAB) natalizumab (25). Three other MABs (alemtuzumab, rituximab, and daclizumab) are therapies in development for treatment of MS (26). Beyond this, there are unproven therapies, but sometimes patients receive experimental treatment like very intensive immunosuppression (autologous bone marrow transplantation, high dose cyclophosphamide) (27) or are treated with combination of therapies in the context of a trial.

Besides these treatments which aim to slow disease progression, there are symptomatic treatments and treatment of exacerbations with intravenous methylprednisolone (IVMP).

Glucocorticoids

GCs are hormones, which are produced in the adrenals under influence of the hypothalamic-pituitary- adrenal (HPA) axis, in response to a variety of stressors. Cortisol, the most abundant endogenous GC in humans, exerts its action via interaction with glucocorticoid receptor (GRs) that are present in nearly every cell in the human body.

Cortisol inhibits the synthesis of cytokines and other inflammatory mediators in such a way that the probability of recovery from relapse is increased (28, 29). It has been thought that it acts as a mediator between the nervous system and the immune system, forming a negative feedback loop.

Glucocorticoid therapy in multiple sclerosis

GCs are widely used for the treatment of chronic inflammatory diseases because of their anti-inflammatory properties. For the treatment of exacerbations in MS high doses of IVMP are



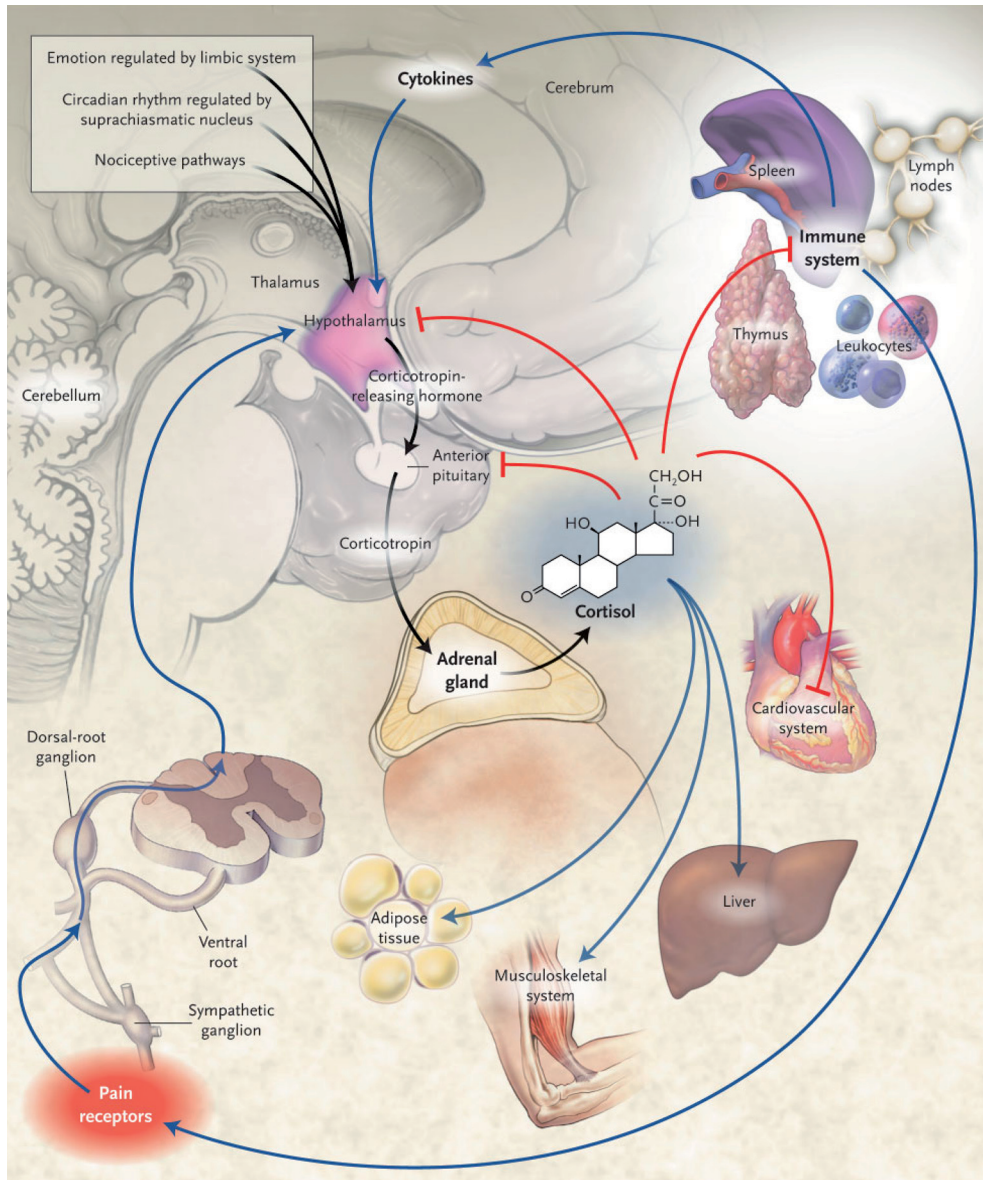


Figure 3. Pathways of communication among the immune system, the hypothalamic-pituitary-Adrenal axis, and other Tissues influenced by immune signals and glucocorticoids.

The diagram also shows other important influences on the hypothalamic-pituitary-adrenal axis. Red lines denote inhibition, and blue and black arrows activation.

From: Rhen T, Cidlowski JA. Anti-inflammatory action of glucocorticoids-new mechanisms for old drugs. *N Engl J Med* 2005



In 1948 Philip Showalter Hench for the first time treated a patient, with rheumatoid arthritis (RA), with cortisone. The effect was almost miraculous. Two years later he was awarded for his work on glucocorticoids with the Nobel Prize. Clues which set Hench to investigate the adrenal cortex were his own observations that pregnancy and jaundice ameliorate RA. He hypothesized that in both conditions the improvement was caused by elevated corticoid levels. This directed him to isolate compounds found in the adrenal cortex. Later side effects became clear and new derivatives, for example ACTH and methylprednisolone were developed. Since then glucocorticoids have been used for a varied and wide therapeutic range, especially for their 'anti-inflammatory' properties. The use of steroids in the treatment of MS is based on the assumption that there is an underlying disturbance of the immune system. Still now, intravenous methylprednisolone is a widely used treatment of MS during relapses to shorten the duration of clinical deficits. References textbox (30, 35, 36)

used. MP is a synthetic GC, a chemical modification of the natural GC hydrocortisone. The exact mechanism by which they influence MS exacerbations is complex, but they are known to inhibit lymphocyte proliferation, the synthesis of most known pro-inflammatory cytokines (i.e. tumornecrosis factor alpha (TNF- α) and IL-1 β) and cell surface molecules required for immune function. Moreover GCs have effects on the blood brain barrier permeability, via an influence on among others adhesion molecules, leading to reduction of tissue oedema. Also they can induce apoptosis of immune cells by stress-induced thymic involution (30, 31).

Response to GC treatment is highly variable between MS patients, as is seen in other inflammatory diseases as well. GC sensitive and relatively GC resistant patients could be identified clinically and by *in vitro* studies of GC sensitivity of peripheral blood cells in asthma (32), ulcerative colitis (33) and RA (34). In this thesis we studied *in vitro* and *in vivo* GC sensitivity in MS patients before and after IVMP treatment.

Side effects of high dose intravenous methylprednisolone

In one of the early placebo controlled studies it was noted that high doses of IVMP was surprisingly free from serious adverse effects (37). Most frequently observed side effects were slight reddening of the face, transient ankle swelling, and metallic taste in mouth during infusion. Psychiatric symptoms are described as elevated mood and insomnia, increasing in frequency after 5 days to 15 days (38). Gastrointestinal side effects were not seen after IVMP but some studies gave prophylactic antacid treatment. Hypertension and infection were not described after IVMP (30). Probably patients in these studies with pre-treatment infections were excluded or treated. Severe side effects of IVMP are rare, but psychosis, acute pancreatitis, and anaphylactic reactions have been reported (39). Patients with diabetes should be monitored during IVMP treatment, avoiding hyperglycaemia or hyperosmolar state. Because GCs are



metabolized by the cytochrome p-450 isoenzymes, interaction with warfarin and anti epileptic drugs have to be taken in mind (40).

Glucocorticoid receptor

The human GR gene is encoded by a member of the nuclear receptor subfamily 3 (group C, member 1, gene: NR3C1) on chromosome 5q31-33. Alternative splicing results in two isoforms GR α and GR β (see below).

GCs exert their function via three mechanisms (figure 2 (41)). Once a GC, for example cortisol has entered the cell, it binds to the GR and subsequently the cortisol-GR complex moves to the nucleus. In the nucleus it can act via two pathways. First, the cortisol-GR complex binds to DNA sequences called glucocorticoid responsive elements (GRE) located within promoters of a wide variety of responsive genes. This results in enhancement or repression (negative GRE's) of transcription of specific target genes. These genes are involved in glucose metabolism, cell adhesion, cell proliferation, cell survival, growth and development. Side effects are presumed to be mainly mediated by GC trans-activation and the effects of the GR on the HPA activity also seem to be exerted via this pathway (42).

Second, the cortisol GR- complex can act via direct protein-protein interaction with other transcription factors such as nuclear factor (NF)-kappa B or activating protein (AP)-1, which leads to inhibition or activation of inflammatory proteins. It is generally accepted that the anti inflammatory properties of GCs involve this pathway (*trans*-repression), and these are extensively used in the treatment of inflammatory diseases.

Third, rapid non-genomic actions, outside the nucleus, which are thought to be mediated through interaction with membrane associated receptors. It has been suggested that this pathway has a role in GC induced immunosuppression (43). All three pathways are supposed to inhibit inflammation (41) and play a role in the pleiotrophic effect of GCs.

Hypothalamic-pituitary- adrenal axis in multiple sclerosis

The production of cortisol is regulated by the HPA axis. An increased activity of the HPA axis leads to an increased production of cortisol. Because of a negative feedback system, mediated via the GRs, elevated levels of cortisol will usually lead to a decrease in HPA axis activity (44). Cytokines and other inflammatory mediators can also directly act on the brain to activate the HPA axis (figure 3 (41)).

Dysregulation of this neuroendocrine loop by hyperactivity or hypo activity of the HPA axis causes systemic changes in inflammation and immunity (41). Active lesions in the hypothalamus can lead to hypo- activation of the HPA axis, such lesions were found post mortem in brains of a subgroup of patients with severe MS (45) and these patients had lower cortisol responses to sepsis.



On the other hand, there is a body of evidence for hyperactivity of the HPA axis in MS (46). Signs of increased basal secretion of cortisol have been found (5, 6). In post-mortem studies in MS patients an increased size of adrenal glands was observed (7). Other post mortem studies revealed higher numbers (8) 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 HPA axis hyperactivity has been associated with disease progression, cognitive dysfunction and global atrophy on MRI (46, 47).

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 (48-52). 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). In this thesis we investigated the hypothesis that immune cells of MS patients are less sensitive to GC compared to healthy controls (HC).

Factors that influence glucocorticoid sensitivity

Alternative splicing processes in the GR gene.

Alternative splicing of the GR has been shown to generate the classic GR mRNA, which is translated into the protein GR α isoform which can bind to cortisol, DNA and other transcription factors, and a variant termed GR β mRNA, which is translated into the protein GR β isoform. GR β has been reported not to bind endogenous or synthetic agonists. In many cells and tissues examined GR β is expressed at low levels when compared to GR α . In vitro studies have indicated that reduction of the GR α : GR β ratio contribute to decreased GC sensitivity. Increased expression of the GR β protein isoform has been associated with GC resistance in other inflammatory diseases (53) (54). Recently, new isoforms have been identified, among others GR α A and B, and they seem to play a role in differences in GC sensitivity as well (43) (Figure 4).

Tissue specific GC sensitivity

Cortisol has a crucial role in maintaining homeostasis. Therefore, the level of intracellular cortisol in different tissues is regulated by the 11 β hydroxysteroid dehydrogenase (11 β HSD) type 1 and 2. Type 1 converts cortisone to cortisol while type 2 converts cortisol to cortisone. For example 11 β HSD2 is important to prevent cortisol from gaining access to high affinity mineralocorticoid responsive cells of the kidney. The 11 β HSD1 ensures bioavailability in the glucocorticoid-responsive metabolic tissue of the liver, lung, fat and the CNS (43). Outside the scope of this thesis are post-translational modifications of the GR.



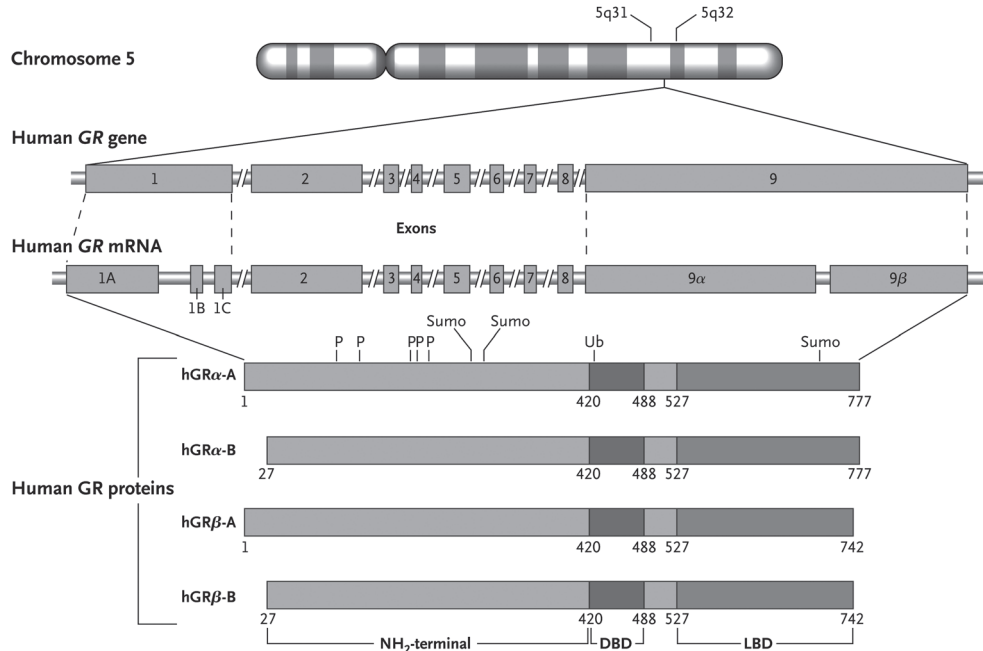


Figure 4. Genomic location and organization of the human glucocorticoid receptor (GR). Diversity in the expression and function of the GR results from alternative sites for the initiation of transcription (exon 1A, 1B, or 1C), as well as alternative splicing of pre-messenger RNA (mRNA) at exon 9 α or 9 β . Additional variation in the structure and function of the protein results from alternative for the initiation of translation with exon 2 and post-transcriptional modifications in the form of phosphorylation (P), ubiquitination (Ub), and sumoylation (Sumo). DBD denotes DNA-binding domain, LBD ligand-binding domain, and hGR human GR.

From: Rhen T, Cidlowski JA. Anti-inflammatory action of glucocorticoids-new mechanisms for old drugs. *N Engl J Med* 2005

Variability in the Glucocorticoid Receptor (GR) gene

The human GR gene is encoded by a member of the nuclear receptor subfamily 3 (group C, member 1, gene: NR3C1) on chromosome 5q31-33 (for the current list of single nucleotides polymorphisms (SNP) see the database on <http://www.ncbi.nlm.nih.gov/SNP/>).

Five SNPs phisms in the GR gene have been associated with changes in GC sensitivity. These are N363S (rs6195) and the *Bcl I C/G* (no rs numbers available), which are associated with increased GC sensitivity, and the ER22/23EK (rs6189 and rs 6190), GR9 β (rs6198), and TthIII (rs10052957) (55) (56) (Figure 5). The functionality of the N363S, ER22/23EK and 9 β has been reported *in vitro* (57).

The 9 β SNP in the GR gene, an adenine to guanine nucleotide substitution, is located in the GR β part of the GR (58). *In vitro* data show that the G allele of this SNP phism leads to a more

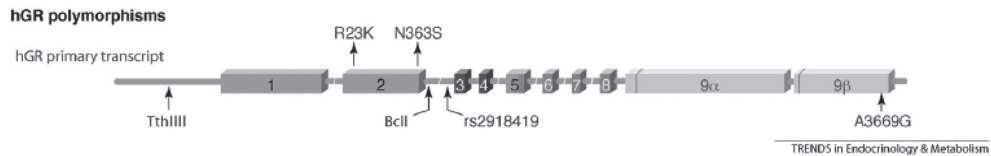


Figure 5. Polymorphisms in the GR gene.
hGR denotes human glucocorticoid receptor.

From K.L. Gross and J.A.Cidlowski. Trend in Endocrinology and Metabolism 2008 (43).

stable GR β mRNA and increased receptor protein expression of the GR β isoform which may contribute to a relative GC resistance (59). However, this finding remains controversial; other studies could not reproduce these results. A higher frequency of 9 β polymorphism was found in patients with RA (58). In men with the 9 β polymorphism, there was a decreased suppression of adrenocorticotrophic hormone (ACTH) and higher salivary cortisol levels after 1 mg DST (60).

The ER22/23EK polymorphism, that occurs in approximately 3% of the population, has been shown to decrease GC sensitivity; 'healthy' carriers were less responsive to DST (0.25mg) and had a beneficial cardiovascular profile (61). It has been elucidated that ER22/23 EK changes the balance between the A and B translational isoforms of the GR α protein, which could explain the relative resistance (62). However in depressed patients and another group of HCs, the association between the effect on DST (1 mg) and the ER22/23EK polymorphism was not found (63) (60). A possible explanation might be, the different populations studied and the dexamethasone dosages that have been used.

Several studies have investigated the role of ER22/23EK polymorphism in combination with other GR-gene polymorphisms in other chronic inflammatory disease in relatively small groups of patients with Crohn's disease (64), Graves ophthalmoplegia (65), psoriasis (66) and RA (58, 67). The results are inconsistent. Not only differences in methodology, studied combinations of SNPs phisms and populations may explain this variability, but also differences in pathogenesis and involved organs may play a role.

Outline of this thesis

Chapter 2 Glucocorticoid sensitivity in MS

In MS there is strong evidence for HPA-axis hyperactivity. 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 *in vitro* in peripheral blood-cells (monocytes) in MS patients. In **chapter 2.1**, we evaluated this hypothesis. GC sensitivity was measured by the *in vitro* suppressive effect of GC on lipopolysaccharide (LPS) stimulated TNF- α production in a whole blood assay.

Various mechanisms can underlie differences in GC sensitivity. One mechanism could be genetically variation in the gene of the GR. In **chapter 2.2** we evaluated the hypothesis that carriers of SNPs of the GR gene, associated with a decreased or increased GC sensitivity have decreased respectively increased sensitivity of peripheral blood-cells *in vitro*. Differences in GC sensitivity have been also been found in HCs, therefore we studies this in patients as well HCs.

Chapter 3 Clinical implications of GC sensitivity

GC sensitivity and clinical effect after IVMP

GCs are widely used for the treatment of chronic inflammatory diseases. In several inflammatory or autoimmune diseases including RA, asthma, M. Crohn, and ulcerative colitis, subgroups of patients who are clinically less responsive to GC have been observed and this has been associated with evidence of decreased GC sensitivity of blood cells. We proposed that patients who respond clinically, with a reduction in disability, to IVMP are sensitive to GCs and vice versa. We evaluated whether *in vitro* as well as *in vivo* GC sensitivity before and after treatment with IVMP during a relapse, did correlate with the clinical effect and whether *in vitro* GC sensitivity before treatment could predict the clinical effect. (**Chapter 3.1**)

Glucocorticoid receptor gene variation and disease course

Endogenous GCs are considered to restrain the immune system in such a way that the probability of recovery from relapse is increased. In MS patients, lifelong decreased GC sensitivity, due to SNPs of the GR gene, may lead to ongoing inflammation and therefore may influence the clinical course. We investigated SNPs of the GR gene, which have been previously associated with altered GC sensitivity *in vivo*, for their potential role in disease course and, in addition, in disease susceptibility in MS (**Chapter 3.2**). In **chapter 3.3** we evaluated previous findings in a larger population and considered the effect of other SNPs by inferring haplotypes.

Chapter 4 Measurement of treatment effect after IVMP

In MS, responsiveness to treatment is complicated to measure, because of the large variation of neurological symptoms, as well less clinical definable complaints as fatigue and cognitive problems. Therefore it is quite complicated to find good outcome measures, to detect which patients do clinically respond to IVMP, and who do not. Since long the EDSS has been used to evaluate treatment effect in MS, or disease progression. In the last two decades the development of new therapy options as well the awareness of the importance of patient-oriented measures, has led to the development of new outcome measures which aim to be more responsive to changes and which measure clinically relevant changes for the patients. .

In this thesis we compared a physician-oriented (MSFC) and patient-oriented (Guys neurological disability scale (GNDS)) clinical outcome measure with the EDSS concerning the



ability to detect improvement after IVMP treatment (**Chapter 4.1**). Furthermore we evaluated the ability to detect a, for the patient, meaningful change of two quantitative tests (Timed 25-foot Walk (T25FW) and the 9-Hole Peg (9-HPT) and the EDSS in a population of MS patients treated with high dose IVMP. The influence of baseline disability at responsiveness was studied (**Chapter 4.2**).

Chapter 5 Discussion and future perspectives

In **chapter 5** the results of this thesis will be summarized and discussed, and placed in the light of the current knowledge about the pathogenesis of MS, and future perspectives will be presented.

