Glucocorticoid Sensitivity in Rheumatoid Arthritis

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Glucocorticoid Sensitivity in Rheumatoid Arthritis
PhD Thesis, Erasmus University Rotterdam, the Netherlands

The studies described in this thesis were performed at the Department of Internal Medicine, Division of Endocrinology, and the Department of Rheumatology, Erasmus Medical Center, Rotterdam, the Netherlands. The tREACH trial comprises the following rheumatology centers: Erasmus MC, Rotterdam; Sint Franciscus Gasthuis, Rotterdam; Maasstad Ziekenhuis, Rotterdam; Vlietland Ziekenhuis, Schiedam; Admiraal de Ruyter Ziekenhuis, Goes and Vlissingen; Zorgsaam Ziekenhuis, Terneuzen; Albert Schweitzer Ziekenhuis, Dordrecht. The Erasmus MC, Sint Franciscus Gasthuis and Maasstad Ziekenhuis participated in the FLARE study. The work in this thesis was funded by the Dutch Arthritis Foundation.

Financial support for the publication of this thesis was kindly provided by: Dutch Arthritis Foundation, Merck Sharp & Dohme B.V., Goodlife Healthcare, Pfizer B.V., Daiichi Sankyo B.V.

ISBN: 978-94-6169-410-2

Cover design, layout and printing: Optima Grafische Communicatie, Rotterdam, the Netherlands.

Glucocorticoid Sensitivity in Rheumatoid Arthritis

Glucocorticoïd gevoeligheid in reumatoïde artritis

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus
Prof. dr. H.G. Schmidt
en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
dinsdag 3 september 2013 om 13.30 uur

door

Rogier Alfons Machiel Quax
geboren te Breda

PROMOTIECOMMISSIE

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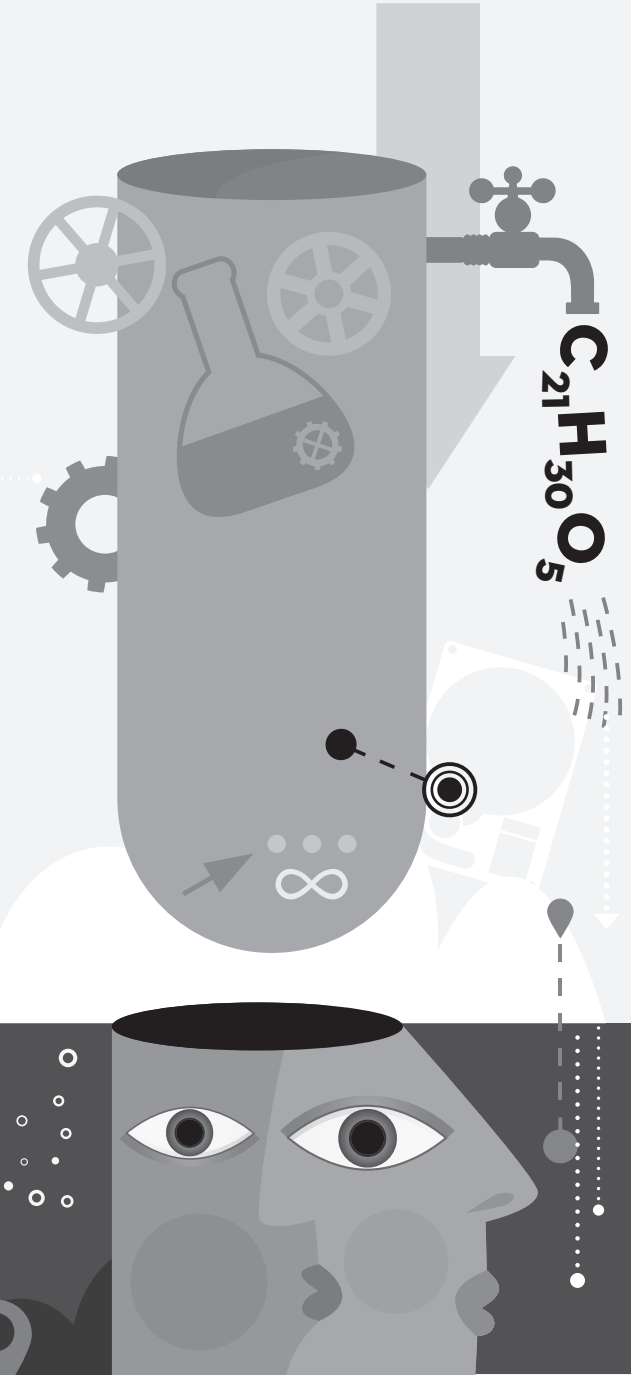

General Introduction

Chapter 1

Partly based on: Quax R.A.M., Manenschijn L., Koper J.W., Hazes J.M.W., Lamberts S.W.J., van Rossum E.F.C., Feelders R.A.

Glucocorticoid sensitivity in health and disease. *Nature Reviews Endocrinology*. *Accepted for publication*.

BACKGROUND

'Dr. Kendall and I decided to use for this first rheumatoid patient daily doses of 100 mg intramuscularly, so that we might not commit the error of underdosage. Thus, on September 21, 1948, Dr. Slocumb began to administer to the above-mentioned patient daily doses of 100 mg of compound E (not the acetate) in the form of a crystalline suspension in saline solution. Within three days the patient was markedly improved and continued to improve until the daily dose was reduced to 25 mg'

Dr Philip Hench, Nobel Prize winner in his Nobel Lecture, December 11, 1950

Accumulating observations of women with rheumatoid arthritis (RA) who 'spontaneously' experienced less active disease during pregnancy led to the growing belief by Philip Hench that a hormonal substance had to be involved in the improving clinical conditions of pregnant patients with RA. In close collaboration with Edward Kendall and Tadeus Reichstein they eventually managed to isolate, identify and produce compound E (i.e. cortison) and administered the compound to RA patients with impressive results.
Ever since, glucocorticosteroids (GC) are being used in a variety of inflammatory and non-inflammatory disorders because of their powerful anti-inflammatory and immunomodulating properties.
As such, GC are also frequently used in early and longstanding RA, although long-term use of GC is also associated with dose- and time-dependent side effects such as changes in body composition (obesity, muscle atrophy, osteoporosis), diabetes and hypertension. However, large interindividual differences in GC sensitivity are present, reflected by different treatment responses and by the degree to which side effects develop. In addition, abnormalities in the hypothalamic-pituitary-adrenal (HPA) axis itself have been recognized in RA which may contribute to its pathogenesis.
This justifies the search for determinants of GC sensitivity, in order to individualize and optimize GC therapy in RA, and to further unravel HPA-axis dysfunction in the pathophysiology of RA.

RHEUMATOID ARTHRITIS

RA is a common autoimmune disease affecting about 0.5-1% of the population worldwide. In the Netherlands, approximately 150,000 patients are known with this disabling disease. As in other autoimmune diseases, women are more often affected than men (ratio approximately 3:1). The disease is characterized by chronic synovial inflammation, ultimately leading, when untreated, to destruction of the joints. In addition, extra-articular manifestations are also

well-known features of RA, including heart (myocarditis, pericarditis), lungs (pleural and parenchymal manifestations), skin, eye ((epi-) scleritis), vasculitis of small to medium blood vessels, kidney (focal glomerulonephritis) and the nervous system (e.g. mononeuritis multiplex) (1).

Although many molecular mechanisms involved in the pathophysiology of RA have been unravelled in the past decades, the cause of RA is still unclear.

Genetic factors contribute up to 60% of RA heritability (2). The association between HLA-DRB1 and RA has repeatedly been confirmed and genome wide association studies (GWAS) have revealed many single nucleotide polymorphisms (SNPs) in genes involved in immune regulation (3-7). Next to genetic variation, several environmental factors have been linked to increased susceptibility to RA. Smoking is associated with a higher risk of developing anti-citrullinated protein antibodies (ACPA) positive RA, dependent on the amount of smoking and the number of HLA-DRB1 shared epitope alleles (8). These findings and the presence of citrullinated proteins in broncheoalveolar lavage cells exclusively in smokers, have led to the hypothesis that smoking might be causative involved in the etiopathophysiology of RA (9). Hormonal factors are also involved given the higher prevalence in women and the intriguing phenomenon of pregnancy-related improvement of disease activity and the subsequent postpartum flare (10). Inappropriately low cortisol levels and HPA-axis dysfunction in RA have been studied thoroughly (see section 'Glucocorticoid Sensitivity in Disease'). Infectious triggers might be implicated in RA pathogenesis as well (11). Another environmental factor which is currently being explored is the effect of the oral microbiome on immune modulation, since (pathological) oral flora might influence citrullination of mucosal proteins (12-13). The above-mentioned gene-environmental interactions may subsequently lead to altered post-transcriptional regulation and increased citrullination of proteins. Loss of tolerance via yet unknown mechanisms may govern the development of auto-antibodies against these citrullinated proteins (ACPA) and the Fc fraction of immunoglobulins (i.e. rheumatoid factor) by B-cells which are diagnostic hallmarks of RA (1). The presence of these auto-antibodies has been detected in blood donor samples years prior to disease onset (14). Interestingly, the so-called seronegative RA (i.e. RF and ACPA negative) patients also feature specific auto-antibodies, such as anti-MCM2 and anti-RPS6 (15).

Adaptive and innate immunity in RA

Inflammatory arthritis is characterized by the influx of leucocytes due to increased expression of adhesion molecules and higher levels of chemokines. B-cells may contribute to RA pathophysiology by the secretion of auto-antibodies (16). They are also able to act as antigen-presenting cells in co-stimulating T-cells (17). The pathological and clinical relevance of B-cells is underlined in B-cell knock-out mice, in which arthritis could not be induced following immunization with collagen type II (18). The success of Rituximab-mediated B-cell

depletion in controlling disease activity in RA emphasizes the importance of B-cells in human disease as well (19).

The importance of T-cells and T-cell signaling has already been demonstrated by the gain-of function polymorphism in the PTPN22 gene (6). Cell-cell interactions are fundamental for the activation of naïve T-cells through ligation of CD28 by antigen-presenting cells (APC, i.e. dendritic cells, macrophages or B-cells). Activated T-cells upregulate CD40-ligand which, upon binding with CD40, stimulates B-cells to synthesize immunoglobulins and APCs to synthesize cytokines (20). The efficacy of Abatacept, interfering with activation of T-cells by inhibiting CD28-ligation, underscores the importance of cell-cell interactions in RA (21). More recent, attention has been focused on the pathological role of Th17-cells in RA (22-23).

Innate immunity is also of utmost importance in the pathophysiology in RA. Most extensively studied in this context are cells of the myelomonocytic lineage (i.e. monocytes/macrophages, osteoclasts and dendritic cells), in particular macrophages (24). High numbers of activated macrophages are found in RA-derived synovial membrane and at the cartilage-pannus junction, where they produce a spectrum of pro-inflammatory cytokines (e.g. IL-1 and IL-6) and chemokines, herewith recruiting other inflammatory cells. Direct cell-cell interactions and cytokine-mediated crosstalk between recruited inflammatory cells and residents of the synovium and cartilage pannus (i.e. fibroblast-like synoviocytes (FLS), chondrocytes), result in a self-perpetuating pro-inflammatory micro-environment. This supports chronic inflammation of the synovium and, eventually, cartilage destruction and the development of erosions (17, 24-27).

Treatment of RA

The modern treatment of RA aims at reaching low disease activity quickly, using intensive combination therapies (28-33). These therapeutic strategies have proven to beneficially alter long-term outcome in terms of radiographic presence of erosions and hence functional disability.

The mainstay in most treatment regimens is methotrexate, although sulfasalazine and hydroxychloroquine are also frequently (co-) prescribed disease modifying antirheumatic drugs (DMARDs). The last two decades are characterized by the emergence of 'biologicals', which interfere with cytokine signaling cascades and cell-cell interactions. Prominent biologicals include the TNF-α blocking agents, the B-cell depleting drug Rituximab, the IL-6 receptor neutralizing antibody Tocilizumab, and Abatacept, which acts through competition with co-stimulatory T-cell signals (1).

Despite these evolving areas of research governing intensive treatment of RA, GC remain one the cornerstones in the treatment of more than half of patients with RA (34-35). GC have been demonstrated to reduce the rate of radiological progression of erosions, even after withdrawal of prednisone therapy (36). Moreover, GC have rapid anti-inflammatory effects

which is the main reason why GC are frequently used as bridging therapy in RA. In spite of the frequent use of GC as bridging therapy, the lack of structured clinical data evaluating the response to GC is remarkable, although the clinical problem of GC resistance is well recognized in approximately 30% of GC treated patients (37). Studies evaluating parameters of the initial response to GC bridging therapy should therefore be undertaken and this is one of the research topics in this thesis.

BIOSYNTHESIS OF CORTISOL AND THE HYPOTHALAMIC PITUITARY ADRENAL AXIS

Blood levels of cortisol are under tight regulation of a defined negative feedback system (Figure 1). Parvicellular neurons in the paraventricular nuclei of the hypothalamus store the peptide corticotrophin releasing hormone (CRH) in secretory granules. Upon receiving appropriate stimuli, CRH is released into the capillary network and transported to the anterior pituitary gland. Corticotroph cells within the pituitary are then stimulated to secrete adrenocorticotropic hormone (ACTH) into the bloodstream. CRH not only stimulates the release of ACTH by the pituitary, but also the synthesis of ACTH by promoting the expression of pro-opiomelanocortin mRNA, the precursor of ACTH. Finally, ACTH enters the bloodstream and can bind to receptors in the zona fasciculata of the adrenal glands. In the zona fasciculata, multiple enzymatic conversions finally result in the synthesis (and secretion) of GC from cholesterol. The rise of cortisol following stimulation by CRH and ACTH leads to inhibition of CRH and ACTH release, providing a classical endocrine negative feedback loop.
This negative feedback loop ensures appropriate levels of GC under normal physiological circumstances. This feedback mechanism has a strict diurnal rhythm with high cortisol levels in the morning (peak levels around 08.00-09.00 am) and a gradual decrease during the day with lowest cortisol levels at night. Furthermore, this circadian rhythm is composed of an underlying ultradian rhythm of cortisol secretion, which is crucial for normal gene transcription (38-39). HPA-axis activity is also greatly influenced by interactions with the nervous system and components of the innate and adaptive immune system in response to inflammatory stimuli. Emotional distress (e.g. fear, pain, emotional trauma/depression) also increases cortisol secretion by stimulating the HPA-axis. Dysregulation of the HPA-axis can lead to a range of clinical symptoms (e.g. hypercortisolemia as in Cushing's disease, hypocortisolemia as in Addison's disease).

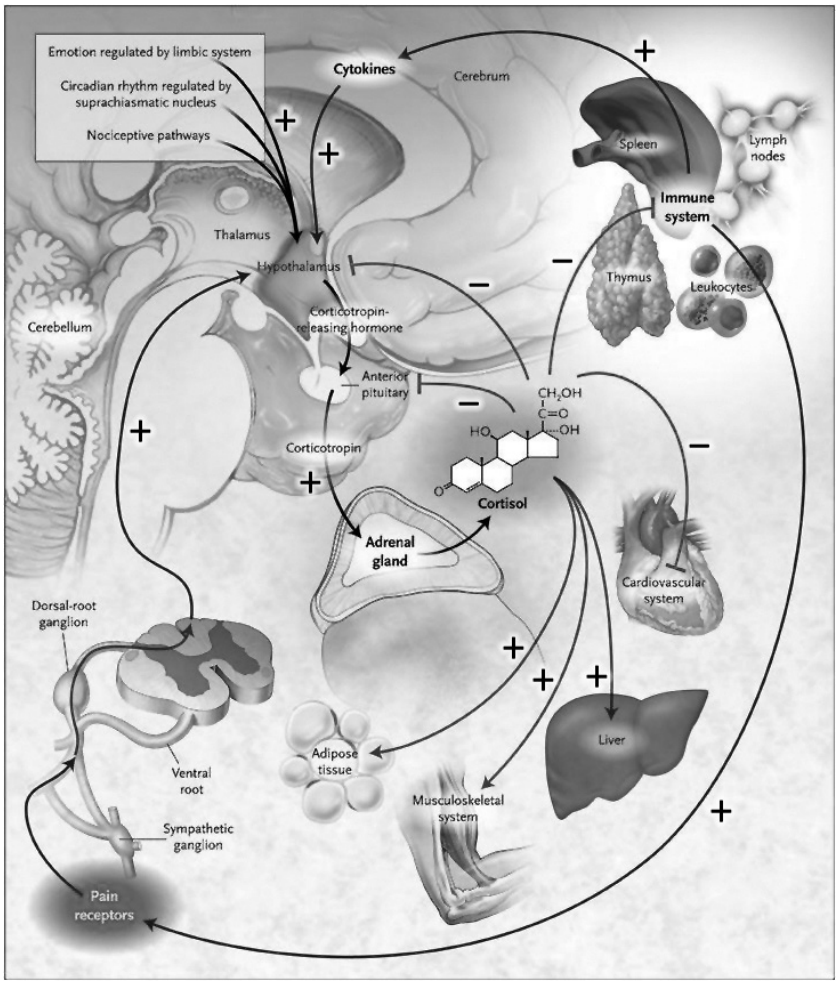

Figure 1. The hypothalamic-pituitary-adrenal axis and the diverse effects of cortisol. Reproduced with permission from N Engl J Med; 2005;353:1711-23, Copyright Massachusetts Medical Society.

GLUCOCORTICOID ACTION

The glucocorticoid receptor

GC are involved in many processes in various tissues and organs, ranging from glucose homeostasis and modulation of the immune and inflammatory responses to their important role in bone metabolism and their effects on mood, behaviour and sleeping patterns (Figure 1). Approximately 1-20% of all genes are estimated to be positively or negatively regulated by GC, illustrating the diversity of GC action (40-43). The actions of GC are mediated by the glucocorticoid receptor (GR), which is expressed in virtually all cells and is essential for life. The

GR is one of the members of the nuclear receptor family and it is encoded on chromosome 5 and consists of nine exons. As all other nuclear receptors, the GR has a N-terminal transactivation domain, a central DNA binding domain and a C-terminal ligand binding domain.

Alternative splicing and translation of the glucocorticoid receptor

The nine exons comprising the GR gene are subject to alternative splicing, giving rise to the alternative splice variants GR-α, GR-β, GR-γ, GR-A and GR-P (44). Research has mainly been focusing on two isoforms derived from alternative splicing of exon 9, GR-α and GR-β (45). GR-α is the biologically active isoform. The GR-β isoform lacks helix 12 and hence is not capable of binding GC. Nevertheless, micro-array analysis of HeLa-cells stably expressing GR-β, and COS-1 and U-2 OS cells transiently expressing GR-β, revealed numerous genes to be regulated by GR-β (46-47). In addition, overexpression of GR-β in an airway epithelial cell line modulated histone deacetylase activity (48). Above all, GR-β is thought to act as a dominant negative inhibitor of GR-α, hereby affecting transcriptional activity of GR-α, although conflicting results have been reported (Table 1) (49-52). Functional diversity of the GR is further extended via alternative translation of the GR. Eight different translation initiation sites lead to as many translational isoforms of GR-α (44). Although ligand affinity and interaction with glucocorticoid responsive elements (GRE) do not seem to differ among translational isoforms, striking differences have been found in their induced gene expression profiles, suggesting regulation of GC signaling at the post-transcriptional level as well (53).

Formation of the glucocorticoid receptor-multiprotein complex

In the absence of ligand, the GR is considered to be located in the cytoplasm as part of a multiprotein complex. This multiprotein complex, composed of several heat shock proteins, immunophilins and other co-chaperones, dynamically regulates the folding, affinity and intracellular/nuclear transport of the GR according to the presence of ligand. Recent literature showed that nuclear recycling – both intranuclear unloading and reloading of ligand and intermittent interaction of the liganded receptor with DNA - is also an important process (39). The default low affinity state of the GR, with closed steroid binding pocket, is reached via binding of heat shock protein (hsp) 70, hsp40 and hsp70-hsp90 organizing protein (hop), mediating conformational changes in protein folding. The low affinity hsp70-hsp40-hop-GR complex can either be 1) directed towards proteolysis if the complex is destabilized by competitive antagonism of hop by bag-1 (a cochaperone of Hsp70) or CHIP (a cytoplasmic protein with E3 ubiquitin ligase activity), or 2) proceed to a high affinity state after binding of hsp90 and hsp90 co-chaperone p23, leading to opening of the steroid binding cleft. In the latter case, binding of ligand will eventually lead to nuclear translocation of the GC-GR-complex, affecting transcriptional regulation (54).

Mechanisms of glucocorticoid signaling

Genomic actions of glucocorticoids

After binding of ligand and the subsequent trafficking towards the nucleus, the GR-GC complex can mediate gene transcription via several different mechanisms (Figure 2). The first mode of transcriptional regulation requires binding of liganded dimerized GR on glucocorticoid-responsive elements (GREs). The subsequent recruitment of several co-activators promotes remodelling of the chromatin and stimulates initiation of transcription by the RNA-polymerase II complex. The process of genes which are transcribed at higher rates upon binding of the liganded GR to GREs, is called transactivation.
Alternatively, GC can bind to negative GREs in the promoter region of target genes, herewith inhibiting gene transcription. The GR-monomer can also interfere with transcriptional regulation of other pro-inflammatory transcription factors (TF), by means of direct protein-protein interactions, controlling chromatin compaction and the presence, or absence, of essential co-factors. The process of genes which are transcribed at lower rates upon binding of the GC-GR complex to GREs, is called transrepression.
Recent micro-array experiments in dexamethasone treated 3134 (a murine mammary adenocarcinoma cell line) and AtT20 (a pituitary cell line) cells have unravelled complex regulation profiles in both induced and repressed genes (subdivided in transiently, continuously and plateau-like profiles) (55). Intriguing new insights have been obtained on gene regulation by the ultradian secretion pattern of cortisol (i.e. pulsatile secretion within the circadian rhythm of cortisol), emphasizing the dynamic processes involved in GC signaling (38). Alternative modes of transcriptional regulation by GC which go beyond the scope of this thesis may include micro-RNAs, phosphomodulation by kinases and phosphatases, methylation of histones and, although largely hypothetical, inhibition of a de-repression step (i.e. preventing release of co-repressors) (56-57). Post-translational modifications of the GR itself may further increase diversity and tissue and cell-type specificity of GC action (44).

Anti-inflammatory actions of glucocorticoids: crosstalk with pro-inflammatory signaling cascades

Major pathways of transrepression by GC involve direct interactions of the GR with activator protein 1 (AP-1) and nuclear factor kappa B (NFκB) (protein-protein contact/tethering mechanisms), two key players in pro-inflammatory signaling cascades (Figure 2 and (58)). Immune modulation and crosstalk of GC also occurs via complex multi-layered interplay with factors upstream of NFκB and AP-1 and other TF (e.g. T-Bet, STAT, NFAT, CREB, PPARγ) and pro-inflammatory cytokines. A major pre-transcriptional mechanism of regulation by GC, mainly explored in the field of asthma and COPD, involves modulation of DNA accessibility via recruitment of histone

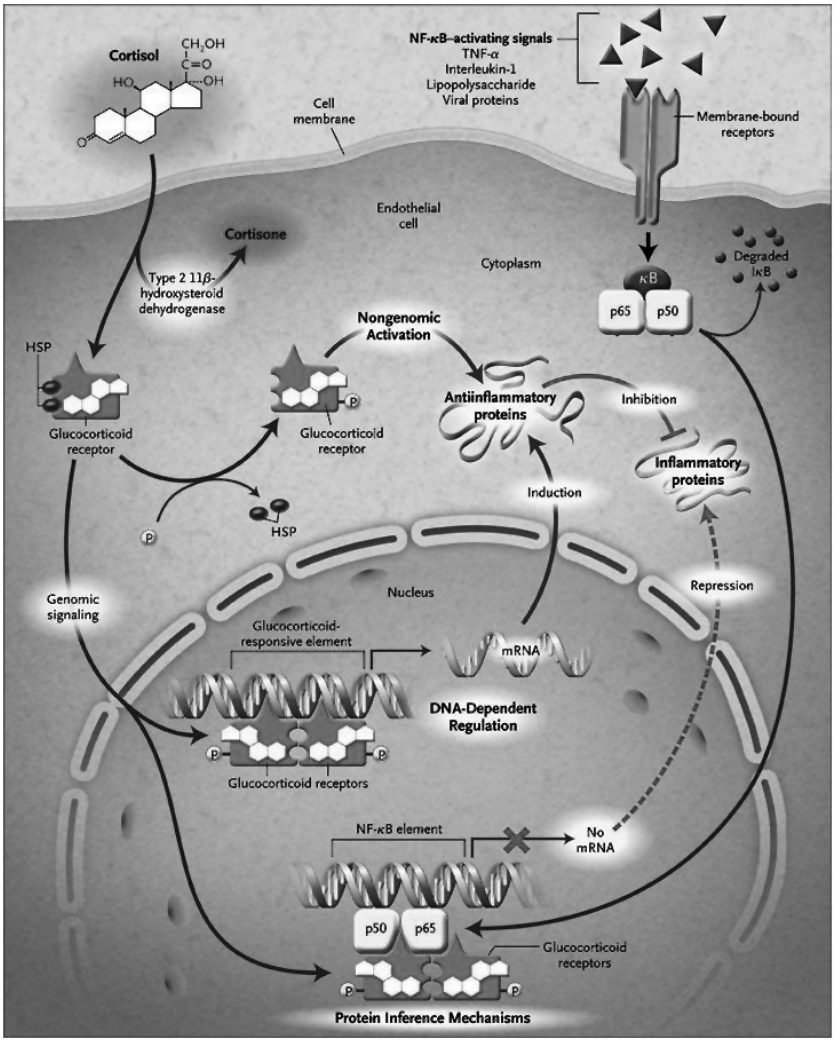

Figure 2. Glucocorticoid signaling pathways counteracting pro-inflammatory cascades via both genomic and nongenomic pathways. Reproduced with permission from N Engl J Med; 2005;353:1711-23, Copyright Massachusetts Medical Society.

deacetylase 2 (HDAC2). The subsequent deacetylation and recondensation of histones located in promoter regions of pro-inflammatory genes inhibits transcription (59). Moreover, GC inhibit histone H3 S10 phosphorylation by repressing mitogen- and stress-activated protein kinase-1 (MSK-1) recruitment, also stimulating a closed format of chromatin (60). In addition, phosphorylation of RNA polymerase II, required for the initiation of transcription, is also tightly regulated by GC (61).

A central factor integrating virtually all signaling cascades involved in inflammation is the phosphorylation and dephosphorylation by mitogen-activated protein kinases (MAPKs) and

phosphatases (57, 62). The MAPK family can be subdivided in three well-characterized pathways, MKK1-2/ERK, p38 and JNK, which can be stimulated by a wide range of inflammatory stimuli. These activated signaling cascades are counterregulated by two GC-induced genes: glucocorticoid-induced leucine zipper (GILZ) and MAPK phosphatase 1 (MKP-1). GILZ inhibits the MKK1-2/ERK pathway by sequestering the upstream MAPK-kinase-kinase Raf, but can also directly interfere with AP-1 and NFκB (63-64). MKP-1 can dephosphorylate and inactivate MAPKs, hereby dynamically regulating inflammatory responses (65-66). Vice versa, MAPKs themselves can directly and indirectly phosphorylate the GR, stimulating nuclear export and proteosomal degradation of the GR (67). Additional mechanisms of GC crosstalk may include GC-mediated induction of 1) SOCS proteins, which are direct inhibitors of Toll-like receptors 2 and 5 (68), 2) tristetraprolin, a zinc finger protein, destabilizing mRNA of some pro-inflammatory cytokine genes, including TNF-α (69), and 3) IκBα, leading to cytoplasmic sequestration of NFκB further adding to GC/NFκB antagonism (70). Finally, GC-mediated induction of annexin-1 and inhibition of cyclooxygenase 2 (COX2) expression both block the formation of arachidonic acid, the precursor of leukotrienes and prostaglandins (71).

Nongenomic actions of glucocorticoids

GC also exert multiple effects which do not require binding of the GR-GC complex to (n)GREs or direct protein-protein interactions (72). These so-called nongenomic effects of GC involve, among effects in other cell types and tissues, immune regulation (72-75). Several mechanisms of nongenomic GC signaling have been postulated. First of all, nongenomic effects may also require binding of the GR but may not require DNA binding. This is exemplified by Croxtall and co-workers who demonstrated that cytosolic phospholipase A_2 (cPLA2α)-mediated release of arachidonic acid could be inhibited by dexamethasone in the presence of actinomycin-D (inhibiting transcription), but not in the presence of the GR antagonist RU-486 (76). Yet unknown factors released from the heterogeneous GC-GR multiprotein complex upon ligand binding of the GR may be involved in secondary signaling cascades. A second mechanism may involve binding of the membrane-bound GR (mGR). Although the origin and function of the mGR is largely unknown, mGR have been demonstrated to influence human T-cell receptor signaling (77-78). Third, high doses of GC (>100mg/day) also increase cellular energy metabolism, possibly via affecting cell membrane properties, cytokine synthesis and migratory capacities of immune cells (79). Finally, ligand-bound GR have been shown to translocate to mitochondria modulating apoptosis in thymocytes (75).

Cellular effects of GC

At the cellular level, GC have profound effects on immune cell function. First of all, GC restrain leukocyte trafficking via reduced expression of adhesion molecules, possibly via a direct ef-

fect of GC (nongenomic effect) or indirectly via suppression of cytokines upregulating adhesion molecules. Furthermore, GC promote the apoptosis of eosinophils, reduce the clearance of opsonized bacteria by the mononuclear phagocyte system, impair the antigen-presenting potency of macrophages and inhibit mast cell degradation.
Acquired immunity is also influenced since GC stimulate apoptosis of dendritic cells, resulting in reduced numbers of circulating dendritic cells and hence compromised antigen-presenting capacity. T-cells are strongly affected by GC, in terms of increased apoptosis and interference with interleukin-2 which is an important T-cell growth factor. Likewise, but to a much lesser extent, the number of circulating B-cells is reduced following GC administration, although the acute effect of GC administration involves temporary increased secretion of immunoglobulins (80).

Glucocorticoid-induced side effects

The limiting factor compromising GC therapy is primarily determined by dose and time dependent side effects of GC (81). These side effects include cardiovascular disease (i.e. hypertension, accelerated atherosclerotic disease), metabolic disturbances (i.e. diabetes mellitus, suppression of the HPA-axis, osteoporosis), neuropsychiatric disorders (both euphoric/manic and depressive episodes are described), increased risk of (opportunistic) infections, cataract and glaucoma, gastro-intestinal problems (e.g. gastritis, peptic ulcera, gastro-intestinal bleeding) and dermatological conditions (skin thinning, acne, striae, hirsutism, Cushingoid appearance).

GLUCOCORTICOID SENSITIVITY IN HEALTH AND DISEASE

Methods to measure glucocorticoid sensitivity

Genetic determinants of glucocorticoid sensitivity

The GR gene harbors 4 well studied functionally relevant single nucleotide polymorphisms (SNPs) (Figure 3) (82). The *Bcl*I polymorphism (rs41423247), located in an intron, embodies a C to G nucleotide change and is very common in the general population (minor allele frequency (MAF): 36.5%). In contrast, the N363S (rs6195) variant is located on exon 2, involves an amino acid change (asparagine → serine) and is relatively rare (MAF 3.7%). Both the *Bcl*I and N363S polymorphisms have been associated with increased sensitivity to GC as exemplified by a disadvantageous metabolic profile and decreased susceptibility to and severity of inflammatory diseases. Their genetic counterparts are the 9β (rs6198; MAF 17.9%) and ER22/23EK (rs6189 and rs6190; MAF 3.4%) variants, associated with decreased GC

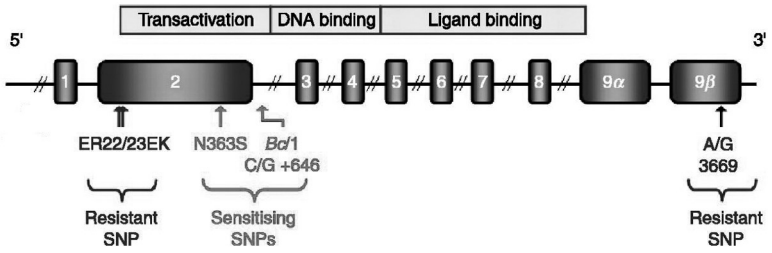

Figure 3. The GR gene comprises 9 exons and harbours 4 well-known functional polymorphisms. Reprinted from Walker B R Eur J Endocrinol 2007; 157 : 545-559.

sensitivity as illustrated by a favorable metabolic profile and body composition and increased susceptibility and more severe disease course in inflammatory disorders. The ER22/23EK SNP is located in the transactivation domain (exon 2), where 2 linked nucleotide changes lead to one amino acid change (arginine to lysine). Situated in the 3'UTR of the GR gene (exon 9β), the 9β variant comprises an A to G substitution without an amino acid change. Other SNPs which have been demonstrated to contribute to differences in GC sensitivity include variants in the glucocorticoid-induced transcript 1 (GLCCI1) and CRH gene (83-85).

Bioassays to measure glucocorticoid sensitivity

Cell proliferation assays, dexamethasone suppression tests, skin blanching assays, cytokine production assays and dexamethasone-induced gene expression assays all have been shown to serve as measures of GC sensitivity (86-92).

In the last decade, accumulating evidence supports the fact that anti-inflammatory and metabolic effects of GC are mediated via both transrepressive and transactivating pathways (93). In our laboratory, these new insights have been implemented in a recently developed bioassay. In this bioassay, dexamethasone-regulated expression of interleukin-2 (IL-2) and

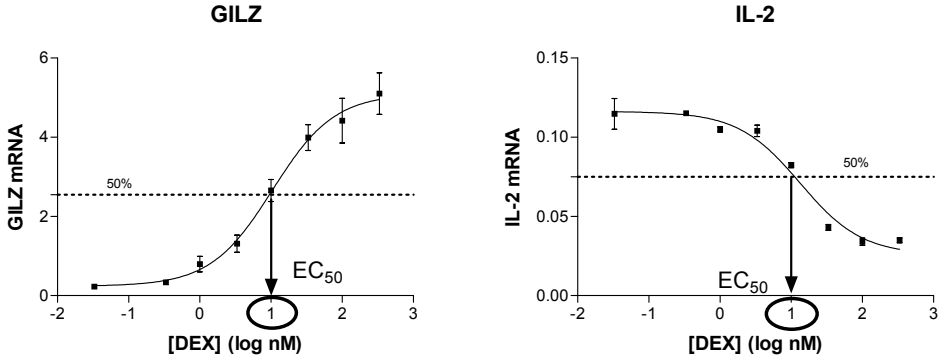

Figure 4. The GILZ and IL-2 bioassays, representing transactivated and transrepressed genes by glucocorticoids respectively. The half maximum effective concentration (EC_{50}) can be used as a read-out for GC sensitivity (91).

glucocorticoid-induced leucine zipper (GILZ) is measured. Transrepressive effects of GC, classically considered to be the predominant mechanism regulating anti-inflammatory actions of GC, are represented by the IL-2 assay. The GILZ assay exemplifies one of the transactivated genes, originally postulated to be responsible for the development of GC-induced side effects (Figure 4). Using these bioassays, a spectrum of GC sensitivity could be demonstrated in healthy individuals (91).

Glucocorticoid Binding Capacity

The number of GR and the affinity of these receptors can be assessed by GC binding capacity assays. The general principle of these assays relies on the fact that GC binding depends on both the number of GR available and the affinity ($1/K_D$) of these receptors for GC. In the context of an excess of unlabeled dexamethasone, increasing doses of [^{3}H]-dexamethasone are added to isolated peripheral blood mononuclear cells (PBMC). Specific binding of [^{3}H]-dexamethasone is calculated by substracting aspecific binding from total binding of [^{3}H]-dexamethasone and can be used to calculate both the number of GR as well as the K_D of the receptor (94).

The low-dose dexamethasone suppression test

Central GC sensitivity can also be studied *in vivo* using a dexamethasone suppression test (DST). A DST interferes with the physiological feedback system, as the body does not discriminate between the body's own cortisol and synthetic GC at sites of central feedback. This principle has been elegantly applied in the low-dose 0.25 mg DST, revealing subtle

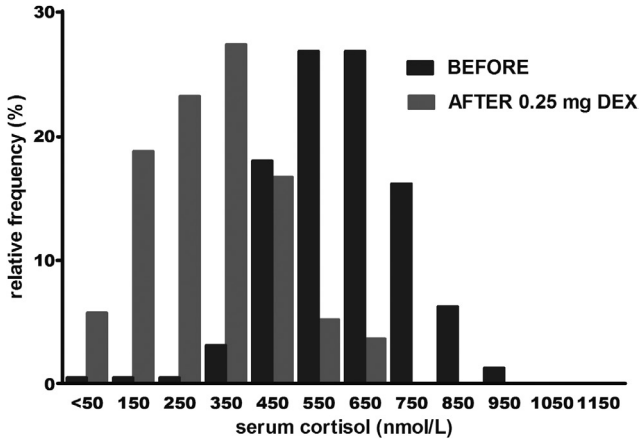

Figure 5. Relative frequency (%) of serum cortisol levels before and after ingestion of 0.25mg dexamethasone (90).

but evident interindividual differences in post-dexamethasone serum cortisol levels while intra-individual variation is limited (Figure 5) (90). Furthermore, salivary cortisol, as has been measured in this thesis, might even better represent the functional plasticity of the HPA-axis since salivary cortisol reflects the free biologically active portion of cortisol (95).

Glucocorticoid sensitivity in health

Glucocorticoid sensitivity refers to the interindividual differential effects of GC, both with respect to endogenously produced cortisol and to therapeutically or experimentally administered GC. In healthy individuals, large interindividual differences in GC sensitivity have been measured, using lymphocyte proliferation assays (87, 89), oral and intravenous DST (87-88, 90), GC binding assays (87) and our previously described bioassays (91). Interestingly, intra-individual GC sensitivity is more or less stable (90), although diurnal and seasonal variation has been demonstrated (86, 96-97). Gender and age may also influence GC sensitivity (98-102).

Furthermore, Ebrecht and co-workers elegantly demonstrated that GC sensitivity is highly tissue-specific using three different assays (skin blanching assay, DST and DEX-mediated cytokine suppression) (103). Cell-type and tissue-specific GC sensitivity might be greatly determined by pre-existing ligand-independent chromatin accessibility, further modulated by local interaction with regulatory factors (e.g. NFκB, AP-1) (104). Interestingly, long-term cortisol levels can be measured in scalp hair (105). By using this method, it is possible to create retrospective timelines of cortisol exposure. Long-term cortisol levels are associated with the body-mass index in healthy controls (106).

Mechanisms of glucocorticoid resistance

GC resistance has been studied extensively and was found to depend on multiple mechanisms including genetic variation, GC availability, GC signaling and GC counteracting processes (107-108).

Genetics

Four functional genetic variants of the GR gene, i.e. 9β, *Bcl*I, N363S, and ER22/23EK, are implicated in differences in GC sensitivity in a variety of diseases (see section '*Genetic determinants of glucocorticoid sensitivity'* and (82)). Using transiently transfected COS-1 cells, it was demonstrated that the 9β gene variant is associated with increased expression and stabilization of the dominant negative GR-β isoform (109). *Ex vivo* analysis in PBMC of two homozygous 9β carriers also showed this SNP to selectively alter transrepression of IL-2 while leaving transactivation of GILZ untouched (110). Functionality studies on the ER22/23EK polymorphism

revealed higher expression of the GR-A translational isoform which is associated with less effective transactivation *in vitro* (111-112).
The mechanisms possibly underlying the beneficial effects on GC sensitivity of the *Bcl*I and N363S polymorphisms have not yet been elucidated. Micro-array studies, however, revealed a distinct gene expression profile in human osteosarcoma cells stably transfected with the N363S variant (113).
More recently, Tantisira and co-workers identified the GLCCI1 SNP as an independent predictor of GC inhalation therapy outcome in asthma. *In vitro* analysis, by using luciferase reporter assays, demonstrated reduced transcriptional activity of the variant rs37973 allele (84). These findings were reproduced in a cohort of COPD patients in which CC-wildtype patients improved more with respect to FEV_1-values following GC inhalation therapy (85). Genetic variants in the Macrophage Migration Inhibitory Factor (MIF) and CRH genes also have been associated with altered response to intra-articular GC (114-116) and differential effects in the insulin tolerance test (ITT) (83).

Glucocorticoid Bioavailability

The majority of secreted GC (90-95%) is bound to cortisol-binding globulin (CBG) (117-118). Since only free unbound circulating levels of GC may exert their actions, variation in the levels of CBG and CBG-bound cortisol may influence the bioavailability of GC. Alternatively, cortisol may be subject to oxidation by the 11 beta hydroxysteroid dehydrogenase (11βHSD) type II enzyme, converting active cortisol into inactive cortisone. As such, an altered equilibrium between 11βHSD type I, converting cortisone into cortisol, and 11βHSD type II following cytokine secretion may change GC availability (119). Recently, 11β HSD type I was shown to be differently regulated by the synthetic glucocorticoid dexamethasone in GC sensitive (induction of 11β HSD1 mRNA) and GC resistant (repression of 11β HSD1 mRNA) leukemia cells (120). Finally, active transport of GC out of the cell by the multidrug resistance P-glycoprotein 1 (MDR1) may inhibit binding of the GR and hence GC action. Higher expression levels of this efflux pump have been associated with DMARD and GC refractory states in RA (121-122).

Glucocorticoid Function and Action

The ultimate effects of GC depend on binding of the GR, the formation of the GC-GR complex, translocation of this complex into the nucleus and the subsequent regulation of transcriptional processes. Interference at one or more of these levels may therefore influence GC action.
Indeed, low numbers and/or reduced affinity of the GR have been correlated with GC resistance in asthma (123), SLE (124) and leukemia (125).

Alternative splicing of the GR gene leading to the dominant negative GR-β isoform is postulated to negatively influence the transcriptionally active GR-α via 1) dimerization of GR-α and GR-β, 2) functional competition for cofactors, 3) tethering and 4) inhibition of histone deacetylase 2 expression (126-128). The GR-β splice variant is upregulated by pro-inflammatory cytokines *in vitro* (129-130). Opposing results on the levels of GR-β in GC resistant states have been found in a wide range of inflammatory and non-inflammatory diseases, driving the ongoing debate concerning the dominant negative effects of GR-β (Table 1 and (50, 131)).
Translational interference by differential expression of micro-RNAs, small non-coding RNAs that induce degradation of the target mRNA or inhibition of its translation, might be an additional level of GC sensitivity regulation. This has been demonstrated *in vitro* in GC resistant leukemia, sepsis and multiple myeloma cell lines (132-134).
Furthermore, MAPK-mediated phosphorylation of the GR, and hence nuclear translocation of the GR, is another modulator and potential marker of GC sensitivity (57, 135-136). Cytokine-mediated downregulation of protein phosphatase 2 might be one of the factors involved in GR phosphomodulation (136).
The cytokine MIF acts as a physiological counterregulator of the anti-inflammatory effects of GC and can hence influence GC sensitivity (137). Postulated mechanisms of GC-opposing actions of MIF include interference with GC-mediated upregulation of MKP-1 (138), activation of MAPK leading to release of arachidonic acids (139), increased ERK MAPK phosphorylation in T-cells and FLS (140-141) and inhibition of the ability of GC to increase IκBα (an inhibitor of NF-κB) (142). Higher levels of MIF, as has been found in serum, synovial fluid and cultured FLS in RA (114-115, 143-144) are therefore likely to interfere with GC signaling (114, 116).
High levels of FKBP51, a co-chaperone involved in cellular signal transduction of GC, reduce ligand affinity of the GR and hamper nuclear translocation of the GR-GC complex (145-146). Indeed, the degree of dexamethasone-induced expression of FKBP51 in PBMC served as marker for clinical response to GC in asthmatic and RA patients (92, 147-149).
Finally, recruitment of histone deacetylase (HDAC) type 2, modulating protein acetylation and gene transcription, is a major mechanism of gene repression by GC (59). Low HDAC2 expression levels have been found in PBMC and alveolar macrophages in GC resistant asthma and COPD patients and may also alter GC sensitivity in RA (150-152).

Glucocorticoid sensitivity in disease

GC posses strong anti-inflammatory effects by modulating cell trafficking, apoptosis and altering transcriptional and translational regulation of genes in virtually all cells. An imbalance in the opposing effects of the immune system and endogenous GC may lead to unrestricted immune reactions eventually becoming clinically evident as autoimmune diseases. Abnormalities in cortisol secretion and GC sensitivity have indeed been found in a variety of both inflammatory and non-inflammatory disorders as outlined below, with emphasis on RA.

Table 1. The human glucocorticoid receptor β in inflammatory and non-inflammatory disorders.

'Positive' association between GR-β and diseased states						
Disease	**Tissue**	**N**	**Method**	**Definition of GC resistance/disease severity**	**Main Findings**	**REF**
Asthma	Airway Tissue	30	Immunocytochemistry	Moderate versus severe asthma*	Higher GR-β in severe GC resistant asthma	(153)
Asthma	BAL cells	15	Immunofluorescence	<15% improvement in FEV1 after 1 wk 40mg pred/day	Reduced nuclear translocation of GR-α in GC-insensitive asthma Increased levels of cytoplasmic and nuclear GR-β	(154)
Asthma	Lung biopsy (PM)	27	Immunocytochemistry	-	More GR-β immunoreactive cells in fatal asthma compared with HC	(155)
Asthma	Skin biopsy	15	Immunocytochemistry	<15% improvement in FEV1 after 2 wk 40mg pred/day	8-fold higher GR-α/GR-β ratio in GC-sensitive patients	(156)
Asthma	PBMC	24	Immunocytochemistry Western blotting, EMSA	<15% improvement in FEV1 after 1 wk 40mg pred/day	Higher GR-β in GC-resistant asthma (IL-2 and IL-4 mediated)	(157)
SLE	PBMC	12	PCR, Western blotting	Not defined	Higher GRβ expression in active SLE patients	(158)
Colitis Ulcerosa	Colonic mucosa	38	Immunocytochemistry, PCR	CAI≥5 after 4 weeks GC (20mg/day) or need to surgery	Higher levels of GR-β in GC-resistant patients	(159)
Colitis Ulcerosa	Colonic mucosa	25	Immunocytochemistry	CAI≥5 after 4 weeks GC, dose of GC not mentioned	GR-α and GR-β are predictors of GC response, no correlation with inflammation	(160)
IBD	PBMC	66	PCR	CAI≥5 after 4 weeks GC (20mg/day) or need to surgery	Higher GRβ mRNA expression in active CU patients, but not in Crohn's disease; no correlation with indices of inflammation	(161)
Colitis Ulcerosa	PBMC	43	PCR, Western blotting	CAI≥5 after 4 weeks GC (20mg/day) or need to surgery	Higher levels of GR-β in GC-resistant patients	(162)
Crohn's disease	PBMC		PCR	CDAI<150 after 4 weeks GC (40mg/day)	High levels of GR-β in active Crohn's disease may predict GC resistance	(163)
Nasal polyps	Nasal tissue biopsy	16	Immunocytochemistry	Continuous outcome variable (see main findings)	Inverse correlation between % GR-β positive cells at baseline and % reduction in EG2-positive eosinophils	(164)
Autoimmune hepatitis	PBMC	27	PCR	severe type PT < 40%; non-severe type PT>40%	GR-β expression in non-severe type 42.9% (9/21) ; 100% (6/6) in severe type	(165)

Table 1. The human glucocorticoid receptor β in inflammatory and non-inflammatory disorders (*continued*).

Disease	Tissue	N	Method	Definition of GC resistance/disease severity	Main Findings	REF
Ankylosing Spondylitis	PBMC	25	PCR	Mean (SD)BASDAI 3.7 (2.1)	Higher levels of GR-β in AS patients. No correlation with disease activity	(166)
Rheumatoid Arthritis	PBMC	22	PCR/flow cytometry	Hydrocortisone EC_{50}>10^{-6} M in a PBMC proliferation assay	Higher GR-β mRNA and protein in GC-resistant patients	(167)
Atopic dermatitis	PBMC	34	PCR	Severe disease (EASI>18); mild disease (EASI<5) Poor response is % reduction in EASI<8%	GR-β in severe AD is higher than in HC, GR-β is increased during topical GC treatment in lymphocytes of patients with GC-insensitive AD	(168)
'Negative' association between GR-β and diseased states						
Disease	**Tissue**	**N**	**Method**	**Definition of GC resistance/disease severity**	**Main Findings**	**REF**
Rheumatoid Arthritis	PBMC	25	PCR	Mean (SD) DAS28 4.3 (0.9)	Similar GR-β in RA and HC, no correlation with disease activity	(166)
Nasal polyps	Nasal tissue biopsy	75	PCR, immunohistochemistry	Based on nasal symptoms score, not further defined	No correlation GR-β and GC therapy outcome	(169)
Nasal polyps	Nasal tissue biopsy	64	PCR	Necessity to surgical removal of polyps	No correlation GR-β and GC therapy outcome, downregulation GR-α after GC	(170)
Mood Disorders	PBMC	167	PCR	Not applicable	Decreased GR-α in bipolar and severe depression. No changes in GR-β	(171)
Interstitial lung diseases	Lung biopsy	72	PCR, immunohistochemistry	No criteria for steroid responsiveness are defined	GR-β did not differ between sensitive and resistant patients, but GR-α did	(172)
Leukemia	PBMC	22	Western blotting	Not applicable	10-15 times lower GR-α expression, but normal GR-β expression in T-cell lymphoblastic leukemia	(173)
Idiopathic inflammatory myopathies	Muscle tissue	46	Western blotting	Not applicable	No differences in GR-isoforms expression between inclusion body myositis versus polymyositis	(174)

PM post-mortem; *according to American Thoracic Society criteria; EMSA electrophoretic mobility shift assay; GR glucocorticoid receptor; GC glucocorticoid; HC healthy controls; PCR polymerase chain reaction; CAI clinical activity index; BASDAI Bath Ankylosing Spondylitis Disease Activity Index; EASI Eczema Area and Severity Index; CDAI Crohn's Disease Activity Index; FEV1 Forced expiratory volume in 1 second; PT prothrombin time; AD Atopic dermatitis, IBD inflammatory bowel disease; SLE systemic lupus erythematosus, CU Colitis Ulcerosa, RA rheumatoid arthritis; PBMC Peripheral Blood Mononuclear Cells, BAL bronchoalveolar lavage; SD standard deviation; DAS28 disease activity score (28 joints); EC_{50} half maximum effective concentration; mRNA messenger ribonucleic acid.

Rheumatoid Arthritis

Several genetic loci haven been associated with susceptibility to develop RA, including the GR gene, where the *BclI* and 9β polymorphisms have been implicated (175-176). Furthermore, patients carrying the ER22/23EK variant more frequently used TNF-α blocking agents reflecting higher disease activity (176). Next to genetic factors influencing RA disease susceptibility and severity, several functional disturbances in GC sensitivity have been observed in RA, as outlined below.

Studies on the number of GR have obtained contradicting results (177-180). Schlaghecke studied 90 patients with early RA and found significantly lower number of GR per cell with normal affinity as compared to healthy controls (180). The same observations were done by the group of Huisman and co-workers but in female patients only (178). In contrast, Neeck et al found higher numbers of GR in early untreated RA (179). With respect to *in vivo* GC therapy outcome, Huisman and co-workers showed that GR levels at baseline did not correlate with clinical or radiological outcome after two years of GC therapy (177). Interestingly, this longitudinal study demonstrated an upregulation in the number of GR, suggesting a compensatory increase in GR to counteract the chronic inflammatory state (177). Furthermore, De and coworkers found a higher proportion of GC resistant persons in patients with RA as compared with healthy controls, according to EC_{50} levels of dexamethasone-mediated secretion of cytokines in PBMC (181).

Subject of many studies comprise HPA-axis abnormalities in RA, since Chikanza and co-workers reported the absence of a rise in cortisol in the presence of a pro-inflammatory profile in RA patients after surgery (182). Although many studies also found serum levels of cortisol that were relatively low in relation to the degree of inflammation, contradicting results are reported about the origin of these HPA-axis abnormalities. Defects at the level of the hypothalamus, the pituitary or the adrenal gland have all been suggested (summarized in Table 2).

Differences in GC sensitivity are also reflected in the wide range in clinical responses seen following treatment with GC. The first reports on GC therapy in RA date back to the late sixties and early seventies where several cross-over trials demonstrated reduction in disease activity after 1 week of oral prednisone (10-15 mg daily), although interindividual differences in response to GC therapy are not mentioned (183-186). Later on, Van Gessel and co-workers noticed that only 12 out of 20 patients (60%) had a significant decrease in disease activity after 4 weeks of treatment with 10 mg prednisone daily (187) and recent data confirm the substantial proportion of patients resistant to GC therapy (37).

Kirkham and co-workers have shown that the degree of inhibition of proliferation of Concanavalin A-stimulated PBMC by methylprednisolone correlated with clinical improvement in patients with RA (203). PBMC from patients resistant to a 10-day methylprednisolone regimen (20 mg daily intravenously) were less sensitive to dexamethasone-mediated reduction in cell proliferation (37).

Table 2. Hypothalamic-pituitary-adrenal axis in rheumatoid arthritis.

	Cohort	N	Medication	Disease Activity	Main findings	REF
CRH-test						
	Recent-onset RA	10	Untreated + 4 pt NSAIDs	ESR 29-123, SJC 4-20	Similar basal cortisol in RA and HC Similar ACTH and cortisol response in RA compared to HC	(188)
	Established RA	10	Use of NSAIDs/DMARDs	6/10 active disease	Similar basal cortisol in RA and HC. Blunted non-significant ACTH response. Cortisol response not different from HC	(189)
	Established RA	18	Withdrawal NSAIDs 3 days prior to study	ESR range 6-97	Similar basal cortisol in RA and HC Intact ACTH response, but reduced rise in serum cortisol in RA	(190)
	Established RA	10	Withdrawal NSAIDs for 6 days	Mean CRP: 18.9	Basal cortisol in HC>RA, AUC-ACTH after CRH: HC=RA Peak ACTH-RA< Peak-ACTH HC	(191)
	Recent-onset RA	20	Withdrawal NSAIDs 7 and DMARDs 14 days prior to study	DAS28>3.5	Similar basal cortisol in RA and HC ACTH and cortisol responses to CRH within normal limits	(192)
	Established RA	10	Use of NSAIDs/DMARDs	Mean ESR 56.9	Similar basal cortisol in RA and non-inflammatory arthritis Similar ACTH and cortisol level following CRH administration	(182)
	Recent-onset RA	5	Withdrawal NSAIDs 5 times $T_{1/2}$	Mean ESR 51.4	Similar basal cortisol in RA and HC (serum, urine) Similar ACTH and cortisol level following CRH administration	(193)
ACTH-test						
	Established RA	10	Withdrawal NSAIDs for 6 days	Mean CRP: 18.9	Basal cortisol RA < HC, AUC-Cortisol after ACTH: HC = RA	(191)
ITT						
	Established RA	10	NSAIDs	ESR 30-110	Similar basal cortisol in RA and HC AUC-Cortisol insulin induced hypoglycemia HC = RA	(194)
	Recent-onset and established RA	50	Withdrawal NSAIDs and DMARDs 14 days prior to study	40 patients DAS28>3.5 10 patients DAS28<1.5	Similar basal cortisol in RA and HC Consistent lower cortisol levels during ITT in RA patients, normal ACTH	(192)
Physical stress						
	Established RA	10	Use of NSAIDs/DMARDs	Mean ESR 56.9	Similar basal cortisol in RA and non-inflammatory arthritis Lowish cortisol levels in active RA, failure to increase cortisol following surgery	(182)

Table 2. Hypothalamic-pituitary-adrenal axis in rheumatoid arthritis (*continued*).

	Cohort	N	Medication	Disease Activity	Main findings	REF
	Recent-onset RA	29	Use of NSAIDs/DMARDs	Mean ESR 25	Basal cortisol RA = HC, less pronounced ACTH and smaller cortisol response	(195)
	Established RA	19	Use of DMARDs/GC	Mean DAS28 3.9	Similar basal cortisol in RA and HC Similar ACTH and cortisol patterns during exercise test	(196)
	Established RA	15	Use of NSAIDs in some patients	Mean RAI 10.6-16.7	Similar basal cortisol in RA and HC (10 AM) AUC-ACTH in untreated patients is higher, similar urinary and serum cortisol	(197)
Suppression test						
	Established RA	10	Withdrawal NSAIDs for 6 days	Mean CRP: 18.9	Basal cortisol RA < HC , similar suppression HC and RA (2mg DEX)	(191)
	Recent-onset RA	20	Withdrawal NSAIDs 7 and DMARDs 14 days prior to study	DAS28>3.5	All patients <0.06 µmol/L after 1 mg DEX	(192)
	Established RA	21	Last GC > 4 months	Mean DAS28 4.5	Similar basal cortisol in RA and HC. Cortisol suppression similar in HC and RA (20 ug i.v. DEX/m^2)	(198)
DEX-CRH test						
	Established RA	20	Use of NSAIDs/DMARDs	Mean DAS28 5.7	17/20 patients normal response 1.5 mg DST, no response to CRH challenge	(199)
Observational studies						
	Recent-onset RA	25	Use of NSAIDs/DMARDs/GC	Mean ESR>19	Similar basal cortisol and early morning rise in RA and HC Active RA persistent high salivary cortisol in the afternoon	(200)
	Recent-onset RA	15	Withdrawal NSAIDs for 3 days	Mean ESR 48	Similar basal cortisol in RA and HC	(201)
	Recent-onset RA	34	No DMARDs or GC	High IL6/TNF-α	Basal cortisol in RA higher than in HC Decreased serum cortisol-to-cytokine ratios in RA	(202)

ESR erythrocyte sedimentation rate; DAS28 disease activity score (28 joints); RAI Ritchie Articular Index; SJC swollen joint count; RA rheumatoid arthritis; HC healthy controls; GC glucocorticoid; NSAIDs non-steroidal anti-inflammatory drugs; DMARDs disease modifying antirheumatic drugs; ACTH adrenocorticotropic hormone; IL6 interleukin 6; TNF-α tumor necrosis factor alpha; AUC area under the curve; CRH corticotropin releasing hormone; ITT insulin tolerance test; DST dexamethasone suppression test; DEX dexamethasone; i.v. intravenous; $T_{1/2}$ half-life.

Interestingly, methotrexate and sulfasalazine, both essential in the treatment of RA, have been demonstrated to increase the GR-α/GR-β ratio in PBMC and human lymphocyte cell lines, herewith modulating GC sensitivity (204). Moreover, TNF-α blocking agents seem to restore the capacity of the HPA-axis to produce cortisol as illustrated by higher levels of cortisol in those patients responding to TNF-α blocking agents (205).
Thus, GC production and sensitivity might be involved in the etiology of RA and the vicious circle of ongoing chronic inflammation in RA as well as in the response to antirheumatic therapy. In the light of recent findings supporting the presence of a 'window of opportunity' and the importance of aggressive initial therapy (28-33, 206), the lack of studies structurally evaluating the initial response to GC is remarkable, especially since regular DMARD therapy is known only to become effective after 6-12 weeks.

Pregnancy-induced amelioration of rheumatoid arthritis and the postpartum flare
Observations dating back to the beginning of the 20th century already indicated reduction of disease activity in RA during pregnancy. The mechanisms underlying this spontaneous amelioration have still not been resolved. Numerous factors mediating disease activity during pregnancy and directly postpartum have been postulated, including immunological factors, biochemical alterations, and changes in hormonal levels (estrogen, progesterone and cortisol).
Although the pregnancy-related rise in cortisol has been indisputably shown, well-designed studies correlating serum or free levels of cortisol with disease activity during pregnancy and postpartum have never been executed. The immunomodulating effects of cortisol and their role in regulating disease activity in pregnant RA patients therefore remain unclear.
Differences in GC sensitivity might become even more evident in the postpartum period, as the HPA-axis is suppressed in the first three months after delivery (207). The clinical relevance of this blunted HPA-axis is shown by the higher postpartum incidence of depression, autoimmune thyroid disease and RA itself (207-210).

GC sensitivity in other inflammatory and non-inflammatory disorders

GC resistance in SLE has been linked to higher levels of MIF (211), differences in P-glycoprotein expression (212) and decreased levels of GR-DNA binding (213). Interestingly, the number of GR in mononuclear leucocytes and the degree of GC-induced apoptosis were shown to be related to GC therapy efficacy in SLE (214-215). Disturbances in the HPA-axis have also been shown in Sjögren's syndrome (216). Great variability of GC sensitivity has been demonstrated in multiple sclerosis (MS) patients as well (217-218). Disorders of GC sensitivity in MS are illustrated by significantly higher levels of non-suppressors (>5 microgram/dl) following the 1-mg dexamethasone suppression test (219) and decreased GC sensitivity of PBMC (220-221). Interestingly, the 9β/ER22/23/EK haplotype of the GR gene is associated with a more

aggressive disease course in MS (222). GC sensitivity of PBMC at baseline correlated with clinical *in vivo* response following methylprednisolone pulse therapy (223). In a large cohort with 173 MS patients, higher baseline cortisol levels and reduced affinity of the GR receptor (while similar number of GR) were found, suggesting a compensatory mechanism (224).

The high prevalence of asthma worldwide and the fact that most asthma treatment regimens contain GC, has led to a large and dynamic research area investigating GC resistance in asthma. *In vitro* decreased sensitivity of lymphocytes has been associated with a clinical GC resistant state (225-227). Furthermore, *in vitro* and *in vivo* studies in GC resistant asthma have demonstrated decreased GR binding affinity and/or decreased GR number (123, 228-229), altered GR-AP-1 interaction (230), increased c-fos expression levels (231-232), higher levels of GR-β (153-154, 156), reduced HDAC and enhanced histone acetyltransferase (HAT) activity (150, 233), higher dexamethasone-induced expression of FKBP51 (147) and increased p38 MAPK activation (234-235). Interestingly, in the pioneer study by Sher et al, reversibility of GC sensitivity of PBMCs was clearly established by pre-treatment with IL-2 and IL-4 (123). Promising results were obtained by the group of Hakanarson who could distinguish GC responders from non-responders (based on clinical parameters) in asthma using the expression patterns of 15 genes, including NF-κB, with 84% accuracy (236). As mentioned previously, GLCCI1 gene variants are associated with efficacy of GC inhalation therapy in asthmatic patients (84). Remarkably, a 3-weekly depot of 40 mg methylprednisolone acetate for 27 weeks in patients with active Behçet's disease, an auto-inflammatory disorder characterized by oral and genital ulcera, did not result in any benefit over placebo-treated patients (237). Interestingly, a case-series reported by Tanaka and co-workers showed that patients with ocular manifestations of BD with low *in vitro* GC sensitivity had a worse clinical course as defined by more frequent relapses of ocular inflammation and higher intra-ocular pressure (238). These functional studies suggest a pivotal role for GC sensitivity in BD as well.

The research area of GC sensitivity in psychiatrical and functional somatic disorders has been thoroughly explored. Genetic diversity in the GR, FKBP51,CRH and mineralocorticoid receptor genes have subtle effects on the risk of developing an depressive episode, hypomania and post-traumatic disorders as well as on the response to antidepressant therapy (239-240). A significantly higher cortisol awakening response has been observed in patients prior to the development of a depressive episode, currently depressed patients and in patients after recovery from a depressive period (241-242). Non-suppression of cortisol in the DST has been observed more frequently in (psychotic) depression (243). A recent meta-analysis evaluating HPA-axis activity in functional somatic disorders, only revealed a significantly lower basal cortisol level in chronic fatigue syndrome and in female patients with fibromyalgia, but not in irritable bowel syndrome (244).

Variations in the GR gene associated with increased GC sensitivity, i.e. the *Bcl*I and N363S SNP, have been associated with abdominal obesity, lower bone mineral density and increased cortisol suppression. Carriers of the 9β and the 9β/ER22/23EK variant, characterized by relatively

decreased GC sensitivity, are overrepresented in patients with a healthy metabolic profile (i.e. low cholesterol, higher insulin sensitivity, more muscle mass in males, lower body weight in females) (82). Interestingly, in a large population-based cohort (N=7983) the 9β variant of the GR gene has been shown to increase the risk of myocardial infarction and coronary heart disease approximately 2-3 fold (245). Furthermore, a large pharmaco-epidemiological study found associations between high-dose GC (>7.5mg/day) and increased risk of heart failure, myocardial infarction, CVA or TIA and overall mortality (246). GC sensitivity modulated at pre-receptor levels by the 11βHSD type I and type II enzymes in the metabolic syndrome is currently receiving much attention and has led to the development of selective 11βHSD1 inhibitors (247).

Because of their apoptosis-inducing properties, GC are included in many treatment regimens for hematological malignancies and solid tumors. Inevitably, clinicians often face GC resistant states in these disorders. GC resistance is possibly related to acquired GR mutations, downregulation of the GR, dysregulation of pro-apoptotic (Bax, Bad) or anti-apoptotic factors (FKBP51, Bcl-2, Bcl-xL, McL-1), overexpression of GR co-repressors (e.g. NCoR) or differential expression of transcriptional (GR-β) or translational GR isoforms (GR-D) (248). Recently, 11βHSDI and GR-α mRNA levels were shown to be regulated differently by dexamethasone in sensitive versus resistant leukemia cells (120).

AIMS AND OUTLINE OF THESIS

Glucocorticoids are essential for the maintenance of metabolic homeostasis and the response to mental and physical (i.e. diseased states) stress. Differences in sensitivity to glucocorticoids have been associated with:

1) Susceptibility to and severity of inflammatory and non-inflammatory disorders
2) Efficacy of therapeutically administered GC in inflammatory and non-inflammatory disorders

A substantial proportion of patients with RA is resistant to GC therapy (approximately 30%). Furthermore, mounting evidence supports the presence of a blunted HPA-axis in RA. Further exploration of factors modulating GC sensitivity could provide more insight in the pathophysiology of RA and may stimulate the development of individualized 'tailor-made' GC therapy.

Therefore, this thesis will focus on determinants of glucocorticoid sensitivity in rheumatoid arthritis. The following research aims were defined:

- ✓ To investigate the incidence and the clinical implications of resistance to GC bridging therapy in early rheumatoid arthritis (Chapter 2).
- ✓ To study the clinical relevance of functional single nucleotide polymorphisms associated with altered GC sensitivity, with respect to disease activity and efficacy of GC bridging therapy in rheumatoid arthritis (Chapter 3).
- ✓ To study the association of *in vitro* GC sensitivity in active RA, by using the IL-2 and GILZ bioassays and the glucocorticoid receptor binding assay, with *in vivo* GC sensitivity, as reflected by the treatment response to GC (Chapter 4).
- ✓ To assess the HPA-axis activity in recent-onset and established RA, using (free) salivary cortisol levels and a low-dose dexamethasone suppression test. Furthermore, the potency of basal and dexamethasone suppressed salivary cortisol levels in predicting efficacy of GC bridging therapy was evaluated (Chapter 5).
- ✓ To study the average cortisol content in the very early phase of rheumatoid arthritis using a newly developed method to measure long-term levels of cortisol in hair (Chapter 6).
- ✓ To address the intriguing questions concerning pregnancy-induced amelioration and the postpartum flare in the nationwide PARA study and the possible role of glucocorticoid receptor gene polymorphisms (Chapter 7).
- ✓ To study GC sensitivity in another inflammatory disorder than rheumatoid arthritis, i.e. Behçet's disease (Chapter 8).

Chapter 9 embodies the general discussion of the findings described in this thesis.

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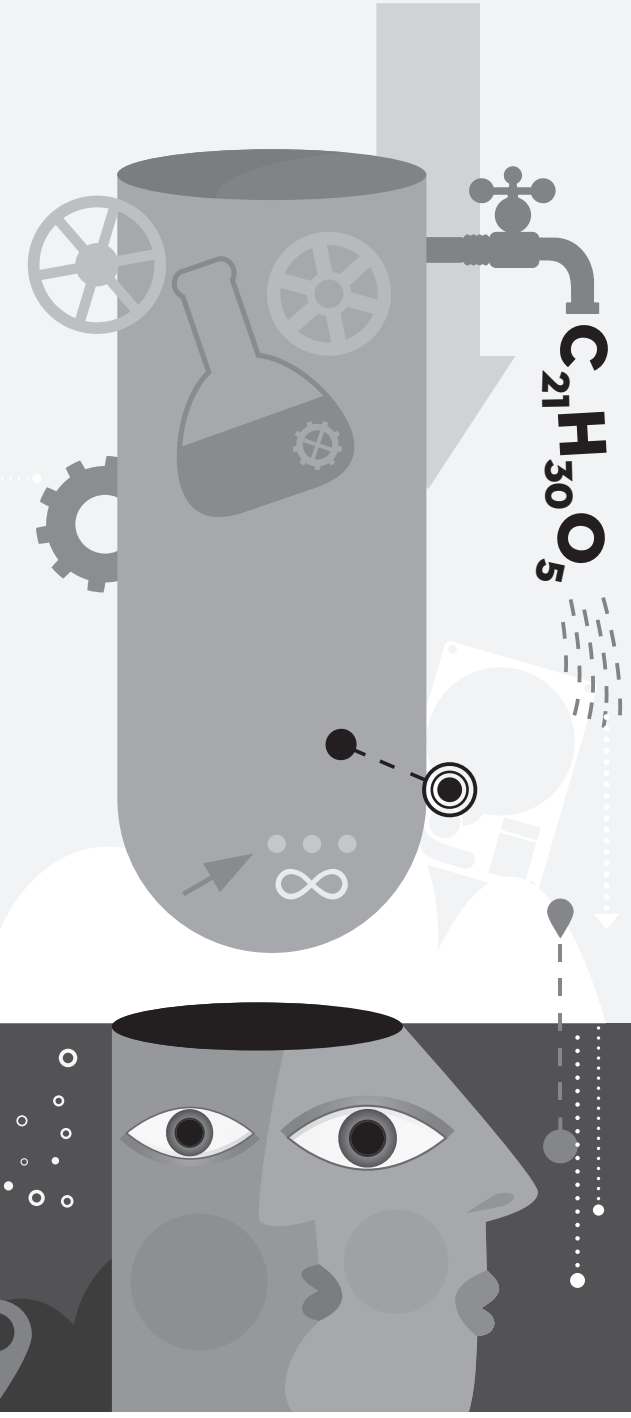

Response to glucocorticoids at 2 weeks predicts the effectiveness of DMARD induction therapy at 3 months: post hoc analyses from the tREACH study

Chapter 2

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Adapted from: Annals of the Rheumatic Diseases, 2012 Oct 31. [Epub ahead of print]

ABSTRACT

Objective

To investigate if the glucocorticoid (GC) response at 2 weeks, defined by EULAR response criteria, can predict active disease (Disease Activity Score (DAS)>2.4) at 3 months.

Methods

For this study, data of the Treatment in the Rotterdam Early Arthritis Cohort study (tREACH), an ongoing clinical trial that evaluates different induction therapies in early rheumatoid arthritis, were used. We selected patients who had a high probability of progressing to persistent arthritis (>70% based on the prediction model of Visser). All patients within the high probability stratum, who had a baseline DAS>2.2 and a DAS assessment at 2 weeks after randomization, were included (N=120). Besides GC response at 2 weeks, we investigated which other factors were associated with having active disease (DAS>2.4) after 3 months of disease modifying antirheumatic drug (DMARD) treatment. All variables with a $p \leq 0.25$ were assessed in our logistic regression model with backward selection. Variables were eliminated until all remaining variables had a significant association ($p \leq 0.05$).

Results

Patients who did not respond to GC bridging therapy at 2 weeks had an overall odds ratio (OR) of having active disease at three months of 10.29 (95% CI: 3.34 to 31.64;$p<0.001$) in comparison with responders. The corrected OR was 14.00 (95% CI: 3.31 to 59.21; $p<0.001$). Our final model predicting response at 3 months included the following variables: gender, GC response, induction therapy arms and baseline DAS, which had an explained variance of 39%.

Conclusions

GC response at 2 weeks is a useful tool for recognising those patients who will probably have active disease (DAS>2.4) after 3 months of DMARD treatment.

INTRODUCTION

The EULAR treatment guideline recommends that rheumatologists strive, in patients with newly diagnosed rheumatoid arthritis (RA), for remission or at least low disease activity within 3 months in order to obtain better functional and radiological outcomes (1-2). Since the time span for the optimal effect of disease modifying antirheumatic drugs (DMARDs) is at least 6-12 weeks (3), the right choice of the initial DMARD has an important role in obtaining recommended treatment goals. The guideline recommends methotrexate (MTX) as anchor drug, but studies show that only about 70% of patients will respond sufficiently to the initial therapy (4-5). Moreover, we recently showed that a combination of DMARDs shows better remission rates than MTX monotherapy in the early phase of RA (5). Therefore it would be helpful to be able to predict treatment response to the initial DMARD treatment as early as possible, ultimately leading to a 'tailor-made' treatment approach.

The huge body of prognostic research till now has mainly focused on predicting long-term destructive and disabling disease in order to guide the initial choice of treatment (6). In contrast, studies evaluating prediction of treatment response are sparse. Aletaha et al (7) demonstrated that high disease activity during the first 3 months of treatment are significantly related to high disease activity at 1 year, which subsequently leads to more destructive and disabling disease. Besides some possible pharmacogenetic markers (e.g. TYMS polymorphisms affect efficacy of MTX in RA), a clinical applicable predictor for treatment response to classical DMARDs in a very early stage, is unknown (8).

In line with studies performed in polymyalgia rheumatica, a clinical applicable predictor for treatment response in a very early stage might be the initial response to glucocorticoids (GCs) (9). It is well known that GCs have a rapid anti-inflammatory effect, and therefore are often used as bridging therapy to treat active disease in the period between initiation of DMARD therapy and onset of their therapeutic effect (10). However, in RA clinical responses to GCs differ between patients. Sliwinska-Stanczyk et al (11) showed that GC sensitivity of peripheral blood mononuclear cells of RA patients is related to their own observed clinical response to GCs. However, clinical data linking the early effect of GCs to DMARD response in RA are missing. Therefore our objective was to investigate whether the GC response at 2 weeks, defined according to the EULAR response criteria (12), predicts DMARD response at 3 months.

PATIENTS AND METHODS

Patients

For this study data were used of a current clinical trial (ISRCTN26791028), Treatment in the Rotterdam Early Arthritis Cohort (tREACH) (13). The tREACH study, a multicenter, stratified

single-blinded trial to evaluate different induction treatment strategies in early RA, is being carried out in eight rheumatology centers in the Netherlands. The medical ethics committee at each participating center approved the study protocol, and all patients gave written informed consent before inclusion.

An extended description of the material and methods section of the tREACH study has already been published (13). Inclusion criteria for the tREACH study are: age ≥18 years, arthritis ≥1 joint and symptom duration <1 year. Eligible patients were stratified into three groups according to their likelihood of progressing to persistent arthritis based on the prediction model of Visser (14). The three strata (low, intermediate and high) correspond with probability tertiles of developing persistent arthritis according to the Visser model. The Visser algorithm and 2010 criteria for RA have similar discriminative abilities to identify patients at risk of persistent arthritis at 1 year (15).

For our analysis we selected all patients who had a high probability of developing persistent arthritis and a disease activity score (DAS) assessment at 2 weeks after randomization. Not all patients in the high probability stratum had a DAS assessment at 2 weeks, because this assessment was part of a substudy, primarily evaluating differences in GC sensitivity, embedded in the tREACH. Furthermore patients with a DAS≤2.2 and/or DAS28≤3.3 were excluded, because the EULAR response criteria are only valid in patients having a baseline DAS>2.2 (DAS28>3.3) (12).

Methods

Patients were randomized, using variable block randomization stratified for center, into one of three initial treatment strategies (later referred to as 'induction therapy arms'):

A. Combination therapy (MTX, sulfasalazine (SSZ) and hydroxychloroquine (HCQ)) with GCs intramuscularly);
B. Combination therapy with an oral GCs tapering scheme;
C. MTX with an oral GCs tapering scheme.

DMARD dosages were: MTX 25 mg/week orally or subcutaneously (starting dose 10mg/week, maximum dosage reached after 3 weeks); SSZ first week 1 g/day, thereafter 2 g/day; HCQ 400 mg/day. GCs were either given as a single intramuscular dose at randomisation (methylprednisolone 120mg or triamcinolone acetonide 80mg) or prednisone in an oral tapering scheme (week 1-4: 15 mg/day, week 5-6: 10 mg/day, week 7-8: 5 mg/day and week 9-10: 2.5 mg/day). We used a treat-to-target approach, with patients being examined every three months. Treatment decisions were based, every three months, upon the DAS thresholds for low disease activity (16). When *'treatment failure'* occurred, defined as DAS>2.4, medication was intensified to MTX with etanercept (50mg/week). Treatment intensifications were the same in each stratum for each treatment arm.

Demographic and disease characteristics of each patient were recorded at baseline. After 2 weeks and after 3 months the following variables were assessed: a 44-joint count for swelling, a graded 53-joint count for tenderness (17), general health and erythrocyte sedimentation rate, which we used to calculate the DAS and 28-joint count DAS (DAS28). At 2 weeks we also determined the EULAR response criteria (12). EULAR response criteria are based on attained level and change in DAS.

Statistical analysis

First, we investigated whether a GC response at 2 weeks, defined according to EULAR response criteria, was associated with DMARD response at 3 months of treatment. Active disease at 3 months was defined as DAS>2.4. The discriminative ability of GC response at 2 weeks for identifying active disease at 3 months was expressed by sensitivity and specificity. To overcome confounding by medication we also carried out a stratified analysis for induction therapy arms. All analyses were also performed for the DAS28; active disease was defined as DAS28>3.2 (18).
Furthermore, we determined which other factors were associated with active disease at 3 months by comparing the baseline characteristics of patients with and without active disease after 3 months of DMARD induction therapy. Statistical comparison between baseline characteristics was made by the student's t-test, χ^2 test, or the Wilcoxon rank-sum test, as appropriate. All variables with a $p \leq 0.25$ together with known prognostic factors (age, gender, disease duration, rheumatoid factor (RF), anti-citrullinated peptide antibodies (ACPA) and baseline DAS) were analyzed using univariate and multivariate logistic regression (with backward selection). In our backward selection procedure the variable with the highest p value was eliminated from the model, until all variables in the model had a significant association ($p \leq .0.05$).
All statistical analyses were carried out using STATA V.11.1. A $p \leq 0.05$ was considered statistically significant.

RESULTS

Of the 281 tREACH patients within the high probability stratum 132 patients (47%) had a DAS assessment at 2 weeks after randomization. Of those patients, 12 (9%) were excluded because of a baseline DAS≤2.2. These 12 patients all had a DAS≤2.4 at 3 months of follow-up. Table 1 shows the baseline characteristics of the 120 patients. Patients were more often female (65%) and had a median symptom duration of 161 days (97 – 210 days, IQR). RF and/or ACPA positivity was present in 92 patients (77%), of those 70 (76%) were both RF and ACPA positive. At baseline 20 patients (17%) had ≥1 erosion typical for RA. Active disease (DAS>2.4) was found in 113 patients (94%).

Table 1. Baseline characteristics for patients with a DAS>2.2.

	Total population (N=120)
Age (years), median (IQR)	54 (44 – 63)
Symptom duration (days), median (IQR)	161 (97 – 210)
Female gender, N (%)	78 (65)
Rheumatoid Factor (IgM) positive, N (%)	78 (65)
ACPA positive, N (%)	84 (70)
Morning stiffness >1 h, N (%)	93 (78)
Presence of Erosions, N (%)	20 (17)
Fulfillment of RA criteria, N (%)	
• 1987	82 (68)
• 2010	114 (95)
DAS, mean (95% CI)	3.43 (3.28 – 3.57)
TJC44, median (IQR)	10 (5 – 15)
SJC44, median (IQR)	8 (4 – 12)
ESR (mm/h), median (IQR)	22 (13 – 39)
General Health (0-100mm), median (IQR)	53 (37 – 66)
Treatment, N (%)	
A. MTX+SSZ+HCQ+GCs IM	43 (35.8)
B. MTX+SSZ+HCQ+GCs oral	39 (32.5)
C. MTX+GCs oral	38 (31.7)

ACPA: anti-citrullinated peptide antibodies; ESR: erythrocyte sedimentation rate; GCs: glucocorticoids; HCQ: hydroxychloroquine; SSZ: sulfasalazine; IM: intramuscular; MTX: methotrexate; RA: rheumatoid arthritis; DAS: Disease Activity Score; SJC44: swollen joint count (44 joints); TJC44: tender joint count (44 joints); IQR: interquartile range.

The relation between GC response at 2 weeks, defined according to the EULAR response criteria, and having active disease after 3 months of induction DMARD therapy is shown in table 2A. A total of 39 out of 78 patients with a DAS≤2.4 after 3 months of DMARD therapy (50%), were classified as good GC responders, whereas this was only the case for 6 out of 42 patients (14%) who still had active disease (DAS>2.4). Vice versa, in patients with a DAS≤2.4 after 3 months, only 12 of 78 patients (15%) did not respond initially to GC bridging therapy as distinct from 19 of 42 patients with active disease at 3 months (45%) who were classified as GC non-responders. Patients who do not respond to GC bridging therapy at 2 weeks had an overall OR of having active disease at three months of 10.29 (95% CI: 3.34-31.64; p<0.001) in comparison with responders.

Table 2B demonstrates the relationship between GC response and disease activity states stratified for induction therapy arms. The OR (95% CI) for active disease after 3 months of being a GC non-responder relative to a good GC responder for treatment arm (A), (B) and (C) is, respectively, 4.2 (0.75 to 23.18); 10.7 (0.98 to 115.7) and infinite. In treatment arm C,

Table 2. Response to GC bridging therapy and the presence of active disease (DAS>2.4) after 3 months of DMARD induction therapy.

A.

	Active disease	
Response to GCs at 2 wks	Yes (N=42)	No (N=78)
• Good, N (%)	6 (13)	39 (87)
• Moderate, N (%)	17 (39)	27 (61)
• None, N (%)	19 (61)	12 (39)

B.

	Active disease	
Response to GCs at 2 wks	Yes (N=42)	No (N=78)
A. MTX+SSZ+HCQ+GCs IM, N (%)		
• Good	3 (17)	15 (83)
• Moderate	3 (21)	11 (79)
• None	5 (45)	6 (55)
B. MTX+SSZ+HCQ+GCs oral, N (%)		
• Good	1 (6)	16 (94)
• Moderate	5 (42)	7 (58)
• None	4 (40)	6 (60)
C. MTX+GCs oral, N (%)		
• Good	2 (20)	8 (80)
• Moderate	9 (50)	9 (50)
• None	10 (100)	0 (0)

The relationship between disease activity after 3 months of induction DMARD therapy and response to GCs at 2 weeks in all patients (A) and stratified for induction therapy arms (B). DAS: Disease Activity Score; DMARDs: disease modifying antirheumatic drugs; GCs: glucocorticoids; HCQ: hydroxychloroquine; IM: intramuscular; MTX: methotrexate, SSZ: sulfasalazine.

with current recommended induction therapy, all GC non-responders had active disease at 3 months. The same analysis was performed for DAS28 instead of DAS, and showed similar results (see supplementary tables 1 and 2).

To determine the discriminative ability of GC response at 2 weeks for identifying active disease at 3 months, the following two cut-offs were used: (1) being a non-responder to GC or not and (2) being a non-responder or moderate responder to GC or not. The sensitivity (95% CI) and specificity (95% CI) of GC response to identify active disease, using the first cut-off point, were, respectively, 45% (30% to 61%) and 85% (75% to 92%). For the second cut-off point the calculated sensitivity (95% CI) and specificity (95% CI) were, respectively, 86% (72% to 95%) and 50% (39% to 62%).

Second, we investigated which other factors were associated with having active disease after 3 months of DMARD therapy (table 3). Besides known prognostic factors (age, gender, disease duration, RF, ACPA and baseline DAS), other possible variables associated with active disease after 3 months of DMARD therapy were: type of induction therapy (treatment arm

Table 3. Baseline characteristics of patients with and without active disease (DAS>2.4) after 3 months of induction DMARD treatment.

	Active disease		p value
	Yes (N=42)	No (N=78)	
Age (years), median (IQR)	55 (45 – 63)	54 (43 – 64)	0.69
Female gender, N (%)	35 (83)	43 (55)	0.002
Symptom duration (days), median (IQR)	139 (92 – 208)	164 (116 – 214)	0.30
Rheumatoid Factor (IgM) positive, N (%)	24 (57)	54 (69)	0.19
ACPA positive, N (%)	27 (64)	57 (73)	0.31
Morning stiffness >1 h, N (%)	33 (79)	60 (77)	0.84
Presence of Erosions, N (%)	4 (10)	16 (21)	0.12
Fulfillment RA criteria, N (%)			
• 1987	28 (67)	54 (69)	0.77
• 2010	42 (100)	72 (92)	0.07
DAS, mean (95% CI)	3.89 (3.65 to 4.14)	3.17 (3.02 to 3.34)	<0.0001
TJC44, median (IQR)	14 (10 – 21)	7 (3 – 14)	<0.0001
SJC44, median (IQR)	8.5 (4 – 12)	8 (4 – 12)	0.95
ESR (mm/h), median (IQR)	29 (17 – 45)	20 (12 – 34)	0.03
General Health (0-100mm), median (IQR)	54 (50 – 70)	51.5 (30 – 65)	0.02
Treatment, N (%)			
A. MTX+SSZ+HCQ+GCs IM	11 (26)	32 (41)	0.11
B. MTX+SSZ+HCQ+GCs oral	10 (24)	29 (37)	0.14
C. MTX+GCs oral	21 (50)	17 (22)	0.002

ACPA: anti-citrullinated peptide antibodies; DMARD: disease modifying antirheumatic drug; ESR: erythrocyte sedimentation rate; GCs: glucocorticoids; HCQ: hydroxychloroquine; IM: intramuscular; MTX: methotrexate; SSZ: sulfasalazine; RA: rheumatoid arthritis; DAS: Disease Activity Score; SJC44: swollen joint count (44 joints); TJC44: tender joint count (44 joints).

(A), (B) or (C)), presence of erosions and the components of the baseline DAS, except swollen joint count. Table 4 shows the univariate logistic regression (4A) and final multivariate model (4B), after backward selection, for the prediction of active disease after 3 months of DMARD induction therapy. The final model had an explained variance of 39%. The same analysis was performed for DAS28 instead of DAS, which showed similar results (see supplementary table 3).

DISCUSSION

We investigated if the GC response at 2 weeks, defined by EULAR response criteria, can predict active disease after 3 months of DMARD induction therapy. Patients who do not respond to GC bridging therapy at 2 weeks have an overall OR of having active disease at three months

Table 4. Predicting active disease (DAS>2.4) at 3 months with (prognostic) variable(s), using univariate logistic regression (A) and logistic regression model with backward selection (B).

A.		OR (95% CI)	p value
	Age (years)	1.01 (0.98 – 1.03)	0.72
	Sex (1=female)	4.07 (1.61 – 10.27)	0.003
	Symptom duration (days)	1.00 (0.99 – 1.00)	0.387
	RF (1=positive)	0.59 (0.27 – 1.29)	0.187
	ACPA (1=positive)	0.66 (0.30 – 1.48)	0.318
	Erosion typical for RA (1=present)	0.41 (0.13 – 1.31)	0.132
	GCs response at 2 wks (ref. = good)		
	• moderate	4.09 (1.43 – 11.72)	0.009
	• none	10.29 (3.34 – 31.64)	<0.001
	Treatment (ref. =MTX+GCs oral)		
	• MTX+SSZ+HCQ+GCs IM	0.28 (0.11 – 0.71)	0.007
	• MTX+SSZ+HCQ+GCs oral	0.28 (0.11 – 0.73)	0.009
	DAS	3.50 (1.95 – 6.30)	<0.001
	TJC44	1.13 (1.06 – 1.19)	<0.001
	ESR (mm/h)	1.01 (1.00 – 1.04)	0.062
	General Health (0-100mm)	1.02 (1.00 – 1.04)	0.017

B.		OR (95% CI)	p value
	Sex (1=female)	5.98 (1.67 – 21.40)	0.006
	GCs response at 2 wks (ref. = good)		
	• Moderate	1.67 (0.48 – 5.88)	0.424
	• none	14.00 (3.31 – 59.21)	<0.001
	Treatment (ref. = MTX+GCs oral)		
	• MTX+SSZ+HCQ+GCs IM	0.25 (0.07 – 0.90)	0.03
	• MTX+SSZ+HCQ+GCs oral	0.18 (0.05 – 0.69)	0.01
	DAS	5.54 (2.55 – 12.04)	<0.001

ACPA: anti-citrullinated peptide antibodies; ESR: erythrocyte sedimentation rate; GCs: glucocorticoids; HCQ: hydroxychloroquine; IM: intramuscular; MTX: methotrexate; RA: rheumatoid arthritis; RF: rheumatoid factor; SSZ: sulfasalazine; DAS: Disease Activity Score; TJC44: tender joint count (44 joints).

of 10.29 (95% CI 3.34 to 31.64; p<0.001) in comparison with responders. If we stratify for induction therapy arms, ORs (95% CI) were 4.2 (0.75 to 23.18); 10.7 (0.98 to 115.7) and infinite for respectively treatment arms (A), (B) and (C). In treatment arm C, MTX with an oral GCs tapering scheme, all GC non-responders had active disease after 3 months of DMARD therapy. Until now a clinical applicable predictor for treatment response of classical DMARDs in a very early stage was missing. However, we have shown that assessment of disease activity at 2 weeks, reflecting the initial response to GCs, might be a predictor of active disease after 3 months of induction DMARD treatment.

Although our data do not necessarily indicate a direct causal association it is tempting to speculate about possible synergistic effects of GCs and DMARDs. GCs and DMARDs have mutual anti-inflammatory pathways. The anti-inflammatory actions of GCs are mediated via the GC receptor and include an transrepressive effect on the transcription factor nuclear factor kappa B (NF-κB) (19). Other studies have shown that SSZ and MTX both suppress activation of NF-κB by inhibiting degradation of IκBα *in vitro* (20-21). Another mutual pathway might be the effect of GCs and DMARDs on the intracellular levels of cyclic AMP (cAMP). SSZ and MTX promote the release of the sympathetic neurotransmitter adenosine (22) and hence the ligation of A2-receptors whereas GCs stimulate the expression of β-adrenoreceptors. Ligation of β-adrenoreceptors and A2 receptors both lead to higher levels of intracellular cAMP which eventually is essential in inhibiting the production of pro-inflammatory cytokines by stimulating CREB-responsive elements (23). Furthermore, the MTX and SSZ-stimulated upregulation of adenosine also inhibits the conversion of cortisol to inactive cortisone (24). Complementary to this reduced level of oxidation of cortisol, MTX and SSZ both have been demonstrated to upregulate the biologically active α-isoform of the glucocorticoid receptor in human monocytic/macrophage and lymphocyte cell lines (25-26). Both mechanisms could possibly potentiate the effects of (exogenously administered) GC. Interestingly, the degree of MTX-induced GR-α upregulation in peripheral blood mononuclear cells was associated with MTX-outcome at 3 months *in vivo* (27). Finally, it could be hypothesized that by reducing the initial inflammatory load, GC facilitate optimal efficacy of DMARDs. In line with this hypothesis is the fact that DMARD failure is associated with high baseline disease activity. Of note, active disease at 3 months is a combined endpoint of GC and DMARD therapy and could therefore still reflect GC insensitivity in combination with DMARD failure, independent of possible synergistic pathways.

Other non-modifiable baseline predictors associated with active disease after 3 months of DMARD induction therapy are gender and baseline DAS. The only modifiable baseline predictor is the choice of induction therapy. First, the relationship between gender and active disease is probably found because women experience more pain, resulting in higher DAS values and more functional impairment than men (28-29). Second, the baseline disease activity is an important predictor for disease activity (states) during follow-up, which is reconfirmed in our study (30). Finally, the choice of induction therapy, which is the only modifiable predictor at presentation, determines the clinical response.

The EULAR treatment guideline recommends a treat-to-target approach in which rheumatologists should strive for remission or low disease activity within 3 months, in patients with newly diagnosed RA with active disease (2). Until the desired target is reached, treatment should be altered every 1-3 months (2). Recommended induction therapy consists of MTX with or without GCs (1). However, some points in the mentioned recommendation can be discussed. First, the choice of induction therapy wherein DMARD monotherapy is preferred over a combination of DMARDs. Current guidelines are based upon a systemic review (31), which

concluded that in DMARD-naïve patients the efficacy/toxicity ratio favours MTX monotherapy over combination therapy. However, in this review, triple DMARD therapy versus MTX monotherapy in DMARD-naïve patients was not compared. Furthermore, trials favouring triple DMARD treatment (BeSt, FIN-RACo and COBRA trial) were excluded from this review (4, 32-33). In a previous publication we have already shown that in patients with early RA a combination of DMARDs is superior to MTX monotherapy in achieving low disease activity after three months (5), which is supported by a recent systematic review by Graudal and Jürgens (34).

Second, the time span for the optimal effect of DMARDs takes at least 6-12 weeks (3), and thus the right choice of induction DMARD treatment has an important role in obtaining recommended treatment goals. Furthermore, several studies have shown that only about 70% will respond sufficiently to the initial treatment (4-5). A tailor-made treatment approach might be preferable, however, no clinical applicable predictors for early treatment response are available.

Therefore in daily practice we advice starting with a combination of DMARDs. However, if MTX monotherapy is preferred, either by the rheumatologist or patient, we recommend combining MTX with a GC bridging scheme and determining the response to GC after 2 weeks. Patients who do not respond to GC after 2 weeks have a higher risk of not reaching the treatment goals and therefore a higher risk of a poorer outcome. It seems sensible to intensify the DMARD treatment, if patients do not respond to GC after 2 weeks.

Our study had certain limitations. First, sample size calculations were not based upon our research question and therefore we had a small sample size, especially restricting the stratified analysis for induction therapy arms. Despite the small sample size we found significant ORs for active disease after 3 months of DMARD therapy of approximately 10 for non-responders relative to good responders.

Second, not all patients in the high probability stratum had a DAS assessment at 2 weeks, which possibly introduces a selection bias. The DAS assessment at 2 weeks was part of a substudy, primarily evaluating differences in GC sensitivity. Inclusion for the tREACH and the mentioned substudy started concurrently, with all randomised patients automatically enrolled in the substudy. The DAS assessment at 2 weeks was terminated, because the substudy had reached its predefined sample size. Therefore we think that a significant selection bias did not arise.

Third, the requirements for EULAR response criteria are a baseline DAS>2.2, as a result of which 12 patients (9%) were excluded from the analyses. Consequently, in daily practice we cannot use a GC response to predict DMARD response in patients with a low baseline DAS. In our study, however, we showed that none of the patients with a baseline DAS≤2.2 had active disease after 3 months of DMARD treatment. Therefore, if adequate DMARD therapy is initiated, we can assume that patients with a baseline DAS≤2.2 will respond to this treatment. Future research is necessary to validate our findings and to evaluate the clinical applicability of GC response as a prediction tool in daily practice.

CONCLUSIONS

Determining GC response at 2 weeks is a useful tool for recognizing those patients who will probably have active disease (DAS>2.4) after 3 months of DMARD therapy.

ACKNOWLEDGEMENTS

We thank all patients who are enrolled in the tREACH trial. Without their active cooperation, our trial would not be possible. The tREACH trial comprises the following rheumatology centers: Erasmus MC, Rotterdam; Sint Franciscus Gasthuis, Rotterdam; Maasstad Ziekenhuis, Rotterdam; Vlietland Ziekenhuis, Schiedam; Admiraal de Ruyter Ziekenhuis, Goes and Vlissingen; Zorgsaam Ziekenhuis, Terneuzen; Albert Schweitzer Ziekenhuis, Dordrecht. We thank the following people from all centers (listed alphabetically) for their contribution to the tREACH trial: Aartsen R, Alfenaar C, Alves C, Arendse R, Arnoldus M, Baak-Dijkstra M, Bal-overzier J, Barendregt P, Basoski N, Beer S, Berkel D, Bonte F, Born van den M, Breukelen van D, Bron S, Buijs H, Buijs N, Cambier M, Claessen S, Colin E, Dekker A, Dolhain R, Donze M, Fodili F, Grillet de B, Haasnoot H, Hamelink B, Han K, Houdt van Y, Hove van L, Jager de J, Jager de M, Jasperse J, Jonkers C, Joziasse S, Klootwijk K, Krommenhoek T, Krugten van M, Lam Tse W, Leemput van H, Legierse C, Lubbe van de P, Maclean P, Man de Y, Matena D, Molenaar A, Mous L, Nijs J, Paassen van H, Reijnierse J, Romme A, Rotte de M, Schaeybroeck B, Schardijn G, Schrauwen S, Sturm L, Sutter T, Tchetverikov I, Tusschenbroek D, Veldman R, Voordt van der A, Voorneveld H, Vroed de M, Walravens M, Walter M, Werff van de N, Westeinde van het A, Wiele J, Willemse M, Wouter J, Zandbergen W, Zeben van D, Zwart H.

Supplementary data. Relationship between GC response at 2 weeks and active disease at 3 months using the DAS28.

Supplementary Table 1. Baseline characteristics for patients with a DAS28>3.3, also stratified for active disease (DAS28>3.2) after 3 months of induction DMARD therapy.

	Total population	**Active disease**		**p value***
	(N=120)	Yes (N=59)	No (N=61)	
Age (years), median (IQR)	55 (45 – 64)	55 (46 – 63)	54 (44 – 66)	0.62
Female gender, N (%)	79 (66)	45 (76)	34 (56)	0.02
Symptom duration (days), median (IQR)	161 (96 – 201)	137 (88 – 197)	172 (133 – 214)	0.07
Rheumatoid Factor (IgM) positive, N (%)	78 (65)	37 (63)	41 (67)	0.61
ACPA positive, N (%)	84 (70)	41 (69)	43 (70)	0.90
Morning stiffness >1hr., N (%)	93 (78)	46 (78)	47 (77)	0.90
Presence of Erosions, N (%)	20 (17)	8 (14)	12 (20)	0.37
Fulfillment RA criteria, N (%)				
• 1987	83 (69)	42 (71)	41 (67)	0.64
• 2010	114 (95)	58 (98)	56 (92)	0.10
DAS28, mean (95% CI))	4.96 (4.78 – 5.14)	5.30 (5.05 – 5.55)	4.63 (4.39 – 4.87)	0.0002
TJC28, median (IQR)	6 (2 – 10)	4 (8 – 13)	4 (2 – 9)	0.001
SJC28, median (IQR)	6 (4 – 10)	6 (4 – 10)	6 (3 – 10)	0.95
ESR (mm/hr), median (IQR)	22.5 (13 – 39)	24 (16 – 44)	20 (13 – 34)	0.07
General Health (0-100mm), median (IQR)	53 (37.5 – 67.5)	55 (49 – 71)	52 (29 – 62)	0.02
Treatment, N (%)				
A. MTX+SSZ+HCQ+GCs IM	42 (35)	18 (30)	24 (39)	0.31
B. MTX+SSZ+HCQ+GCs oral	40 (33)	17 (29)	23 (38)	0.30
C. MTX+GCs oral	38 (32)	24 (41)	14 (23)	0.04

*p value= testing difference in baseline characteristics between patients with and without active disease after 3 months of induction DMARD therapy.

ACPA: Anti-citrullinated peptide antibodies; CI: Confidence Interval; ESR: erythrocyte sedimentation rate; GCs: glucocorticoids; HCQ: hydroxychloroquine; IM: intramuscular; IQR: Interquartile range; MTX: methotrexate; SSZ: sulfasalazine; DAS28: Disease Activity Score (28 joints); SJC28: swollen joint count (28 joints); TJC28: tender joint count (28 joints).

Supplementary Table 2. Response to GC bridging therapy and the presence of active disease (DAS28>3.2) after 3 months of DMARD induction therapy.

A.		**Active disease**	
	GCs response at 2 wks	Yes (N=59)	No (N=61)
	• Good, N (%)	11 (23)	36 (77)
	• Moderate, N (%)	26 (53)	23 (47)
	• None, N (%)	22 (92)	2 (8)

B.		**Active disease**	
	GCs response at 2 wks	Yes (N=59)	No (N=61)
	MTX+SSZ+HCQ+GCs IM, N (%)		
	• Good	4 (25)	12 (75)
	• Moderate	6 (35)	11 (65)
	• None	8 (89)	1 (11)
	MTX+SSZ+HCQ+GCs oral, N (%)		
	• Good	2 (11)	16 (89)
	• Moderate	11 (65)	6 (35)
	• None	4 (80)	1 (20)
	MTX+GCs oral, N (%)		
	• Good	5 (38)	8 (62)
	• Moderate	9 (60)	6 (40)
	• None	10 (100)	0 (0)

The response after GC bridging therapy, defined according to EULAR response criteria, and the relationship with active disease (DAS28≥3.2) after 3 months of induction DMARD therapy in all patients (A) and stratified for induction therapy (B). DAS28: Disease Activity Score (28 joints); DMARD: disease modifying antirheumatic drugs; GCs: glucocorticoids; HCQ: hydroxychloroquine; IM: intramuscular; MTX: methotrexate, SSZ: sulfasalazine.

Supplementary Table 3. Predicting active disease (DAS28>3.2) at 3 months with (prognostic) variable(s), using univariate logistic regression (A) and logistic regression model with backward selection (B).

A.	**OR (95% CI)**	**p value**
Age (years)	1.01 (0.98 – 1.03)	0.68
Sex (1=female)	2.55 (1.17 – 5.59)	0.02
Symptom duration (days)	1.00 (0.99 – 1.00)	0.14
Rheumatoid Factor (IgM) (1=positive)	0.82 (0.39 – 1.74)	0.61
ACPA (1=positive)	0.95 (0.44 – 2.08)	0.91
Erosion typical for RA (1=present)	0.64 (0.24 – 1.70)	0.37
GCs response at 2 wks (ref. = good)		
• moderate	3.70 (1.54 – 8.90)	0.003
• none	36 (7.29 – 177.82)	<0.001
Treatment (ref. = MTX+GCs oral)		
• MTX+SSZ+HCQ+GCs IM	0.44 (0.18 – 1.07)	0.07
• MTX+SSZ+HCQ+GCs oral	0.43 (0.17 – 1.07)	0.07
DAS28	2.10 (1.39 – 3.18)	<0.001
TJC28	1.12 (1.04 – 1.21)	0.003
ESR (mm/hr)	1.02 (1.00 – 1.04)	0.05
General Health (0-100mm)	1.02 (1.00 – 1.04)	0.02

B.	**OR (95% CI)**	**p value**
GCs response at 2 wks (ref. = good)		
• moderate	2.29 (0.87 – 6.00)	0.09
• none	30.35 (6.00 – 153.45)	<0.001
DAS28	1.96 (1.20 – 3.18)	0.007

ACPA: anti-citrullinated peptide antibodies; ESR: erythrocyte sedimentation rate; GCs: glucocorticoids; HCQ: hydroxychloroquine; IM: intramuscular; MTX: methotrexate; RA: rheumatoid arthritis; SSZ: sulfasalazine; DAS28: Disease Activity Score (28 joints); TJC28: tender joint count (28 joints).

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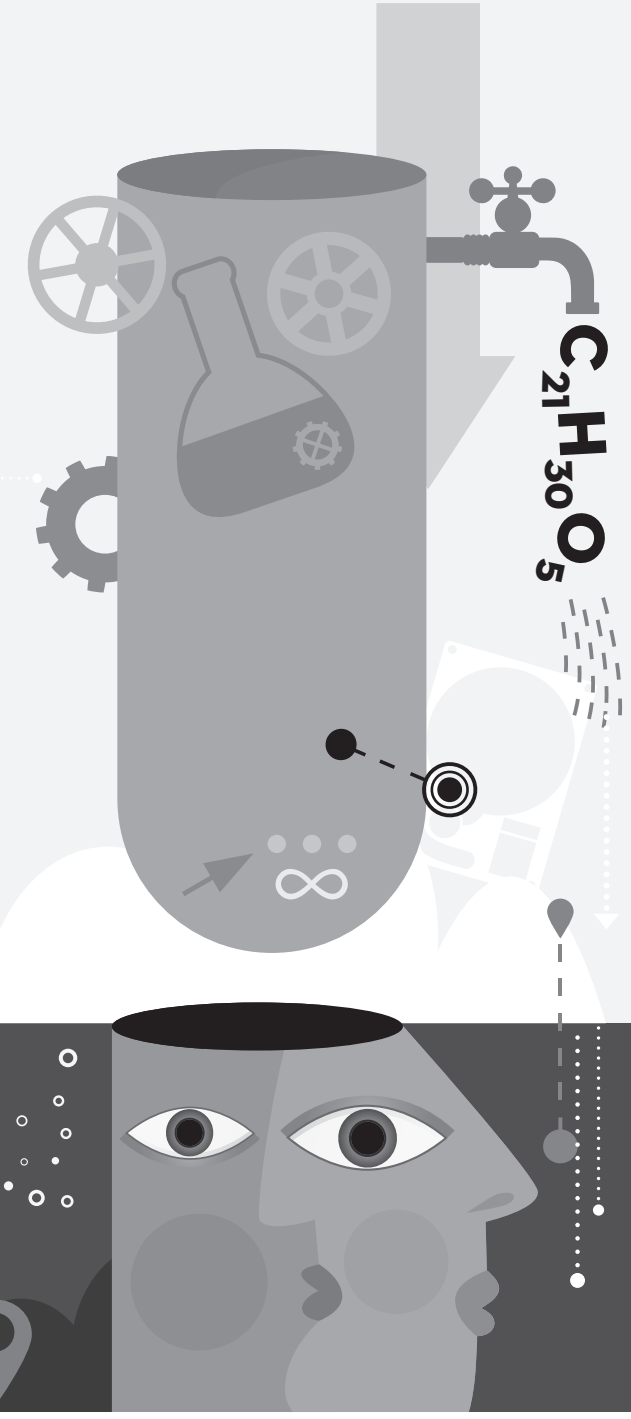

Polymorphisms in the glucocorticoid receptor gene and in the glucocorticoid-induced transcript 1 gene are associated with disease activity and response to glucocorticoid bridging therapy in rheumatoid arthritis

Chapter 3

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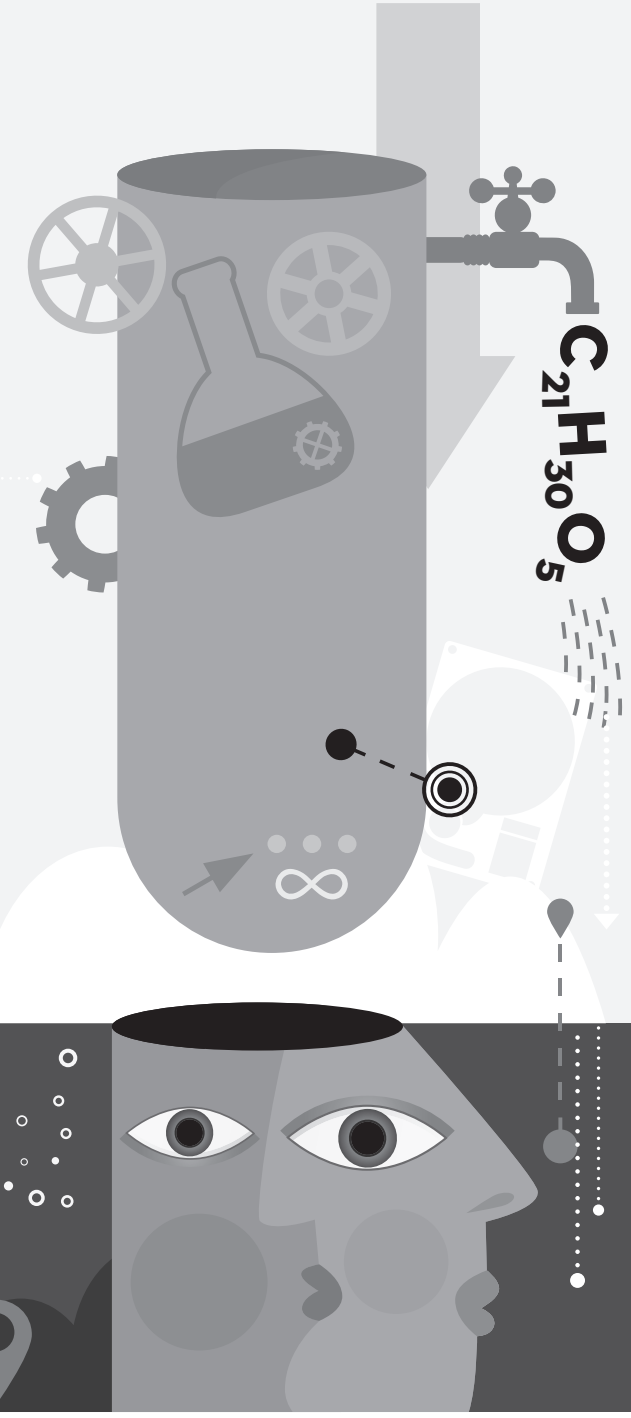
Submitted



In vitro glucocorticoid sensitivity is associated with clinical glucocorticoid therapy outcome in rheumatoid arthritis

Chapter 4

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Arthritis Research & Therapy 2012, 14:R195

ABSTRACT

Objective

Genetic and disease-related factors give rise to a wide spectrum of glucocorticoid (GC) sensitivity in rheumatoid arthritis (RA). In clinical practice, GC treatment is not adapted to these differences in GC sensitivity. *In vitro* assessment of GC sensitivity prior to start of therapy could allow more individualized GC therapy. The aim of the study was to investigate the association between *in vitro* and *in vivo* GC sensitivity in RA.

Methods

Thirty-eight early and 37 established RA patients were prospectively studied. *In vitro* GC sensitivity was assessed by dexamethasone-induced effects on interleukin-2 (IL-2) and glucocorticoid-induced leucine zipper (GILZ) messenger RNA expression in peripheral blood mononuclear cells (PBMCs). A whole cell dexamethasone binding assay was used to measure number and affinity ($1/K_D$) of glucocorticoid receptors (GRs).
In vivo GC sensitivity was determined by measuring the disease activity score (DAS) and health assessment questionnaire disability index (HAQ-DI) score prior to and after two weeks of standardized GC treatment.

Results

GR number was positively correlated with improvement in DAS. IL-2-EC_{50} and GILZ-EC_{50} values both had weak near-significant correlations with clinical improvement in DAS in intramuscularly treated patients only. HAQ-responders had lower GILZ-EC_{50} values and higher GR number and K_D.

Conclusions

Baseline cellular *in vitro* GC sensitivity is modestly associated with *in vivo* improvement in DAS and HAQ-DI score after GC bridging therapy in RA. Further studies are needed to evaluate whether *in vitro* GC sensitivity may support the development of tailor-made GC therapy in RA.

INTRODUCTION

Rheumatoid arthritis (RA) is a common autoimmune disorder characterized by chronic synovial inflammation leading to joint destructions. Based on their anti-inflammatory properties, glucocorticoids (GCs) have an important role in first-line treatment regimens of RA in combination with disease-modifying antirheumatic drugs (DMARDs). However, upon administration of GCs, a wide spectrum of clinical responses is observed with up to 30% of patients being relatively GC resistant (1-3). In addition, it is well known that in some patients side effects rapidly develop during GC therapy, whereas others tolerate GCs well, independent of dose and treatment duration. This indicates that GC sensitivity is highly variable among patients.

Determinants of individual GC sensitivity include both genetic and acquired factors. Functional polymorphisms of the glucocorticoid receptor (GR) gene have been identified that modulate GC sensitivity (4). Recently we found that these polymorphisms are also associated with RA susceptibility and disease severity (5). Acquired, disease-related factors include the effects of inflammation, mediated by pro-inflammatory cytokines, on cellular GC sensitivity, resulting in systemic or tissue-specific GC resistance of immunocompetent cells at the site of inflammation (6).

Despite this wide variety in individual GC sensitivity, RA patients are mostly treated with standardized schedules, by using fixed GC dose and treatment duration, inevitably leading to under- or overtreatment in subsets of patients. Considering the detrimental effects of prolonged synovial inflammation in undertreated patients and the potential severe burden of GC side effects in overtreated patients, it is obvious that a need exists for tools measuring individual GC sensitivity, allowing more tailor-made GC therapy.

GC binding capacity (i.e. number and affinity of GRs) has proven its potential as a possible predictor of GC therapy outcome, as has been shown for asthma (7), systemic lupus erythematosus (SLE) (8), and leukemia (9). In RA, both higher and lower GR expression levels have been reported (10-13). With respect to *in vivo* GC therapy outcome, Huisman and co-workers showed that GR levels at baseline did not correlate with clinical or radiological outcome after 2 years of GC therapy (11). However, this outcome may have been influenced by concomitant use of other antirheumatic drugs.

In addition, studies in patients with inflammatory bowel disease (14), asthma (15) and RA (16), using *in vitro* functional assays, have shown that the degree of GC-mediated suppression of proliferation of peripheral blood mononuclear cells (PBMCs) may predict *in vivo* GC sensitivity. More recently, a diminished inhibitory effect of GCs on PBMC proliferation *in vitro* was shown in a larger cohort of GC resistant RA patients (3).

Recently, we developed *in vitro* bioassays to measure individual cellular GC sensitivity (17). In these bioassays, dexamethasone-regulated expression of interleukin-2 (IL-2) and glucocorticoid-induced leucine zipper (GILZ) are measured. Transrepressive effects of GC, traditionally

considered to be the predominant mechanism regulating anti-inflammatory actions of GC, are represented by the IL-2 assay. The GILZ assay is an example of genes which transcription is transactivated by GCs. Originally such genes were postulated to be responsible for the development of GC-induced side effects (18-19). By using these bioassays, a spectrum of GC sensitivity could be demonstrated in healthy individuals.
The aim of this study was to examine whether *in vitro* assessment of GC sensitivity of PBMCs, using both these bioassays and measurement of GC binding capacity, is associated with the *in vivo* response to GC treatment in patients with RA.

PATIENTS AND METHODS

Patients

This study was embedded in a multicenter randomized clinical trial studying persons older than 18 years presenting with recent-onset arthritis, the so-called tREACH study (treatment in the Rotterdam early arthritis cohort) (20). The primary aim of this study is to establish the best treatment strategy for patients with early arthritis.
Patients were included if arthritis in at least one joint was observed by a rheumatologist, and complaints were present for less than 12 months. With a prediction model developed by Visser et al (21), patients were stratified according to their risk of having persistent erosive disease after a follow-up period of 2 years (high, intermediate, and low probability). We studied 41 patients in the high-probability group. These patients were randomized to three different treatment strategies, all including GC, either oral GC (15 mg prednisone/day, 2 treatment arms) or intramuscular GC (single depot of methylprednisolone, 120 mg, or triamcinolone acetonide, 80 mg, 1 treatment arm). All tREACH patients were naïve to GCs and DMARDs (Figure 1).
After a minimum of one year of follow-up, the diagnosis of the patients was verified in medical documentation or, if necessary, in consultation with the treating rheumatologist.
In an independent cohort, 37 patients with established RA and active disease (FLARE study) were recruited. Active disease was defined as disease activity requiring GC therapy according to the treating rheumatologists (22). All patients received a single intramuscular depot of GC (methylprednisolone, 120 mg, or triamcinolone acetonide, 80 mg). None of the FLARE patients had used GC in the last 3 months and were taking stable DMARD therapy (Figure 1). As a control group, we studied healthy laboratory employees (N=20). None of the controls was using GC.
Of the 41 high-probability patients included via the tREACH study, 38 were ultimately diagnosed as having definite RA. In this group of early RA, two patients were lost to follow-up, leaving 36 patients for complete analysis. After randomization, oral GCs were prescribed to 22 patients, and 14 patients were given a single depot of intramuscular GC. In the FLARE study, two patients were lost to follow-up for logistic reasons. In 10 patients, only one of the

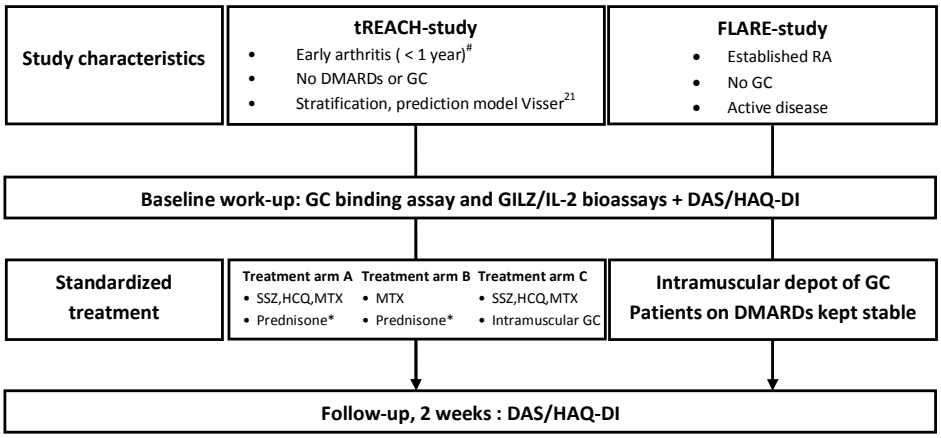


Figure 1. The baseline work-up in tREACH patients included the GILZ/IL-2 assays. In FLARE patients both a GC binding assay and GILZ/IL-2 assays could be performed. In all patients disease activity score (DAS) was measured at baseline and after two weeks of GC bridging therapy. #Only patients in the high-probability group eventually fulfilling the 1987 ACR criteria for RA were included in the final analysis. *Prednisone is tapered according to the following schedule: week 1-4 15 mg/day, week 5-6 10 mg/day, week 7-8 5 mg/day, week 9-10 2.5 mg/day. Intramuscular GC could be either methylprednisolone 120 mg or triamcinolone acetonide 80 mg. Baseline work-up and start of standardized treatment occurred on the same day. HAQ-DI health assessment questionnaire disability index, GC glucocorticoids, SSZ sulfasalazine, HCQ hydroxychloroquine, MTX methotrexate.

assays could be performed due to limited amount of PBMCs. Ultimately, 32 patients could be evaluated for binding capacity of the GC receptor, and 32 patients for the bioassay (in 27 patients, both assays were performed). Patients lost to follow-up were included in the baseline analysis (2 patients in each cohort).

Methods

Assessment of in vitro glucocorticoid sensitivity

In the tREACH cohort, *in vitro* GC sensitivity was assessed by the GC bioassays (for logistic reasons, only enough PBMCs were available for the GC bioassays). In patients participating in the FLARE study, *in vitro* GC sensitivity was assessed by both the GC bioassays and GC binding capacity.

The GC bioassays were performed as described previously (17). In short, peripheral blood was drawn in all patients before start of treatment using Cell Preparation Tubes with Sodium Heparin (Becton Dickinson, Breda, the Netherlands) allowing isolation of PBMCs. Cells were resuspended in RPMI 1640 medium containing L-glutamine supplemented with penicillin (100 U/ml) and streptomycin (100µg/ml) and 10% fetal calf serum (FCS) and precultured overnight in a 48-well plate (Costar, Amsterdam, the Netherlands, 5.0 x 10^5 cells/well in du-

plicate, density of 4.0 x 10^6/ml). A single batch of FCS was used throughout. Before use, this batch was analyzed for cortisol content, which was found to be below the detection limits. Trypan blue staining revealed the viability of isolated cells to be greater than 95%. The next day, cells were incubated with dexamethasone 0, 0.33, 1, 3.3, 10, 33, 100 and 333 nmol/L dexamethasone and stimulated with 10µg/ml phytohemagglutinin (Sigma-Aldrich, Zwijndrecht, the Netherlands). After 4h in the incubator, total RNA of the cells was collected (Total RNA isolation Kit, Roche, Almere, the Netherlands). Reverse transcription was performed using 100 ng total RNA per reaction. Quantitative real-time PCR analysis was carried out on a 7900HT Taqman machine (Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands), according to the manufacturer's instructions. Data were analyzed using the SDS 2.4 software (Applied Biosystems). GC-specific transactivation of the GILZ gene and transrepression of the IL-2 gene were measured while correcting for the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) using the ΔΔCT method, primers and probes were obtained from Biolegio, Nijmegen, the Netherlands (Supplementary Table 1). Half-maximal effective concentration (EC_{50}) was calculated using nonlinear regression in GraphPad Prism 5.0 and used as a read-out for *in vitro* GC sensitivity. The EC_{50} values of GILZ and IL-2 in PBMC were not significantly influenced by the cellular composition (percentages lymphocytes and monocytes) of the PBMCs (data not shown).

GC binding capacity was measured using a whole-cell dexamethasone binding assay, as described previously, with minor modifications (23). In brief, by using PBMCs from the same isolation procedure, incubation was started in a volume of 200 µl (0.5 to 2 x 10^6 cells) containing [^{3}H] dexamethasone at concentrations of 1 to 30 nmol/l with and without a 400-fold excess of unlabeled dexamethasone reflecting nonspecific and total binding of [^{3}H] dexamethasone, respectively. Two tubes without labeled dexamethasone were incubated under the same conditions for determination of cell number and viability at the end of the procedure. The PBMCs were incubated during 1 h at 30°C in a shaking water bath. The incubation was stopped by the addition of 2 ml cold saline, followed by centrifugation and two washing steps. Finally, the PBMCs were resuspended in 250 µl saline. Radioactivity in 200 µl of this suspension was counted in a liquid scintillation counter. Specific binding was calculated by subtracting nonspecific binding from total binding. EC_{50} values, receptor number, and ligand affinity ($1/K_D$) were calculated using the nonlinear regression method (GraphPad Prism, version 5.0, La Jolla, CA, USA).

In vivo glucocorticoid sensitivity

Trained research nurses examined patients before and after two weeks of standardized GC treatment. Disease Activity Score (DAS, 44 joints) was calculated according to the following formula: DAS= 0.54 x √RAI + 0.065 x SJC44 + 0.33 x ln(ESR) + 0.007 x GH (RAI=Ritchie Articular Index, SJC44 = 44 swollen joint count, ESR = erythrocyte sedimentation rate, GH = general

health at a 100 mm scale). As primary outcome, the relative decrease in DAS (100 x (($DAS_{baseline}$ – $DAS_{after\ 2\ weeks}$) /$DAS_{baseline}$)) was used as an index for *in vivo* GC sensitivity. Using this continuous outcome variable, a floor effect in patients with relatively low disease activity was prevented. In addition, continuous variables represent the full information, in contrast to (arbitrary) categorical data (i.e. response criteria). We chose a 2-week interval for follow-up in tREACH patients to minimize the influence of the disease-modifying effects of the other antirheumatic drugs on the DAS. A similar follow-up period was chosen in the FLARE study to make comparisons between the groups possible. During the study period, the dose of DMARD(s) already being used was not changed and no additional antirheumatic therapy was started.
To further explore effectiveness of GC therapy, the impact of GC treatment on performing activities of daily living was assessed using the health assessment questionnaire disability index score (HAQ-DI). The HAQ-DI is a widely used and validated tool to quantify functional disability in RA (24) and comprises questions about different aspects of daily life. In particular, the minimal import difference (MID) in HAQ-DI score is the smallest difference in HAQ-DI score that patients sense as a difference. In clinical trials, the MID in HAQ-DI improvement ranged from 0.22 to 0.24 (25). As a result, patients were classified as responder ($HAQ\text{-}DI_{baseline}$ – $HAQ\text{-}DI_{2wks} \geq 0.25$) or non-responder ($HAQ\text{-}DI_{baseline}$ – $HAQ\text{-}DI_{2wks} < 0.25$).

Glucocorticoid-induced side effects

We measured blood pressure and body weight before and after 3 months of GC therapy in tREACH patients. Furthermore, glycosylated hemoglobin (HbA_{1c}) was measured at baseline and after 3 months in tREACH patients (HbA_{1c} analyzer, type Adams A1c HA-8160, Menarini Benelux).

Statistical Analysis

Differences in continuous variables between the cohorts were tested using analysis of variance (ANOVA). GILZ-EC_{50} values were normally distributed (Kolmogorov-Smirnoff $p>0.20$) whereas IL-2-EC_{50} was square-root transformed and number of receptors and K_D were both natural logarithm transformed to normalize the data. Bonferroni post hoc tests were used to correct for multiple testing.
Pearson or Spearman correlation coefficients were used to describe the bivariate relationships between *in vitro* parameters of GC sensitivity and DAS at baseline and relative decrease in DAS.
ANOVA analysis was applied to test for differences in *in vitro* parameters of GC sensitivity between HAQ-responders and non-responders. Paired t-test or Wilcoxon Signed Ranks Test were used for analysis of alterations in DAS, HAQ-DI scores, blood pressure, body weight and HbA_{1c} values.

To test for potential confounders, each of the individual *in vitro* parameters of GC sensitivity (i.e. IL-2- and GILZ-EC_{50}, K_D and number of GR) and selected covariates were modeled using linear regression (relative decrease in DAS as dependent variable). These selected covariates included gender, age and, based on potential synergistic immunomodulating properties with GCs, use of NSAIDs, number of DMARDs and use of anti-TNF-α agents.
Orally and intramuscularly treated patients were analyzed separately because of non-equivalent cumulative dosages of GC (cumulative GC-dosage: oral > intramuscular). We considered differences statistically significant if p≤0.05 (2-sided).

Ethical Approval

All subjects signed informed consent and the study was approved by the medical ethics committee of the Erasmus Medical Center.

RESULTS

Thirty-eight tREACH patients and 37 FLARE patients were prospectively studied. Patients in the FLARE study had a significantly higher disease activity at baseline, a longer duration of

Table 1. Patient characteristics.

	Controls (N=20)	tREACH (N=38)	FLARE (N= 37)
Female gender, N (%)	10 (50)	25 (65.8)	25 (67.6)
Age in years, mean (SD)	31.8 (9.7)	53.3 (13.98)##	53.7 (13.40)##
Disease duration in months, median (range)	-	5.4 (2-12)	73.0 (0-414) †
Presence of Joint Erosions, N (%)	-	10 (26.3)	20 (54.1)#
Anti-CCP positive, N (%)	-	30 (78.9)	24 (85.7)*
Rheumatoid Factor (IgM) positive, N (%)	-	31 (81.6)	27 (73.0)
DAS44 at baseline, mean (SD)	-	3.05 (0.92)	3.57 (0.95)#
HAQ-DI at baseline, mean (SD)	-	-	1.43 (0.62)
Use of NSAIDs, N (%)	-	25 (65.8)	19 (51.4)
Use of methotrexate, N (%)	-	-	22 (59.5)
Use of hydroxychloroquine, N (%)	-	-	11 (29.7)
Use of sulfasalazine, N (%)	-	-	5 (13.5)
Number of DMARDs, median (range)	-	-	1 (0-3)**
Use of anti-TNF-α therapy, N (%)	-	-	5 (13.5)

DAS44: Disease Activity Score, 44 joints; HAQ-DI: Health Assessment Questionnaire Disability Index; TNF-α: tumor necrosis factor alpha; anti-CCP: anti-cyclic citrullinated protein; NSAIDs: non steroidal anti-inflammatory drugs; DMARDs: disease modifying antirheumatic drugs; # p<0.05, ## p<0.001 as compared to healthy controls; † p<0.001 as compared to tREACH patients; *anti-CCP was not routinely analyzed, % is based on 28 patients with known anti-CCP status; ** 7 patients were not using any DMARD at time of assessment.

disease and a higher percentage of erosions compared to tREACH patients. Further baseline characteristics are summarized in Table 1.

Baseline *in vitro* glucocorticoid sensitivity in RA and healthy controls

Overall, patients with early (tREACH cohort) and established RA (FLARE cohort) had higher mean EC_{50} values in the IL-2 assay than healthy controls (although not statistically significant in the FLARE cohort), indicating that RA patients needed a higher dosage of dexamethasone to suppress IL-2 mRNA expression *in vitro*. In contrast to this, similar EC_{50} values were measured in the GILZ assay (Figure 2A and 2C). Patients participating in the FLARE study had a higher number of GR compared to healthy controls, while having comparable affinity ($1/K_D$) of the receptor (Figure 2E and 2F). The percentage monocytes was measured in subsets of FLARE patients and healthy controls and did not differ significantly (mean ± SD: 24.6 ± 9.2 in FLARE patients versus 20.9 ± 5.0 in healthy controls). Ligand affinity of monocytes and lymphocytes did not differ significantly. The number of glucocorticoid receptors per cell was about three fold higher in monocytes as compared to lymphocytes (data not shown). The maximum induction of GILZ and repression of IL-2 tended to be lower in the established RA cohort as compared to healthy controls (p=0.068 and p=0.101 respectively) (Figure 2B and 2D).

There were no correlations between the DAS and parameters of *in vitro* GC sensitivity. Of the variables used to calculate the DAS, a negative association was observed between the RAI and IL-2-EC_{50} ($\rho = -0.465$, P=0.005), but only in the early RA patients. No gender differences were noted at the mean level of the IL-2- EC_{50} and GILZ-EC_{50}, number of GRs, or the affinity of the receptor.

HAQ-DI sum scores before start of treatment did not show any correlations with *in vitro* parameters of GC sensitivity. Male and female individuals did not differ significantly in HAQ-DI sum scores.

Correlation between *in vitro* parameters of glucocorticoid sensitivity

GILZ-EC_{50} and IL-2-EC_{50} were positively correlated, but only in the patients with early RA ($\rho = .383$, $p = 0.028$). In patients with established RA, the number of GR was inversely correlated with GILZ-EC_{50} and IL-2-EC_{50} ($\rho = -.401$, $p = 0.042$ and $\rho = -.462$, $p = 0.020$ respectively). K_D was also inversely correlated with GILZ-EC_{50} ($\rho = -.413$, $p = 0.032$), but not with IL-2-EC_{50}. Finally, K_D and GR-number were correlated ($\rho = .627$, $p < 0.001$, Supplementary Figure 1).

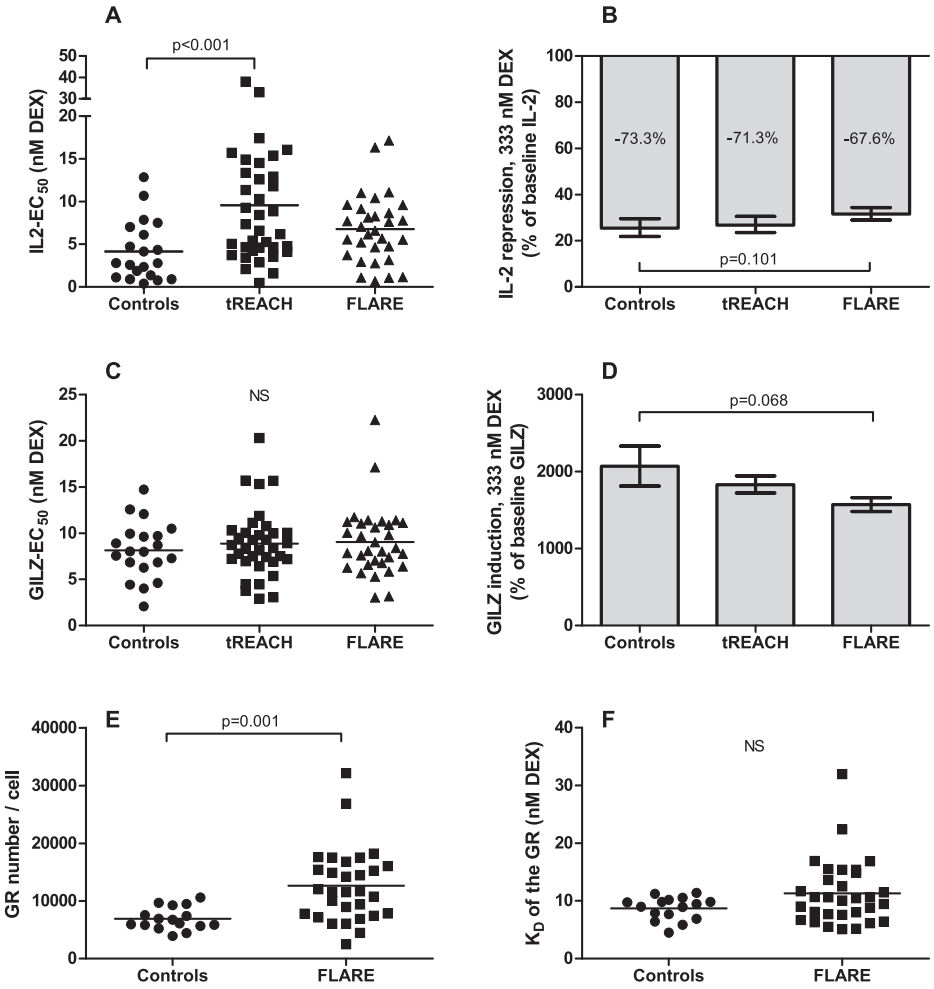


Figure 2. Baseline *in vitro* parameters of GC sensitivity in healthy controls, tREACH and FLARE patients. The IL-2 assay (A) and GILZ assay (C) were performed in both tREACH and FLARE patients (bioassays in 32 patients, GC binding assay in 32 patients, bioassays and GC binding assay in 27 patients; control groups for the bioassays (N=20) and binding assay (N=16) were not the same). As secondary outcome IL-2 repression (B) and GILZ induction (D) was calculated as follows:

$$\text{IL-2 repression} = 100 \times \frac{(\text{IL2-expression, PHA}) - (\text{IL2-expression, 333nM})}{(\text{IL2-expression, PHA})}$$

$$\text{GILZ induction} = 100 \times \frac{(\text{GILZ-expression, 333nM})}{(\text{GILZ-expression, PHA})}$$

The number of GR (E) and the affinity of the receptor (F) was measured in FLARE patients only.
EC_{50} = half maximal effective concentration. P values were calculated using ANOVA and Bonferroni post-hoc correction, normalized data were used where appropriate.

Pre-treatment *in vitro* glucocorticoid sensitivity and disease activity in RA after two weeks of glucocorticoid therapy

After two weeks of GC treatment, a significant decrease in disease activity was measured in both orally and intramuscularly treated patients (ΔDAS_{oral} = 0.92, p<0.001, $\Delta DAS_{intramuscular}$ = 0.89, p <0.001 and Supplementary Table 2). The interquartile range in relative decrease in DAS was 22% and 43% in orally and intramuscularly treated patients respectively, indicating greater variability in relative decrease in DAS in the intramuscularly treated group.

In patients treated with a single intramuscular depot of GC (all FLARE patients and a proportion of tREACH patients) a modest inverse relation was found between *in vitro* GC sensitivity as reflected by IL-2-EC_{50} values and the percentage improvement in DAS after two weeks

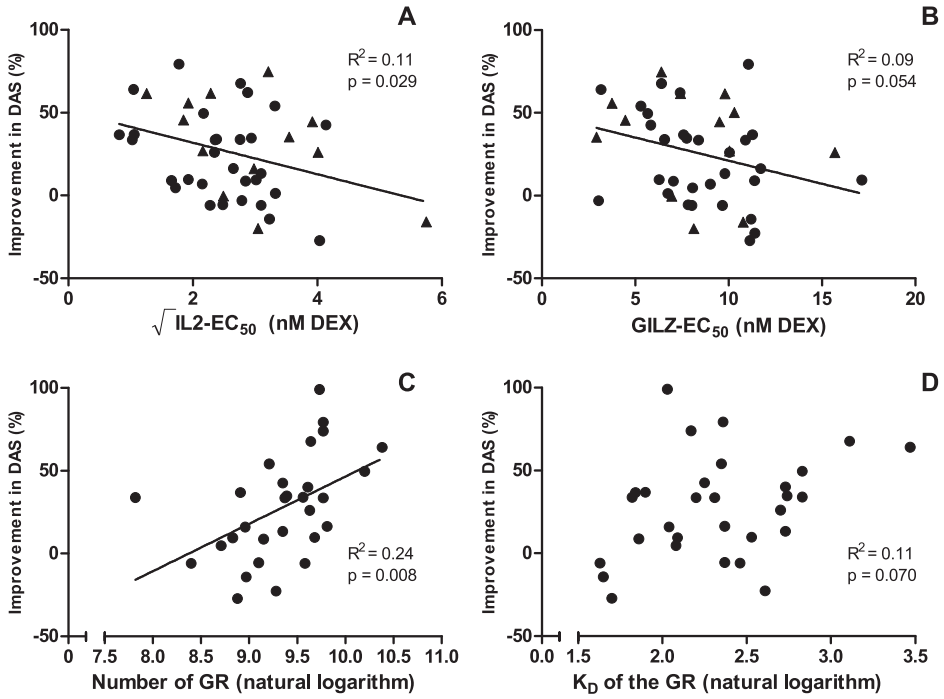


Figure 3. Correlation between *in vitro* and *in vivo* glucocorticoid sensitivity in intramuscularly treated RA patients. *In vivo* glucocorticoid sensitivity is presented as percentage improvement in DAS according to the following formula:

$$100 \times \frac{\text{DAS, baseline} - \text{DAS, after 2 weeks}}{\text{DAS, baseline}}$$

Correlations between √IL-2-EC_{50} values (A), GILZ-EC_{50} values (B), natural logarithm of the number of GR per cell (C) and natural logarithm of the K_D of the receptor (D) and percentage improvement DAS. R^2 = square of the Pearson correlation coefficient; proportion explained variability. Triangles (▲) represent the tREACH patients and closed circles (●) represent FLARE patients.

Table 2. *In vitro* parameters of GC sensitivity and relative decrease in DAS.

BIOASSAYS			GC BINDING ASSAY		
	β (95% CI)[a]	**P value**		**β (95% CI)[a]**	**P value**
IL2-EC_{50}	-0.014 (-0.028-0.001)	0.058	K_D	0.03 (0.014-0.046)	0.001
Age	0.005 (-0.002-0.110)	0.161	Age	0.009 (0.002-0.017)	0.020
Gender	0.128 (-0.057-0.312)	0.169	Gender	0.199 (0.013-0.385)	0.037
Use of NSAIDs	0.087 (-0.087-0.261)	0.316	Use of NSAIDs	0.212 (0.025-0.400)	0.028
Number of DMARDs	0.023 (-0.079-0.125)	0.647	Number of DMARDs	0.112 (0.004-0.221)	0.043
Use of anti-TNF-α	0.008 (-0.283-0.299)	0.955	Use of anti-TNF-α	0.222 (-0.010-0.455)	0.060
GILZ-EC_{50}	-0.023 (-0.046-0.001)	0.062	GR number/1000	0.027 (0.012-0.042)	0.001
Age	0.006 (-0.001-0.014)	0.079	Age	0.010 (0.002-0.018)	0.015
Gender	0.116 (-0.075-0.308)	0.225	Gender	0.017 (-0.188-0.223)	0.862
Use of NSAIDs	0.181 (-0.004-0.366)	0.055	Use of NSAIDs	0.203 (0.006-0.400)	0.044
Number of DMARDs	0.021 (-0.085-0.126)	0.695	Number of DMARDs	0.084 (-0.027-0.195)	0.132
Use of anti-TNF-α	0.023 (-0.270-0.320)	0.874	Use of anti-TNF-α	0.129 (-0.121-0.378)	0.297

In all four models, relative decrease in DAS was the dependent variable. The data represent the combined bioassays from intramuscularly treated patients with early (N=14) and established RA (N=31). GILZ-EC_{50} and IL2-EC_{50} were not associated with relative decrease in DAS in orally treated patients in recent-onset RA (N=22). The GC binding assay is only performed in established RA (N=30, all intramuscular GC). a) Values represent adjusted β-coefficients and 95% confidence intervals (95% CI). TNF-α: tumor necrosis factor alpha; NSAIDs: non steroidal anti-inflammatory drugs; DMARDs: disease modifying antirheumatic drugs.

(p=0.029, Figure 3A). Similarly, near-significance was reached for GILZ-EC_{50} values and the relative decrease in DAS, but also only in intramuscularly treated patients (p=0.054, Figure 3B). In addition, the number of GR displayed a modest positive relationship with the improvement in DAS in patients with intramuscular depots of GC and a positive trend was observed for the K_D of the GR (p=0.008 and p=0.070 respectively, Figure 3C-D).

Using multiple regression however, both the number of GR and K_D of the receptor were significant factors contributing to the relative decrease in DAS. The negative association between IL-2-EC_{50} and GILZ-EC_{50} values and relative decrease in DAS persisted, although only near-significance was reached (Table 2).

Of note, in the subgroup of patients with evaluation of GC binding capacity (FLARE study), age and use of NSAIDs were also independent predictors of improvement of disease activity after two weeks of GC treatment. Age and use of NSAIDs both had positive β coefficients, indicating a better response with older age and use of NSAIDs.

Pre-treatment *in vitro* glucocorticoid sensitivity and functional disability in RA after two weeks of glucocorticoid therapy

After two weeks of GC treatment, a significant decrease in HAQ-DI sumscores was measured (ΔHAQ-DI = −0.40, p<0.001). However, 12 out of 34 patients still had to be classified as non-responder. Responders had lower EC_{50} values of GILZ and higher number of GR with higher K_D (Figure 4). IL2-EC_{50} values tended to be lower in responders.

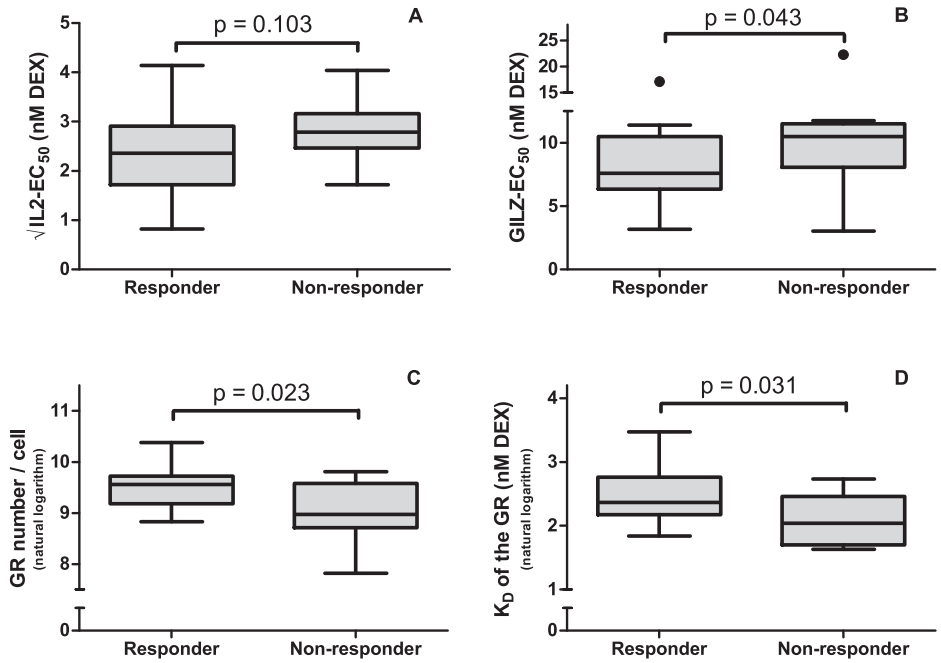


Figure 4. *In vitro* glucocorticoid sensitivity and improvement in HAQ-DI score in patients with established rheumatoid arthritis (FLARE study). Boxplots (each box shows the mean and interquartiles) and outliers (●) of IL-2-EC_{50} (A), GILZ-EC_{50} (B), number of GR (C) and K_D of the GR (D) in HAQ-DI responders and HAQ-DI-non-responders. Patients are defined as responders if their HAQ-DI sum score was at least 0.25 lower after GC therapy.

In vitro glucocorticoid sensitivity and development of glucocorticoid-mediated metabolic side effects

The mean systolic and diastolic blood pressure of patients was lowered at their 3 months follow-up visit (systolic $RR_{baseline}$ 145.0 mmHg, systolic $RR_{3\ months}$ 134.4 mmHg, p=0.005 and diastolic $RR_{baseline}$ 87.4 mmHg, diastolic $RR_{3\ months}$ 82.6 mmHg, p=0.003). This decrease was observed in both orally and intramuscularly treated patients. Body mass index did not change significantly after 3 months ($BMI_{baseline}$ 26.9, $BMI_{3\ months}$ 26.7).

At baseline, the mean HbA_{1C} was 5.51% (reference range: 4.5-6.0%). 3 patients had HbA_{1C} values above the upper limit of the normal range. After 3 months, the mean HbA_{1C} was even somewhat lower (5.31%, p = 0.016). 9 patients had a higher HbA_{1C}, 4 patients had an equal percentage of HbA_{1C} and 13 patients had an improvement. No relation was found between alterations in HbA_{1C} and blood pressure and *in vitro* GC sensitivity as measured by the bioassay.

DISCUSSION

We examined whether *in vitro* GC sensitivity is associated with the clinical response to GC treatment in RA. Our results show that in particular the number of GR in PBMC and the K_D of the GR correlated with *in vivo* GC sensitivity as reflected by the relative decrease in DAS. Near-significant associations were found between dexamethasone-mediated changes in IL-2- and GILZ-mRNA expression levels and the relative decrease in DAS. Similar patterns between clinically relevant improvement in HAQ-DI sumscores and *in vitro* parameters of GC sensitivity were observed.

Remarkably, PBMC of RA patients have a decreased *in vitro* capacity for transrepression which is most pronounced in the early RA cohort. This transrepression of pro-inflammatory cytokine production by (endogenous) GC is an important mechanism to counteract the inflammatory response (26). Consequently, reduced transrepression might hamper the resolution of acute inflammation, governing the evolution into a chronic phase of inflammation, a central feature of many autoimmune diseases. Interestingly, polymorphisms of the GR gene associated with reduced (i.e. 9β) or increased (i.e. *Bcl*I and N363S) GC sensitivity are associated with increased respectively decreased susceptibility to RA (5). Next to decreased GC sensitivity, a blunted hypothalamic-pituitary-adrenal axis has been postulated to be part of the pathophysiology of RA (27).

Importantly, we did not find a relationship between disease activity and *in vitro* GC sensitivity, suggesting that the impaired GC sensitivity is not just due to increased levels of pro-inflammatory cytokines. This is in accordance with the study performed by Hearing and co-workers who also did not find a relationship between disease activity and *in vitro* GC sensitivity in inflammatory bowel disease (14).

In contrast to this reduced GC sensitivity at the transcriptional level, we found a higher number of GR in patients with established RA. A large study by Schlaghecke et al showed lower numbers of GR in RA (13). In contrast, Eggert and co-workers found increased expression of GR, which dramatically decreased following long-term GC treatment (10). Interestingly, the only study with longitudinal data on GR expression in RA reports an increase in GR expression over time in female RA patients, suggesting a compensatory mechanism for the ongoing inflammatory state (11). In addition to this concept, the higher number of GRs in our cohort

might be interpreted as a counterbalancing mechanism for the reduced GC sensitivity. In line with this hypothesis, we found a correlation between higher numbers of GR and lower EC_{50} values of GILZ and IL-2.

GC exert their anti-inflammatory properties via the GR. Upon binding of GC to the GR, the receptor-ligand complex migrates to the nucleus to interact with GC responsive elements of target genes. During inflammation, cellular GC sensitivity can be modulated by cytokines via effects on GR number and affinity, GR translocation to the nucleus, interaction with inflammatory transcription factors (e.g. NF-κB, AP-1) and expression of the GR-β splice variant (28). The assessment of GC-mediated gene expression, as performed in our bioassay, may have the advantage of integrating all post-receptor downstream factors that modulate GC sensitivity. Originally, the immunosuppressive effects of GC were attributed to transrepression of immune genes. We indeed found that IL-2-EC_{50} values are moderately associated with the relative decrease in DAS. However, in the last decade, increasing evidence has been obtained pointing toward immunomodulating effects of GC-activated genes (29) .

In this perspective, the GILZ gene studied in our bioassay is of particular interest. GILZ can directly interfere with the AP-1 complex (30) and can also inhibit NF-kB nuclear translocation and DNA binding *in vitro* (31). Recently, GILZ has been demonstrated to function as an endogenous inhibitor of chronic inflammation in a murine model of RA (32). In addition, GILZ transgenic mice are less prone to develop T-helper 1 mediated colitis (33). We extend these observations by demonstrating that GILZ-regulation by dexamethasone *in vitro* might be a potential marker for *in vivo* effects of GC therapy in humans.

Remarkably, the predictive value of the GILZ and IL-2 assays is only found in the intramuscularly treated patients and not in the orally treated patients. A possible explanation is that the higher dosage of GC used in the orally treated patients masks subtle differences in GC sensitivity. This is supported by the fact that the interquartile range in relative decrease in DAS was higher in the intramuscularly treated patients. Furthermore, a lack of compliance in orally treated patients could play a role, whereas this problem is obviously not present in intramuscularly treated patients. Finally, differences in pharmacokinetics and duration of disease could also be causes adding to observed differences between orally and intramuscularly treated patients.

In our group of patients with established RA, both the number of GR and the K_D were positively correlated with improvement in disease activity. From a biological point of view, higher numbers of receptors correlating to better response seems plausible. Indeed, GR levels have been shown to serve as possible markers of GC therapy outcome in SLE and leukemia (8-9). On the other hand, our observations concerning the K_D of the GR are in contrast with other reports (7, 34). In this perspective, it is important to note higher numbers of GR were accompanied by lower affinity of the receptor (i.e. a higher K_D) in several other conditions (7, 34-38). Whether this phenomenon truly occurs *in vivo* or represents an artificial correlation (since K_D and GR number are calculated from the same data) is yet unclear. Analysis of GR number and

K_D separately using different techniques could possibly give more insight in this intriguing observation. Clearly, the interpretation of binding assays should be done with caution.

Although we did not measure serum levels of the exogenously administered GCs in our patients, the (average) serum concentrations of these GC, in the doses administrated, are reported to be in the same (equipotent) range as the GILZ and IL-2-EC_{50} values and the K_D of the GR, suggesting that *in vitro* parameters of GC sensitivity may reflect *in vivo* GC sensitivity reasonably well (39-40).

Unexpectedly, the IL-2/GILZ assays, integrating all determinants of GC sensitivity up to the transcriptional level, showed a weaker correlation with the *in vivo* response than the more upstream GR. However, GC also have effects that do not require gene transcription, also referred to as nongenomic effects of GC (41). Also in RA nongenomic actions are important, as illustrated by rapid inhibition of leukocyte recruitment in inflamed joints after GC administration (42). GR levels may therefore be a better predictor of *in vivo* GC effects, since both genomic and nongenomic actions of GC are taken into account.

Our study clearly highlights the potential of *in vitro* (bio) assays as possible clinical markers for GC treatment of RA patients. Recently it was shown that assessment of early arthritis patients by a rheumatologist within 12 weeks was associated with less joint destruction and a higher chance of DMARD-free remission as compared with patients assessed after this so-called window of opportunity (43). This favorable outcome of early treatment could be further substantiated by effective (tailor-made) GC treatment in the window of opportunity and emphasizes the need for biomarkers of GC sensitivity prior to start of GC treatment.

However, there are several limitations in our study that need to be addressed. A relatively weak correlation was found between the GILZ and IL-2 assays and *in vivo* glucocorticoid sensitivity, restricting the usefulness of these assays in the clinical context at this moment. Further, presumably due to the restricted period of GC treatment, we could not evaluate the potency of our bioassay and binding assay to predict susceptibility for GC-mediated side effects. Also, since GC sensitivity is highly tissue-specific, extrapolation of our findings to other inflammatory disorders should be done with caution. As prednisone is a pro-drug requiring reduction by 11β-HSD type 1 and methylprednisolone and triamcinolone acetonide are active 11-hydroxysteroids, it is possible that differences in the cortisol-cortisone shuttle, mediated by the pro-inflammatory state, might also have influenced *in vivo* GC sensitivity (44). Furthermore, local steroid metabolism in the synovial cells may play a role in increasing local cortisol and prednisolone concentration as shown by Hardy and co-workers (45). Considering this tissue-specificity and the sample size of our RA-cohort, validation of these *in vitro* assays should be done both in cohorts with RA and other autoimmune disorders.

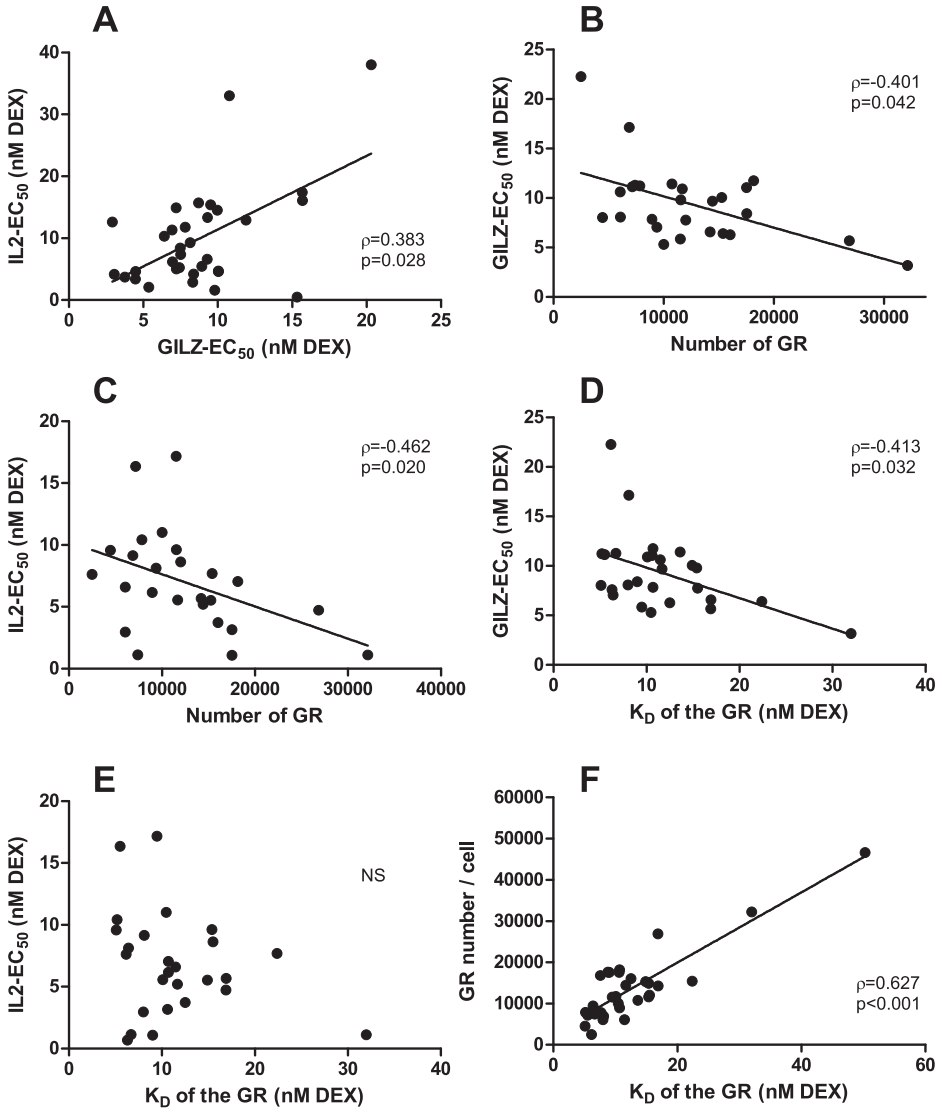
CONCLUSIONS

We show that upon two weeks of GC treatment of patients with RA, the relative decrease in DAS *in vivo* is modestly associated with the number and affinity of GR. Near-significant associations were found with EC_{50} values of IL-2 and GILZ. *In vitro* identification of hypo- or hypersensitive subgroups of RA patients may facilitate a more individual GC therapy for these particular patients in order to maximize therapeutic efficacy and minimize time- and dose-dependent side effects. Further studies evaluating the number and affinity of GR in PBMC at baseline in relation to improvement in DAS are needed to establish whether assessment of *in vitro* GC sensitivity can support individualized therapeutic management of RA patients treated with GC.

ACKNOWLEDGEMENTS

The authors would like to acknowledge all patients and rheumatologists for their contribution to the tREACH study and the FLARE study. We are thankful to all research assistants for their help in data collection and Karolina Sikorska for her excellent assistance in the statistical analysis of data. This study was supported by the Dutch Arthritis Foundation.

Supplementary Figure 1.



The correlations between IL-2-EC_{50} and GILZ-EC_{50} in early RA (A), GILZ-EC_{50} and number of GR (B), IL-2-EC_{50} and number of GR (C), GILZ-EC_{50} and K_D of the GR (D), IL-2-EC_{50} and K_D of the GR (E) and K_D of the GR and number of GR (F) are depicted. NS denotes non-significant.

Supplementary Table 1. Primer and probe sequences for GILZ, IL-2 and HPRT.

GILZ: forward primer	5′-GCACAATTTCTCCATCTCCTTCTT-3′
GILZ: reverse primer	5′- TCAGATGATTCTTCACCAGATCCA -3′
GILZ: probe	5′-6FAM-TCGATCTTGTTGTCTATGGCCACCACG-BHQ1-3′
IL-2: forward primer	5′-TTTGAATGGAATTAATAATTACAAGAATCC-3′
IL-2: reverse primer	5′-TCTAGACACTGAAGATGTTTCAGTTCTGT-3′
IL-2: probe	5′-6FAM-CCAGGATGCTCACATTTAAGTTTTACATGCCC-BHQ1-3′
HPRT: forward primer	5′-CACTGGCAAAACAATGCAGACT-3′
HPRT: reverse primer	5′-GTCTGGCTTATATCCAACACTTCG T-3′
HPRT: probe	5′-6FAM-CAAGCTTGCGACCTTGACCATCTTTGGA-TAMRA-3′

Primer and probe sequences for GILZ, IL-2 and HPRT as used in the bioassays to measure messenger RNA levels of GILZ, IL-2 and HPRT.

Supplementary Table 2. DAS and individual measures of the DAS in tREACH and FLARE patients.

	tREACH	**p value**	**tREACH**	**p value**	**FLARE**	**p value**
	oral GC (N=23)		*intramuscular GC (N=15)*		*intramuscular GC (N=37)*	
DAS, baseline (mean; SD)	3.12 (1.05)	<0.001	2.94 (0.69)	<0.001	3.57 (0.95)	<0.001
DAS, 2 weeks (mean; SD)	2.20 (0.96)		1.84 (0.80)		2.70 (1.39)	
SJC, baseline	5 (1-18)	0.013	6 (1-19)	0.013	7 (2-25)	<0.001
SJC, 2 weeks	3 (0-11)		2 (0-9)		3 (0-26)	
RAI, baseline	6 (0-50)	0.001	4 (0-9)	0.048	7 (0-31)	0.040
RAI, 2 weeks	2 (0-19)		0 (0-9)		6 (0-35)	
ESR, baseline	22 (4-80)	<0.001	23 (9-69)	0.142	22.5 (1-85)	0.002
ESR, 2 weeks	13 (1-60)		16 (4-69)		18 (1-75)	
GH, baseline	53 (9-92)	0.002	40 (11-77)	0.451	69 (9-99)	<0.001
GH, 2 weeks	28 (0-70)		31 (0-80)		50 (3-100)	

One patient each in the orally and intramuscularly treated tREACH group was lost-to-follow-up and two patients in the FLARE study did not have a second DAS. Values are given as median (range) unless otherwise stated. P values refer to the 0-2 weeks change of the different variables. SJC: Swollen Joint Count; RAI: Ritchie Articular Index; ESR: Erytrocyte Sedimentation Rate; GH: general health at a 100 mm scale.

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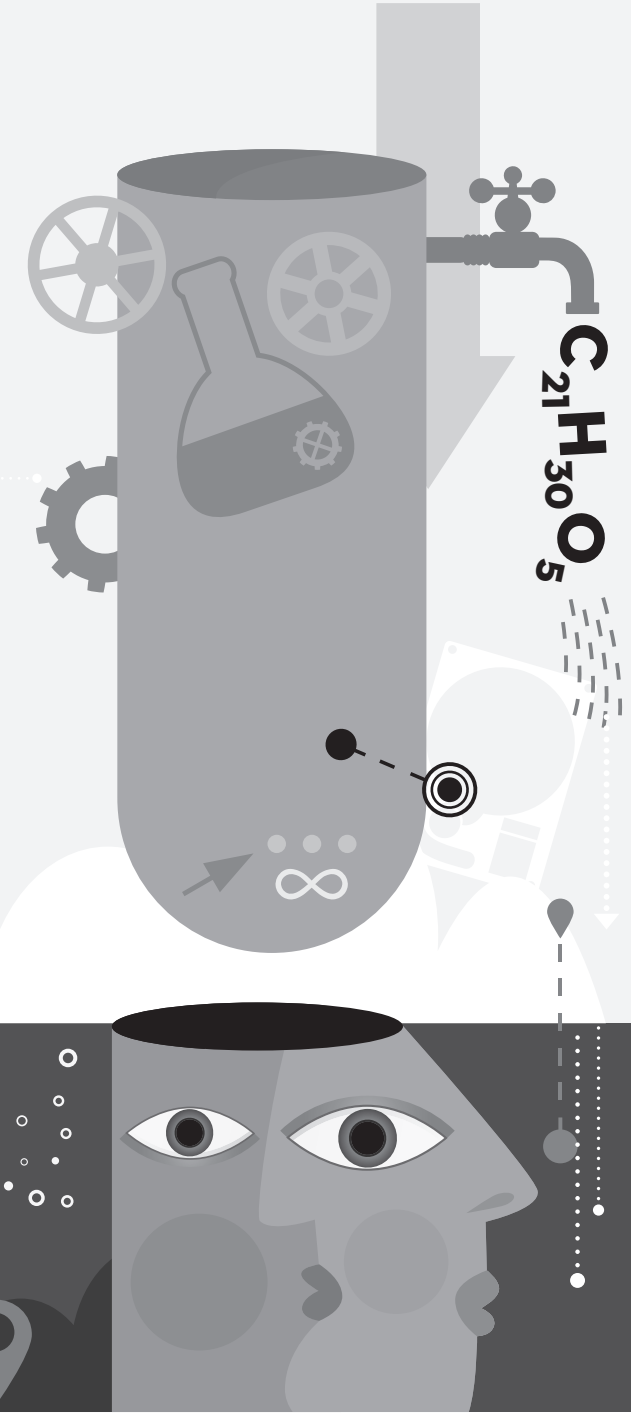
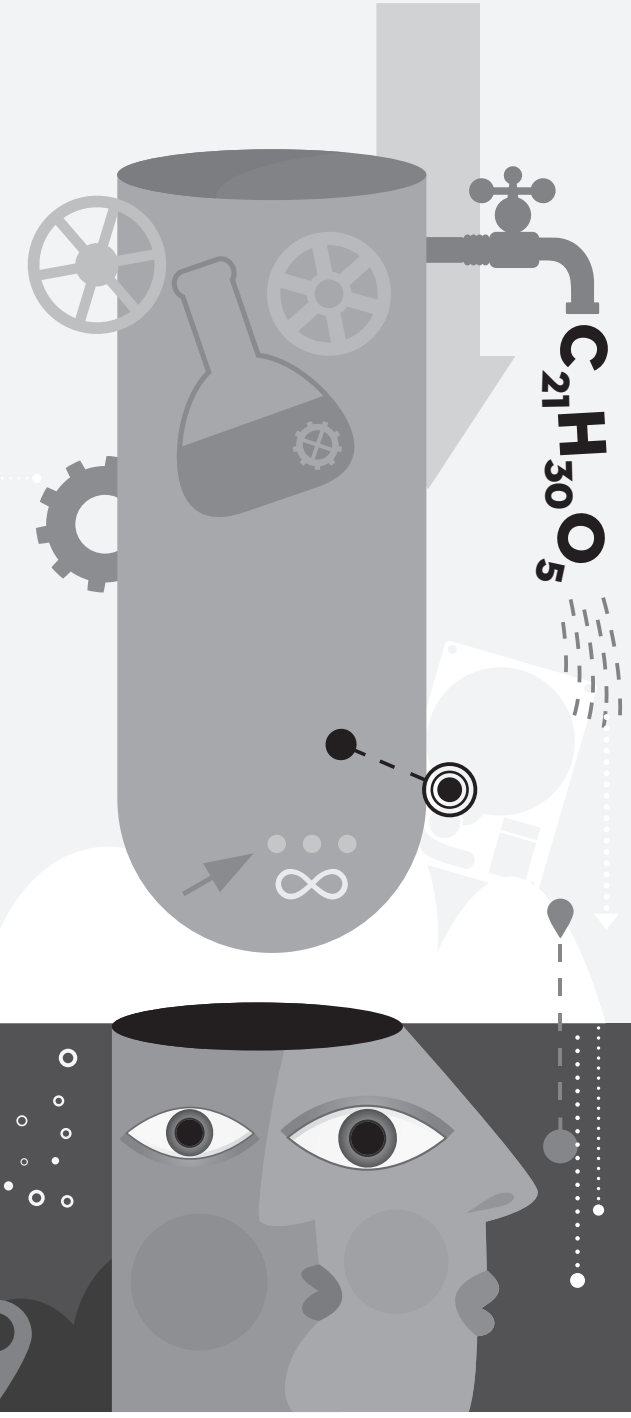


Recent-onset and longstanding active rheumatoid arthritis are associated with relatively low salivary cortisol levels and decreased dexamethasone-mediated cortisol suppression

Chapter 5

Quax R.A.M., de Jong P.H.P., Koper J.W. , van der Wal R., Hop W.C., den Dulk A.C., Weel A.E., Huisman A.M., van Zeben D., de Jong F.H., Hazes J.M.W., Lamberts S.W.J., Feelders R.A.

Submitted



Long-term cortisol levels in scalp hair in recent-onset and established rheumatoid arthritis

Chapter 6

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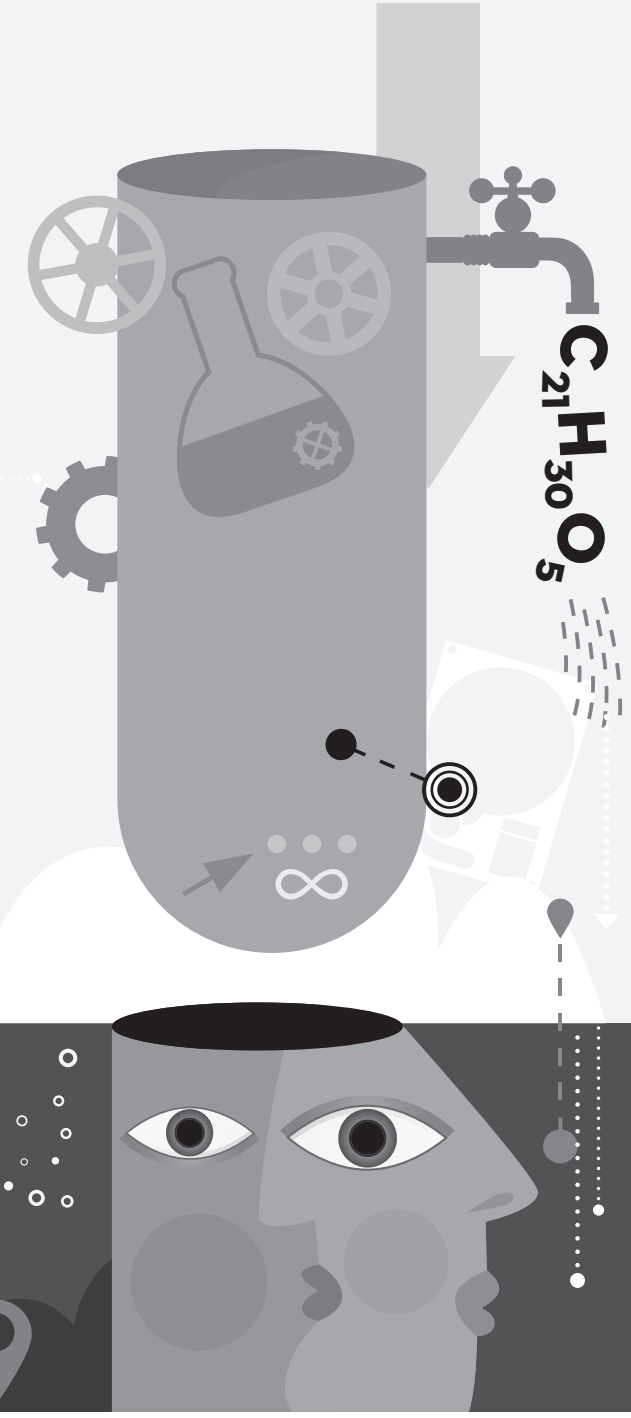

Glucocorticoid receptor gene polymorphisms and disease activity during pregnancy and the postpartum period in rheumatoid arthritis

Chapter 7

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Arthritis Research & Therapy, 2012 Aug 13;14(4):R183.

ABSTRACT

Objective

The mechanism underlying the spontaneous improvement of rheumatoid arthritis (RA) during pregnancy and the subsequent postpartum flare is incompletely understood and disease course varies widely between pregnant RA patients. In pregnancy, total and free levels of cortisol increase gradually, followed by a decrease to pre-pregnancy values postpartum. The glucocorticoid receptor (GR) polymorphisms *Bcl*I and N363S are associated with relatively increased glucocorticoid (GC) sensitivity whereas the 9β and ER22/23EK polymorphisms of the GR gene are associated with a relatively decreased GC sensitivity. We examined the relationship between the presence of these GR polymorphisms and level of disease activity and disease course of RA during pregnancy and postpartum.

Methods

We studied 147 participants of the PARA study (Pregnancy-Induced Amelioration of Rheumatoid Arthritis study), a prospective study investigating the natural improvement during pregnancy and the postpartum flare in women with RA. Patients were visited, preferable before pregnancy, each trimester and at three time points postpartum. At all occasions disease activity was scored using DAS28. All patients were genotyped for the GR polymorphisms *Bcl*I, N363S, 9β and ER22/23EK and divided in groups harboring either polymorphisms conferring increased GC sensitivity (*Bcl*I and N363S; GC-S patients) or polymorphisms conferring decreased GC sensitivity (9β or 9β + ER22/23EK; GC-I patients). Data were analyzed using a mixed linear model, comparing GC-S patients to GC-I patients with respect to improvement during pregnancy and the postpartum flare. The cumulative disease activity was calculated using time-integrated values (area under the curve, AUC) of DAS28 in GC-I patients versus GC-S patients. Separate analyses were performed according to the state of GC use.

Results

GC-S patients treated with GC had a significantly lower AUC of DAS28 in the postpartum period than GC-I patients. This difference was not observed in patients who were not treated with GC. During pregnancy, GC-S and GC-I patients had comparable levels of disease activity and course of disease.

Conclusions

Differences in relative GC sensitivity, as determined by GR polymorphisms, are associated with the level of disease activity in the post-partum period in GC treated patients, but they do not seem to influence the course of the disease per se.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic inflammatory disorder characterized by chronic synovitis leading to joint destruction. During pregnancy, spontaneous reduction of disease activity in RA is common; a phenomenon which is also observed in other autoimmune disorders (1-5). Postpartum however, RA deteriorates in the majority of women (3-4, 6). Pregnancy is supposed to have immunomodulatory effects but the exact mechanisms underlying the spontaneous amelioration during pregnancy and the subsequent postpartum flare have still not been elucidated. Several hypotheses have however been put forward including the beneficial effect of maternal-fetal HLA-incompatibility (7-8) and of increased galactosylation of immunoglobulin G (9-11). Also shifts in T-cell cytokine secretion profiles have been proposed as a potential mechanism underlying the improvement of RA during pregnancy and deterioration postpartum (12-15).

In healthy pregnancy, total and free levels of cortisol rise progressively reaching a peak in the second and third trimester (16-18). The improvement in RA starts in the first trimester and almost half of patients have at least low disease activity (DAS28 ≤ 3.2) in the third trimester (4). Nevertheless, prospectively studied cohorts of pregnant RA patients concurrently evaluating reduction of disease activity with accompanying (free) cortisol levels on an individual basis are lacking. It is known from daily clinical practice however, that interindividual differences in the degree of pregnancy-induced remission and the postpartum deterioration do exist, with some women reaching complete remission during pregnancy while others have persistent active disease. This discrepancy was already noticed in two early case series in which cortisol metabolites (i.e. 17-hydroxycorticosteroid (17-OHCS)) were measured in pregnant RA women and found that high levels of 17-OHCS only related to improvement of disease activity in a subset of patients (19-20). This variation in clinical responses does not depend solely on the absolute levels of cortisol but might also be explained by differences in individual GC sensitivity.

In the healthy population, a considerable variation in GC sensitivity has been demonstrated by low-dose (0.25 mg) dexamethasone suppression tests and functional *in vitro* assays (21-22). In diseased states, these differences in GC sensitivity are reflected by a wide spectrum of GC therapy efficacy, which may partly be explained by four functional single nucleotide polymorphisms (SNPs) in the glucocorticoid receptor (GR) gene. The minor alleles of the polymorphisms N363S (rs6195) and *Bcl*I (rs41423247) are associated with a relative hypersensitivity to GC, whereas the ER22/23EK (rs6189 and rs6190) and 9β (rs6198) SNPs are associated with a relatively decreased GC sensitivity (23). Previously, we have demonstrated that carriers of the ER22/23EK variant had more often erosive disease and more frequently needed tumor necrosis factor-alpha (TNF-α) blocking therapy (24). Similarly, these GR polymorphisms could explain differences in disease course during pregnancy and postpartum in RA.

Therefore, the aim of our study was to investigate the association between GR gene polymorphisms and level of disease activity and disease course during pregnancy and in the postpartum period in RA patients.

PATIENTS AND METHODS

Patients

All patients were participants of the PARA study (Pregnancy-Induced Amelioration of Rheumatoid Arthritis study), a nationwide prospective study investigating the natural improvement of RA during pregnancy and the postpartum flare (4). If possible, patients were visited before conception. Patients were visited at their home address at each trimester and at 6 weeks, 12 weeks and 26 weeks after delivery. In the present study, women who had a miscarriage were excluded from further analysis and no woman was included twice.

Methods

Data collection

Trained research nurses or physicians examined all patients using a standardized 28-joint count for swelling and pain. Disease activity was calculated using the disease activity score (DAS28) with three variables (swollen joint count, tender joint count and C-reactive protein (CRP) level) (25), since this variant of the DAS has been shown to reflect disease activity most reliably during pregnancy (26). Current medication use at each visit was recorded. Postpartum, all mothers provided information on breastfeeding, since this may interfere with resumption of methotrexate (MTX) therapy after delivery.
Improvement of disease activity during pregnancy was defined according to the EULAR criteria as 'responders' ('moderate' and 'good' response combined) versus 'non-responders' and could in accordance with the EULAR criteria only be applied to those patients with a baseline DAS28≥3.2 at first trimester (N=71) (25). The 'reversed' EULAR criteria were used to define a very early flare, (deterioration between visit at third trimester and 6 weeks postpartum), early flare (deterioration between visit at 6 weeks and 3 months postpartum) and late flare (deterioration between visit at 6 weeks and 6 months postpartum) as described previously (4) with minor modifications (Supplementary Table 1).

Glucocorticoid receptor polymorphisms

All patients were genotyped for 4 functional polymorphisms of the GR gene (ER22/23EK, rs6189 and rs6190; N363S, rs6195; *Bcl*I, rs41423247 and 9β, rs6198), using DNA extracted from samples of peripheral venous blood. Genotyping was performed using Taqman allelic discrimination assays (Applied Biosystems), following protocols described by the supplier. Results were analyzed using the sequence detection system 2.2 software (Applied Biosystems).

Data and statistical analysis

Mann-Whitney U tests and Pearson $\chi 2$ tests were used to determine differences in baseline characteristics.

We estimated DAS28 in patients who used GC versus patients who did not use GC using a linear mixed model (LMM). Using this model we compared the area under the curve (AUC) of DAS28 in the two groups on the whole trajectory, during pregnancy and in the postpartum period. We used the DAS28 score as the response, and 'time' and the 'use of glucocorticoid' x 'time' interaction as covariates. Time is used as a categorical variable denoting one of the seven measurement occasions. Similarly, we then estimated separate linear mixed models for each individual polymorphism, using 'time' and the interaction of 'time' x 'carriage of minor alleles' as covariates. Because of the low frequencies of the N363S (4.1%) and the ER22/23EK (7.5%) carriers, no AUC of DAS28 could be calculated for these models. Subjects were therefore further analyzed as carriers of a polymorphism associated with increased sensitivity for GCs (*Bcl*I and/or N363S, referred to as the GC-S group) versus carriers of a polymorphism associated with reduced sensitivity to GCs (9β or 9β + ER22/23EK, referred to as the GC-I group). Patients who were heterozygous for both the *Bcl*I and 9β polymorphisms or the N363S and 9β variants were excluded from the GC-S/GC-I groups. In this final model we again tested whether the average DAS28 was equal between the GC-S and GC-I groups on the whole profile, during pregnancy and postpartum. In all models we used a person specific intercept and assumed the residual covariance structure was autoregressive heteroskedastic.

Pearson $\chi 2$ analysis was applied to compare rates of response during pregnancy and the presence of a very early, early or late flare. All above-mentioned analyses were performed in patients who used GC and patients who did not use GC separately. Patients were designated as GC-users when patients used GC during pregnancy and used GC at the time of at least 2 out of 3 postpartum visits. No correction for multiple comparisons was applied. Differences in the median daily dosage of prednisone given during pregnancy and postpartum were calculated using the Mann-Whitney test. Statistical analysis was performed using SPSS version 17.0 and SAS version 9.2. We considered differences statistically significant if $P \leq 0.05$ (2-sided).

Ethical Approval

All subjects signed informed consent and the study was approved by the medical ethics committee of the Erasmus Medical Center. This study is in compliance with the Declaration of Helsinki.

RESULTS

Baseline characteristics

In total, 147 patients participating in the PARA study were enrolled in the current study. More than 60% of patients had active disease in the first trimester of their pregnancy and all women fulfilled the ACR 1987 revised criteria for RA (Table 1).
As shown previously, sulfasalazine and prednisone were the most used treatment regimens during pregnancy (4). Approximately 40% of patients did not use any antirheumatic drug (Supplementary Table 2). Disease activity scores were available in 69, 115, 133, 142, 140, 137 and 131 women at the seven different study visits before conception, during pregnancy and postpartum respectively.

Table 1. Patients characteristics.

	N = 147
Age at delivery in years, mean (SD)	32.4 (3.8)
Disease duration in years, median (range)	5.5 (0.1-28.4)
Gestational age at delivery in weeks, mean (SD)	39.3 (1.9)
Anti-CCP positive, N (%)	90 (61.2)
Rheumatoid Factor (IgM) positive, N (%)	110 (74.8)
Presence of erosions, N (%)	105 (71.4)
Number of DMARDs* prior to conceive, median (range)	2 (0-6)
Breastfeeding (6 weeks postpartum), N (%)	60 (40.8)
DAS28-CRP3≥3.2 in first trimester, N (%)**	71 (61.7)
Moderate/good response during pregnancy, N (%) ***	32 (45.1)
Very early flare, N (%)#	27 (20.0)
Early flare, N (%) ##	29 (22.0)
Late flare, N (%) ###	37 (30.1)

*disease modifying antirheumatic drugs, including prednisone; ** in 115 patients DAS28 in first trimester was available; ***according to EULAR response criteria, DAS28 ≥ 3.2 in the first trimester is required. Data were available in #135 , ## 132 and ### 123 patients respectively, according to reversed EULAR response criteria; very early flare: deterioration between visit at third trimester and 6 weeks postpartum; early flare: deterioration between visit at 6 weeks and 3 months postpartum; late flare: deterioration between visit at 6 weeks and 6 months postpartum; anti-CCP: anti-cyclic citrullinated protein.

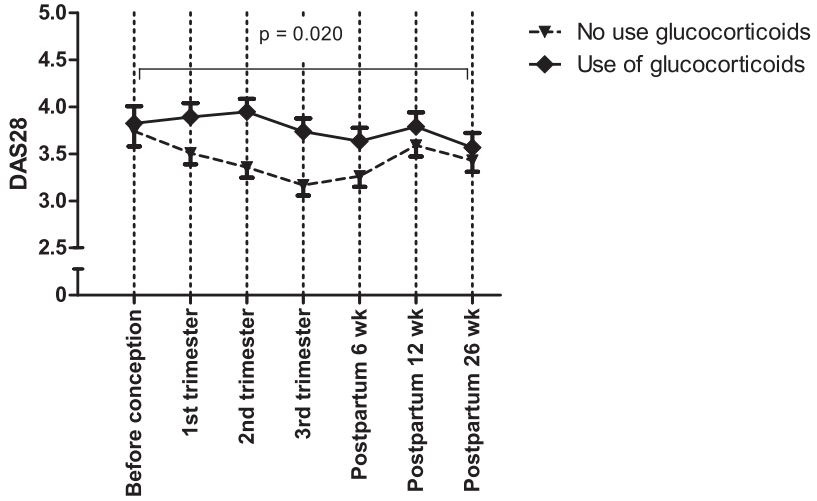

Figure 1. Disease activity (DAS28 ± SEM) among pregnant women with (N=57) and without (N=90) use of glucocorticoids.

In general, patients treated with GC (N=57) had significantly higher disease activity than patients not treated with GC (N=90, Figure 1). Patients who used GC had a significantly shorter duration of gestation and had erosions more frequently (Table 2). Analyses were therefore performed separately according to the state of GC use.

Table 2. Patients characteristics stratified according to use of GC.

	Use of GC (N=57)	No use of GC (N=90)
Age at delivery in years, mean (SD)	33.15 (3.80)	31.93 (3.67)
Disease duration in years, median (range)	6.07 (0.22-28.57)	5.18 (0.14-28.54)
Gestational age at delivery in weeks, mean (SD)	38.44 (2.30)	39.85 (1.27)†
Anti-CCP positive, N (%)	39 (68.4)	51 (56.7)
Rheumatoid Factor (IgM) positive, N (%)	47 (82.5)	63 (70.0)
Presence of erosions, N (%)	49 (86)	56 (62.2)†
Dosage of prednisone (mg/day), median (range)	7.5 (2.5-20)	-
Number of DMARDs* prior to conceive, median (range)	2 (0-5)	1 (0-4)†
Breastfeeding (6 weeks postpartum), N (%)	13 (22.8)	47 (52.2)†
DAS28 ≥3.2 in first trimester, N (%)**	33 (70.2)	38 (55.9)
Moderate/good response during pregnancy, N (%) ***	15 (45.5)	17 (43.6)
Very early flare, N (%) #	9 (17.3)	18 (21.7)
Early flare, N (%) ##	9 (18.0)	20 (24.4)
Late flare, N (%) ###	10 (21.3)	27 (35.5)

*disease modifying antirheumatic drugs, excluding prednisone;** in 115 patients DAS28 in first trimester was available; ***according to EULAR response criteria, DAS28 ≥ 3.2 in the first trimester is required, N=71 out of 115. # Data were available in 135 patients, ## 132 patients and ### 123 patients, according to reversed EULAR response criteria; † p<0.05 as compared to patients using GC; anti-CCP: anti-cyclic citrullinated protein.

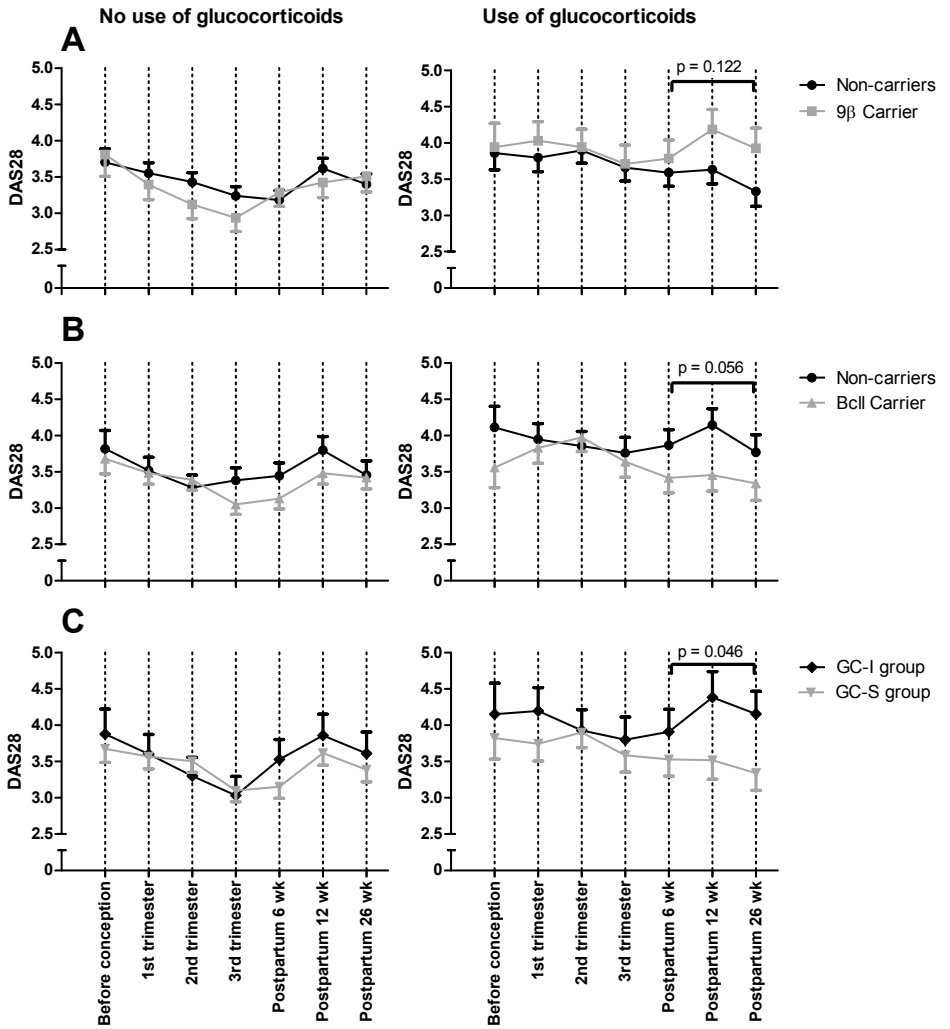

Figure 2. Disease activity according to carriage of GR polymorphisms. A) Disease activity in carriers of 9β (N=29) versus non-carriers (N=61) in patients not using GC (left panel). Of patients using GC, there were 19 carriers of the 9β polymorphism and 37 non-carriers (right panel). In one patient the 9β-genotype could not be determined. B) Disease activity in carriers of *BclI* (N=55) versus non-carriers (N=34) in patients not using GC (left panel). In one patient the *BclI*-genotype could not be determined. Of patients using GC, there were 29 carriers of the *BclI* polymorphism and 28 WT carriers (right panel). C) Disease activity in carriers of polymorphisms conferring increased GC sensitivity (N=44, GC-S group) versus patients carrying polymorphisms conferring decreased GC sensitivity (N=15, GC-I group) not using GC (left panel). Of patients using GC, there were 24 in the GC-S group and 13 in the GC-I group (right panel). Disease activity is presented as DAS28 ± SEM.

Glucocorticoid receptor polymorphisms and disease course during gestation and postpartum

We found 84 patients (57.1%) who were heterozygous or homozygous carrier of the *Bcl*I polymorphism. The 9β polymorphism was present in 48 (32.7%) patients.

Analysis of the level of disease activity in carriers versus non-carriers of these polymorphisms showed that 9β carriers did not differ significantly in AUC of DAS28 compared with non-carriers (Figure 2A). *Bcl*I carriers treated with GC had a near-significant lower AUC of DAS28 postpartum compared with non-carriers (p = 0.056, Figure 2B, right panel). No differences in the AUC of DAS28 postpartum were observed in non-GC treated patients.

Nineteen patients (12.9%) were heterozygous carrier of both the *Bcl*I and 9β polymorphisms or the N363S and 9β variants. These patients were excluded in the final analysis to enable

Table 3. Clinical characteristics of patients in the GC-S and GC-I group according to the use of glucocorticoids.

	Use of GC		No use of GC	
	GC-S (N=24)	**GC-I (N=13)**	**GC-S (N=44)**	**GC-I (N=15)**
Age at delivery in years, mean (SD)	34.1 (3.1)	34.1 (3.6)	31.6 (3.9)	31.2 (3.0)
Disease duration in years, median (range)	4.6 (0.2-28.6)	6.8 (1.0-22.7)	5.3 (0.1-28.5)	2.4 (0.2-28.4)
Gestational age at delivery in weeks, mean (SD)	39.0 (1.9)[A]	37.4 (2.2)	39.8 (1.3)	39.9 (1.3)
Anti-CCP positive, N (%)	16 (66.7)	8 (61.5)	24 (54.5)	6 (40)
Rheumatoid Factor (IgM) positive, N (%)	17 (70.8)	10 (76.9)	31 (70.5)	9 (60.0)
Presence of erosions, N (%)	22 (91.7)	13 (100)	28 (63.6)	8 (53.3)
Dosage of prednisone (pregnancy;mg/day), median (range)	6.25 (2.5-15)	8.75 (5-20)	-	-
Dosage of prednisone (postpartum;mg/day), median (range)	8.75 (2.5-15)	10.0 (5-15)	-	-
Number of DMARDs* prior to conceive, median (range)	2 (0-4)	2 (1-5)	2 (0-4)	2 (0-3)
Moderate/good response during pregnancy, N/N_{total} (%)	5/11 (45.5)	4/9 (44.4)	8/16 (50)	5/11 (45.5)
Very early flare, N/N_{total} (%)	5/21 (23.8)	3/13 (23.1)	10/41 (24.4)	5/13 (38.5)
Early flare, N/N_{total} (%)	4/21 (19.0)	2/12 (16.7)	12/40 (30.0)	3/13 (23.1)
Late flare, N/N_{total} (%)	4/19 (21.1)	4/13 (30.8)	15/37 (40.5)	4/12 (33.3)
Breastfeeding (6 weeks postpartum), N (%)	8 (33.3)	1 (7.7)	20 (45.5)	8 (53.3)
Use of NSAIDs 6 months postpartum[§], N/N_{total} (%)	7/22 (31.8)[A]	10/13 (76.9)	13/40 (32.5)	6/13 (46.2)
Use of MTX 6 months postpartum[§], N/N_{total} (%)	11/22 (50.0)	9/13 (69.2)	10/40 (25.0)	5/13 (38.5)
Use of sulfasalazine 6 months postpartum[§], N/N_{total} (%)	6/22 (27.3)	2/13 (15.4)	17/40 (42.5)	6/13 (46.2)
Use of anti-TNF-α 6 months postpartum[§], N/N_{total} (%)	3/22 (13.6)	3/13 (23.1)	2/40 (5.0)	0/13 (0)

Data concerning response during pregnancy, very early flare, early flare and late flare were present in 47, 88, 86 and 81 patients respectively. [§] available in 88 patients. [A] $p<0.05$ compared to GC-I, use of GC; anti-CCP: anti-cyclic citrullinated protein; TNF-α: tumor necrosis factor alpha; ; DMARDs: disease modifying antirheumatic drugs.

an appropriate comparison between patients carrying a polymorphism associated with increased sensitivity to GCs (*BclI* and/or N363S, GC-S group) and patients harboring a genetic variant associated with reduced sensitivity to GCs (9β or 9β + ER22/23EK, GC-I group). The results of this analysis, shown in Figure 2C, indicate that GC treated patients in the GC-I group had a significantly higher AUC of DAS28 in the whole postpartum period, i.e. up to 26 weeks, than patients in the GC-S group (p = 0.046). In patients not treated with GCs, these differences did not exist.

The AUC of DAS28 during pregnancy, the course of the disease, EULAR response during pregnancy and the presence of a very early flare, early flare or late flare using 'reversed' EULAR response criteria, were not associated with any GR genotype, although the DAS28 was lower in the GC-S group than in the GC-I group at all time points in GC treated patients (Figure 2C). The GR genotypes were equally distributed among GC-users and non-GC-users. The clinical characteristics between GC-S and GC-I patients, stratified according to the use of GC, did not differ, except for the more frequent use of non-steroidal anti-inflammatory drugs (NSAIDs) in the GC-I group (p = 0.01; Table 3). The median daily dosage of prednisone given during pregnancy, taking the highest dosage needed at any time during pregnancy, tended to be higher in GC-I patients (8.75 mg daily versus 6.25 mg daily, p = 0.157). GC-S patients could more frequently reduce the daily needed GC dose during pregnancy than GC-I patients, possibly reflecting higher GC sensitivity to the pregnancy-related rise in cortisol in GC-S patients, although this was not statistically significant (N=7, 29.2% versus N=1, 7.7%, p=0.130). In the postpartum period, prednisone daily dosages did not differ between GC-S and GC-I patients.

DISCUSSION

In this nationwide prospective study including 147 pregnant RA patients, we examined for the first time whether GR polymorphisms that modulate GC sensitivity are associated with the level of disease activity and disease course during pregnancy and the postpartum period. We show that GC treated patients in the GC-S group (i.e. those with the *BclI* or N363S or both polymorphisms, associated with relatively increased GC sensitivity) have a significantly lower disease activity in the postpartum period than patients in the GC-I group (9β or 9β + ER22/23EK, associated with relatively decreased GC sensitivity) as measured by the AUC of the DAS28. In patients not treated with GC, the level of disease activity and disease course during pregnancy or in the postpartum period does not seem to be influenced by differences in GR genotype.

Gestational-induced remission of RA has been recognized for a long time (27) and may in part be attributed to the increase in cortisol production which in turn enhances endogenous immunosuppression. Pregnancy is indeed considered to be a natural variant of hypercortisolism (28-29) and serum (free) cortisol, urinary free cortisol, salivary cortisol and cortisol

content in hair all have been demonstrated to rise progressively during gestation, followed by a rapid decrease in cortisol levels postpartum (17-18, 30-37).

Apart from cortisol availability, the ultimate biological effects of GC also depend on GC sensitivity which is modulated by GR polymorphisms (23).

Based on the course of cortisol levels during pregnancy and after delivery, we hypothesized that differences in glucocorticoid sensitivity might in part explain why the beneficial effect of pregnancy on RA disease activity does not occur in all RA patients.

Polymorphisms of the GR gene have been demonstrated to influence disease course in several inflammatory disorders, including Graves ophthalmopathy (38), Crohn's disease (39) and multiple sclerosis (40). We recently demonstrated that the minor alleles of *Bcl*I and 9β were associated with respectively decreased and increased susceptibility to develop RA. In addition, ER22/23EK carriers had a worse disease phenotype and needed more frequently TNF-α blocking therapy (24). We extend these data by demonstrating higher levels of disease activity in the postpartum period in GC treated patients in the GC-I group, despite the more frequent use of NSAIDs.

Interestingly, the differences in disease activity between carriers of GC-sensitive and GC-resistant polymorphisms were observed only in women treated with GC. The GC treated patients involve a subgroup of women with high disease activity, as reflected by observed higher DAS28. Our observations may imply that in the postpartum phase, when endogenous cortisol levels fall, patients with polymorphisms associated with increased GC sensitivity benefit more from GC therapy. Therefore in states of relative glucocorticoid deficiency, differences in GC sensitivity due to genetic variability may in part determine variations in disease activity. Vice versa, in patients with low disease activity, as characterized by the absence of glucocorticoid therapy in our cohort, endogenous levels of cortisol apparently can prevent uncontrolled inflammatory processes independent of genetic variations of the GR gene, although we did not measure cortisol levels in our patients.

This concept of a 'relative glucocorticoid deficiency' might also explain why the observed variation in disease activity seems to be restricted to the postpartum period, since Magiakou and co-workers have shown that hypothalamic CRH secretion in healthy pregnant women is transiently suppressed at three and six weeks only recovering at 12 weeks postpartum (41). This suppression of the hypothalamic-pituitary-adrenal (HPA) axis in the postpartum period, which could be even more pronounced in RA in which a pre-existing blunted HPA-axis is described in non-pregnant states (42), might even further attenuate the ability of the HPA-axis to produce sufficient levels of cortisol.

The clinical relevance of this blunted HPA-axis in the first three months after childbirth is illustrated by a higher incidence or exacerbation of several autoimmune diseases, including postpartum depression, autoimmune thyroid disease and RA itself (41, 43-46). The lack of differences between GC-I and GC-S patients in disease activity during pregnancy could also be explained by altering levels of glucocorticoid sensitivity as was suggested by Majzoub

and co-workers (47-48). Alternatively, patients in the GC-I group tended to need higher daily dosages of GC during pregnancy which could have masked a higher level of disease activity in this subgroup of patients. Although we have focused on GC, absolute levels of estrogens and progesterone also rise progressively during gestation. Both estrogens and progesterone possess anti-inflammatory properties and are therefore likely to have substantially influenced disease course (49). Similar to differences in GC sensitivity, one could speculate that variation in sensitivity to the immunosuppressing effects of estrogens and progesterone might also contribute to the wide clinical spectrum of changes in disease activity observed in pregnancy and after delivery in RA.

Interestingly, the difference in disease activity between GC-I and GC-S patients persisted during the entire postpartum follow-up period, i.e. up to 26 weeks. Future studies should examine at which time points disease activity patterns of both groups converge to pre-pregnancy levels.

It should be noted that our study also has some limitations. First of all, genetic association studies usually require larger numbers of patients. Although this is the largest prospectively studied cohort of pregnant RA patients, additional studies are needed to validate our findings. Second, the presented data are based on Caucasian patients only, who may differ from patients from other geographical areas with different genetic and environmental backgrounds. Third, parameters of HPA-axis activity were not measured in this study which could have provided additional information in the non-GC treated patients.

Although the pattern of cortisol levels in pregnancy and after delivery has been extensively documented (17-18, 30-37), large prospective studies evaluating cortisol levels along with clinical responses during pregnancy and postpartum in RA are currently lacking. Together with new insights in the past two decades supporting a blunted HPA-axis in RA, this justifies renewed interest in the precise role of GC in pregnant RA patients and course of disease (50). In this context, long-term indices of HPA-activity as measured by means of cortisol in hair, together with dynamic functional assays to assess GC sensitivity (i.e. GR number, affinity of the GR receptor and GR-mediated gene transcription) are promising techniques to further unravel the role of GC and their precise contribution to pregnancy-associated alterations in disease activity in RA.

CONCLUSIONS

We demonstrate that differences in GC sensitivity, as determined by GR polymorphisms, might influence the level of disease activity in the postpartum period in GC treated women. The course of the disease itself does not seem to be associated with polymorphisms of the GR. In the light of the relatively small numbers of patients in each genotype group however, our data should be regarded as an interesting new hypothesis possibly adding to the eluci-

dation of the multi-factorial mechanisms underlying pregnancy-induced amelioration and the postpartum flare, but do not necessarily prove the genetic association. Therefore, future (larger) studies should validate our hypothesis and examine both parameters of glucocorticoid availability as well as parameters of glucocorticoid sensitivity in relation to individual disease courses of pregnant RA patients.

ACKNOWLEDGEMENTS

The authors would like to acknowledge all patients and rheumatologists for their contribution to the PARA study. We are thankful to all research assistants for their help in data collection. This study was supported by the Dutch Arthritis Foundation.

Supplementary Table 1. 'Reversed' EULAR response criteria for the definition of deterioration postpartum.

	Increase of DAS28 with		
DAS28 at 6*,12 or 26*** weeks postpartum**	**>1.2**	**>0.6 and ≤1.2**	**≤0.6**
>5.1	Severe flare	Moderate flare	No flare
>3.2 and ≤5.1	Moderate flare	Moderate flare	No flare
≤3.2	Moderate flare	No flare	No flare

Deterioration of disease activity is studied from trimester 3 to 6 weeks postpartum (*very early flare), 6 weeks postpartum to 12 weeks postpartum (**early flare) and from 6 weeks postpartum to 26 weeks postpartum (***late flare). No baseline disease activity is required.

Supplementary Table 2. Medication use.

Medication	Before pregnancy (N=69)	1st trimester (N=115)	2nd trimester (N=133)	3rd trimester (N=142)	PP-1 (N=139)	PP-2 (N=137)	PP-3 (N=129)
Prednisone	28 (40.6)	45 (39.1)	51 (38.3)	51 (35.9)	50 (36.0)	47 (34.3)	44 (34.1)
Sulfasalazine	24 (34.8)	33 (28.7)	38 (28.6)	40 (28.2)	40 (28.8)	45 (32.8)	42 (32.6)
Hydroxychloroquine	2 (2.9)	2 (1.7)	3 (2.3)	2 (1.4)	4 (2.9)	8 (5.8)	9 (7.0)
Methotrexate	0 (0)	0 (0)	0 (0)	0 (0)	21 (15.1)	38 (27.7)	51 (39.5)
TNF-α blocking agents	1 (1.4)	0 (0)	0 (0)	0 (0)	6 (4.3)	12 (8.8)	13 (10.1)
NSAIDs	1 (1.4)	7 (6.1)	5 (3.8)	3 (2.1)	29 (20.9)	57 (41.6)	49 (38.0)
Other	3 (4.3)	1 (0.8)	1 (0.8)	1 (0.7)	1 (0.7)	4 (2.9)	5 (3.9)
No medication	23 (33.3)	47 (40.9)	53 (39.8)	63 (44.4)	46 (33.1)	24 (17.5)	19 (14.7)

Values are presented as N(%). PP-1: postpartum visit after 4-6 weeks, PP-2: postpartum visit after 12 weeks, PP-3: postpartum visit after 26 weeks. The percentages presented here do not add up to 100%, as patients may use more than one antirheumatic drug. Data about use of medication were missing in 1 and 2 patients at postpartum visit 1 and 3 respectively.

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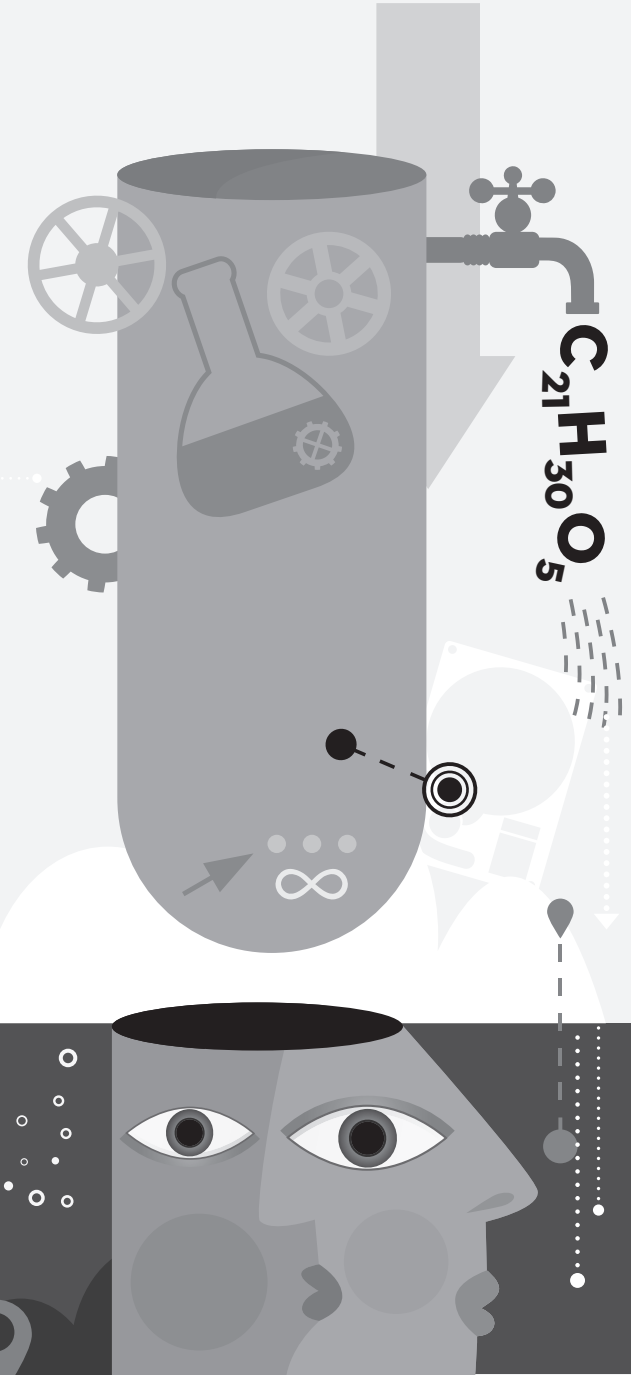

Glucocorticoid sensitivity in Behçet's disease

Chapter 8

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Endocrine Connections 2012 vol. 1 no. 2 103-111

ABSTRACT

Objective

Glucocorticoid (GC) sensitivity is highly variable among individuals and has been associated with susceptibility to develop (auto-) inflammatory disorders.
The purpose of the study was to assess GC sensitivity in Behçet's disease (BD) by studying the distribution of four glucocorticoid receptor (GR) gene polymorphisms and by measuring *in vitro* cellular GC sensitivity.

Methods

Three independent cohorts of patients with BD and controls were genotyped for four functional GR gene polymorphisms. To gain insight into functional differences in *in vitro* GC sensitivity, 19 patients with BD were studied using two bioassays and a whole cell dexamethasone binding assay. Finally, mRNA expression levels of GR splice variants (GR-α and GR-β) were measured.

Results

Healthy controls and BD patients in the three separate cohorts had similar distributions of the four GR polymorphisms. The *Bcl*I and 9β minor allele frequency differed significantly between Caucasians and Mideast and Turkish individuals.
At the functional level, a decreased *in vitro* cellular GC sensitivity was observed. GR number in PBMC was higher in BD compared to controls. The ratio of GR-α/GR-β mRNA expression levels was significantly lower in BD.

Conclusions

Polymorphisms in the GR gene are not associated with susceptibility to BD. However, *in vitro* cellular GC sensitivity is decreased in BD, possibly mediated by a relative higher expression of the dominant negative GR-β splice variant. This decreased *in vitro* GC sensitivity might play an as yet unidentified role in the pathophysiology of BD.

INTRODUCTION

Behçet's disease (BD) is an inflammatory disorder characterized by recurrent episodes of orogenital ulcers, uveitis, arthritis and skin lesions. Less frequent symptoms include gastrointestinal lesions and involvement of the central and peripheral nervous system. The onset of disease is typically in the third or fourth decade of life and equally affects men and women.
BD is common along the ancient Silk Road, which extends from Eastern Asia to the Mediterranean area. The highest prevalence occurs in Turkey (80-420 cases per 100,000), followed by Middle and Far Eastern countries (13.5-20 cases per 100,000). In contrast, the prevalence in Western countries is much lower; approximately 0.12-5.1 per 100,000 (1-3).
The etiology of BD is unknown. It is mainly considered a multi-factorial disease and it is associated with the presence of human leukocyte antigen (HLA) variants, in particular HLA-B51 (2). Variations in several other genes like the tumor necrosis factor alpha (TNF-α), interleukin-10 and the nucleotide-binding oligomerization domain containing 2 (NOD2) genes are also associated with BD (4-6). Immunohistochemically, BD is characterized by the presence of vasculitis and infiltration of tissue by neutrophils and mononuclear cells (7).
Interestingly, BD also displays some features distinctive of autoimmune processes. First, cellular immunity is disturbed in BD as exemplified by autoreactive T cells targeting heat shock protein 60 (8). For many autoimmune disorders, autoreactive T cells are a primary culprit in their pathogenesis. Second, several auto-antibodies have been described targeting numerous antigens, including CTLA-4 (9) (enhancing T cell proliferation), retinal S-antigen (10) and antikinectin (11), providing evidence for a dysfunctional humoral immune response. In general, BD might be regarded an immune-mediated inflammatory disease.
Glucocorticoids (GC) play a key role in mediating a balanced inflammatory response. GC exert their effects via interaction with the glucocorticoid receptor (GR). After binding of its ligand, the GR-GC complex migrates to nucleus to induce ("transactivation") or to suppress ("transrepression") expression of target genes. The ultimate biological effects of (endogenous) GC depend on the GC sensitivity of an individual, which is influenced by both genetic and acquired (disease-related) factors. Hence, decreased GC sensitivity could lead to unrestricted immune activation and facilitation of a chronic inflammatory process, a hallmark of many autoimmune disorders.
Indeed, decreased GC sensitivity has been shown to be involved in several autoimmune diseases. For instance, carriers of polymorphisms of the GR gene associated with reduced (i.e. 9β) or increased (i.e. *Bcl*I and N363S) GC sensitivity have increased respectively decreased susceptibility to develop rheumatoid arthritis (RA). In addition, carriers of the ER22/23EK allele of the GR gene, which is associated with decreased GC sensitivity, had a more severe disease course (12). At the functional level, van Winsen and co-workers showed that in multiple sclerosis patients, higher doses of dexamethasone were required to suppress LPS-induced TNF-α production in peripheral blood mononuclear cells (PBMC) when compared

with healthy controls (13). Also in active RA, PBMC were less sensitive to dexamethasone *in vitro* (14). Finally, an increased expression of the GR splice variant GR-β, the dominant negative inhibitor of the biologically active GR-α, is associated with GC resistance in several inflammatory disorders (15-20).

Since decreased GC sensitivity may contribute to immune-mediated inflammatory diseases, we hypothesized that decreased GC sensitivity is involved in the pathophysiology of BD. To test our hypothesis, we genotyped three independent cohorts of patients with BD for the prevalence of four functional GR polymorphisms. Furthermore, *in vitro* GC sensitivity was assessed by measurement of GR binding capacity (GR number and affinity) and by two bioassays (21). In these bioassays, dexamethasone-regulated expression of interleukin-2 (IL-2) and glucocorticoid-induced leucine zipper (GILZ) in PBMC is measured. Transrepressive effects of GC, traditionally considered to be the predominant mechanism regulating anti-inflammatory actions of GC, are represented by the IL-2 assay. The GILZ assay embodies all transactivated genes, mediating both anti-inflammatory effects of GC as well as (metabolic) side effects (22-23). Using these bioassays, a spectrum of GC sensitivity could be demonstrated in healthy individuals (21). Finally, we measured mRNA expression levels of GR-α and GR-β.

PATIENTS AND METHODS

Patients

To study the prevalence and distribution of the GR polymorphisms, three cohorts of in total 290 unrelated BD patients were included in the study (56 patients from the Erasmus MC, Rotterdam; 109 patients from The Jordan Hospital, Amman, Jordan and St John's Ophthalmic Hospital, Jerusalem, Israel; 39 patients from St Thomas' Hospital, London, UK and 86 patients from the University of Cukurova, Adana, Turkey. Of those, 55 were Caucasians, 125 of Middle Eastern (ME) origin or Arab descent and 110 patients were of Turkish descent.

The control population consisted of 150 Turkish and 75 ME individuals. Caucasian controls (N=5295-5413, depending on polymorphism) were participants in the Rotterdam Study, a population-based prospective cohort study on determinants of disease and disability in persons, aged 55 years and older, living in Rotterdam, the Netherlands.

To study functional differences in *in vitro* GC sensitivity in patients with BD, 19 consecutive BD patients from our outpatient clinic were included in the study. Experienced clinical immunologists (J.v.L. and M.v.H.) examined all patients and assessed disease activity using the validated Behçet's disease current activity form (BDCAF) (24). As a control group, we studied 20 healthy Caucasian laboratory employees. None of the patients or controls used GC in the last 3 months. All patients described in this study fulfilled the International Study Group criteria for the diagnosis of BD (25).

Methods

Glucocorticoid receptor polymorphisms

All patients and controls were genotyped for four functional polymorphisms of the GR gene (ER22/23EK, rs6189 and rs6190; N363S, rs6195; *Bcl*I, rs41423247 and 9β, rs6198) (26). DNA was extracted from samples of peripheral venous blood samples using standard techniques. DNA (1-2 ng) was dispensed in 384-well plates. PCR amplification (initial denaturation at 95°C for 15 min and 40 cycles with denaturation of 15 sec at 95°C and annealing and extension at 60°C) and genotyping was performed using the Taqman allelic discrimination assay. Results were analyzed by Taqman Prism 7900HT using the sequence detection system 2.2 software (Applied Biosystems).

Assessment of in vitro glucocorticoid sensitivity

Functional in vitro assays

Recently, two bioassays to determine GC sensitivity were developed in our laboratory (21). In short, peripheral blood was drawn in all patients using Cell Preparation Tubes with Sodium Heparin (Becton Dickinson) allowing isolation of peripheral blood mononuclear cells (PBMC). Cells were resuspended in RPMI 1640 medium containing L-glutamine supplemented with penicillin (100 U/ml) and streptomycin (100µg/ml) and 10% fetal bovine serum and precultured overnight in a 48-well plate (Costar) at a density of 4.0 x 10^6/ml. Trypan blue staining revealed the viability of isolated cells to be greater than 95%. The next day, cells were incubated with increasing doses of dexamethasone (range 0-333 nM dexamethasone) and stimulated with phytohemagglutinin 10µg/ml (Sigma-Aldrich). After four hours in the incubator total RNA of the cells was collected (Total RNA isolation Kit, Roche). cDNA was synthesized using 100 ng RNA and Taqman® Reverse Transcription Reagent (N808-0234, Applied Biosystems). For quantitative real-time PCR analysis, the Taqman technology was applied according to the manufacturer's instructions. GC-specific transactivation of the GC-induced leucine zipper (GILZ) mRNA and transrepression of the interleukin-2 (IL-2) mRNA were measured. Half maximal effective concentration (EC_{50}) was used as a read-out for *in vitro* GC sensitivity. The EC_{50} values of GILZ and IL-2 in PBMC were comparable when different compositions of lymphocytes and monocytes were tested (data not shown).

In addition, we measured the affinity and number of GR using a whole cell dexamethasone binding assay, as described previously (27).

Gene expression levels of glucocorticoid receptor isoforms

Immediately after isolation of PBMC as described above, 1x10^6 PMBC (in duplicate) were lysed and total RNA was extracted (Total RNA isolation Kit, Roche). cDNA was synthesized

using 200 ng RNA and Taqman® Reverse Transcription Reagent (N808-0234, Applied Biosystems) in a total volume of 50 µl. Gene expression levels of GR-α and GR-β were measured using pre-manufactured assays (Applied Biosystems, Hs00230818_m1 and Hs00354508_m1 respectively). All results were corrected for the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT).

Statistical Analysis

To analyze possible associations between GR genotypes and risk of having BD, we calculated odds ratios and 95% confidence intervals for hetero- and homozygous individuals separately (wildtype allele as reference). Given the low number homozygous carriers of the N363S and ER22/23EK minor allele, hetero- and homozygous carriers were analyzed together. Pearson χ^2 tests were performed to test for differences in distribution of the polymorphisms between the various ethnic groups. Differences in continuous variables between the cohorts were tested using Mann-Whitney U-tests and analysis of variance (ANOVA). IL-2-EC_{50} was square-root transformed and number of receptors and K_D were both natural logarithm transformed to normalize the data. All statistical analyses were performed using SPSS for Windows, release 17.0 (SPSS, Chicago, IL, USA) and we considered differences statistically significant if p values were ≤0.05 (2-sided).

Ethical Approval

This study was approved by the medical ethics committee of the Erasmus Medical Center and all subjects signed informed consent.

RESULTS

Glucocorticoid receptor polymorphisms

Healthy controls and BD patients in the three separate cohorts had similar distributions of the four GR polymorphisms (Table 1). Prevalence of the *Bcl*I minor allele was significantly higher in Caucasians compared to both Turkish and ME persons (37.1% in Caucasians versus 21.5% and 21.0% in Turkish and ME persons respectively, p <0.001). In contrast, the 9β minor allele was less prevalent in Caucasians compared to the Turkish and ME persons (17.2% in Caucasians versus 28.5 % and 29.6% in Turkish and ME persons respectively, p <0.001).

Table 1. Frequencies of GR polymorphisms in Behçet's disease and healthy controls.

	Caucasian group			Turkish group			Mid-East group		
Polymorphism	Case, N(%)	Control, N(%)	OR (95% CI)	Case, N(%)	Control, N(%)	OR (95% CI)	Case, N(%)	Control, N(%)	OR (95% CI)
ER22/23EK									
Non-carriers	55 (100)	4959 (93.7)	Reference	104 (94.5)	144 (97.3)	Reference	124 (99.2)	73 (97.3)	Reference
Carriers	-	336 (6.4)[1]	NA	6 (5.5)[3]	4 (2.7)[3]	2.07 (0.57-7.55)	1 (0.8)[3]	2 (2.7)[3]	0.29 (0.26-3.30)
N363S									
Non-carriers	52 (94.5)	4932 (92.7)	Reference	107 (97.3)	147 (98.7)	Reference	124 (99.2)	75 (100)	Reference
Carriers	3 (5.5)[3]	388 (7.3)[2]	0.74 (0.23-2.39)	3 (2.7)[3]	2(1.3)[3]	2.06 (0.34-12.55)	1 (0.8)[3]	-	NA
BclI									
Non-carriers	25 (46.3)	2133 (39.4)	Reference	67 (60.9)	89 (60.1)	Reference	76 (60.8)	50 (66.7)	Reference
Carriers	29 (53.7)	3280 (60.6)	0.75 (0.44-1.29)	43 (39.1)	59 (39.9)	0.97 (0.58-1.60)	49 (39.2)	25 (33.3)	1.29 (0.71-2.35)
Heterozygous carriers	23 (42.6)	2539 (46.9)	0.77 (0.43-1.36)	40 (36.4)	53 (35.8)	1.00 (0.60-1.68)	43 (34.4)	21 (28.0)	1.35 (0.71-2.54)
Homozygous carriers	6 (11.1)	741 (13.7)	0.69 (0.28-1.69)	3 (2.7)	6 (4.1)	0.66 (0.16-2.75)	6 (4.8)	4 (5.3)	0.99 (0.27-3.67)
9β									
Non-carriers	38 (71.7)	3681 (68.5)	Reference	53 (50.5)	76 (51.4)	Reference	62 (52.1)	33 (44.0)	Reference
Carriers	15 (28.3)	1692 (31.5)	0.86 (0.47-1.57)	52 (49.5)	72 (48.6)	1.04 (0.63-1.71)	57 (47.9)	42 (56.0)	0.72 (0.40-1.29)
Heterozygous carriers	13 (24.5)	1531 (28.5)	0.82 (0.44-1.55)	41 (39)	63(42.6)	0.93 (0.55-1.58)	49 (41.2)	34 (45.3)	0.77 (0.42-1.41)
Homozygous carriers	2 (3.8)	161 (3.0)	1.20 (0.29-5.00)	11 (10.5)	9 (6.0)	1.75 (0.68-4.52)	8 (6.7)	8 (10.7)	0.53 (0.18-1.55)

[1] Eight homozygous carriers, [2] Five homozygous carriers, [3] All heterozygous carriers. NA = not applicable (zero cases in one of the groups). OR = Odds Ratio.

In vitro glucocorticoid sensitivity

We included 19 BD patients from our outpatient clinic. These patients used a wide spectrum of anti-inflammatory agents, including NSAIDs (N=7, 36.8%), colchicine (N=5, 26.3%), hydroxychloroquine (N=4, 21.1%), TNF-α blockers (N=2, 10.5%) and pentoxifylline (N=3, 15.8%). Thalidomide, methotrexate, interferon-alfa and octreotide were each used by one patient. Further baseline characteristics and clinical features (present at any time in the disease course) are summarized in Table 2. None of the patients had involvement of the central nervous system.

Table 2. Patient characteristics.

	Healthy Controls (N=20)	Behçet's disease (N=19)
Female gender	10 (50)	12 (63.2)
Age in years, mean (SD)	31.8 (9.7)	43.3 (10.6)
Caucasian ethnicity	20 (100)	5 (26.3)
BDCAF, median (range)	-	12 (0-30)
Phenotype of disease (ever)		
Oral Ulcers	-	19 (100)
Genital Ulcers	-	17 (89.5)
Arthralgia/Arthritis	-	15 (78.9)
Gastro-intestinal Involvement	-	15 (78.9)
Uveitis/Vasculitis Retinae	-	11 (57.9)
Positive Pathergy Test	-	4 (21.1)*
Erythema Nodosum	-	11 (57.9)
Pustulopapular Skin Lesions	-	15 (78.9)

Values are presented as number (%), unless otherwise stated. BDCAF: Behçet's Disease Current Activity Form, * 3 patients never underwent a pathergy test.

Patients with BD had higher mean EC_{50} values in both the IL-2 assay and GILZ assay compared to healthy controls (mean IL-2-EC_{50} (95% CI): 10.80 (7.91-14.15) nM in BD versus 3.48 (2.16-5.10) nM in HC, p<0.001; mean GILZ-EC_{50} (95% CI): 12.16 (10.91-13.42) nM in BD versus 8.13 (6.69-9.58 nM) in HC, p<0.001) indicating decreased *in vitro* GC sensitivity in BD (Figure 1). The maximum induction of GILZ and repression of IL-2 did not differ significantly (data not shown). The GR number in PBMC (mean, 95% CI) was higher in BD (10380, 8593-12539 GR/cell) compared to controls (6652, 5719-7738 GR/cell, p=0.001), whereas the mean K_D (95% CI) of the receptor did not differ between patients (8.34, 6.62-10.50 nM) and controls (8.46, 7.37-9.71 nM). Importantly, the EC_{50} values of GILZ and IL-2 and the number of GR did not differ significantly between Caucasian and Turkish-ME patients (Figure 1). Patients and healthy controls had comparable percentages of monocytes (mean ± SD: 18.9 ± 5.5 in BD versus 20.9 ± 5.0 in healthy controls). Ligand affinity of monocytes and lymphocytes did not differ signifi-

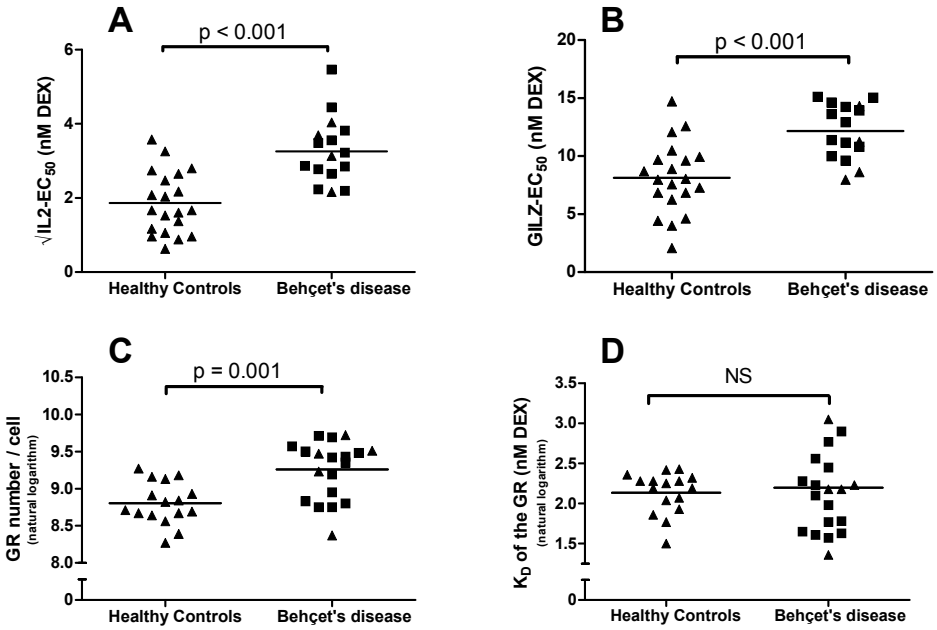

Figure 1. Cellular GC sensitivity in Behçet's disease and healthy controls. EC_{50} (nM DEX) values of interleukin-2 (A) and Glucocorticoid-Induced Leucine Zipper (B) of dexamethasone-treated PBMC. Number of glucocorticoid receptors per cell and K_D of the glucocorticoid receptor using a whole cell binding assay are depicted in C and D respectively. Triangles (▲) are Caucasian subjects; squares (■) represent Turkish-ME subjects. Please note the square-root transformed Y-axis in figure (A) and the logarithmically transformed Y-axis in figure (C) and (D).

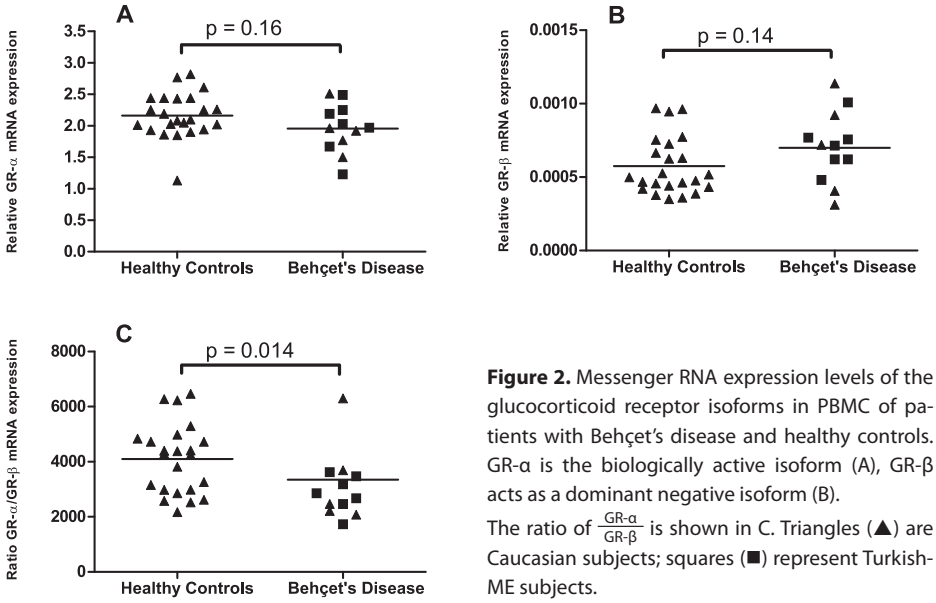

Figure 2. Messenger RNA expression levels of the glucocorticoid receptor isoforms in PBMC of patients with Behçet's disease and healthy controls. GR-α is the biologically active isoform (A), GR-β acts as a dominant negative isoform (B).

The ratio of $\frac{\text{GR-}\alpha}{\text{GR-}\beta}$ is shown in C. Triangles (▲) are Caucasian subjects; squares (■) represent Turkish-ME subjects.

cantly. The number of GR per cell was about three fold higher in monocytes as compared to lymphocytes (data not shown).
No correlations were found between the BDCAF-score and parameters of *in vitro* GC sensitivity. Men and women had equal mean levels of IL-2-EC_{50} and GILZ-EC_{50}. Likewise, there were no gender differences at the level of the number of GR or the affinity of the GR.
In 12 BD patients and healthy controls, mRNA expression levels of GR-α and GR-β were measured. GR-α mRNA showed a trend toward lower expression in patients with BD while a tendency toward higher mRNA levels of GR-β was observed. Combined, the GR-α/GR-β ratio was significantly lower in patients (p=0.014; Figure 2).

DISCUSSION

The results of our study suggest that decreased GC sensitivity might play a role in the pathophysiology of BD. More specifically, both transactivating and transrepressing pathways of GC action seem to be affected in BD, together with an altered expression of the GR in PBMC. At the transcriptional level, a lower GR-α/GR-β ratio was observed in BD.
We examined the prevalence of four functional GR polymorphisms in three independent cohorts. None of the GR polymorphisms was associated with susceptibility to BD, consistent with two recent genome-wide association studies from Turkey and Japan (5, 28). However, we found significant differences in the prevalence of GR polymorphisms between the Caucasian and Turkish-ME cohort, which have not been reported before. The *Bcl*I minor allele is associated with increased GC sensitivity (26) and is present at a lower frequency in the Turkish-ME cohort. The *Bcl*I minor allele is also less prevalent in other areas with relatively high prevalence of BD (e.g. China, Korea), as compared to allele frequencies observed in Caucasian populations (29-30). On the other hand, the 9β minor allele, which is associated with decreased GC sensitivity (26), showed a lower prevalence in the Caucasian population. The clinical relevance of these observations is yet unclear, but they do not directly support the concept of a "glucocorticoid-resistant" genetic profile contributing to the development of BD since a comparable prevalence of the *Bcl*I and 9β minor alleles was found in the Turkish and ME patients and healthy controls. Future studies should examine whether the different prevalence pattern of GR polymorphisms in the Turkish-ME population is associated with other immune-mediated disorders.
To further explore the role of GC sensitivity in BD, we assessed transactivating and transrepressing capacity of GC *in vitro* by measuring the EC_{50} values of two representative GC-mediated genes, GILZ and IL-2. We measured higher EC_{50} values of both genes in BD, indicating decreased *in vitro* GC sensitivity compared to healthy controls. In contrast, we found higher numbers of GR per cell, which might reflect a compensatory upregulation of the GR. Importantly, most patients in our study had relatively low BDCAF scores, suggesting that the

higher EC_{50} values are not solely influenced by higher levels of pro-inflammatory cytokines, a well-known mechanism of acquired GC resistance (31). Diminished (counterbalancing) cellular effects of GC on the immune system could allow for the development of chronic (auto)-inflammatory processes as in BD. A point of future attention is that in this relatively small group of patients there was considerable variation with respect to the use/not use of disease modifying drugs. In this setting it was not possible to analyze the possible effects of these drugs on the outcome of the assays.

In clinical practice, GC are widely used in BD. However, the only randomized clinical trial studying the effects of GC in BD showed a lack of efficacy of GC treatment. In this study, a 3-weekly depot of 40 mg methylprednisolone acetate for 27 weeks in patients with active Behçet's disease demonstrated no benefit over placebo-treated patients with respect to oro-genital ulcers, folliculitis and arthritis, although lesions with erythema nodosum did improve following GC treatment (32). Interestingly, in asthma and RA approximately one-third of patients are also GC resistant (33-34). In addition, a case-series reported by Tanaka and co-workers showed that patients with ocular manifestations of BD with low *in vitro* GC sensitivity had a worse clinical course as defined by more frequent relapses of ocular inflammation and higher intra-ocular pressure (35). Therefore, the observed decreased *in vitro* GC sensitivity in BD may not only contribute to an increased understanding of the (etio) pathophysiology of the disease, but could also have direct clinical implications. Obviously, it would be of great interest to study whether assessment of *in vitro* GC sensitivity, as measured by the IL-2, GILZ and whole cell dexamethasone binding assays, correlates with *in vivo* response to GC therapy in BD. Insights in the patients response to exogenously administered GC prior to start of therapy could then be used to facilitate more individualized GC therapy.

In order to examine possible mechanisms underlying this decreased GC sensitivity in BD, we determined mRNA expression levels of the α and β splice variant of the GR. GR-β is thought to act as a dominant negative inhibitor of the biologically active GR-α by means of competition for co-factors, formation of inactive heterodimers with GR-α and possibly competition for GRE *in vitro* (15-16, 19). High expression of GR-β *in vivo* has been associated with GC-resistant states in inflammatory bowel disease, asthma and RA (17-18, 20, 36-38). In our cohort, the ratio of GR-α/GR-β was significantly lower in patients with BD and could therefore partially explain the decreased cellular GC sensitivity in BD, although the clinical relevance of the very low expression levels of GR-β are still subject of debate. Other mechanisms possibly underlying the decreased cellular GC sensitivity in BD may include disturbed nuclear trafficking of the GR via phosphomodulation by kinases and phosphatases, interference with the transcriptional machinery by histone deacetyltransferases (HDAC) modulating protein acetylation or transcriptional blocking by altered expression of microRNAs.

It must be kept in mind that our data represent relatively small groups, with mixed ethnic background. In this perspective, it is important to note that the Caucasian and Turkish-ME patients with BD were equally distributed with respect to the bioassays, GR assay and the

gene expression levels. Therefore, we assumed that ethnic background is not a major factor determining outcomes of the bioassay, GR assay or gene expression levels and analyzed Caucasian and Turkish-ME patients together. Also, messenger RNA levels of GR-α and GR-β do not necessarily represent protein expression levels in our patients. Finally, the interpretation of cross-sectional data is limited with respect to dynamic processes as the pathogenesis of BD. Therefore, longitudinal studies evaluating GC sensitivity at various stages of BD, including recent-onset disease, and different levels of disease activity will provide more insight in the importance of GC sensitivity and the development of BD.

CONCLUSIONS

Polymorphisms of the GR gene are not associated with susceptibility to BD. However, our *in vitro* data indicate decreased cellular GC sensitivity in BD. This altered GC sensitivity could play an as yet unidentified role in the etiopathophysiology of BD. A decreased GR-α/GR-β ratio may in part explain this decreased GC sensitivity.

ACKNOWLEDGEMENTS

The authors would like to acknowledge all patients and healthy volunteers for their contribution to this study. This work was supported by a grant from The Dutch Arthritis Foundation.

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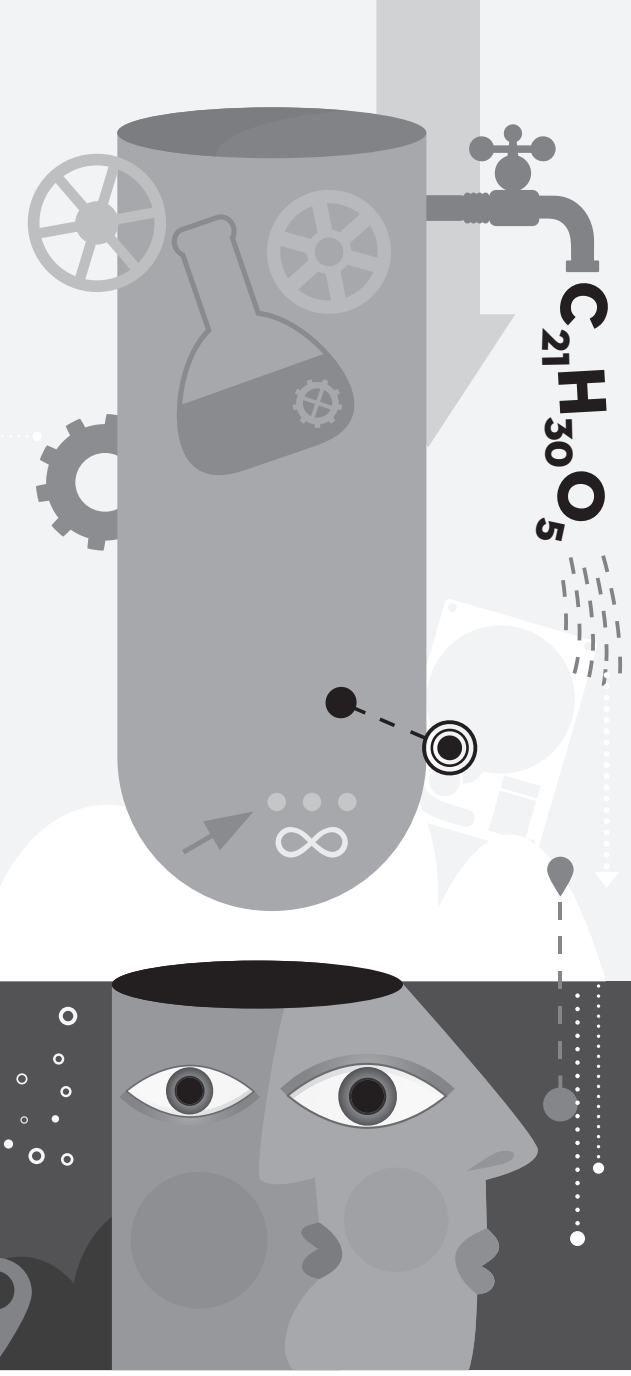

General discussion

Chapter 9

Partly based on:

Quax R.A.M., Peeters R.P., Feelders R.A. *Selective glucocorticoid receptor modulators: future of glucocorticoid immunosuppressive therapy? Endocrinology. 2011 Aug;152(8):2927-9.*

Quax R.A.M., Manenschijn L., Koper J.W., Hazes J.M.W., Lamberts S.W.J., van Rossum E.F.C., Feelders R.A. *Glucocorticoid sensitivity in health and disease. Nature Reviews Endocrinology. Accepted for publication.*

RATIONALE OF THE THESIS

Since the discovery of glucocorticoids (GC) in the late forties of the 20th century, the important functions of GC in glucose homeostasis, bone metabolism, regulation of mood and behavior and immunity and many other processes have been thoroughly explored. Given the close interaction between GC and the immune system, disorders in GC function are likely to influence the susceptibility to and the disease course of autoimmune diseases. Indeed, disturbances in the hypothalamic-pituitary-adrenal (HPA) axis have clearly been demonstrated in rheumatoid arthritis (RA), although the exact mechanisms are still not completely understood. Even more important is the fact that it is not really known whether disorders in GC sensitivity contribute to the development of autoimmunity, or, that once autoimmunity has developed, the subsequent inflammatory environment alters GC sensitivity, or both. Studies evaluating GC sensitivity in the very early phase of RA, or even prior to clinical presentation of the disease, could contribute to solve this intriguing question.
Due to their favorable effects on inflammation, GC soon found their way into daily clinical practice. Nowadays, GC are indispensable for the treatment of numerous inflammatory and non-inflammatory disorders, ranging from classic autoimmune diseases such as RA and systemic lupus erythematosus (SLE) to diseases that lay a heavy burden on the cost of healthcare such as COPD and asthma as well as life-threatening hematological malignancies. However, during more than 60 years use of GC as anti-inflammatory therapy, no or little progress has been made in the development of tools to 1) identify patients who will or will not benefit from GC treatment, or to 2) adjust GC dose according to an individuals' GC sensitivity. Indeed, a substantial proportion of patients experiences a lack of, or suboptimal, anti-inflammatory effect of GC therapy, interfering with a favorable disease outcome. Determination of GC sensitivity prior to GC therapy, allowing individual-dosed treatment schedules, could further optimize GC therapy in these patients. Alternatively, patients with proven GC resistance may be treated with alternative (more aggressive) immunomodulatory agents, which also increases the likelihood of successful initial treatment.

PART I. GLUCOCORTICOID SENSITIVITY: SUSCEPTIBILITY TO AND SEVERITY OF INFLAMMATORY DISORDERS

Genetic variation: impact on susceptibility and severity of inflammatory disorders

The field of research exploring the genetic background of diseases has been greatly pushing on since the emergence of genome-wide association studies (GWAS). This hypothesis-free approach enables researchers to study thousands of genes at once. Among hundreds of known and unknown genetic variants within the glucocorticoid receptor (GR) gene, four

single nucleotide polymorphisms (SNPs; i.e. the *Bcl*I, N363S, ER22/23EK and 9β variants) have proven to alter GR function and/or the GR transcriptome (1-3). These *in vitro* effects are also translated *in vivo*. Previously, our group found a higher prevalence of the ER22/23EK and 9β variants in patients with RA and also that patients carrying the minor allele of these SNPs were more likely to be treated with tumor necrosis factor alpha (TNF-α) blocking agents, probably reflecting a more severe disease course. These findings are compatible with the concept that the ER22/23EK and 9β variants are associated with decreased GC sensitivity and may 1) confer an increased risk to develop RA, and, once disease is clinically evident, 2) increase susceptibility to a more aggressive disease course. Vice versa, carriers of the *Bcl*I and N363S minor alleles were less susceptible to develop RA (4). This large RA cohort (N=368) was studied retrospectively, however, and disease activity was assessed using a surrogate marker (i.e. the use of biologicals). In Chapter 3, we prospectively studied a cohort of 138 early RA and established RA patients. Carriers of the 9β and/or ER22/23EK minor allele had significantly higher disease activity at initial presentation than patients with either the *Bcl*I and N363S minor allele, or both. These findings further support the concept that the four outlined SNPs of the GR gene functionally modulate the effects of endogenously produced GC. The current study could however not reproduce the previous association between the four GR SNPs and the likelihood of developing RA, probably due to the smaller study cohort. The clinical relevance of the *Bcl*I, N363S, 9β and ER22/23EK SNPs has been further substantiated by our findings in the PARA study, as described in Chapter 7. In this unique, prospectively studied cohort of pregnant RA patients, we found that the disease course in the postpartum period differed significantly among the carriers of the various GR SNPs. Overall disease activity was higher in carriers of the 9β and/or ER22/23EK variant, but only in GC treated patients in the postpartum period. These observations are highly interesting, suggesting a certain threshold of GC need before subtle differences in GC sensitivity by GR SNPs become clinically evident. Thus, the GC imbalance in patients with high GC need (as in active disease) and relatively low GC production (i.e. a blunted HPA-axis postpartum (5)), seems to put more emphasis on otherwise less potent factors such as the GR SNPs.

We hypothesized that the influences on GC sensitivity by the GR SNPs would not be restricted to RA alone, but rather reflect a general mechanism applicable to many inflammatory disorders. To test this hypothesis, we studied almost 300 patients diagnosed with Behçet's disease (BD), an inflammatory disorder common along the Silk Route and primarily characterized by oral and genital ulcers (Chapter 8). Interestingly, large differences in minor allele frequency (MAF) of GR SNPs were found between the different ethnic subgroups. The clinical relevance of these remarkable differences (much higher prevalence of 9β and much lower prevalence of *Bcl*I in Turkish and Middle-East persons as compared to Caucasian people) is not yet clear. The only prospective study evaluating the effect of systemic GC treatment in Turkish patients did, surprisingly, not favor GC treatment over placebo, although GR genotype or other *in vitro* parameters of GC sensitivity were not measured (6). Unfortunately, information about

the disease course in our patients with BD was not available, still leaving the question unanswered whether GR SNPs might influence disease severity. In the subsequent forced stratified analysis, we could not detect an association between GR gene variants and susceptibility to BD, possibly because of lack of power.
We also evaluated a novel genetic variant of the glucocorticoid-induced transcript 1 gene (GLCCI1), which was recently shown to be associated with the response to GC inhalation therapy in COPD and asthma (7-8). Interestingly, newly diagnosed and established RA patients carrying the GLCCI1 variant had significantly higher disease activity (Chapter 3). This implies that variants of the GLCCI1 gene, whose protein functions are largely unexplored, might be involved in modulation of GC sensitivity for both exogenous and endogenous GC.

Glucocorticoid sensitivity dynamics and consequences for susceptibility to and severity of inflammatory disorders

In contrast to the fixed genetic background, GC sensitivity is also influenced by a variety of other factors, and is highly variable between and within individuals (9-14). GC sensitivity is a composite of numerous factors (e.g. number and affinity of the GR, counteracting effects of NFκB, deacetylation of histones by HDAC) which ultimately determines transcriptional regulation of target genes by GC. This key principle has been translated in two bioassays developed in our laboratory, measuring two representative genes of transrepression (IL-2) and transactivation (GILZ), respectively. Interestingly, transrepression of IL-2 was significantly lower in early RA patients than in healthy controls, while there was no association with disease activity (Chapter 4). This suggests a decreased anti-inflammatory capacity in peripheral blood mononuclear cells (PBMC) of early RA patients. At least in established RA patients, we found a higher number of GR, which might act as a compensatory mechanism to (partly) overcome decreased GC sensitivity.
Moreover, we found that basal early morning salivary cortisol levels in patients with active disease are inappropriately low, further attenuating the ability to counteract the (auto-) inflammatory response (Chapter 5). Interestingly, a negative association was found between the degree of suppression of salivary cortisol (in %) by low-dose dexamethasone and disease activity, suggesting that feedback relationships within the HPA-axis are modulated by the inflammatory response in RA. This, in turn, may point towards a compensatory mechanism to increase cortisol secretion in the setting of high levels of pro-inflammatory cytokines. One could object that a single salivary cortisol value, measured in the dynamic window of the cortisol awakening response and possibly influenced by salivary gland expression of 11-beta hydroxysteroid dehydrogenase type 2 (11βHSD2) (15), is insufficiently reliable. In a subsequent (pilot) study, however, we also found relatively low levels of cortisol in hair in early RA patients (Chapter 6). Since cortisol in hair represents the average secretion of cortisol over a longer time frame (16), these findings support the idea of relative cortisol deficiency

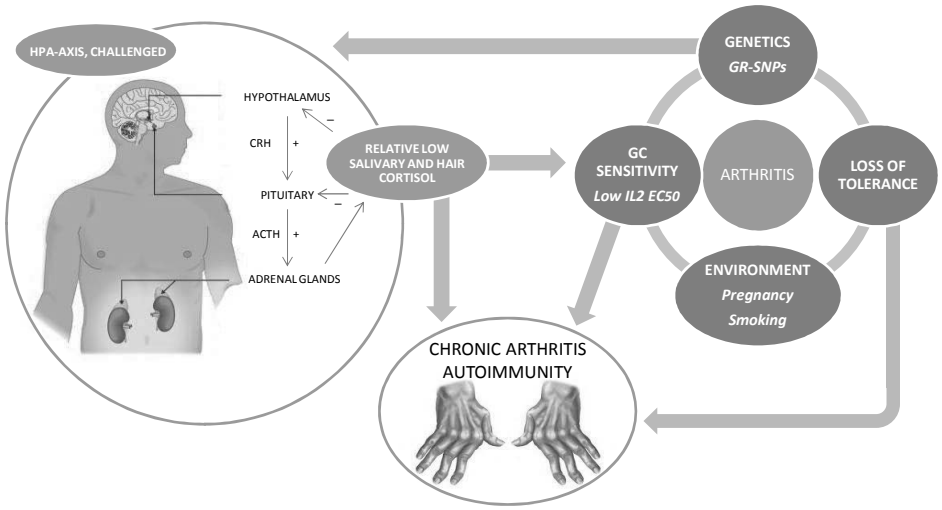

Figure 1. Hypothetical representation of the role of GC sensitivity in the initial development of arthritis and the evolution into chronic arthritis. Mounting evidence supports the concept of relative deficiency of cortisol in early RA, possible influenced by a GR-genotype mediated altered setpoint of the HPA-axis. In interaction with low cellular GC sensitivity and co-existing risk factors, this may initiate arthritis and stimulate chronic arthritis/autoimmunity.

in the very early phase of RA. An intriguing observation is that the cortisol levels in hair are slightly lower (compared to healthy controls) prior to joint complaints reported by the patients. These observations may support the hypothesis of primary relative GC deficiency in recent-onset RA. RA is considered to be a multi-factorial disease and decreased GC sensitivity in the 'right' context (environmental factors, genetic predisposition, loss of tolerance) may add to the initial development of arthritis and contribute to perpetuation of inflammation (Figure 1).

PART II. GLUCOCORTICOID SENSITIVITY: PREDICTION OF GC THERAPY EFFICACY

Clinical relevance of predicting GC sensitivity

Consensus is reached advocating initial intensive treatment of early RA, since persistent high disease activity in the first time period has been associated with adverse long-term outcome parameters (e.g. presence of joint erosions) (17-19). In line with these studies are the observations in the early arthritis cohort in the Leiden area by van der Linden and co-workers (20). They showed that those patients who were referred within less than 12 weeks, had less erosive disease and more frequently reached remission without using disease modifying antirheumatic drugs (DMARDs) over a follow-up period of 6 years. These data illustrate the

presence of a critical period in which the disease course of RA can be modulated most effectively, the so-called 'window of opportunity'.
These new insights in the field of rheumatology further stimulated the initiation of numerous randomized clinical trials. Different kinds of DMARD combinations and variable 'step-up' flow charts encouraging early treatment with biologicals are being used to reach low disease activity as quickly as possible. Nevertheless, it is generally known that all currently used DMARDs take some time (6-12 weeks) to become clinically effective (21), which largely comprises the important 'window of opportunity'. The anti-inflammatory actions of GC are much quicker, and it is highly likely therefore, that GC are one of the most important modulators of disease activity within the 'window of opportunity'. Our findings as described in Chapter 2 further strengthen this hypothesis. We show that those patients with active disease after 2 weeks despite GC bridging therapy, have an adjusted odds ratio of approximately 14 to proceed to DMARD failure at 3 months. This might reflect the fact that DMARDs are less effective in an inflammatory environment and that possible synergism between GC and DMARDs is suboptimal (see Part III, section 'Combination therapy'). It is not known yet whether the lack of GC response at 2 weeks also predicts differences in long-term disease outcome as follow-up of these patients is still ongoing.
In sharp contrast to the logical tenet that GC are likely to be modulators of disease outcome, is the observation that the field of efficacy of GC bridging therapy has hardly been explored. Treating RA means aiming to reach low disease activity as soon as possible and there is only one chance for the individual patient. This also requires knowledge of GC sensitivity, and preferably identification of precise predictors of the efficacy of GC (bridging) therapy.

Predicting GC therapy efficacy

Genetic markers

Pharmacogenetics is the field of research evaluating genetic variation and the subsequent differential (individual) responses upon drug administration. The most extensively used DMARD in RA, methotrexate, can count on broad attention in this research area (22-23). Efficacy of TNF-α blocking therapy also seems to be partly genetically determined (24). In contrast, to the best of our knowledge, no pharmacogenetic studies evaluating GC response in RA have been published. In Chapter 2, we describe for the first time that the GLCCI1 minor allele is associated with a less favorable GC treatment outcome in male patients with RA. This remarkable gender difference could in part be explained by differences in pain experience, which is relatively important in the disease activity score (DAS). Nevertheless, an objective index as difference in the number of swollen joints also lacked any correlation with GLCCI1 genotype in female patients, suggesting a true gender specific effect. It must be noted, however, that the current study was not primarily designed to challenge our hypothesis.

Therefore, as recommended in all genetic studies, replication studies are needed to provide a more definite answer. The same cohort of 138 patients was also used to study the predictive value of GR SNPs. We did not find an association between GR genotype and GC response, which is in contrast to our findings in the PARA study (Chapter 7). This may relate to methodological issues, since 1) both early and established RA patients were studied and 2) oral and intramuscular GC were administered to tREACH and FLARE patients. The abrupt fall in cortisol concentration postpartum together with a blunted HPA-axis restraining adequate cortisol secretion, may also more easily reveal subtle clinically relevant differences in GC sensitivity modulated by GR SNPs, as observed in the PARA study.

Functional assays

Efficacy of GC therapy is only determined by the genetic background of the patient to a small extent. GC therapy outcome will also depend on a combined interplay between GC availability, GR binding capacity and efficacy of GC transduction leading to gene transcription and protein translation, most of which are counteracted by pro-inflammatory signaling cascades (25-26).

Basal salivary cortisol and central GC sensitivity

We hypothesized that the absolute amount of salivary (free) cortisol before treatment would be a predictor of GC therapy outcome. This appeared not to be true in a mixed group of early and established RA patients with active disease (Chapter 5). Similarly, we did not find a relationship between GC therapy outcome and central GC sensitivity (as assessed by the low-dose DST). This suggests that the balance between the absolute level of (endogenous and synthetic) GC and local GC sensitivity (i.e. in the inflamed joint) is probably more important in predicting GC therapy outcome. Furthermore, the discrepancy between central GC sensitivity and local GC sensitivity might reflect the known cell-type and tissue-specific effects of GC therapy (27).

GR binding capacity

Glucocorticoid receptors (GR) are centrally positioned in the GC signaling cascade. In the FLARE study we could demonstrate that the number of GR was positively associated with GC therapy outcome (Chapter 4). This implies that the number of GR is an important determinant of the ultimate anti-inflammatory effects of GC. Previous studies only report (almost) 100% saturation of GR at very high doses of GC (>100mg/day) (28), suggesting a non-linear association between saturation of GR and GC exposure. The labor-intensive character of the assay and their suboptimal correlation with disease outcome limits large-scale implication in the clinics at this moment.

Gene transcription: the GILZ and IL-2 assays

Rather than aiming at one single variable, the GC bioassays integrate all factors that influence the ultimate response to GC up to the transcriptional level. Indeed, we found a clear association between the number of GR and EC_{50} values of GILZ and IL-2 respectively. Unexpectedly, the individual EC_{50} values of GILZ and IL-2 correlated much weaker with *in vivo* GC sensitivity than did GR number which is positioned more upstream in the GC signaling pathway. One explanation could be the robustness of the assays, with intra-individual variation of GILZ-EC_{50} and IL2-EC_{50} values of approximately 27% and 51%, respectively (29). It could also be postulated that dexamethasone was used in the bioassays, whereas the patients were treated with prednisone, methylprednisolone or triamcinolone acetonide. Whereas large differences are not to be expected, patients could have different sensitivity for the various types of synthetic GC. Furthermore, timing of blood processing may influence gene transcription. Although all blood samples were transported as quickly as possible, we cannot rule out some degree of variation in our bioassays by time-dependent factors. We did not find associations between GC therapy outcome and the degree of GILZ-induction and IL-2-repression, which previously was shown to have less intra-individual variation (29). Thus, measurement of dexamethasone-modulated GILZ and IL-2 gene transcription demonstrates the potency of bioassays to predict *in vivo* GC therapy outcome, but needs refinement to improve accuracy.

In general, individual factors may act synergistically and increase the accuracy of predicting GC therapy outcome. Prediction models incorporating multiple variables, however, require a large study cohort since each variable of interest needs a sufficient number of events (i.e. GC responder or non-responder) for a proper statistical analysis. The cohorts described in the tREACH and FLARE study are relatively small and assays could not be performed in all patients, restricting its suitability for multivariate modeling. Clearly, such models would be extremely interesting, but are not easy to achieve considering that the used assays are very labor-intensive.

The past decades of research have identified many factors influencing GC sensitivity, which is even more complicated by cell-type and tissue-dependent GC sensitivity. Some overarching factors, however, are likely to influence GC sensitivity in general, independent of cell-type and tissue-specificity (30-31). A schematic representation of known and suggested factors, supplemented with insights from the current thesis, influencing GC sensitivity and RA disease course is shown in Figure 2. Of note, GC-mediated side effects also vary substantially between individuals. Due to the relatively short duration of GC treatment in the tREACH and FLARE studies, however, it was not possible to study the association between GC-related side effects and interindividual differences in GC sensitivity.

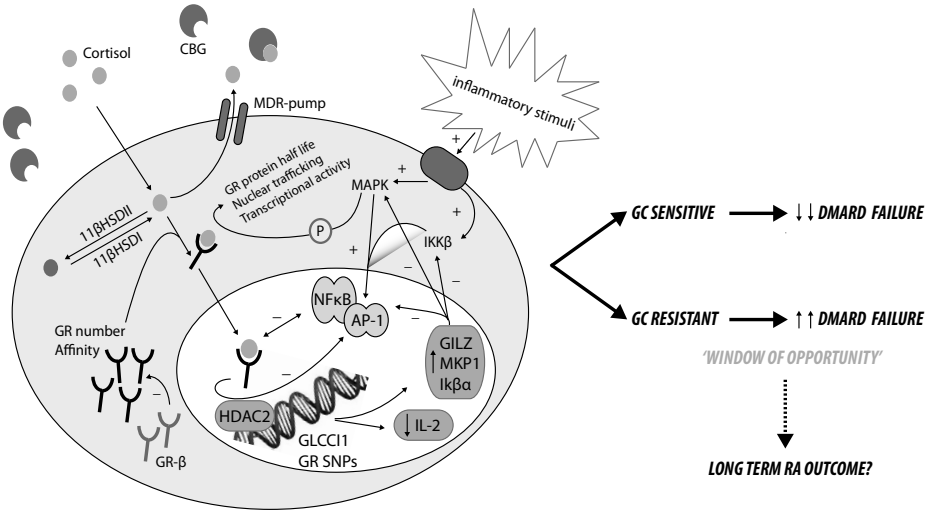

Figure 2. Determinants of GC sensitivity in rheumatoid arthritis. The amount of intracellular cortisol depends on the balance between unbound cortisol, active transport out of the cell by the MDR-pump and the balance between 11βHSD type I and II. The subsequent formation of the GC-GR complex relies upon GC binding capacity (i.e. number of GR, affinity), negatively influenced by overexpression of the GR-β isoform. Phosphomodulation (P) may alter GR protein half-life, nuclear trafficking and hence transcriptional activity depending on which serine residue is phosphorylated. Multiple direct and indirect interactions between GC and the two major pro-inflammatory transcription factors, NFκB and AP-1, eventually modulate gene transcription and hence GC sensitivity. Recruitment of HDAC2 by GC stimulates deacetylation and recondensation of histones, restricting transcription of genes encoding pro-inflammatory cytokines. GC resistance is associated with DMARD failure in the 'window of opportunity' and may even influence long-term disease outcome.

PART III. FUTURE PERSPECTIVES

Glucocorticoids and etiology of RA: chicken or egg?

One of the most intriguing questions remains which factor(s) triggers the process clinically leading to RA. The role of (decreased) GC sensitivity and/or relative cortisol deficiency in this process is still incompletely understood. Relatively low cortisol levels (i.e. cortisol in saliva and hair) in combination with reduced transrepressive capacity (high IL-2-EC_{50}) of PBMC as observed in early RA, are likely to contribute to the chronic inflammatory state in RA (Figure 1). Assuming a multi-hit theory for the development of RA, a decreased GC sensitivity may also be involved in the initial process of autoimmunity in persons with already circulating auto-antibodies and/or a certain genetic background. This decreased sensitivity may only become relevant when the HPA-axis is challenged, for example by inflammatory/emotional stimuli. To clarify this interesting hypothesis, one should measure GC sensitivity prior to and

at the moment of the first symptoms. This could be prospectively studied in large population based cohorts, perhaps by selecting persons at risk (e.g. first degree relatives of patients with RA), although this will be very time-consuming and expensive. Alternatively, sequential measurements of cortisol in hair in a (large) cohort with early RA could provide more insight in this hypothesis. If such studies reveal that a relative GC deficiency is one of the cornerstones for the development of RA, it is tempting to speculate that prompt treatment with GC might even prevent evolution into chronic arthritis.
Our findings concerning the IL-2 bioassay, the low-dose DST and cortisol in hair all support HPA-axis dysfunction and decreased GC sensitivity in the very early phase of RA (median disease duration 3-5.4 months). Nonetheless, the pro-inflammatory environment in early RA will also affect the HPA-axis (Chapter 5 and (32)) and is likely to contribute to the described alterations in GC sensitivity in RA.

From laboratory to clinical practice

In the last decades several methods to measure GC sensitivity have been developed (9, 11-14, 29, 33). Nevertheless, most assays are labor-intensive and are so far used in the experimental setting only. Another major drawback of these assays is the relative poor correlation with clinical outcome parameters which hamper their introduction in clinical practice. Thus, clinical applicability of an assay guiding individual GC treatment requires 1) accurate prediction of an individual's GC sensitivity, 2) a low degree of labor-intensity and 3) cost-effectiveness. An interesting approach could be gene profiling of PBMC which has been demonstrated to predict GC response with 84% accuracy in a cohort with asthma patients (34). In general, it is likely that multiple variables must be combined to predict GC sensitivity and GC response with reasonable precision. Candidate parameters which deserve further investigation with regard to their clinical usefulness in assessing GC sensitivity include histone deacetylase type 2 (HDAC2), macrophage migration inhibitory factor (MIF), FK506 binding protein 51 (FKBP51), the GR-β splice variant and the almost unexplored field of regulation of GC sensitivity by microRNAs. It would also be fascinating to evaluate GC sensitivity at the 'crime scene', for example by measuring GC sensitivity of fibroblast-like synoviocytes in the inflamed joint obtained by biopsy. This may correlate even better with the clinical course after GC therapy, when important local intracrine regulatory mechanisms by the cortisol-cortisone shuttle are taken into account (35-36). In this thesis, the most promising parameter to reach the clinics is the number of GR per cell in PBMC. A less time-consuming and possibly more accurate method to measure the number of GR could be flow-cytometry using monoclonal GR antibodies. In addition, the clinical response after two weeks of GC treatment, clearly associated with DMARD failure at 3 months in the tREACH trial (Chapter 2), could be used to adapt treatment regimens. The next step would be to randomize early RA patients non-responsive to GC therapy after 2 weeks to 1) more intensive therapy, e.g. biologicals or 2) continuation of their

DMARD regime, and evaluate treatment failure at 3 months and long-term disease outcome parameters such as erosive disease.

Modulation of GC sensitivity and GC action

GC sensitivity is determined by a wide range of known and yet unknown factors. Interference with one or more of these factors is therefore likely to influence GC sensitivity. This knowledge could be applied to improve GC sensitivity and GC therapy outcome. Some promising strategies are outlined below.

Histone deacetylase (HDAC) inhibitors

HDAC inhibitors (HDACi) modulate DNA accessibility and have been demonstrated to influence cytokine profiles of PBMC and macrophages and to attenuate inflammatory diseases *in vivo* in rodent models of lupus, colitis and sepsis. Their clinical applicability has been established mainly in the treatment of various solid and hematological malignancies (37-38). In RA, several HDACi (MS-275, suberoylanilide hydroxamic acid, ITF2357 and trichostatin A) exhibited profound effects on synovial fibroblasts by modulating cell growth, cytokine release, angiogenesis, release of MMPs and joint destruction (39-41). Currently there are no registered trials investigating HDACi (mono) therapy in RA.

MIF inhibitors

Since GC-induced expression of MIF counteracts GC action, one could speculate that the ultimate effects of GC could be facilitated by inhibiting MIF. Several animal studies indeed demonstrated a more favorable disease course of experimental autoimmune encephalomyelitis (EAE), SLE, type 1 diabetes and concanavalin-A induced hepatitis in MIF knock-out mice (42). Modulation of inflammatory disease by using MIF inhibitors has not reached the clinical setting yet, although the applicability of MIF antibodies in treating lupus nephritis is currently being investigated (http://clinicaltrials.gov; NCT01541670).

Mitogen-activated protein kinase (MAPK) inhibitors

Higher levels of stress-activated protein kinases are associated with the clinical entity of GC resistance in IBD (43) and asthma (44). The central role of MAPKs in perpetuating inflammation was therefore the main rationale for the development of MAPK inhibitors as new molecular target in the combat against inflammation (30, 45). Inhibition of MAPK prevented onset of arthritis if used prophylactically and substantially suppressed the progression of joint destruction when used in a therapeutical setting (46). These promising results were

in sharp contrast with the lack of clinical effectiveness of the subsequently developed p38 MAPK inhibitors (Pamapimod, VX-702 and SCIO-469) *in vivo* in RA (47).

P-glycoprotein inhibitors

The problem of GC resistance might also reflect high expression levels of the multi-drug transmembrane efflux pump P-glycoprotein (P-gp). Low intracellular dexamethasone levels (IDL) combined with high expressions levels of P-gp were associated with high disease activity in SLE and RA, suggesting P-gp expression levels could be used as a marker of drug resistance (48-49). Treatment of PBMC of these patients with P-gp inhibitors (e.g. tacrolimus, cyclosporine) did indeed restore IDL to that of healthy controls and patients without drug resistance. The use of P-gp inhibitors however, is also accompanied by potential serious side effects (e.g. bone marrow suppression), which limit routine clinical implementation of P-gp inhibitors in the treatment of inflammatory disorders at this moment.

Vitamin D

In addition to the well established role of 1,25-dihydroxy vitamin D3 in calcium and phosphate homeostasis, the knowledge of immunomodulating effects of vitamin D is still expanding. Interestingly, e*x vivo* data acquired by the laboratory of Lubberts and co-workers demonstrated synergistic effects of vitamin D and dexamethasone on IL-17A, interferon-γ and TNF-α production by PBMC derived from early RA patients. In contrast, vitamin D reduced the dexamethasone-mediated downregulation of IL-4 (50). Clinical studies should prove the potential efficacy of vitamin D, in particular in combination with GC, on RA disease activity.

Selective glucocorticoid receptor agonists (SEGRAs)

The complex genomic actions of GC are classically divided into transrepressive and transactivating effects on gene transcription. Based on findings in GR dimerization defective mice, the process of transrepression was mainly held responsible for the anti-inflammatory properties of GC. In the past 15 years, several compounds have been developed which exhibit strong dissociative properties in favor of transrepression *in vitro* and *in vivo*. None of these substances has reached the clinic yet (51), although ZK245186 is currently being tested to treat atopic dermatitis (www.clinicaltrials.gov; NCT00944632). Moreover, some GC-induced genes also have anti-inflammatory capacity, whereas other induced genes are involved in both repression of inflammation as well as GC-mediated side effects (depending on tissue and/or cell type). This challenges the transrepression-transactivation model and questions the clinical applicability of SEGRAs (52-53).

Combination therapy

The major drawback of using (high doses of) GC as anti-inflammatory agents are their widespread side effects. Concurrent use of other immunomodulating drugs could improve GC therapy outcome, i.e. increase GC sensitivity, by either 1) antagonizing processes which lower GC sensitivity or 2) acting synergistically with GC.
Beck and co-workers demonstrated synergistic effects of GC and inhibitors of p38 MAPK and mitogen- and stress-activated protein kinase 1 (MSK1) in fibroblasts (54). Furthermore, frequently used antirheumatic drugs such as MTX and SSZ have been shown to 1) inhibit degradation of IκBα *in vitro* (55-56), 2) upregulate adenosine herewith inhibiting the conversion of cortisol to inactive cortisone (57) and 3) upregulate GR-α in human monocytic/macrophage and lymphocyte cell lines (58-59). All of these processes facilitate GC availability and support GC action. Although not necessarily causatively related, our findings in GC resistant patients displaying a significantly higher prevalence of DMARD failure at 3 months do suggest synergism between affected signaling cascades by GC and DMARDs. Another recent study reported that the degree of MTX-induced GR-α upregulation in PBMC at baseline was positively associated with ACR response after 24 weeks, further indicating synergism between GC and DMARDs (60).
These findings combined, GC are likely to act in concert with other immunomodulating drugs. The recent CAMERA-II study elegantly demonstrated that initial combination therapy with MTX and GC (10 mg/day) significantly reduces the need for additional biologicals (Adalimumab) as compared to MTX monotherapy (61). These findings underscore that GC therapy is not only relevant in the bridging period of DMARD therapy but can also be used as long-term treatment. Especially in the Netherlands, with ever increasing costs in health care, the reduced proportion of patients needing biologicals in GC (co-) treated patients would be of exceptional interest (biologicals cost approximately €15.000,- per year).
Alternative interesting methods to improve GC therapy efficacy include nitration of GC, targeted drug delivery by liposomal GC and modified-release prednisone (62). The latter one is likely to enter the clinical setting soon because this compound has greater efficacy than immediate release prednisone to decrease disease activity parameters, as was demonstrated in the CAPRA-1 and 2 studies (63-64).

CONCLUDING REMARKS

This thesis has covered different aspects of GC sensitivity in rheumatoid arthritis; from variations in single nucleotides to numbers of glucocorticoid receptors per cell and from basic *in vitro* laboratory assays to direct clinical relevant findings in the tREACH trial.
The following new insights concerning rheumatoid arthritis have been obtained in this thesis:

1) The early phase of active rheumatoid arthritis is characterized by relatively low cortisol levels in saliva and hair and low *in vitro* GC sensitivity.
2) Preliminary data derived from cortisol in hair support the hypothesis that early RA patients are not able to increase their cortisol secretion appropriately for the level of inflammation. Clinical disease onset of RA might be preceded by lower cortisol levels in hair.
3) Predictors of GC bridging therapy efficacy include the number of glucocorticoid receptors/cell, GILZ and IL-2 EC_{50} values in the GC bioassays and the GLCCI1 genotype.
4) The postpartum RA disease course in GC treated patients is influenced by GR genotypes.
5) Early RA patients who are classified as GC non-responder after two weeks, are at increased risk of DMARD failure at 3 months.

As with most research, answering questions raises new questions. Does altered GC sensitivity precede the formation of anti-CCP antibodies and the development of arthritis? Could immediate GC treatment prevent development of chronic inflammation? Which combination of parameters will best predict the effect of GC bridging therapy and GC side effects? How to guide treatment in patients already on GC therapy? How 'small' is the window of opportunity? What is the role of simple, but rational combination therapies (e.g. GC and vitamin D) to induce remission?

These questions, and many others, need to be elucidated in the near future. The complex but intriguing world of GC sensitivity in rheumatoid arthritis deserves further exploration.

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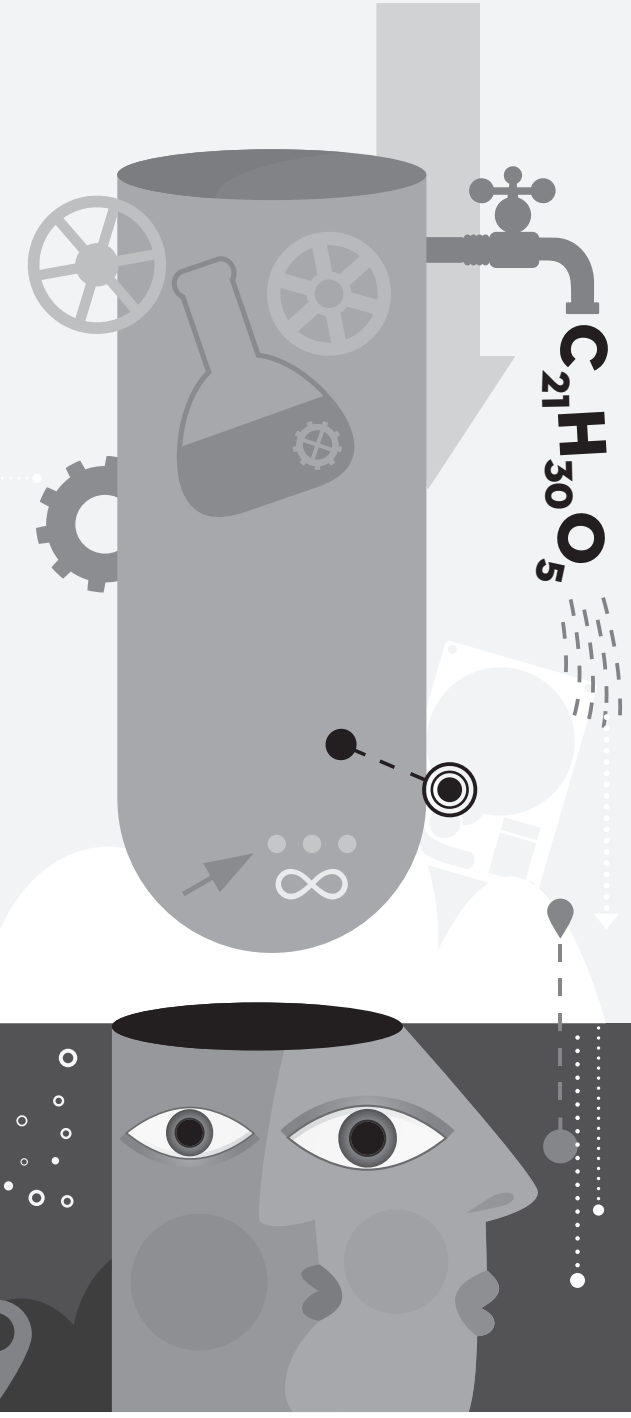

Chapter 10

SUMMARY

Rheumatoid arthritis (RA) is a very common autoimmune disease characterized by synovial inflammation, as well as many extra-articular manifestations. Uncontrolled RA will lead to substantial damage to the joints, disability and a subsequent decrease in quality of life. Moreover, RA is associated with an increased prevalence of cardiovascular disease, further increasing the burden of RA on healthcare. Accumulating evidence points towards a blunted hypothalamic-pituitary-adrenal (HPA) axis in RA. Furthermore, the rapid anti-inflammatory effects of glucocorticoids (GC) are widely applied in attempts to control disease activity in recent-onset RA or flares of established RA. Nevertheless, the exact role of the HPA-axis in RA is still insufficiently clarified. Moreover, structural data on GC therapy efficacy in RA and their determinants are still scarce.

This thesis on GC sensitivity in RA further clarifies the degree of GC resistance and its direct clinical relevance, uncovers some new aspects of HPA-axis activity in (recent-onset) RA and provides new insights concerning prediction of GC therapy efficacy.

An overview of RA pathophysiology, glucocorticoid receptor (GR) physiology, GC signaling cascades, mechanisms of GC resistance and methods to measure GC sensitivity has been outlined in **Chapter 1**. The clinical problem of GC resistance has been put in a broader perspective, covering a wide spectrum of inflammatory and non-inflammatory disorders.

In **Chapter 2**, the results of the 'treatment in the Rotterdam Early Arthritis Cohort' (tREACH) study are described. In this randomized clinical trial we studied patients with a high-probability of developing persistent (erosive) arthritis (according to the model of Visser). In practice, the majority of patients (95%) fulfilled the newly developed 2010 American College for Rheumatology (ACR) criteria for RA. All patients in this high-probability group were treated with oral or intramuscular GC and disease activity was reassessed after 2 weeks. Remarkably, a quarter of these patients were classified as GC non-responders according to EULAR response criteria. Moreover, GC non-responders had a much higher chance of having active disease after 3 months. This latter observation is highly clinically relevant, since mounting evidence suggests a 'window of opportunity' to treat RA most effectively. Thus, patients with recent-onset RA 'at risk' of having an unfavorable disease course, may be identified as soon as after 2 weeks, according to their response to GC bridging treatment.

As GC responsiveness may affect long-term outcome in RA, we aimed to identify determinants of GC sensitivity. In this context, we evaluated whether the carriage of known functional single nucleotide polymorphisms (SNPs), associated with altered GC sensitivity, are related to efficacy of GC bridging therapy. In **Chapter 3** we present our findings on 4 GR SNPs and the recently discovered variant in the glucocorticoid-induced transcript 1 (GLCCI1) gene. Interestingly, carriers of the variants associated with reduced GC sensitivity had higher disease activity at baseline. This suggests that the effects of endogenously produced cortisol are modulated by these SNPs. Furthermore, we found a significant association between the

GLCCI1 minor allele and decreased response upon GC bridging therapy in male patients with RA.

The search for predictors of GC sensitivity was extended by evaluating the recently developed GILZ and IL-2 bioassays and the GR binding assay in relation to the *in vivo* response to GC therapy, as described in **Chapter 4**. Recent studies have shown that both glucocorticoid-induced (exemplified by the GILZ assay) and glucocorticoid-repressed genes (reflected by the IL-2 assay) are involved in the ultimate anti-inflammatory effects of GC. Indeed, significant though moderate associations were found between EC_{50} values in the GILZ and IL-2 assays and the relative decrease in disease activity in patients. The number of GR per cell, however, was associated more strongly with the actual response in patients. These findings underscore the potential of *in vitro* assays to predict clinically relevant responses following GC therapy in RA. Nevertheless, the limited accuracy of these assays and their labor-intensive character limit their introduction in daily clinical practice at this moment.

A balanced HPA-axis tightly regulates the production and secretion of cortisol, depending on the interplay between inflammatory and non-inflammatory factors. One hallmark of RA is the relative shortage of cortisol, as has been demonstrated in several studies mostly in serum and urinary samples. This might not represent the biologically active fraction of cortisol since cortisol is mainly bound to cortisol-binding globulin. In contrast, salivary cortisol represents the free, unbound and hence active fraction of cortisol. Therefore, we measured salivary cortisol levels in a mixed cohort of recent-onset and established RA with active disease (**Chapter 5**). We found that salivary cortisol levels are also in the normal range. The same patients also underwent a low-dose (0.25 mg) dexamethasone suppression test to evaluate feedback mechanisms within the HPA-axis. Interestingly, there was less suppression of post-dexamethasone cortisol levels in those patients with most active disease, suggesting a compensatory mechanism to counteract inflammation. None of these parameters, however, were related to the response to GC bridging therapy *in vivo*.

It could be argued that single-value measurements of HPA-axis activity are subject to intra-individual and diurnal/time-dependent variation. To overcome this problem, we evaluated long-term cortisol levels by a recently validated assay measuring cortisol in scalp hair. In **Chapter 6** we present data from our pilot study measuring hair cortisol levels in recent-onset RA, including average levels of cortisol prior to clinical disease onset. Interestingly, these patients could not increase the average levels of cortisol, although it may be presumed that high (systemic) levels of pro-inflammatory cytokines are present. Thus, our findings with regard to salivary and hair cortisol levels further underscore the presence of relative GC deficiency in the very early phase of RA. The slightly lower hair cortisol levels in the pre-clinical phase of RA may support the hypothesis of primary relative GC deficiency in early RA.

Our findings in the unique PARA study, comprising the largest prospectively studied cohort of pregnant RA patients, are reported in **Chapter 7**. In this natural 'experiment', with gradually increasing levels of cortisol during pregnancy and a blunted HPA-axis postpartum, we

could demonstrate that GR SNPs modulate disease course in the postpartum period in those patients requiring GC therapy. These findings suggest that under circumstances of high glucocorticoid need (i.e. active disease requiring GC therapy) and reduced plasticity of the HPA-axis (i.e. blunted HPA-axis postpartum), GR SNPs may subtle alter disease course.

Finally, we hypothesized that disorders in GC sensitivity are not restricted to RA alone but rather contribute to the pathogenesis of diseases characterized by (auto) inflammatory processes in general. Therefore, we evaluated GC sensitivity in Behçet's disease. As described in **Chapter 8**, our patients with Behçet's disease displayed a significantly reduced GC sensitivity for both GILZ and IL-2, whereas the number of GR per cell was significantly higher. This strengthens our hypothesis that GC sensitivity is probably involved in many immune-mediated diseases.

In **Chapter 9** the findings in this thesis are discussed in a broader perspective, also visualized in hypothetical models concerning the potential role of the HPA-axis and GC sensitivity in RA pathogenesis and disease course. Special attention is paid to future perspectives in the field of GC sensitivity in RA, with emphasis on modulation of GC sensitivity.

NEDERLANDSE SAMENVATTING

Reumatoïde artritis (RA) is een veel voorkomende auto-immuunziekte die zich met name kenmerkt door inflammatie van het synoviale weefsel, maar die ook vele extra-articulaire ziekteverschijnselen kent. Ongecontroleerde (actieve) RA zal tot blijvende substantiële schade aan de gewrichten leiden, met lichamelijke beperkingen en verlies van kwaliteit van leven als gevolg. Bovendien is RA geassocieerd met een verhoogd risico op hart- en vaatziekten, wat de reeds aanzienlijke belasting op het gezondheidszorgstelsel alleen maar verder vergroot. Er is steeds meer bewijs dat er bij RA sprake is van een suboptimaal werkende ('blunted') hypothalamus-hypofyse-bijnier (HPA) as. De exacte rol van de HPA-as in RA is echter nog steeds niet volledig opgehelderd. Glucocorticoïden (GC) worden veelvuldig toegepast vanwege de snelle anti-inflammatoire werking bij de behandeling van (actieve) nieuw ontstane en langer bestaande RA. Kwalitatief goede data over de effectiviteit van GC in RA zijn echter schaars. Behandeling met GC kan gepaard gaan met ernstige bijwerkingen. De respons op GC behandeling en het optreden van bijwerkingen wordt waarschijnlijk in belangrijke mate bepaald door individuele gevoeligheid voor GC, waarbij genetische en ziekte-gerelateerde factoren een rol spelen.

Dit proefschrift over GC gevoeligheid in RA schept met name duidelijkheid over de mate van GC resistentie en de klinische gevolgen hiervan, brengt nieuwe aspecten naar voren betreffende de HPA-as in (vroege) RA en bespreekt enkele factoren die voorspellende waarde hebben voor de respons op (exogeen) toegediende GC.

Een overzicht van de pathofysiologie van RA, de fysiologie van glucocorticoïd receptoren (GR), GC signaal cascades, mechanismen van GC resistentie en methoden om GC gevoeligheid te meten is uiteengezet in **Hoofdstuk 1**. Het klinische probleem van GC resistentie wordt in de breedste zin van het woord besproken in een scala van inflammatoire en non-inflammatoire ziektes.

In **Hoofdstuk 2** worden de resultaten van de 'treatment in the Rotterdam Early Arthritis Cohort' (tREACH) studie besproken. In deze gerandomiseerde klinische trial werden patiënten bestudeerd met een hoge a priori kans op een persisterende erosieve aandoening (risicoinschatting aan de hand van het Visser model). In de praktijk bleken bijna alle patiënten (95%) aan de nieuwe door de American College for Rheumatology (ACR) in 2010 opgestelde criteria voor RA te voldoen. Alle patiënten in deze zogeheten high-probability groep werden behandeld met orale danwel intramusculaire GC waarna de ziekte-activiteit na 2 weken opnieuw werd beoordeeld. Volgens de officiële EULAR respons criteria blijkt een kwart van deze patiënten als GC non-responder te kunnen worden geclassificeerd. Bovendien blijken deze GC non-responders ook een veel hogere kans te hebben op persisterend actieve ziekte na 3 maanden. Dit is klinisch zeer relevant aangezien er steeds meer bewijs is dat succesvolle behandeling van RA op langere termijn sterk afhangt van de behandelingsresultaten in de eerste fase van het ziekteproces. Patiënten met een potentieel ongunstig ziektebeloop

zouden dus wellicht al herkend kunnen worden aan de hand van hun initiële respons op GC behandeling.

Aangezien GC gevoeligheid mogelijk dus lange termijn gevolgen heeft voor het ziektebeloop in RA, stelden we ons vervolgens als doel om factoren te identificeren die GC sensitiviteit mogelijk beïnvloeden. Met deze gedachte hebben we enkele bekende functionele 'single nucleotide polymorphisms' (SNPs), reeds geassocieerd met verschillen in GC gevoeligheid, bestudeerd met betrekking tot de respons op GC bridging therapie. In **Hoofdstuk 3** presenteren we onze bevindingen over 4 GR SNPs en de recent beschreven variant in het glucocorticoid-induced transcript 1 (GLCCI1) gen. Dragers van de polymorfismen geassocieerd met een verlaagde GC gevoeligheid hadden een hogere ziekte-activiteit bij de eerste klinische presentatie. Dit suggereert dat de effecten van het endogeen geproduceerde cortisol door deze polymorfismen worden gemoduleerd. Verder vonden we dat het GLCCI1 minor allel geassocieerd is met een verminderde respons op GC behandeling bij mannen met RA.

De zoektocht naar voorspellers van GC gevoeligheid werd voortgezet door met behulp van de recent ontwikkelde GILZ en IL-2 bioassays en de GR bindingsassay te onderzoeken wat de relatie is tussen de *in vitro* en de *in vivo* respons na GC behandeling (**Hoofdstuk 4**). Recente studies hebben aangetoond dat zowel GC-geïnduceerde genen (zoals GILZ) als genen waarvan de transcriptie wordt onderdrukt door GC (zoals IL-2) van belang zijn voor de netto anti-inflammatoire werking van GC. We vonden inderdaad significante, maar beperkte, associaties tussen de EC_{50} waarden in de GILZ en IL-2 assay en de relatieve verbetering in ziekte-activiteit. Het aantal glucocorticoïd receptoren (GR) per cel was krachtiger geassocieerd met de verbetering in ziekte-activiteit. Deze bevindingen bevestigen de potentie van *in vitro* assays om klinisch relevante voorspellingen betreffende respons op GC therapie te doen. De beperkte accuratesse van deze assays en hun arbeidsintensieve karakter beperken op dit moment nog de introductie in de dagelijkse klinische praktijk.

Een gebalanceerde HPA-as reguleert nauwkeurig de productie en secretie van cortisol, afhankelijk van de interactie tussen inflammatoire en non-inflammatoire stimuli. Een van de kenmerken van RA is het relatieve tekort aan cortisol, dat wil zeggen een cortisolconcentratie die opvallend laag is in het licht van de inflammatoire staat van de patiënt, zoals in meerdere studies is aangetoond in vooral serum- en urinemonsters. Dit hoeft echter niet per se de biologisch actieve fractie te weerspiegelen aangezien het grootste gedeelte van cortisol gebonden is aan cortisol-binding globuline. Daarentegen weerspiegelt speekselcortisol de vrije, ongebonden en derhalve biologisch actieve fractie van cortisol. Zodoende hebben we speekselcortisolen gemeten in een gemengd cohort met vroege en reeds langer bestaande RA, zoals beschreven in **Hoofdstuk 5**. We vonden dat ook speekselcortisol waarden binnen de normaalwaarden vielen. Bij dezelfde patiënten werd tevens een lage-dosis (0.25 mg) dexamethason suppressie test verricht om het terugkoppelingsmechanisme van de HPA-as te evalueren. Het bleek dat patiënten met de actiefste ziekte minder suppressie van cortisol hadden, wat een compensatoir mechanisme om de inflammatie te onderdrukken suggereert.

Geen van deze parameters echter, was geassocieerd met de *in vivo* respons op GC bridging therapie.

Men zou kunnen opperen dat dit soort eenmalige metingen ter beoordeling van de HPA-as activiteit onderhevig zijn aan intra-individuele en tijdsgerelateerde variatie. Om dit probleem te ondervangen, evalueerden we de gemiddelde cortisolwaarden middels een recent gevalideerde assay om cortisol in haar te meten. In **Hoofdstuk 6** presenteren we onze bevindingen omtrent de haarcortisol waarden in vroege RA, inclusief de gemiddelde waarden van cortisol in perioden dat patiënten nog klachtenvrij waren. Opmerkelijk genoeg konden patiënten hun (gemiddelde) cortisol spiegels niet ophogen, ondanks dat mag worden aangenomen dat er hoge niveaus van pro-inflammatoire cytokines circuleren. Onze bevindingen in speeksel- en haarcortisol onderschrijven dus de algemene concensus dat er sprake is van een relatieve GC deficiëntie in vroege RA. De lagere gemiddelde cortisol waarden in de klachtenvrije periode van vroege RA patiënten kunnen duiden op een primair cortisol tekort in de eerste (subklinische) fase van RA.

Onze bevindingen in de unieke PARA studie, de grootste prospectief bestudeerde groep zwangere RA patiënten, zijn uiteengezet in **Hoofdstuk 7**. In dit natuurlijke 'experiment', met stijgende cortisolwaarden gedurende de zwangerschap en een 'blunted' HPA-as postpartum, konden we aantonen dat GR polymorfismen het ziektebeloop beïnvloeden in de periode na de bevalling bij patiënten die met GC werden behandeld. Onze bevindingen veronderstellen dat onder omstandigheden met een hoge GC behoefte (actieve ziekte met noodzaak tot GC behandeling) en een verminderde plasticiteit van de HPA-as (blunted HPA-as postpartum), GR polymorfismen een effect hebben op het ziektebeloop.

We veronderstelden dat stoornissen in GC gevoeligheid niet beperkt blijven tot RA, maar kunnen bijdragen aan de pathogenese van tal van (auto) inflammatoire aandoeningen in het algemeen. Zodoende bestudeerden we GC gevoeligheid ook in de ziekte van Behçet. Zoals gerapporteerd in **Hoofstuk 8**, vonden we in ons cohort met Behçet patiënten een verminderde GC gevoeligheid in de GILZ and IL-2 assays, alhoewel het aantal GR per cel significant hoger was. Deze bevindingen ondersteunen onze hypothese dat GC gevoeligheid van belang is in vele immuun-gemedieerde aandoeningen.

In **Hoofdstuk 9** worden alle bevindingen zoals beschreven in dit proefschrift als geheel beschouwd, en eveneens gevisualiseerd in hypothetische modellen over de mogelijke rol van de HPA-as en GC gevoeligheid in RA pathogenese en ziektebeloop. Suggesties voor toekomstig onderzoek in het veld van GC gevoeligheid in RA worden geopperd, in het bijzonder over modulatie van GC gevoeligheid.

ABBREVIATIONS

ACPA	anti-citrullinated peptide antibody
ACR	American College of Rheumatology
ACTH	adrenocorticotropic hormone
AD	atopic dermatitis
ANCOVA	analysis of covariance
ANOVA	analysis of variance
Anti-CCP	anti-cyclic citrullinated protein
AP-1	activator protein 1
AUC	area under the curve
BAG-1	BCL2-associated athanogene
BAL	bronchoalveolar lavage
BASDAI	Bath Ankylosing Spondylitis Disease Activity Index
BD	behçet's disease
BDCAF	behçet's disease current activity form
BMI	body mass index
cAMP	cyclic adenosine monophosphate
cPLA2α	cytosolic phospholipase A2
CAI	clinical activity index
CBG	cortisol binding globulin
CDAI	Crohn's Disease Activity Index
CHIP	Carboxyl Terminus of HSP70-interacting protein
CI	confidence interval
COPD	chronic obstructive pulmonary disease
COX2	cyclooxygenase 2
CREB	cAMP responsive element binding protein
CRH	corticotropin releasing hormone
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CU	Colitis Ulcerosa
DAS28	disease activity score, 28 joints
DAS44	disease activity score, 44 joints
DEX	dexamethasone
DMARD	disease modifying antirheumatic drug
DST	dexamethasone suppression test
EAE	experimental autoimmune encephalomyelitis
EASI	Eczema Area and Severity Index
EC_{50}	half maximal effective concentration
EMSA	electrophoretic mobility shift assay

ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
FEV1	forced expiratory volume in 1 second
FKBP51	FK506 binding protein 51
FLS	fibroblast-like synoviocyte
GC	glucocorticoid(s)
GC-S group	carriers of the *BclI* and/or N363S variant
GC-I group	carriers of 9β or 9β + ER22/23EK variant
GC-SS group	GC-S group + homozygous carriers of the GLCCI1-C allele
GC-II	GC-I group + carriers of one or two GLCCI1-T alleles
GH	general health at a 100 mm scale
GILZ	glucocorticoid-induced leucine zipper
GLCCI1	glucocorticoid-induced transcript 1
GR	glucocorticoid receptor
GRE	glucocorticoid-responsive element
GWAS	genome-wide association study
HAQ-DI	health assessment questionnaire disability index
HAT	histone acetyltransferase
HbA1c	glycosylated hemoglobin
HC	healthy controls
HCQ	hydroxychloroquine
HDAC2	histone deacetylase 2
HDACi	histone deacetylase inhibitor
HLA	human leukocyte antigen
HOP	hsp70-hsp90 organizing protein
HPA-axis	hypothalamic-pituitary-adrenal axis
HPRT	hypoxanthine phosphoribosyltransferase
HSD11B2	11β-hydroxysteroid dehydrogenase type 1
HSD11B1	11β-hydroxysteroid dehydrogenase type 2
HSP40	heat shock protein 40
HSP70	heat shock protein 70
HSP90	heat shock protein 90
IgM	immunoglobulin, class M
IκBα	nuclear factor kappa B inhibitor alpha
IBD	inflammatory bowel disease
IDL	intracellular dexamethasone levels
IL-1	interleukin-1
IL-2	interleukin-2

IL-6	interleukin-6
IM	intramuscular
IQR	interquartile range
ITT	insulin tolerance test
LPS	lipopolysaccharide
LMM	linear mixed model
mGR	membrane-bound glucocorticoid receptor
mRNA	messenger ribonucleic acid
MAF	minor allele frequency
MAPK	mitogen-activated protein kinase
MCM2	minichromosome maintenance 2
MDR	multi-drug resistance
ME	middle eastern
MIF	macrophage migration inhibitory factor
MKP-1	MAPK phosphatase 1
MMP	matrix metalloproteinase
MS	multiple sclerosis
MSK1	mitogen- and stress-activated protein kinase 1
MTX	methotrexate
NF-κB	nuclear factor kappa B
NFAT	nuclear factor of activated T-cells
NOD2	nucleotide-binding oligomerization domain-containing 2
NS	non-significant
NSAID	non-steroidal anti-inflammatory drug
OAC	oral anticonceptives
OR	odds ratio
P-gp	P-glycoprotein
PARA study	Pregnancy-Induced Amelioration of Rheumatoid Arthritis study
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PDSC%	post-dexamethasone salivary cortisol concentration as percentage of basal salivary cortisol
PPARγ	Peroxisome proliferator-activated receptor gamma
PSS	perceived stress scale
PT	prothrombin time
PTPN22	protein tyrosine phosphatase, non-receptor type 22
P23	co-chaperone of hsp90
RA	rheumatoid arthritis
RAI	Ritchie Articular Index

RF	Rheumatoid Factor
RPS6	ribosomal protein S6
SD	standard deviation
SEGRA	selective glucocorticoid receptor agonist
SE	standard error
SEM	standard error of the mean
SJC28	swollen joint count, 28 joints
SJC44	swollen joint count, 44 joints
SLE	systemic lupus erythematosus
SOCS protein	suppressor of cytokine signalling protein
SNP	single nucleotide polymorphism
SSZ	sulfasalazine
STAT	signal transducer and activator of transcription
T-Bet	T-box transcription factor 21
TF	transcription factor
TJC28	tender joint count, 28 joints
TNF-α	tumor necrosis factor-alpha
tREACH	treatment in the Rotterdam early arthritis cohort
UNIANOVA	univariate analysis of variance

PUBLICATIONS

Quax R.A.M., Manenschijn L., Koper J.W., Hazes J.M.W., Lamberts S.W.J., van Rossum E.F.C., Feelders R.A. *Glucocorticoid sensitivity in health and disease*. Nature Reviews Endocrinology. Accepted for publication.

de Jong P.H., **Quax R.A.M.**, Huisman M., Gerards A.H., Feelders R.A., de Sonnaville P.B., Luime J.J., Weel A.E., Hazes J.M. *Response to glucocorticoids at 2 weeks predicts the effectiveness of DMARD induction therapy at 3 months: post hoc analyses from the tREACH study.* Annals of the Rheumatic Diseases, 2012 Oct 31. [Epub ahead of print]

Quax R.A.M., van Laar J.A.M., van Heerebeek R., Greiner K., Ben-Chetrit E., Stanford M., Wallace G.R., Fortune F., Ghabra M., Soylu M., Hazes J.M.W., Lamberts S.W.J., Kappen J.H., van Hagen P.M., Koper J.W., Feelders R.A. *Glucocorticoid sensitivity in Behçet's disease.* Endocrine Connections, 2012 vol. 1 no. 2 103-111

Quax R.A.M., Koper J.W., de Jong P.H., van Heerebeek R., Weel A.E., Huisman A.M., van Zeben D., de Jong F.H., Lamberts S.W.J., Hazes J.M.W., Feelders R.A. *In vitro glucocorticoid sensitivity is associated with clinical glucocorticoid therapy outcome in rheumatoid arthritis.* Arthritis Research & Therapy. 2012 Aug 24;14(4):R195.

Quax R.A.M., de Man Y.A., Koper J.W., van Rossum E.F.C., Willemsen S.P., Lamberts S.W.J., Hazes J.M.W., Dolhain R.J.E.M., Feelders R.A. *Glucocorticoid receptor gene polymorphisms and disease activity during pregnancy and the postpartum period in rheumatoid arthritis.* Arthritis Research & Therapy. 2012 Aug 13;14(4):R183.

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DANKWOORD

Toen ik in 2007 aan dit wetenschappelijke project begon, overzag ik niet dat het zo'n enorme klus zou worden. Vele mensen hebben ertoe bijgedragen, dat ik het onderzoek met succes heb kunnen vertalen in dit proefschrift en dat ik met ontzettend veel plezier op deze bijzondere periode terugkijk.

Allereerst wil ik graag mijn beide co-promotoren bedanken. Dr. R.A. Feelders, beste Richard, half slaperig kwam ik vanuit de nachtdienst in het Ikazia ziekenhuis bij je op sollicitatie. Ondanks deze slaapdeprivatie werkte jouw enthousiasme over het project over glucocorticoid gevoeligheid in reumatoïde artritis aanstekelijk op mij. Jouw onbegrensd optimisme, enthousiasme en wetenschappelijke drive hebben een grote bijdrage geleverd aan de uiteindelijke inhoud van dit proefschrift. Ik ben je dankbaar dat je mij de kans hebt gegeven in de wondere wereld van de medische wetenschap te kijken, ook buiten de grenzen van ons kikkerlandje. Het was altijd heerlijk om op werkbesprekingen, in volgorde van prioriteit, eerst de voetbaluitslagen door te nemen en dan pas de onderzoeksresultaten! Ik kijk uit naar onze hernieuwde samenwerking als ik straks terugkom in het Erasmus MC!

Dr. J.W. Koper, beste Jan Willem, ik zou jou met heel veel respect willen benoemen als de 'stille motor' van mijn promotie-onderzoek. Hoewel je waarschijnlijk wel even aan mij moest wennen als 'gezonde chaoot', heb ik onze samenwerking als ontzettend prettig ervaren. Je stond altijd voor me klaar, hebt me de finesses van het laboratoriumvak geleerd en op het eind alle manuscripten met chirurgische precisie doorgenomen en verbeterd. Jouw persoonlijke touch in de begeleiding en de rust die je uitstraalt hebben me altijd op mijn gemak laten voelen. Ontzettend bedankt voor dit alles!

Verder ben ik veel dank verschuldigd aan mijn beide promotoren. Prof. dr. S.W.J. Lamberts, beste Steven, het is een waar genoegen geweest om onder uw begeleiding te werken en in uw grenzeloze wetenschappelijke ervaring te mogen delen. Uw enthousiasme bij onze werkbesprekingen deden wetenschappelijke 'dipjes' in de kiem smoren en waren juist altijd een enorme stimulans om er nog een schepje bovenop te doen.

Prof. dr. J.M.W Hazes, beste Mieke, als 'doorgewinterde' klinische wetenschapper op het gebied van reumatoïde artritis heb ik veel van u geleerd. De combinatie met de endocrinologie is, denk ik, voor ons allebei een interessante en vruchtbare samenwerking geweest. Met veel plezier kijk ik terug op mijn tijd op de '9e verdieping'.

Prof.dr. T.J. Visser, Prof.dr. J.W.J. Bijlsma en Prof.dr. P.M. van Hagen, hartelijk dank voor het plaatsnemen in de kleine commissie. Beste Theo, dank voor alle gezelligheid en uw scherp wetenschappelijk oog. Prof. dr. F.H. de Jong en Prof. dr. Bootsma wil ik graag bedanken voor het plaatsnemen in de grote commissie. Beste Frank, uw wetenschappelijke kennis én gevoel voor humor zijn beiden van hoog niveau. Het is een eer dat u wilt opponeren op deze mooie dag.

En dan mijn paranimfen, Jeroen en Aimee.
Beste Jeroen, huisgenoten vanaf het begin van de studie, heel vaak mét maar ook tegen elkaar gevoetbald (die bekerfinale zal me altijd achtervolgen), maar vooral ook al meer dan 12 jaar ontzettend goede vrienden. Ik vind het dan ook fantastisch dat je deze bijzondere dag letterlijk aan mijn zijde staat! Veel succes met je eigen promotie-onderzoek, dat loopt al als een trein, en natuurlijk ook heel veel geluk met Ella, 'je aanstaande'.
Beste Aimee, ik leerde je als een ontzettend gezellige (en 'eigenwijze') vrouw kennen toen je nog als student kwam werken op het 'neuro-endo' lab. Al gauw liet je zien veel in je mars te hebben, met naast affiniteit met en talent voor de wetenschap, een onuitputtelijke bron van energie. Het is ook mooi om deze eigenschappen weer terug te zien nu je als dokter (en doctor) in het Maasstad ziekenhuis werkt. Dank voor je vriendschap en collegialiteit, hier hebben we al vaak op geproost en gedanst!
Mijn 'roomie' Laura, heel erg bedankt voor jouw gezelligheid. Fantastisch dat we op het laatste moment onze krachten nog hebben kunnen bundelen in een gezamenlijke pilotstudie. Waarschijnlijk zul jij een van de dikste boekjes van de afgelopen tijd gaan afleveren eind dit jaar. Ik kijk dan ook uit naar jouw verdediging! Maar eerst natuurlijk nog jouw trouwerij in september, 2013 wordt gewoon een topjaar voor jou!
Beste Rob, jouw droge humor en relativerend vermogen hebben je tot een ontzettend gewaardeerde collega gemaakt. Dank voor alle gezelligheid de afgelopen jaren en succes met jouw verdediging net een week na mij!
Als oud-collega's van het eerste uur wil ik graag Chris, Michel, Hans en Marieke nog bedanken. Chris, 6 tripel bier op de lege maag was toch echt te veel van het goede, we hadden het kunnen weten. Wat een fantastisch avontuur ga je aan in de USA; het past bij je en ik weet zeker dat het een succes gaat worden! Michel, nadat ik dacht dat ik alles wel met je had meegemaakt, zag ik je pas in één keer voor pakweg 1000 man even Lee Towers imiteren, het moet niet gekker worden. Je bent een gouden kerel en ik wens je heel veel succes in het Ikazia ziekenhuis. Hans, ook jij gaat je geluk beproeven over de grens. Gezien je eerdere wetenschappelijke prestaties zal dit ongetwijfeld een prachtig vervolg gaan worden van je carrière. La Jolla was slechts één van de fantastische ervaringen die we de laatste jaren samen hebben meegemaakt! Marieke, vanaf het begin een 'klik' waardoor we samen ook altijd over heel veel zaken buiten de wetenschap hebben kunnen praten en lachen. Jouw verhalen als je terugkwam van vakantie/congres waren altijd meer dan de moeite waard ;).

Gelukkig zat ik niet in mijn eentje op het 'neuro-endo' lab. In het bijzonder wil ik Diana, Marlijn en Peter als 'hoeders' van de passanten (lees promovendi) bedanken. Marlijn, toen ik je even oud schatte als Diana kon het natuurlijk al niet meer stuk tussen ons. Geniet lekker van jouw pensioen en het door jou zo geliefde Frankrijk! Diana, ondanks deze onvergeeflijke 'leeftijds'-misser, hebben we heel wat afgelachen de afgelopen jaren. Het doet me goed om te zien hoe je nu volop geniet van al het moois in het leven na je werk. Peter, op het door vrouwen

gedomineerde neuro-endo lab werkte jij met je heerlijke droge humor aanstekelijk op mijn lachspieren. Fadime, ik heb er alle vertrouwen in dat je de 'erfenis' van Diana en Marlijn goed zal invullen, heel veel succes. Leo, ik kijk met veel plezier terug op onze tijd in Napels waar ik als jouw 'Foster Parent kindje' werd gezien! Stephanie en Marije, nog heel veel succes met jullie promotie-onderzoeken. Cristina and Max, thinking of the great hospitality and delicious Italian dishes in Naples still puts a smile on my face and is one of many precious moments we have spent together. All the best for the both of you. Federico, thanks for all the fun we have had! Ramona, bedankt voor al je hulp bij de vele bioassays.
Ronald, al jouw inspanningen met de speekselcortisol metingen worden ook zeer gewaardeerd! Ellenlang praten over het wielrennen met jou was soms ook een welkome afwisseling op de veelal lange dagen op het lab, dank! Michael, dank voor je hulp met de voorbereidingen voor de genotyperingen! Ook alle andere collega's op de 5e verdieping wil ik bedanken voor de leuke lab-uitjes, wetenschapsdagen, Tour de France poules en de vele borrels. Ik heb genoten!

In het kader van de tREACH en FLARE studie heb ik met veel mensen mogen samenwerken. Allereerst wil ik alle patiënten en gezonde vrijwilligers bedanken die belangeloos hebben meegewerkt aan deze studies. Pascal, het is je fantastisch gelukt om een gigantisch project als de tREACH studie goed te coördineren, chapeau! Dank voor al jouw extra inspanningen om ook de verschillende substudies in de tREACH soepel te laten verlopen. Gaaf dat onze eigen projecten nog tot een mooie gezamenlijke paper hebben geleid. Maurits, 'mister MTX', jouw goedlachse persoonlijkheid heeft er voor gezorgd dat we het al snel goed konden vinden samen, ik heb veel met je gelachen! Succes met jouw laatste loodjes en de opleiding tot klinisch chemicus. Celina, ik heb veel respect voor je hoe je de (voor) opleiding tot reumatologe combineert met jouw gezin en het afronden van jouw promotie. Ik hoop dat die extra vrije dag in de week je wat extra lucht verschaft. Nog even volhouden!
Alle goedwillende promovendi zijn echter nergens zonder de enthousiaste medewerking van medisch specialisten. Ik wil dan ook mijn dank uitspreken naar alle reumatologen die patiënten hebben geïncludeerd voor de tREACH en FLARE studie. In het bijzonder wil ik Margriet Huisman bedanken voor haar nimmer aflatende enthousiasme en bereidwilligheid om mee te denken en te werken. Beste Jan, vrijwel alle geïncludeerde Behçet patiënten in onze gezamenlijke paper staan op jouw conto, bedankt! Maar het 'echte' werk wordt toch vooral gedaan door alle researchnurses, reumaconsulentes en doktersassistenten. Alle DAS metingen, bloedafnames, dexamethason suppressie testen, haarmonsters en tal van vragenlijsten zijn met bloed, zweet en tranen door hen verzameld. Mijn dank voor deze immense inspanningen is dan ook groot!
Radboud, Yaël en Fleur, bedankt dat ik de vruchten heb mogen plukken van het unieke PARA cohort wat jullie hebben opgezet. Patrick, Anne-Marie, Nadine, Ferry, Christiaan en Jan Piet, jullie hulp met het isoleren van de PBMCs heeft me ook ontzettend veel tijd bespaard. On-

danks dat de tijd ontbrak om ook op de 9e verdieping een mooi stuk basaal onderzoek neer te zetten, ben ik jullie collegialiteit en behulpzaamheid zeker niet vergeten. Patrick, jouw 'ontgroening' op de OK was echt lachen, en jouw passie voor het analistenvak is hartverwarmend. Ik wil alle promovendi van de afdelingen endocrinologie en reumatologie die nog hard aan het zwoegen zijn om ook hun boekje af te ronden heel veel succes toewensen.

Inmiddels ben ik al weer 2.5 jaar in de kliniek werkzaam en de goede en prettige sfeer in het Maasstad ziekenhuis heeft er zeker toe bijgedragen dat ik de dubbelfunctie van specialist-in-opleiding en promovendus goed heb kunnen blijven combineren. Cynthia, Marijke, Christien, Casper, Femke en Mark; dank voor jullie flexibiliteit als 'poli-maatjes' zodat ik de eindsprint voor het afronden van dit boekje in kon zetten!

Mannen van de voetbal (en daarbuiten): Joost, Vincent, Bas, Peter, Erik, Hugo, Jeroen, Jeroen, Jorrit, Yorick, Menno, Albert, Chris, Theun, Thijs, Remco, Fred, Serge, Arnoud, Hans en Robert-Jan! 11 fantastische seizoenen met de Rotterdam Rhino's, Leonidas 7 en de selectie van SDV, memorabele voetbalmomenten tegen Ouwe Schoen, fantastische feesten bij de Knickerbockers in Groningen en de beruchte mosselavonden en uitstapjes in Plan C. Ondanks dat onze drukke levens niet meer te combineren zijn met het wekelijks 90 minuten hollen op de groene mat, hoop ik dat we elkaar nog vaak zullen zien onder het genot van een biertje!
Beste Tom, Thijs en Maarten! Vanaf de wieg zijn jullie al mijn vrienden voor het leven!! Juist door jullie niet-medische achtergrond en door met enige regelmaat te vragen wanneer die 'afstudeerscriptie' nou eens af was, hebben jullie me (onbewust?) geholpen de relativiteit van de wetenschap en de medische wereld in te zien. Heerlijk praten over NAC, bier, reizen en al het andere wat het leven zo de moeite waard maakt! Jullie vriendschap is van onschatbare waarde en hoop ik nog tot in lengte van dagen te mogen ervaren!

Uiteraard is het dankwoord niet compleet zonder de mensen te bedanken die me waarschijnlijk het beste kennen en het dichtst bij me staan. Beste Guido, grote broer, en lieve Marieke, schoonzussie! Daar waar ik dit boek juist ga afsluiten, gaan jullie juist aan een nieuw hoofdstuk beginnen in jullie leven. Ik kijk ernaar uit om oom te worden! Ik geef ontzettend veel om jullie.
Lieve pap en mam, 2013 zal vanwege meerdere redenen als een bewogen jaar de boeken in gaan. Woorden schieten tekort om jullie te bedanken voor de door jullie geboden mogelijkheden en voor de onvoorwaardelijke steun, zeker de laatste maanden. Ik hou van jullie en ik hoop jullie nog lang in goede gezondheid bij me te mogen hebben!

Het zit erop. Heerlijk!

PhD PORTFOLIO

PhD candidate: Rogier Alfons Machiel Quax
Erasmus MC department: Internal Medicine / Endocrinology
PhD period: 2007-2013
Promotor: prof.dr. S.W.J. Lamberts, prof. dr. J.M.W. Hazes
Co-promotor: dr. R.A. Feelders, dr. J.W. Koper

	Year
Research skills, in-depth courses and workshops	
Photoshop and Illustrator CS4 for PhD-students	September 2010
Regression Analysis, NIHES-institute, Rotterdam	August 2010
Scientific Writing in English for Publication	Jan-Febr 2010
Master Class, Prof. Cidlowski, Maurius Tausk visiting professor	November 2009
Endocrine Trainee day, Endocrine Society	June 2008
Cambridge Advanced English, level C1	Jan-June 2008
Classical Methods for Data Analysis, NIHES-institute, Rotterdam	Sept-Okt 2007
Radioactivity Safety Course (5B)	April 2007
Clinical Courses	
Evidence Based Medicine, Desiderius	Feb-March 2013
DESG course 'Diabetes Mellitus'	May 2013
Rotterdam Course in 'Electrolyte and Acid-Base Disorders'	March 2012
Video training on the job	2012-2013
Dutch Internal Medicine Days	2011-2013
Basic Course, MRI for rheumatologists	January 2009
Fundamental Critical Care Support (FCCS) course	March 2006
National and International Conferences	
Endocrine Society Meeting, Boston, USA (poster presentation).	June 2011
European Society of Endocrinology Meeting, Rotterdam (poster presentation).	April 2011
Dutch Endocrine Meeting, Noordwijkerhout (oral presentation).	February 2011
Nederlandse Vereniging voor Reumatologie (poster presentation).	Oktober 2010
Endocrine Society Meeting, San Diego, USA (poster presentation).	June 2010
Dutch Endocrine Meeting, Noordwijkerhout (oral presentation).	January 2010
Science Days Internal Medicine, Erasmus MC, Antwerp (oral presentation).	January 2010
Nuclear Receptor Meeting, Leiden (oral presentation).	November 2009
IMID meeting, Lissabon, Portugal (poster presentation).	Oktober 2009
Endo Retreat, Rotterdam (oral presentation).	May 2009
Science Days Internal Medicine, Erasmus MC, Antwerp (poster presentation).	January 2009
Science Days Internal Medicine, Erasmus MC, Antwerp (poster presentation).	January 2008
Teaching activities	
Supervision of medical student	2009-2010

Workshop thyroid: basic	2008-2009
Workshop thyroid: clinical	2008-2009
Workshop adrenal gland	2008-2009
Other activities	
Organization of the ski-weekend of the department of Internal Medicine	2010, 2013
Organization of the labday of the department of Internal Medicine	2008

ABOUT THE AUTHOR

Rogier Quax werd geboren op 15 juli 1981 te Breda. In 1999 deed hij eindexamen atheneum aan de Katholieke Scholengemeenschap Etten-Leur. Vervolgens studeerde hij geneeskunde aan de Erasmus Universiteit te Rotterdam. In 2004 richtte zijn afstudeeronderzoek zich op de rol van surfactant eiwitten in neonatale longziekten. In 2005 liep hij een deel van zijn co-schappen op de afdeling traumatologie in het Groote Schuur Hospitaal in Kaapstad (Zuid-Afrika) en in hetzelfde jaar behaalde hij het artsexamen. In 2006 was hij werkzaam als arts-assistent interne geneeskunde in het Ikazia ziekenhuis te Rotterdam. In maart 2007 startte hij als arts-onderzoeker aan de Erasmus Universiteit op het project 'Determinants of Glucocorticoid Sensitivity in Rheumatoid Arthritis' onder de supervisie van Prof.dr. J.M.W. Hazes, Prof.dr. S.W.J. Lamberts, Dr. R.A. Feelders en Dr. J.W. Koper. Sinds januari 2011 is hij is in opleiding tot internist en werkzaam in het Maasstad ziekenhuis te Rotterdam (opleider Dr. M.A. van den Dorpel).

Rogier Quax was born on July 15th 1981, in Breda, The Netherlands. In 1999, he completed grammar school at the 'Katholieke Scholengemeenschap Etten-Leur'. He then started his medical training at the Erasmus University Rotterdam. In 2004, his graduation research was performed which focused on the role of surfactant proteins in neonatal lung diseases. In 2005, he attended a part of his internships at the traumatology department of the Groote Schuur Hospital, Cape Town (South-Africa), and in the same year he obtained his medical degree. In 2006 he worked as a resident at the Ikazia hospital, Rotterdam. In March 2007 he started the work presented in this thesis at the Department of Internal Medicine and the Department of Rheumatology at the Erasmus Medical Center under the supervision of Prof. dr. J.M.W. Hazes, Prof.dr. S.W.J. Lamberts, Dr. R.A. Feelders and Dr. J.W. Koper. In January 2011 he started his training residencies in Internal Medicine at the Maasstad hospital in Rotterdam (supervisor Dr. M.A. van den Dorpel).