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THE GLUCOCORTICOID RECEPTOR AS A REGULATOR OF CORTISOL RESPONSES IN CORTISOL RESISTANT PATIENTS AND HEALTHY SUBJECTS

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Gracias a la vida que me ha dado tanto
Me dio dos luceros que cuando los abro
Perfecto distingo lo negro del blanco
Y en el alto cielo su fondo estrellado
Y en las multitudes el hombre que yo amo.

Gracias a la vida que me ha dado tanto
Me ha dado el sonido y el abedecedario
Con él las palabras que pienso y declaro
Madre amigo hermano y luz alumbrando,
La ruta del alma del que estoy amando.

Gracias a la vida que me ha dado tanto
Me ha dado la marcha de mis pies cansados
Con ellos anduve ciudades y charcos,
Playas y desiertos montañas y llanos
Y la casa tuya, tu calle y tu patio.

Gracias a la vida que me ha dado tanto
Me dio el corazón que agita su marco
Cuando miro el fruto del cerebro humano,
Cuando miro al bueno tan lejos del malo,
Cuando miro al fondo de tus ojos claros.

Gracias a la vida que me ha dado tanto
Me ha dado la risa y me ha dado el llanto,
Así yo distingo dicha de quebranto
Los dos materiales que forman mi canto
Y el canto de ustedes que es el mismo canto
Y el canto de todos que es mi propio canto.

Gracias a la vida
Gracias a la vida
Gracias a la vida
Gracias a la vida

Violeta Parra 1917-1967

To F, E, M, M and J

ABSTRACT

Glucocorticoids are essential for life, and are involved in growth, reproduction, intermediary metabolism, immune and inflammatory reactions as well as central nervous system and cardiovascular functions. Glucocorticoids are also used as treatment of many diseases. Resistance to exogenous glucocorticoids is sometimes seen in patients treated with glucocorticoids. Resistance to endogenous glucocorticoid is seen in some patients causing a syndrome called primary generalized glucocorticoid resistance.

Glucocorticoids exert their effect through the glucocorticoid receptor, which belongs to the nuclear hormone receptor superfamily. The receptor consists of three functional domains, the N-terminal, the DNA binding domain and the ligand binding domain.

The overall aim of this thesis was to study the glucocorticoid receptor in patients with primary generalized resistance to glucocorticoids i.e. resistance to endogenous glucocorticoids.

In 12 unrelated patients with primary generalized glucocorticoid resistance we identified two novel mutations in the glucocorticoid receptor gene in two different patients, R477H and G679S respectively, situated in the DNA binding domain and in the ligand binding domain of the receptor. The R477H mutation is the only mutation described in the DNA binding domain of the human glucocorticoid receptor.

We characterized these two mutations *in vitro* in terms of ligand binding, DNA binding, transactivation and transrepression as well as studies of crosstalking with the inflammatory transcription factor NFκB. We could demonstrate that the phenotype of the two patients expressing these two mutations correlated to the *in vitro* findings.

We further demonstrated that the R477H and G679S were true mutations and not present as polymorphisms among healthy individuals.

Glucocorticoid sensitivity among healthy individuals was also compared between two groups characterized as low and high secretors of urinary free cortisol studied with respect to their responses to a low dose of exogenous glucocorticoid. We concluded that individuals with a low cortisol profile, though still in the normal range, seems to be more sensitive to exogenous cortisol than those with high profile. This could have impact on the response to treatment with exogenous glucocorticoids and the prediction of therapeutic effect and adverse side effects.

LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to by their Roman numerals:

I. Mini Ruiz, Ulrika Lind, Mats Gåfvels, Gösta Eggertsen, Jan Carlstedt-Duke, Lennart Nilsson, Martin Holtmann, Pontus Stierna, Ann-Charlotte Wikström, Sigbritt Werner
Characterization of two novel mutations in the glucocorticoid receptor gene in patients with primary cortisol resistance. Clinical Endocrinology. 2001 Sep. 55(3):363-71.

II. Mini Ruiz, Mats Gåfvels, Gösta Eggertsen, Ann-Charlotte Wikström, Pontus Stierna, Sigbritt Werner
The R477H mutation and the G679S mutation of the glucocorticoid receptor gene associated with glucocorticoid resistance are not found among healthy controls or in individuals investigated for thrombophilia. Manuscript

III. Mini Ruiz, Erik Hedman, Mats Gåfvels, Gösta Eggertsen, Sigbritt Werner, Hans Wahrenberg, Ann-Charlotte Wikström
Further characterization of human glucocorticoid receptor mutants, R477H and G679S, associated with primary generalized glucocorticoid resistance. Scand J Clin Lab Invest. 2013 Feb 8. [Epub ahead of print]

IV. Mini Ruiz, Urban Knutsson, Ann-Charlotte Wikström, Mats Gåfvels, Claude Marcus, Pontus Stierna, Sigbritt Werner
Effects of dexamethasone on endogenous cortisol and on its metabolic targets osteocalcin, leptin, glucose and insulin: a study on individual cortisol sensitivity in healthy men. Manuscript

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LIST OF ABBREVIATIONS

11-βHSD	11- β hydroxi steroid dehydrogenase
ACTH	Adenocorticotropic hormone
ADH	Anti diuretic hormone
AF	Activation function
AIS	Androgen insensitivity syndrome
AP1	Activator protein 1
AR	Androgen receptor
BMI	Body mass index
bp	Basepair
CAIS	Complete androgen insensitivity syndrome
CBG	Corticosteroid binding protein
cDNA	Core DNA
CNS	Central nervous system
CYP	Cytochrome P450
DBD	DNA binding domain
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
EC	Effective concentration
EMSA	Electro Mobility Shift Assay
GC	Glucocorticoid
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HDL	High density lipoprotein
hGR	Human glucocorticoid receptor
HMG-CoA	Hydroxy methyl-CoA reductase
HOMA	Homeostatic model assessment
HPA	Hypothalamic-pituitary-axis
hsp	Heat shock protein
HVDDR	Hereditary vitamin D dependent rickets
LBD	Ligand binding domain
LDL	Low density lipoprotein
MAIS	Mild androgen insensitivity syndrome
MC2-R	Melanocortin receptor type 2
mRNA	Messenger ribo nuclear acid
NFκB	Nuclear factor κ B
NLS	Nuclear localization signal
PAIS	Partial androgen insensitivity syndrome
PCR	Polymerase chain reaction
PGGR	Primary generalized glucocorticoid resistance
POMC	Propiomelanocortin
PVN	Paraventricular nucleus
RelA	Protein 65
StAR protein	Steroidogenic acute regulatory protein
TA	Triamcinolone acetonide
THR	Thyroid hormone resistance
Tk	Tyrosine kinaseh
TNFα	Tumour necrosis factor α
TR	Thyroid receptor
TSH	Thyroid stimulating hormone
UFC	Urinary free cortisol
VDR	Vitamin D receptor
wt	Wild type

1 BACKGROUND

1.1 GLUCOCORTICOIDS

In 1950 Philip Hench, Edward Kendall and Tadeus Reichstein received the Nobel prize for the discovery of the potent anti-inflammatory and immunosuppressive effect of glucocorticoids and the synthesis of cortisone in the 1940,s [1]. Glucocorticoids still have an important role in the treatment of inflammatory, autoimmune diseases and lymphoproliferative disorders. Sixty years after the first chemical synthesis of cortisone, corticosteroids remain among the top ten most commonly used prescriptions and over the counter drugs [2]. The worldwide market in oral and topical glucocorticoids is estimated to be worth more than USD 10 billion per year [3].

Although glucocorticoids are highly effective for therapeutic purposes, long-term or /and high dose glucocorticoid administration is associated with adverse side effects, like hyperglycaemia, abdominal obesity, muscle wasting, hypertension, osteoporosis, depression and decreased immunological function. Patients can also develop reduced glucocorticoid sensitivity and resistance [4].

Cortisol is the principal glucocorticoid (GC) hormone produced by the adrenal cortex, i.e. zona fasciculata and zona reticularis in humans. The production of cortisol results from a series of reactions involving seven different enzymes within the adrenals. The first step, which is also the rate-limiting step is the uptake of cholesterol from cellular stores into the mitochondria by the action of StAR (Steroidogenic acute regulatory) protein [5]. A number of different steroid hormones are generated with differences in their molecular structure that gives them different functions [6]. *Figure 1.*

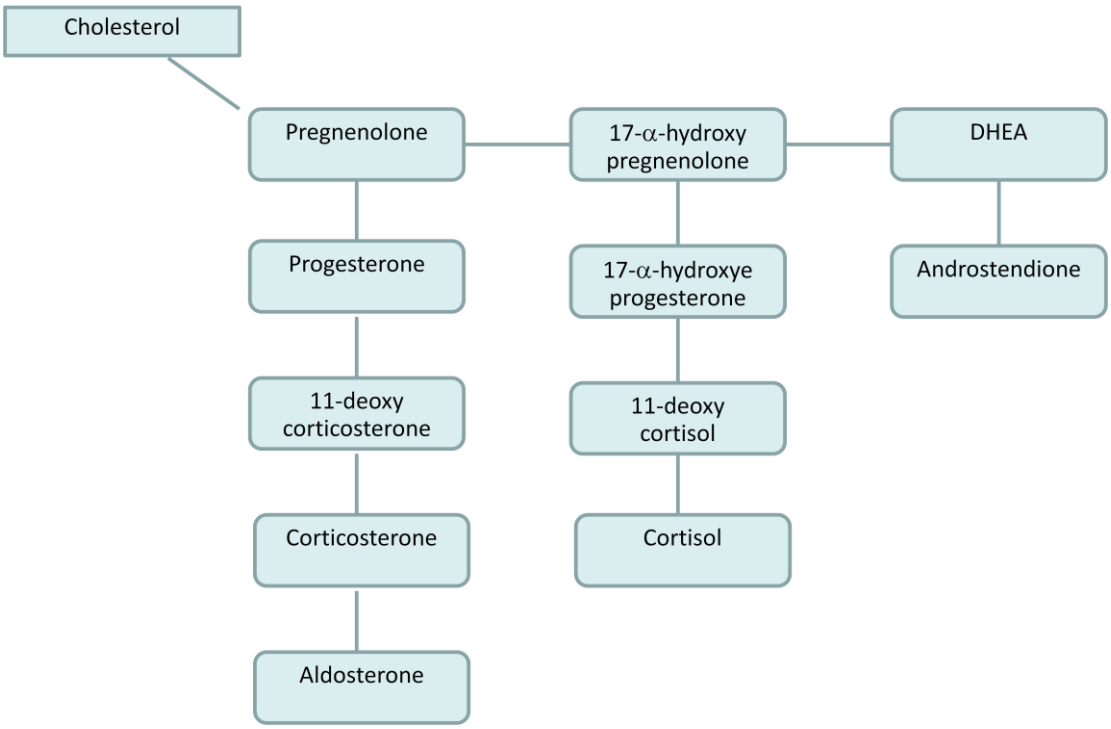

Figure 1

Synthesis of the steroid hormones in the adrenal cortex.

Aldosterone and cortisol are exclusively synthesized in the adrenal cortex and are therefore collectively referred to as corticosteroids. The term glucocorticoid refers to the actions on glucose metabolism whereas the term mineralocorticoid refers to actions on renal electrolyte excretion. Androstendione and dehydroepiandrosterone (DHEA) are weak androgen steroids that can be converted to the more potent testosterone and dihydrotestosterone (DHT).
Glucocorticoids, together with mineralocorticoids, androgens, progestins and estrogens belong to the steroid hormone family.

Glucocorticoids (GCs) exert physiological effects essential for life. Adrenalectomy in humans is fatal unless GCs are administrated. GCs play an essential role in maintaining basal and stress-related homeostasis and have many effects on development and cell differentiation [7, 8]. GCs are involved in growth, reproduction, intermediary metabolism, immune and inflammatory reactions as well as central nervous system and cardiovascular functions [9]. *Figure 2*

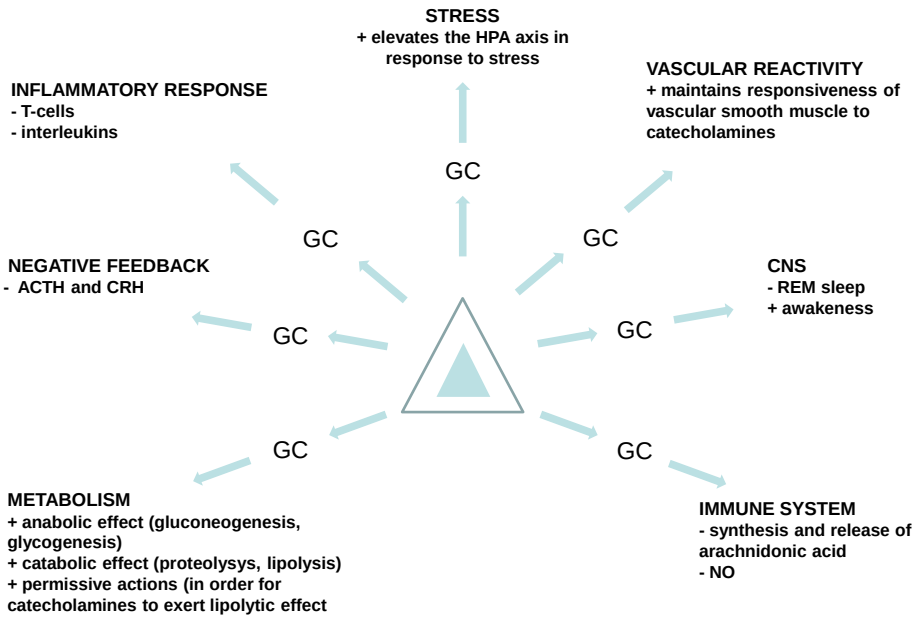

Figure 2

Schematic drawing of the effects exerted by glucocorticoids.

1.2 THE HPA AXIS

The regulation of serum GC is under the influence of the hypothalamus-pituitary-adrenal (HPA) axis, the autonomic nervous system and the endocrine glands together with paracrine and autocrine effects locally [10, 11]. The HPA axis consists of several hypothalamic nuclei, the anterior pituitary gland and the adrenal gland.

The paraventricular nucleus (PVN) is the principal CNS source of corticotrophin-releasing hormone (CRH), a 41 amino acid peptide. CRH together with the nona peptide arginine-vasopressin (ADH) synergistically, in a pulsatile and circadian way, stimulate the 39-aminoacid peptide adrenocorticotropic hormone (ACTH) secretion which in turn induces secretion of GC from the zona fasciculata of the adrenal cortex [11].

ACTH stimulates the release of cortisol from the adrenal cortex by binding to the MC2-R (type 2 melanocortin receptor) on the cell surface. GCs are released immediately into the circulation by diffusion [12]. The effects of ACTH on the adrenal include immediate and chronic impact. The acute effects, within minutes, occur by stimulating steroidogenesis via a StAR mediated increase in cholesterol delivery to the first enzyme step (CYP11A1). The chronic effect, within 24-26h is mediated through increase in the

synthesis of all steroidogenic CYP enzymes (CYP 11A1, CYP17, CYP21A2, CYP11B1). ACTH also stimulates synthesis of the LDL and HDL-receptors and possibly HMG-CoA reductase, all the latter to increase the uptake and production of cholesterol [13].

The secretory pulses of CRH occur in a constant frequency but with varying amplitude and affect the ACTH and cortisol release as well. Under basal conditions approximately 18-25 pulses of ACTH are observed over 24 h [14].
The amplitude increases early in the morning and also as a response to stress [15]. The highest plasma cortisol levels occur between 06 and 10 am with the lowest levels occurring after midnight [16].

All mammalian species studied so far have been detected having this pulsatile secretion of cortisol [17]. The pulses come with an hourly interval with very low nadir values in between. The pattern is found to vary with puberty [18], gender [19], lactation [20] and aging [21]; all normal physiological changes. Changes in frequency, amplitude and pulse mass [14, 22, 23] have been described in affective disorders as well as in chronic stress conditions [24].

However, the thought of a hypothalamic pulse generator was questioned already in 1990 when it was shown that pulsatile secretion of corticosteroids in sheep could still continue after hypothalamic disconnection [25]. A mathematical model was developed by Walker and co-workers where the oscillatory system lies between the pituitary and the adrenals solely, and that the CRH drive from the hypothalamus thus provides the amplitude [26]. Spiga et al have shown in vivo that the HPA axis responds to pulsatile rather than tonic stimuli in the adrenal cortex in rats [27].

The HPA axis is maintained in balance by a negative-feedback mechanism, where cortisol inhibits the synthesis and secretion of CRH and through inhibition of the ACTH precursor peptide propiomelanocortin (POMC), thereby also the ACTH levels [28]. The GC negative feedback regulation of the HPA axis occurs acutely by a relatively rapid inhibition, seconds to minutes of CRH release and chronically by down-regulating CRH expression in the PVN [28]. *Figure 3.*

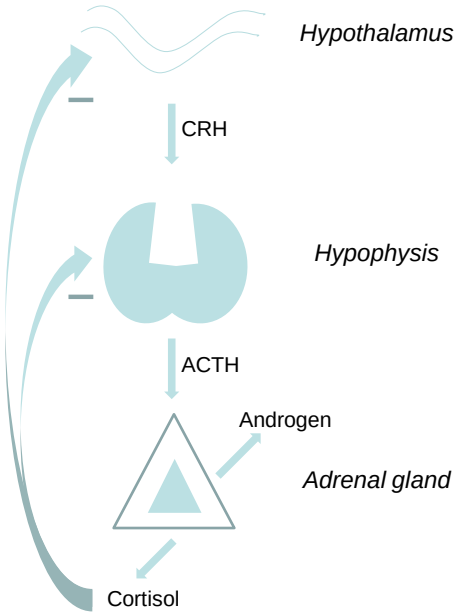

Figure 3

Schematic drawing of the negative feedback mechanism of the HPA axis.

The levels of GC´s secreted into the bloodstream as a response to stress do not only depend on the duration and intensity of the exposure to stress but might also depend on the setting of the fetal HPA axis, and potentially on the functional capacity of the axis to respond to acute or chronic stress in later life [29].

Cortisol has a half-life of 60-90 minutes and is metabolized in the liver. The majority of circulating cortisol is reduced to dihydrocortisol, and thereafter to tetrahydrocortisol which in turn then is conjugated to glucuronic acid. Some cortisol is converted to cortisone in the liver and thereafter undergoing the same reduction and conjugation as cortisol. The tetrahydroglucuronide derivates of cortisol and corticosterone are water soluble and excreted through the urine.

Dysfunction of the HPA axis characterized by a cortisol level increase occur in Cushing´s syndrome, morbid obesity, psychiatric disorders, poorly controlled diabetes mellitus and alcoholism [30]. Functional hypercortisolism occurs in pregnancy and with physical activity [31, 32]. Low levels of cortisol are observed in patients with Addison’s disease, Congenital Adrenal Hyperplasia, stress-related disorders as chronic fatigue, fibromyalgia and post traumatic stress disorder [33-35].

1.3 EVALUATION OF THE HPA AXIS

The function of the HPA axis can be evaluated through a number of tests, from baseline levels to dynamic tests, evaluating the ability of the HPA axis to respond to different stimuli with endogenous cortisol.

Cortisol can be measured in blood, urine and saliva. None of these measurements though reflect the circadian variation or pulsatility in the HPA axis described earlier.

Measurement of *midnight cortisol* has a less individual variability than morning cortisol, however it requires hospitalization [36]. Very low values of serum cortisol < 80-110 nmol/L at 8 am strongly indicate adrenal insufficiency. Values of serum cortisol above 470-500 nmol/L rule out adrenal insufficiency. However values in between need to be further evaluated with provocative testing [37].

An increase in blood cortisol is reflected by a change in the *salivary cortisol* concentration within a few minutes. The cortisol analyzed in saliva is free and not bound to CBG [38]. Measurement of salivary cortisol is non-invasive and easy to perform especially when collection occurs outside the hospital. Limitations of this analysis include insufficient quantities of saliva obtained and varying sleeping patterns as well as various assays having different diagnostic cut-offs [39].

Urinary free cortisol (UFC) is collected for 24 hours and represents approximately 1 % of the daily cortisol production. The unbound free cortisol in the circulation is filtered at the glomerulus and excreted in the urine. Conditions affecting CBG, such as oral estrogens, do not affect the UFC measurement. The UFC is reproducible and robust, however the compliance might be a problem affecting the result. Combining the UFC with creatinine gives a more reliable test that also can be corrected for lean body mass [40].

Long term HPA activity can be measured by analysis of *cortisol in hair*. However, though still not in clinical practice many questions remain to be addressed such as how the cortisol is derived in hair and if it originates from free cortisol in the plasma [41].

Dynamic tests include stimulation tests as well as suppression tests. The *ACTH stimulation test* assesses basal and reserve function, ruling out primary adrenal failure.

A single dose of ACTH is given and cortisol in plasma is measured at baseline and after 30 minutes. An increase in cortisol to > 500 nmol/L is found to be a normal adrenal response [42].

The *insulin tolerance test (ITT)* tests the entire HPA axis by induced hypoglycaemia that triggers the CRH-ACTH-cortisol cascade. After fasting the patient is given an intravenous bolus of insulin in the morning. Baseline plasma cortisol levels is measured followed by measurements after a set time interval. An adequate cortisol response is defined as an increase > 170 nmol/L is required as well as well as a rise greater than 550 nmol/L. This test is uncomfortable for the patients and requires medical supervision [43].

The *CRH stimulation test* is used for differential diagnosis in Cushing's syndrome, to establish the site of hormone excess in patients with documented cortisol excess. The use of CRH in this setting is based on the principle that pituitary tumours are responsive to exogenous CRH, whereas ectopic and adrenal tumours are not. Human or ovine CRH is given as an intravenous bolus after fasting and baseline cortisol and ACTH levels are measured before and during the test after a set time interval. The test is well tolerated by the patients [44].

Dexamethasone is a potent glucocorticoid, about 30 to 40 times more potent than cortisol. In the overnight *dexamethasone suppression test*, 1 mg Dexamethasone, which is a supraphysiological dose, is given at 11 pm and cortisol is measured at 8 am the following day. Inability to suppress cortisol secretion under the cut-off 50 nmol/L is defined as a pathological test [45] .

1.4 FACTORS REGULATING GLUCOCORTICOID BIOAVAILIBILITY

At a cellular level the activity of circulating glucocorticoids are determined by factors that regulate the access of free hormone to its receptor. Such factors include CBG, (Cortisol binding globulin), MDR (Multidrug resistance) protein and 11β-HSD (11β-Hydroxy Steroid Dehydrogenase which will be commented below. These factors modulate the actions of glucocorticoids at a pre-receptor level.

1.4.1 CBG

In the blood only 2-5% of the total cortisol circulates as free cortisol. The majority circulates bound to carrier proteins, predominantly corticosteroid binding globulin (CBG) and to some degree to albumin. The CBG levels stay relatively constant after adolescence and have no diurnal variations [46, 47]. CBG is produced by the liver and the production is stimulated by estrogens [48].

Bound steroids seem to be physiologically inactive and probably function as a reservoir for cortisol.CBG has a low saturation point and therefore at the physiological peaks of cortisol CBG is already saturated [49]. In sepsis, plasma CBG levels fall acutely by 50% approximately, thus increasing the free fraction of cortisol [50]. Glucocorticoid resistance in New World primates due to defect ligand binding is associated with a compensatory reduced CBG –cortisol binding due to lower affinity [51]. The CBG seems to have different affinities for corticosterone at different temperatures [52]. Mutations in the CBG gene causing decreased binding to CBG have been associated with low blood-pressure [53, 54]. However, altered CBG levels have been seen in hypertension and in the metabolic syndrome. The mechanism underlying this is still unclear. One hypothesis is that IL-6 levels are elevated in obesity and IL-6 and insulin may reduce CBG synthesis, if so the altered CBG levels are not causative in these diseases [55].

1.4.2 MDR

The multidrug resistance (MDR) P-glycoprotein is an ATP-dependent multidrug efflux pump that decreases intracellular concentrations of potentially toxic chemicals, i.e. drugs and hormones. It is expressed in both human and rodent tissues, including the adrenal gland, kidney liver, colon, small intestine, and brain and testis capillary endothelial cells [56]. The MDR pump at the blood-brain barrier, in both mice and humans, transports cortisol and the synthetic glucocorticoid dexamethasone, but not corticosterone, out of endothelial cell lining in the brain [57]. Humans with autoimmune diseases, such as RA, Crohn´s disease and lupus exhibit high lymphocytic MDR activity and/or expression. In subpopulations of colitis and RA, in patients resistant to steroid therapy increased MDR expression has been found to be more common [58-64]. Polymorphisms within the MDR gene are associated with GC resistance in GC resistant inflammatory bowel disease and rheumatoid arthritis [65].

1.4.3 11β-HSD

There are two isoforms of the enzyme 11β-HSD, type 1 and type 2. 11β-HSD-2 breaks down naturally occurring glucocorticoids as they enter the cell, thus inactivating cortisol to cortisone in humans, whereas 11β-HSD-1 catalyzes the opposite reaction; cortisone to cortisol.

11β-HSD-1 converts cortisone to cortisol and is found in many tissues, including liver, adipose tissue, muscle, brain and the vascular system, whereas 11β-HSD-2 converts cortisol to cortisone and is found in aldosterone sensitive tissue [66, 67]. The latter enzyme protects the mineralocorticoid receptor from the competitive action of the 1000-fold more abundant cortisol, which has the same binding affinity for this receptor as aldosterone, thus securing a mineralocorticoid effect only by aldosterone.

Pro-inflammatory cytokines such as TNF-α have been shown to up regulate 11β-HSD-1 and/or down regulate 11β-HSD-2 expression/activity in various cell types thus enabling the formation of active cortisol [68]

Studies in transgenic mice have demonstrated that over-expression of 11β-HSD-1 selectively in adipose tissue results in visceral obesity, hyperlipidemia, and insulin resistant diabetes. In contrast mice in which the 11β-HSD-1 gene has been disrupted are protected from obesity-induced hyperglycaemia, insulin resistance and dyslipidemia [67]. In humans however there is no such relationship demonstrated when comparing obese metabolic women with lean healthy women [69].

1.5 NUCLEAR RECEPTORS

To exert its effect the free cortisol, since it is lipophilic, diffuse over the cell membrane into the cytoplasm of the target cells where it eventually docks into the intracellular glucocorticoid (GR) receptor. Both physiological and pharmacological actions of GCs are mediated by the GR, which is a member of the protein super family of hormone nuclear receptors. The receptors for estrogens, androgens, vitamin D, progesterone, mineralocorticoids, thyroid hormone and retinoic acid are also found within this family [70]. Till today 48 nuclear receptors have been found in humans. The super-family also contains orphan receptors, i.e. receptors for which the ligand is still unknown [71].

1.5.1 Glucocorticoid receptor

The GR is a 94 kDa protein and in humans the gene is located on the short arm of chromosome 5 and consists of nine exons. Exon 1 forms the 5´-untranslated region, while exon 2-9 code for the GR protein. The human cDNA for GR was sequenced in 1985 [72].

Studies have identified 9 alternative first exons (1A, 1B, 1C, 1D, 1E, 1F, 1H, 1I and 1J) that are generated from unique promoter sites and probably account for tissue-specific expression of GR [73].

Due to alternative splicing of exon 9, two isoforms of GR, GRα and GRβ are translated. These receptors are identical up to amino acid 727, but then diverge at their carboxy termini, where each has a unique sequence at the end of LBD. In GR α the sequence consists of 50 aa and in GRβ the sequence consists of 15 aa [74]. The GRβ is unable to bind ligand due to its lack of a complete ligand binding domain (LBD). In the absence of ligand GRα is located in the cytoplasm bound to a heat shock protein (hsp) complex, whereas GRβ is found mainly in the nucleus where it has been suggested to inhibit transcriptional activity of GRα [75]. Increased expression of the GR β isoform can cause relative glucocorticoid resistance [76].

Cidlowski et al have identified N-terminal isoforms of GR, which are generated by alternative translation initiation and thus have different kDa. They are named GRα-A, B. C1, C2, C3, D1, D2 and D3.All are hGRα isoforms and since all the alternative start codons are located in the N-terminal of the protein, they have identical DNA binding domains and ligand binding domains. GRα-A corresponds to the 94kDa protein [77].

Alternative splicing of other exons result in GR-A, GR-γ and GR-P. GR-A and GR-B do not bind ligand due to a disrupted LBD [78]. The role of GR β, GR-A, GR-γ and GR-P *in vivo* is still yet controversial and will not be further discussed in this thesis. GRα-A is denoted GR through this thesis. *Figure 4.*

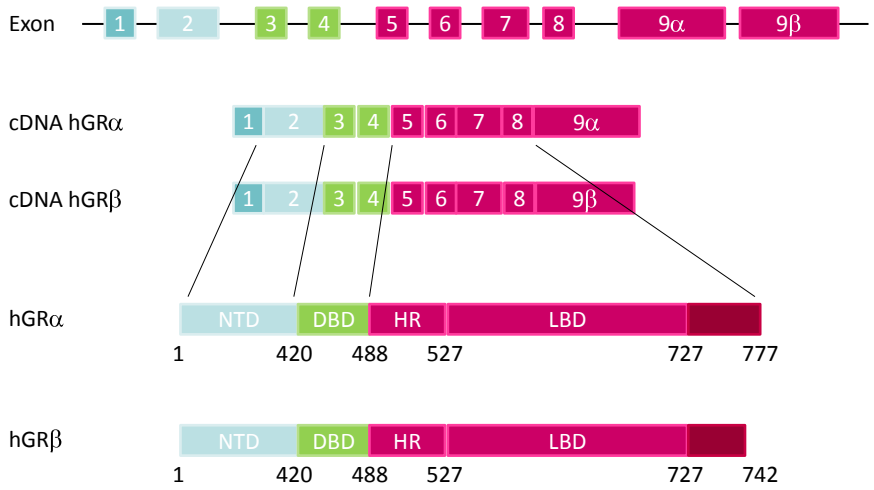

Figure 4

Schematic drawing showing the organization of the hGRα and hGRβ. Adapted from Ramamoorthy et al 2013 [79].

The GR consists of 777 amino acids (aa) divided into three functional domains, an N-terminal domain (NTD), a DNA binding domain (DBD), and a ligand-binding domain (LBD) [80]. Between the DBD and the LBD the hinge region (HR) is located. *Figure 5.*

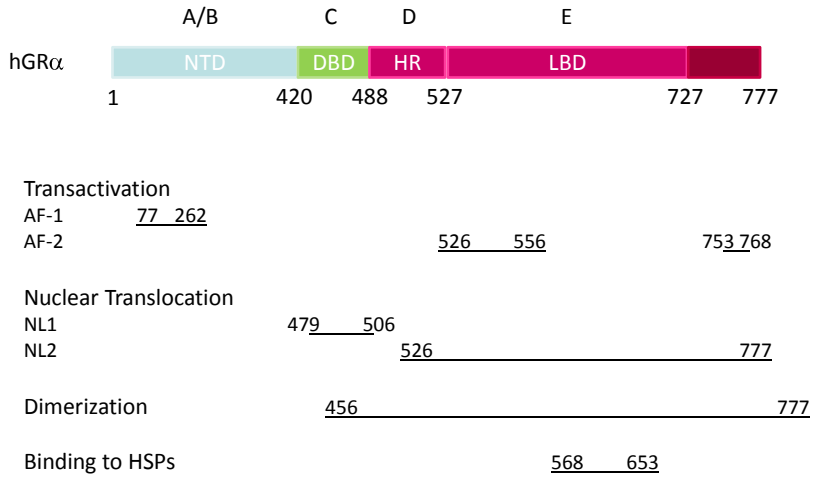

Figure 5

Schematic drawing of the hGRα and the functional domains. The GR consists of 777 amino acids (aa) divided into three functional domains, an N-terminal domain (NTD), a DNA binding domain (DBD), and a ligand-binding domain (LBD) [80]. Between the DBD and the LBD the hinge region (HR) is located. Regions involved in transcriptional activation (AF1 and AF2), dimerization, nuclear localization and chaperone hsp90 binding are indicated. Adapted from Ramamoorthy et al 2013 [79].

The N-terminal domain, aa 1-420, is the most variable domain in terms of length and amino acid sequence among the steroid hormone receptor domains, containing a powerful transactivation region (AF-1) located between aa 77 and 262 required for maximal transcriptional enhancement [81]. However, the GR AF-1 region is not essential for life [82]. The N-terminal domain contains the major known sites of phosphorylation in the GR [83], and is also highly immunogenic with many antigenic sites that have been used to raise antibodies against GR [84].

The DBD, aa 421-488, is responsible for recognizing specific palindromic sequences, glucocorticoid responsive elements (GREs), within the genome. The DBD contains two zinc-fingers, where the N-terminal finger is mainly involved in site-specific recognition, with the amino acids in the P-box being of great interest, P standing for proximal [85]. In the C-terminal finger, the D-box, D standing for distal, functions as a dimer interface and mutations in this region result in a receptor unable to bind as a dimer. This region has also been demonstrated to be involved in interference with NFκB and AP-1, possibly via protein-protein interaction [86]. *Figure 6.*

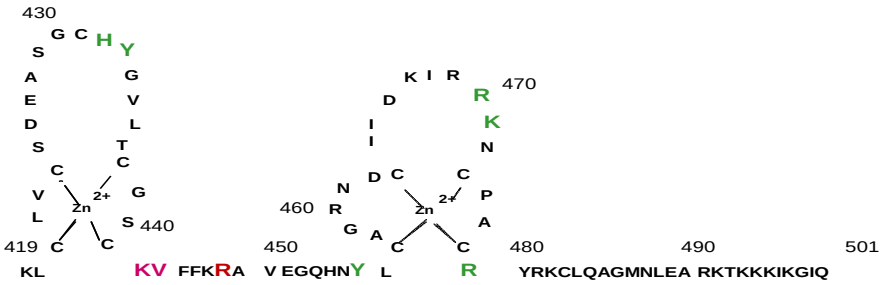

Figure 6

Schematic drawing of the two zinc-fingers in the DBD. Green letters indicate those amino acids interacting with phosphate groups and those amino acids that interact with DNA. Modified from Zilliacus 1994.

The hinge region, (HR) or D-domain, aa 487-526, is a flexible moiety in which the DBD and the LBD can rotate and therefore adopt different conformations. Nuclear localization site 1 (NLS 1) is located in the junction between the hinge region and DBD.

The LBD, aa 528-777, consists of 12 α-helixes and four β-sheets and contain functions for ligand binding, dimerization, binding of co-regulators and transcriptional activation. The LBD is folded into a hydrophobic pocket that binds ligand with high specificity in its interior [87]. The LBD also encompasses the highly conserved protein-protein interaction site on the LBD surface, the AF 2.pocket, which is a ligand dependent transactivation domain involved in the recruitment of co activators [88]. The binding function 3 (BF 3) is also located within the LBD and might be involved in recruitment of co-activators as well [89]. NLS-2 spans the entire LBD [89].

1.5.2 Molecular mechanism of action

In the absence of ligand the GR is located in the cytoplasm in a large multiprotein complex made up of chaperone molecules such as hsp90, hsp70 and p23 [90]. The chaperones have been found to hold the GR in a conformation that has a high affinity

for ligand, so the LBD lies with its ligand binding cleft accessible to ligand binding and subsequent GR activation [91].

Lipophilic glucocorticoids diffuse across the plasma membrane and bind to GR. Upon ligand binding and activation, the GR undergoes a conformational change that leads to dissociation of the chaperone complex thus revealing nuclear localization sequences (NLS) in the hinge region and the LBD [92].
Importin molecules then bind to these NLS sequences and facilitates active transport into the nucleus through the nuclear pore. Shuttling between the nucleus and the cytoplasm has been reported for both activated and non-activated forms of GR [93].

Once in the nucleus GR regulates gene expression via two main molecular pathways;
(1) dimerization and direct binding to positive or negative glucocorticoid response elements (GREs) in the promoter regions of genes leading to transcriptional increase or decrease [94] and
(2) GR monomers interfering with other transcription factors such as activator protein 1 (AP-1) and nuclear factor κB (NFκB) on the cytoplasm and/or acting in a tethering or composite manner when binding to DNA [95]. *Figure 7.*

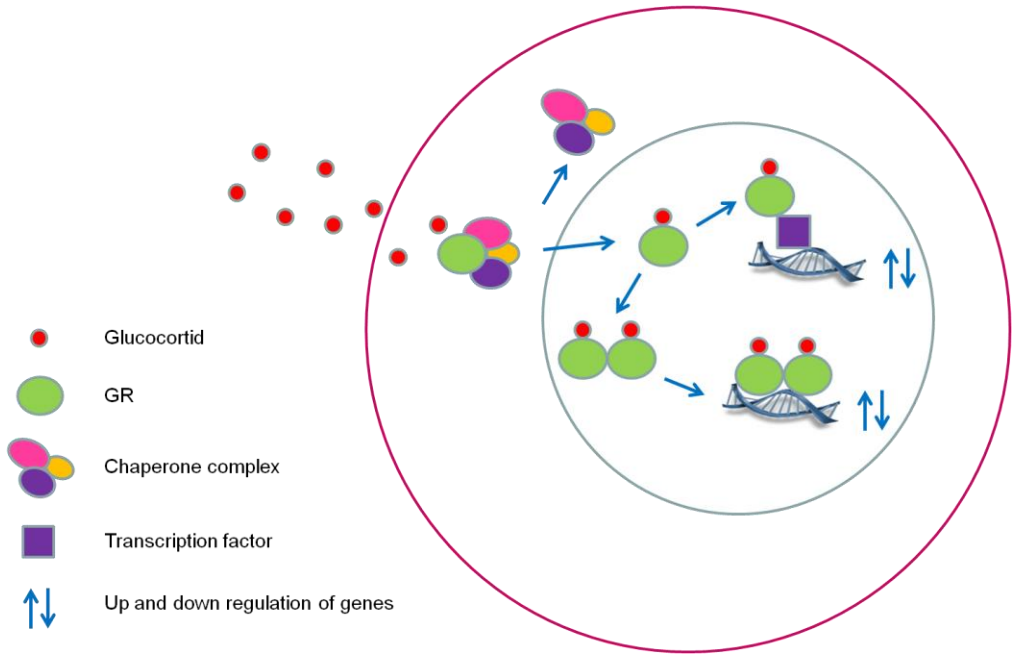

Figure 7

Schematic drawing of the mechanism of action of the GR. In the cytoplasm the GR is bound to chaperones like hsp90 and hsp70. Lipophilic glucocorticoids diffuse across the plasma membrane and bind to GR. Upon ligand binding and activation, the GR undergoes a conformational change that leads to dissociation of the chaperone complex. The bound GR enters the nucleus. Once in the nucleus GR regulates gene expression via two main molecular pathways.

The GRE consists of imperfect palindromic, hexameric half sites separated by 3 base-pair (bp) spacers [96]. Within these 15 bps five positions are nearly invariant, whereas the remaining positions can vary among functional GREs with little effect on receptor binding [97]. However, recent studies [98] have shown that not only do GR dock with GRE, but that the varying bp´s in the GRE, upon GR docking, differently affect GR conformation and subsequently has regulatory effects.

The protein-protein interaction mechanism might be more important than the effects mediated via direct GRE binding, as indicated by studies showing that mice harbouring a mutant GRα, which still can take part in protein in protein-protein interactions but that cannot dimerize or transactivate via GRE survive, in contrast to mice with a deletion of the entire GR gene who die upon birth [99, 100]. However, Kleiman et al have shown that GR dimerization is required for survival in septic shock as it is necessary for suppression of interleukin-1 in macrophages in mice [101].

The liganded GR can interact with many components of the transcriptional machinery; co activators, co repressors and chromatin remodelling proteins. No correlation between ligand affinity for the GR and cofactor interaction has been found [102] which indicates that steps downstream of the ligand binding to the receptor, which differs for transrepression and transactivation can alter the sensitivity EC_{50} [103].

The GR also undergoes several post-translational modification of the receptor protein including phosphorylation and acetylation that influence the transcriptional activity.

The GR displays a basal level of phosphorylation and becomes hyperphosphorylated upon ligand binding, with the extent of phosphorylation dependent on the bound ligand [104].This phosphorylation changes the transcriptional activity in a gene-specific manner. Phosphorylation of Serine 404 for instance diminishes the interaction between GR and nuclear factor κB [105]. Phosphorylation also modulates the cellular trafficking of the receptor and the level of the receptor by modulating its half-life [106-109].
The pattern of phosphorylation in the GR among species is variable, suggesting that species-specific differences may influence functions of the GR.[110].

The GR becomes acetylated upon ligand binding at the acetylation motif of KxKK, where X is any aa and K is lysine. This motif is shared with most of the steroid

hormone receptors [111]. GR becomes acetylated after ligand binding, and HDAC2-mediated GR deacetylation enables GR binding to the NF-kappaB complex. In GR residue K494 and K495 are acetylated. Knockdown of HDAC2, which is responsible for deactylation of K494 and K495, rendered GR insensitive to NFκB, while maintaining nuclear translocation and DNA binding intact [112].

In very rapidly responding genes, increases in GR mRNA have been detected in cultured cell lines as early as 15 minutes after the addition of steroid [113].
Once activated, GR mRNA expression is subject to negative regulation by GCs [114]. This might occur through negative GC response element (nGRE) and NFκ B regulatory motifs which are negatively regulated by GCs [78]. Ligand-activated GR is also degraded upon prolonged exposure to GCs [115].

1.6 NFKB

The transcription factor NFκB plays a key role in inflammation, apoptosis, and oncogenesis [116, 117].

The NFκB family encompasses five members; p65 (RelA), RelB, c-REL, NFκB1 (p50/p105) and NFκB2 (p52/p100). All members can form homo-or heterodimers with each other forming 15 dimeric forms where 12 are able to bind to DNA and regulate transcription [118, 119]. The main research target has been the prevalent p65-p50 heterodimer, where p50 increases DNA-binding and p65 confers transcriptional regulation [120].
The p65-p50 dimer is situated in the cytoplasm, held by an inhibitory IκB molecule. When a pro-inflammatory signal such as TNFα reaches the cell, via a sequence of events, the IκB molecule becomes phosphorylated and ubiquitinated and subsequently degraded, leading to a nuclear translocation of NFκB [120].
NFκB is thereafter modulated by for example acetylation and phosphorylation where the latter has been studied extensively.
NFκB bind to NFκB –specific promoter recognition sites and enhance gene expression of multiple pro inflammatory genes [121].
The NFκB is subject to a negative feedback loop as it stimulates the transcription of IκB [120].

1.7 NFKB AND GR

The transcription factor NFκB is one of the key mediators of inflammation and is also a target of GC-dependent repression of pro-inflammatory gene transcription [122]. The nuclear interaction of the GR with the C-terminal of activation domain of NFκB is crucial to the GC mediated repressive effect on NFκB regulated gene expression [123, 124]. Although GR does not need to bind the promoter of its target genes, mutation analysis has revealed that a functional DBD of the GR is necessary for repression of NFκB regulated genes [123, 125]. The GR LBD has also been suspected to participate in the repression of NFκB regulated genes [126]. Additionally GC can directly affect histone modification, thus influencing chromatin accessibility [127, 128].

1.8 GLUCOCORTICOID RESISTANCE

The cellular response to GC is not homogenous, showing variability in magnitude and specificity [129]. The sensitivity to GC differs not only among individuals but also within tissues of the same individual and also within the same cell during the cell cycle [130]. Tissue specific resistance may also develop in patients with long-term GC therapy. This interindividual difference in HPA axis function, within the normal range or a dysfunctional reactivity of the HPA axis may affect the response to GC therapy and consequently the susceptibility to systemic and/or adverse side effects.

The resistance is defined as the inability of the cells to respond to all or some of the responses to glucocorticoids. At a molecular level GC resistance can be ascribed to reduced expression of GR, altered affinity for the ligand, reduced ability of GR to bind DNA, or increased expression of other transcription factors that compete with GR for DNA-binding. The mechanism behind individual differences in glucocorticoid sensitivity is most certainly multifactorial including bioavailability, of the ligand, variations in liver metabolism, intercurrent diseases as well as tissue-specific effects.

A polymorphism is defined as DNA sequence variation that is found in at least 1% of the population. If the variation is less frequent the DNA sequence variation is defined as a mutation. Interindividual variations in tissue sensitivity to glucocorticoids have been described within the normal population, and have been partly attributed to polymorphisms in the hGR gene [131, 132].

The ER22/23EK polymorphism consists of two linked, single nucleotide mutations in codon 22 and 23. The first nucleotide replacement is silent, i.e. not resulting in an aa change, whereas the second results in arginine (R) to Lysine (K) change at position 23 [133] This polymorphism is present in 3% of the population. ER22/23EK is associated with decreased GR transcription activity in reporter assays and decreased expression of endogenous glucocorticoid responsive genes when compared to wild-type GR. Adult carriers of the ER22/23EK polymorphism were shown to have a lower tendency to develop impaired glucose tolerance, type 2 diabetes and cardiovascular disease [134, 135]. At higher ages this polymorphism is more common and is associated with lower risk of developing dementia compared to non-carriers [132]. The molecular mechanism underlying this phenotype might involve a higher expression of hGRα-A isoform at the expense of hGRα-B, leading to a decrease in transcriptional activity [136].

The N363S polymorphism, resulting in serine (N) to aspargine (S) substitution is associated with higher sensitivity to glucocorticoids in vivo, increased insulin response to exogenous dexamethasone administration [137], higher body mass index, higher waist-to-hip ratio and more coronary artery disease [138-140].

Polymorphisms of the GR gene may be an important marker for diseases of metabolic origin.

1.9 NUCLEAR RECEPTOR INSENSITIVITY SYNDROMES

Hormone resistance is a condition caused by a reduced or absent end-organ responsiveness to a biologically active hormone, which may be due to alterations in the hormone receptor or in the signalling pathways down streams of the receptor (post-receptor alterations). Apart from the GR insensitivity syndrome that will be discussed below, nuclear receptor insensitivity syndromes have been reported in the thyroid hormone receptor, androgen receptor, mineralocorticoid receptor and vitamin D receptor and will also be briefly be described below.

1.9.1 Primary Generalized Glucocorticoid Resistance

1.9.1.1 Patophysiology

Primary glucocorticoid resistance (PGGR) is a rare familial or sporadic syndrome characterized by decreased sensitivity to cortisol signalling. The patients have compensatory elevations of cortisol and ACTH, which maintain circadian rhythmicity

but show resistance to adrenal suppression by dexamethasone in the absence of the clinical stigmata of Cushing´s syndrome. The affected subjects are mostly adults and do not demonstrate overt clinical signs of cortisol hypo/hypercortisolism. The elevated cortisol concentrations are appropriate in these patients. However, the compensatory increase in corticotropin releasing hormone (CRH), adenocorticotropin hormone (ACTH) and cortisol production is often accompanied by increased secretion of glucocorticoid precursors with mineralocorticoid activity, e.g. 11-deoxycorticosterone, corticosterone, aldosterone and increased secretion of adrenal androgens such as dehydroepiandrosterone, androstendione which could be converted in the periphery to the more potent androgen testosterone. *Figure 8.*

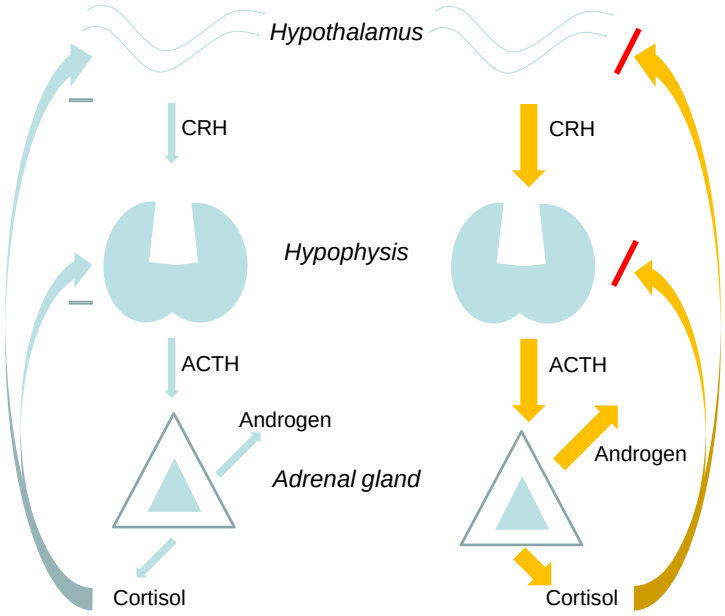

Figure 8

Schematic drawing of the normal HPA axis negative feedback mechanism and the pathophysiological HPA axis seen in PGGR. Elevations of cortisol, ACTH and CRH lead to increased secretion of glucocorticoid precursors with mineralocorticoid activity, e.g. 11-deoxycorticosterone, corticosterone, aldosterone and increased secretion of adrenal androgens such as dehydroepiandrosterone, androstendione which could be converted in the periphery to the more potent androgen testosterone

1.9.1.2 Symptoms

PGGR is seldom recognized due to the diffuse nature of the symptoms. The clinical manifestations of PGGR reflect the pathophysiological manifestations mentioned above and vary from fatigue, hypoglycaemia, hirsutism, oligomenorrhéa, infertility, obesity, pubertas praecox to severe hypertension with hypokalemic alkalosis [141-143]. The

manifestations are mostly due to mineralocorticoid and/or androgen excess. The secondary overproduction of adrenal androgens gives rise to signs of hyperandrogenism only in females since males have much higher gonadal testosterone production in comparison with the weak androgen produced by the adrenals. Clinical manifestations of GC deficiency has been reported in one child with hypoglycaemic seizures and in a newborn baby presenting with hypoglycaemia, excessive fatigability during feeding [143-145], as well as in a few adult patients with chronic fatigue [146, 147]. Fatigue may stand for an insufficient overproduction of cortisol leading to a de facto hypocortisolism, probably due to a post-receptor defect.

The spectra of symptoms are broad, ranging from most severe to mild forms with some patients being asymptomatic. The variance in the phenotype could be due to variations in tissue sensitivity of cortisol/androgen and mineralocorticoid sensitivity in receptor pathways and/or activity of key activator enzymes such as 11 HSD [148].

PGGR has been reported in only a limited number of cases since the first patient who suffered from hypertension and hypokalemic alkalosis, was described in 1976 by Vingerhoeds et al [149].

1.9.1.3 Molecular defects up to date

PGGR has been linked to biochemical distortions as well as to genetic alterations of the glucocorticoid receptor (GR). Cloning of the hGR cDNA and structural characterisation of the hGR gene [72] have made it possible to search for mutations that might correlate to specific phenotypes within the primary glucocorticoid resistance syndromes. Glucocorticoid resistance associated to mutations in the gene for the GR has been documented in 14 patients each having a different mutation [146, 147, 150-161].

Thus 11 mutations and three splice site deletions have hitherto been found, one within the DBD and the others within the LBD. *Figure 9.*

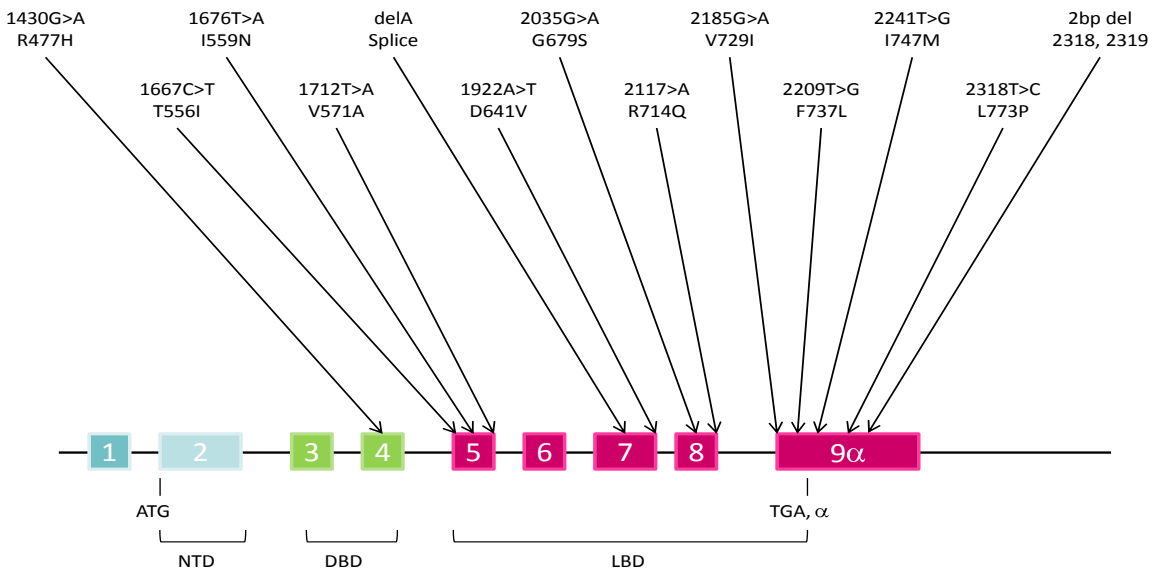

Figure 9

Schematic drawing of the hGRα and the mutations described associated with PGGR.

Four of the 11 mutations have been homozygous and seven heterozygous. No connection between severity of symptoms and homo/heterozygosity has been seen. When tested in vitro all mutations showed, when compared to wild type (wt) receptor a reduced ability to transactivate glucocorticoid-responsive genes in response to cortisol. All mutant receptors where the mutation lies in the LBD, i.e. all but one, showed a reduced affinity for ligand. The R477H, the only mutation situated in the DBD demonstrated a normal ligand affinity. Most mutant receptors were observed in the cytoplasm in the absence of ligand. When exposed to dexamethasone the mutants showed a slow translocation to the nucleus ranging from 20 to 180 minutes compared to the wt that only required 12 minutes for its complete translocation. All mutants except the DBD mutant R4777H showed a preserved ability to bind to DNA, whereas the R477H mutant showed a reduced ability to bind to DNA. [144, 145, 151, 152, 154-163].

Because of the large variation in clinical manifestations of PGGR it has been speculated that the disease may be occurring more frequently than previously thought. In 2000 Huizenga et al identified five patients with clinical and/or biochemical PGGR that did not show any alteration in the glucocorticoid receptor gene [164]. The

explanation might be found in alterations in pre-receptor binding, or alterations in conformational changes that are required for a functional receptor as well as alterations in various post-receptor steps.

1.9.1.4 Treatment

The aim of the treatment of PGGR is to suppress the excess secretion of ACTH, thereby suppressing the increased production of adrenal steroids with mineralocorticoid and androgenic activity. Treatment is preferably done with synthetic glucocorticoids such as Dexamethasone. In one case, the patient identified with I559N mutation, later developed an ACTH-secreting adenoma which might have been due to an over stimulated HPA-axis [158].

1.9.2 Other nuclear hormone resistance syndromes

The nuclear receptors share the same functional structure, an N-terminal domain, a DBD and a LBD. They bind to ligand and form homo or hetero-dimers and interact in the nucleus with specific responsive elements. They are all regulated through a feed-back mechanism. Insensitivity syndromes caused by mutations in the genes coding for the nuclear receptors described below have also been reported. The insensitivity syndromes vary with respect to frequency, inherital pathway, severity and molecular pathophysiology.

1.9.2.1 Androgen insensitivity syndrome

The androgen insensitivity syndrome (AIS) is an X-linked recessive disorder in which affected males are underandrogenized despite normal to high levels of androgens. The first patient was described in 1802, “woman with testes” [165].
AIS results from the incapacity for testosterone and dihydrotestosterone to virilise male embryos and is mainly attributed to molecular defects of the androgen receptor (AR). The degree of AIS varies from a complete form, CAIS, which is characterised with female exterior genitalia to partial, PAIS, which is characterised by a broad spectrum of symptoms such as hypospadias and a micropenis to the mild form, MAIS, where the patients suffer from infertility [165]. More than 800 different mutations have hitherto been found in the androgen receptor. [166]. 25% of the mutations are localised on five amino acid position, hot spots [166]. 30% of the mutations are de novo mutations [167].

Approximately 40 % of the reported patients with AIS lack a mutation in the AR gene [166]. In spite of extensive in vitro studies of mutations found in the AR gene it has not been able to establish a distinct correlation between genotype and phenotype [168].

1.9.2.2 Thyroid hormone resistance

Thyroid hormone resistance syndrome (THR) is an inherited syndrome of reduced tissue sensitivity to thyroid hormone, first described in 1967. The hallmark of THR is a raised serum thyroid hormone level associated with non-suppressible thyroid stimulating hormone (TSH). Other clinical signs are goitre, short stature, decreased weight, tachycardia, osteoporosis, hearing loss, attention-deficit hyperactivity disorder, decreased IQ and dyslexia [169].

Two hormone receptor genes located on separate chromosomes, code for 2 different isoforms which in turn generate four different proteins, thyroid receptor, (TR) α1, α2, β1 and β2. TR α1 lacks the C-terminal and thus cannot bind ligand. TR α2 is predominant in brain, bone and heart, whereas TRβ1 is predominant in liver, kidney and thyroid and TR β2 in the hypothalamic-pituitary-thyroid axis.

15 % of patients lack a mutation in either receptor isoforms [170]. To date approximately 170 different mutations in the TRβ gene have been reported and 2 in the TRα gene in more than 500 families [171]. Therefore most patients with THR express euthyroid and hyperthyroid symptoms simultaneously.

Tissues with predominant TRβ expression are in a euthyroid state whereas tissues expressing TRα, such as the heart are in a hyperthyroid state.

Most mutations are found within the LBD situated around three "hot spots" [172]. The clinical manifestations vary between families with different mutations, between families with the same mutation and also between members of the same family with identical mutations [173].

1.9.2.3 Vitamin D –dependent rickets

Hereditary vitamin D resistant rickets (HVDRR) is a rare recessive genetic disorder caused by mutations in the vitamin D receptor (VDR) gene. This defect leads to an increase in the circulating ligand 1, 25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$). Patients with HVDRR exhibit early-onset rickets, hypocalcaemia, and secondary hyperparathyroidism and generally have a history of consanguinity in the family [174]. The first patient was described in 1978 [175]. Since then 57 families have been reported [174].

The first genetic defect on a nuclear receptor was identified in 1988 on the DBD of VDR. Over 34 heterogeneous mutations have been reported since. The mutations are spread over the entire gene [176].

1.9.2.4 Pseudohypoaldosteronism

Pseudohypoaldosteronism (PHA) is a rare heterogeneous syndrome of mineralocorticoid resistance resulting in salt loss resistant to mineralocorticoid treatment. The systemic PHA is caused by mutations of genes coding for epithelial sodium channels and patient are more severely affected than patients with renal PHA caused by mutations in the mineralocorticoid receptor [177]. The clinical spectrum ranges from unaffected patients with only biochemical elevation of renin and angiotensin to patients with hyponatremia, hyperkalemia and metabolic acidosis [178]. Till today 50 mutations have been reported, spread over the whole gene. No genotype-phenotype relationship has been established [178].

2 AIMS

2.1 GENERAL AIM

The general aim of this thesis was to correlate genotype to phenotype in patients with cortisol resistance. In order to approach this objective the specific aims of this thesis were:

2.2 SPECIFIC AIMS

1. To identify mutations and polymorphisms within the glucocorticoid receptor gene in patients with glucocorticoid resistance and in normal young subjects.

2. To characterize mutations found in order to correlate mutation to clinical phenotype

3. To study the sensitivity to cortisol in various cortisol mediated responses in relation to the expression of GR among healthy individuals with high and low cortisol excretion in urine.

3 SUBJECTS, STUDY DESIGN AND METHODS

3.1 SUBJECTS

In *paper I* we studied 12 female patients. All filled the criteria for primary cortisol resistance characterized as an inability to repress endogenous cortisol below 70 nmol/L 8 h after administration of 1 mg Dexamethasone at 23 hours and they all lacked the clinical signs of hypercortisolism seen in Cushing´s syndrome.
Hirsutism was the most common sign that had motivated the dexamethasone inhibition test. Hirsutism was seen in 10 of 12 patients, obesity was seen in 3 patients, fatigue in 3 patients and hypertension in 2 patients.

In *paper III* we further characterized the mutant GR of two of the patients from paper I, R477H and G679S respectively.

In *paper II* we analyzed young healthy individuals, medical students, male and female n=40 and individuals, male and female who were tested for trombophilia but who had a normal protrombin gene, n=40. All the participants samples were deidentified before analysis.

In *paper IV* we analyzed blood samples from healthy young men who were free from any medical illness, organic, endocrinologic or allergic, as determined by history, physical examination and routine clinical laboratory tests. The subjects were instructed to maintain a regular life-pattern during the course of the study, eating normal meals and avoiding extreme physical exercise. From the total of 104 screened individuals the six with the lowest UFC and the six individuals with the highest UFC were selected. The selected 12 men had a mean age of 30±5 years and BMI of 23±2 kg/m^2.

3.2 STUDY DESIGN

In *paper IV* the subjects were admitted to the hospital for 40 hours on 2 different occasions. Blood sampling was initiated at 0800 hours the following day after admission through a previously inserted heparinized needle. Further samples were collected every half hour during 24 hours. The procedure was then repeated after the subjects had pre-medicated with 0.1 mg dexamethasone orally twice daily at 08 h and 20 h for two weeks. The protocol was reversed for 5 individuals after a 6 months wash-

out period. The plasma was kept frozen until analysis of the specific hormones was performed

3.3 METHODS

The methods used in *Paper I, II and III* are visualized in the flowchart below. *Figure 10.*

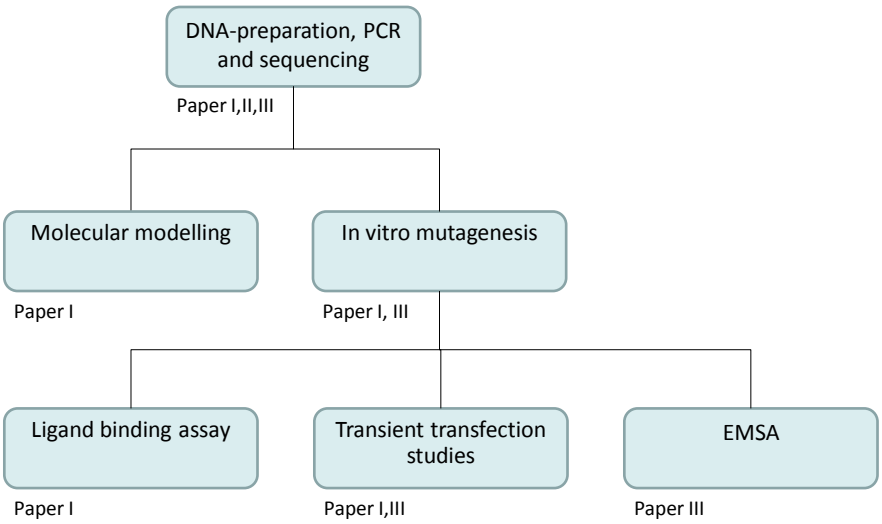

Figure 10

Flowchart of methods used in *paper I, II and III*.

3.3.1.1 DNA preparation, PCR and sequencing

DNA-preparation, PCR and sequencing was performed of exon 2-9 on the 12 patients included in *paper I*. Primers were designed to encompass one exon each, allowing the inclusion of exon-intron boundaries. However, exon 2 was due to its length, divided into 4 parts.

DNA preparation, PCR and sequencing of exon 4, 8 and 9 was done in n=40 + n =40 healthy individuals. When a mutation was found in an exon, both strands of the PCR product were sequenced. Obtained sequences were compared with the published sequence of the hGR cDNA.

The two mutations found were consequently created by in vitro mutagenesis of the wild type GR, expressed in COS-7 cells and thereafter functionally tested.

3.3.1.2 In vitro mutagenesis

To create the mutant GRs Kunkel mutagenesis was used. This is an efficient *in vitro* method to obtain high mutagenesis frequency [179]. The GR template to be mutated is expressed as single stranded DNA from a phagemid vector in a mutant E Coli strain, which allows incorporation of Uracil into the DNA. The method involves annealing an oligonucleotide containing the desired mutation to single stranded DNA from the E. Coli, followed by elongation and ligation to obtain a double stranded plasmid. This plasmid is then introduced to a normal E.Coli strain that degrades the uracil containing strain, leaving the mutation containing strain intact. The mutant strand is thereafter purified by a plasmid preparation.

3.3.1.3 Transacivation studies

The ability to transactivate was tested in COS-7 cells, which do not contain endogenous GR. The receptors are expressed using a CMV vector that is replicated in COS-7 cells. Thus only a small amount of plasmid is needed for transfection. Wt and mutant receptor/s were transfected together with a reporter gene containing two GREs upstream of the luciferase gene. The transfections were carried out using liposomal agents that fuse to the cell membrane and deliver the plasmids into the cytoplasm. Following transfection hormone, either dexamethasone or triamcinolone acetonide was added. After incubation for 24 hours cell extracts were prepared and the luciferase activity was measured by adding luciferase substrate to the cell extracts. A yellow-green light is emitted, and the intensity of this light is measured in a luminometer. The transcriptional activity of GR is directly correlated to the intensity of the emitted light. Luciferase reporter constructs are very sensitive and the assay to measure is fast and simple. A constant amount of βGAL was used to standardize the amount of DNA that actually was transfected.

In *paper I* we transiently co-transfected COS-7 cells with either wt or mutant receptor together with a reporter gene consisting of two GRE:s upstream of the luciferase gene driven by the tk promoter. Dose response curves were then created by adding increasing amount of TA or cortisol. The luciferase activity was measured after

harvesting the cells by adding luciferase substrate. Thus the transcriptional activity of GR is directly correlated to the intensity of the emitted light measured.

In *paper III* we co transfected COS-7 cells with a constant amount of wt and increasing amount of either R477H or G679S mutant together with a luciferase reporter gene. A silent vector PSG stop was added to maintain a constant level of transfected DNA. Luciferase activity was measured as described above.

In *paper III* we also transfected COS-7 cells with a constant amount of Rel-A together with either wt, mutant R477H or G679S respectively in order to study the mutants effect on NFκB activity. The luciferase activity was now transmitted by an NFκB driven reporter gene, which was activated by adding TNFα together with TA to induce also the NFκB reporter gene system.

3.3.1.4 Ligand binding assay

The ligand binding affinity was tested on both the R477H and G679S mutant. Cytosolic COS-7 cell extracts containing either wt or mutants were prepared by lysating and thereafter various concentrations of [^{3}TA] (0,1-4,5nM) were added to the cell extracts and incubating overnight. Bound [^{3}TA] was after being separated from free [^{3}TA] using a gel-filtration column, was then measured in a scintillation counter. The affinity was calculated using Scatchard analysis [180].

3.3.1.5 Electro mobility shift assay

Electro mobility shift assay, EMSA, was used in paper III to study the protein-DNA interaction *in vitro*. EMSAs with whole GR protein are notoriously difficult and do not work well in native systems. When using the DBD, the presence of agonist does not have any impact on the outcome of the EMSA. Purified wt and R477H DBD´s were incubated with radioactively labelled oligonucleotides. Thereafter the DNA-DBD mixture was separated with electrophoresis.

3.3.1.6 Molecular modelling

Molecular modelling of mutant R477H was constructed based on the X-ray structure of GR DBD. The arginine side chain was replaced by a histidine side chain in a standard rotamer conformation using the molecular mechanics program CHARMM.

3.3.1.7 Hormone and blood analysis

Cortisol, leptin, osteocalcin and insulin were analyzed using specific standardized radio immune assay kits. Glucose was measured using automated routine methods.

3.3.1.8 GR mRNA assay

GR mRNA from lymphocytes was retrieved with phenol/chloroform, analyzed after adding a human GR probe and thereafter using hybridization analysis. The quantity and quality of RNA were determined by absorbance at 260 and 280 nm.

The amount of GR mRNA in a sample was calculated from a linear standard curve constructed from incubations with known amounts of *in vitro* synthesized cRNA, complementary to the ^{35}S-labeled probe.

4 RESULTS

4.1 PAPER I

We performed a genetic analysis of the glucocorticoid receptor of 12 unrelated patients with primary cortisol resistance, defined by a pathological dexamethasone suppression test i.e. failure to suppress endogenous cortisol below 70 nmol/L after oral administration of 1 mg Dexamethasone at 23 h. They all lacked the clinical signs of hypercortisolism seen in Cushing's syndrome. The most common symptom was hirsutism seen in10/12 patients. Obesity was seen in 3/12 patients, fatigue was seen in 3/12 patients and hypertension was seen in 2/12 patients.

In two of the patients we found mutations altering the amino acid sequence of the GR, R477H and G679S respectively. Both mutations were heterozygous. A polymorphism at position 766 was found in four other patients changing the triplet AAT to AAC, not altering the amino acid.

The first mutation, R477H, a point mutation at nucleotide position 1430, CGC to CAC, is located in exon 4 and results in the replacement of arginine with histidine at aa 477. The patient having this mutation is referred to as patient 1 in the paper, was at the time of the study 41-years of age. She suffered from notable obesity with a BMI (kg/m^2) of 35, waist: hip ratio > 0,9. Her hirsutism required daily shaving. Her blood pressure was normal.

The second mutation G679S, is also a point mutation, GGT to AGT, at nucleotide position 2035 in exon 8, leading to replacement of glycine with serine at aa 679. This mutation is located in the LBD of the receptor. At the time of this study the patient was 31 years and suffered from hirsutism. Her hirsutism was alleviated with replacement with oral dexamethasone. Her blood pressure was normal.

The polymorphism AAT to AAC at nucleotide position 2226, aa 766, was present in 4 patients. The patients were unrelated and presented with various symptoms. The polymorphism was heterozygous in all four patients.

Using site directed mutagenesis of the hGR in the expression vector pCMVhGR and subsequent expression in COS-7 cells, the function of the GR protein from each of the mutated genes could be explored.

Transactivation capacity was tested, co-transfecting either mutant or wt with a luciferase reporter gene containing two GRE´s. Mutant R477H showed no transactivation capacity at all whereas mutant G679S showed an impaired transactivation capacity having EC_{50} at least 10-fold higher than wt.

Ligand binding affinity of the mutants was tested using cytosolic COS-7 cell extracts and Scatchard analysis. The R477H mutant had the same affinity for the ligand triamcinolone acetonide (TA) as the wt GR. The G679S mutant showed a 50% less affinity to TA compared to wt GR.
A model of the R477H mutant was constructed based on the X-ray structure of the GR-DBD and using the molecular mechanics program CHARMM a histidine chain was replaced with an arginine chain. According to the model the wt GR has direct contact with the phosphate groups of the GRE whereas the R477H mutant as no contact.

4.2 **PAPER II**

To further investigate the significance of the genetic and molecular findings of the above-mentioned mutations R477H and G679S as well as the polymorphism N766N we wanted to study the prevalence of these in a normal population.

We therefore performed a genetic screening of exon four, eight and nine of the glucocorticoid receptor gene since these were the exons were the alterations were located. 40 unrelated healthy individuals as well as 40 unrelated patients being investigated for an unrelated disorder, trombophilia were studied.

PCR and subsequent sequencing of exon four, eight and nine revealed neither of the two mutations. However, the polymorphism in exon 9, N766N, was relatively frequent, found in 12/40 individuals in each subgroup. No other polymorphisms were found in these exons.

4.3 PAPER III

To further study the molecular mechanism underlying the phenotype of the two mutations previously found we studied their effect on glucocorticoid signal transduction.

Our previous molecular modelling of the R477H mutant indicated an impaired DNA binding of this mutant, thus we studied the DNA binding capacity of the mutant R477H compared to the wt GR with electric mobility shift assay (EMSA). The R477H mutant showed a reduced capacity to bind DNA compared to wt GR.

Due to our previous observation of an impaired transactivation capacity of the G679S mutant and neglible transactivation capacity of the R477H mutant we tested the repressive effect of these mutants. Co transfection assays with wt GR and either mutant were performed using a luciferase reporter gene containing two GRE´s. Both mutants inhibited transactivation of the wt GR equally. These findings suggest a dominant negative effect exerted by the two mutants on wt GR transcriptional activity.

Cross-talk between GR and NFκB was studied with transfection studies where a constant amount of RelA was co-transfected together with either wt GR, R477H mutant or G679S mutant respectively together with a luciferase reporter gene containing three NFκB binding sites. There was no significant difference in the capacity to repress NFκB mediated gene transcription between wt and the two mutants.

4.4 PAPER IV

To determine whether differences between healthy individuals in urinary cortisol secretion are associated with differences in tissue specific metabolic responses to short term low dose GC administration and associated with differences in GR mRNA levels in circulating mononuclear cells. Leptin was studied to estimate possible effects on fat metabolism and energy balance. Ostecalcin was analyzed to study possible catabolic effects of GC on bone metabolism. Insulin, glucose and HOMA index were analyzed to study the effect on insulin sensitivity. All these parameters were studied under basal conditions as well as after 2 weeks of low-dose dexamethasone administration.

The subjects were healthy men earlier characterized by their urinary free cortisol (UFC) excretion on two separate occasions. The six subjects with the lowest UFC and the six

subjects with the highest UFC were selected, thus defining a high and low base-line cortisol group.

During basal conditions plasma cortisol was still significantly lower in the low cortisol group. After dexamethasone administration both groups showed significantly lower levels of plasma and urinary cortisol.
Only the low cortisol group showed a significant increase of leptin and a significant decrease of osteocalcin after dexamethasone administration.
In the low cortisol group the GR mRNA levels showed diurnal rhythmicity. Glucose, insulin and HOMA index were similar in both groups and were not altered during dexamethasone administration.

Pat no	Age(y) / sex	dU cortisol	dU aldosterone	Symptoms and signs	S-cortisol 8 h after1 mg of dexamethasone (ref. < 70 nmol/L)	Nucleotide change	Amino acid substitution	Transactivating capacity	Ligand binding capacity	DNA binding capacity	Transrepression capacity	Dominant negative effect
1	41 F	↑	↑	Hirsutism, Obesity	130 nmol/L	CGC→CAC	Arginine→Histidine R477H	No transactivating capacity	No difference in ligand affinity compared to wt GR	↓	intact	+
2	32 F	↑	↑	Hirsutism	680 nmol/L	GGT→AGT	Glycine→Serine G679S	Less transactivating capacity compared to wt GR	2.2 x less ligand affinity compared to wt GR	Not tested	intact	+
3	51 F	↑	↑	Hirsutism, Fatigue	95 nmol/L	AAT→AAC	No aa substitution at position 766	Not tested	Not tested	Not tested	Not tested	Not tested
4	27 F	normal	normal	Hirsutism	105 nmol/L	AAT→AAC	No aa substitution at position 766	Not tested	Not tested	Not tested	Not tested	Not tested
5	24 M	normal	not done	Dilated cardiomyopathy , Gonadotropin and TSH insufficiency	244 nmol/L	AAT→AAC	No aa substitution at position 766	Not tested	Not tested	Not tested	Not tested	Not tested
6	62 F	normal	normal	Florid metabolic syndrome	210 nmol/L	AAT→AAC	No aa substitution at position 766	Not tested	Not tested	Not tested	Not tested	Not tested

Table 1

Summary table of clinical data, molecular findings and in *vitro* characterization of found mutations and polymorphisms presented in paper I and III.

5 GENERAL DISCUSSION

Impairment at any level of the HPA axis, at the CRH level, ACTH level, or adrenal glucocorticoid secretion could lead to overall hypocortisolism, or impairment in local factors affecting glucocorticoid availability and function, including the GR, which can render a state of glucocorticoid resistance by preventing cells and tissues in the body from responding adequately to glucocorticoids.

Once glucocorticoids are released in the blood, the availability at the cellular level is influenced by various factors, including CBG, MDR, and 11β-HSD.

The effect of the glucocorticoid is mediated through the GR. An impaired GR, whether as a consequence of reduced expression, binding affinity to its ligand, nuclear translocation, DNA binding, or interaction with other transcription factors (i.e. NFκB) could also lead to a state of glucocorticoid resistance.
Hence, an observed glucocorticoid resistance can occur through many different molecular defects at various steps in hormone production, signalling or responsiveness.

The work of this thesis has focused primarily on the glucocorticoid receptor in patients with a generalized resistance to glucocorticoids.

At the time of publication of paper I, only three mutations and one splice site deletion had been described in patients with primary generalized glucocorticoid resistance. In 2013, 14 mutations have hitherto been described, although not all have been characterized *in vitro*. All mutations, but one has been reported in the LBD. Two different bp deletions and one point mutation at codon 773 [145, 160, 162] could indicate that this specific region represents a mutation hot spot.

Of the 14 reported mutations I have identified two of them in two different patients, one of the mutations being the only one described in the DBD of the human GR. Both mutations are heterozygous. They differ in clinical presentation as well as *in vitro* characterization. The phenotype of each patient however, correlates to the *in vitro* results of the mutant GRs presented in paper I and III.

The G679S mutation has also been reported by Raef and co workers in a kindred suffering from hyperkalemia and hypertension as well as a history of precocious puberty [181]. High doses of Dexamethasone were needed to partially suppress the endogenous cortisol and improve blood pressure. The difference in severity affecting this patient from the patient described in paper I, who suffered from hirsutism, could be explained by the fact that the severe phenotype was associated with a homozygous mutation whereas the less severe phenotype was associated with a heterozygous phenotype.
All family members with the homozygous G679S mutation demonstrated clinical and biochemical cortisol resistance. In family members with heterozygous G679S mutation and absent ER22/23EK polymorphism had a pathological response to Dexamethasone suppression test, whereas family members with the G679S mutation and the presence of the ER22/23EK polymorphism had a normal response to Dexamethasone suppression test suggesting a possible modulating effect of the ER22/23EK

Much of the work in this field has been done by Chrosous and co workers. Pathological hGR mutants reported in the literature have been functionally characterized by Chrousos and co workers systematically including transcriptional activity, ability to exert a dominant negative effect, ligand affinity, subcellular localization and translocation, ability to bind to GRE, interaction with glucocorticoid receptor protein 1 co activator and motility of the mutant receptors inside the nucleus.
The two novel mutations reported in paper I, have also later been characterized by Chrousos and co workers [182] where they conclude the functional studies of the R477H and G679S mutations presented in paper I; i.e. transactivation and ligand binding results of the mutants. Our findings that the expressed DBD of the R477H mutant showed a decreased capacity to bind to DNA were also in accordance to the findings of Charmandari and co workers. However, the dominant negative effect on the transcriptional activity of wt GR by both the R477H and G679S mutant receptors, reported in paper III, differs from the one demonstrated by Charmandari et al where they did not observe a dominant negative effect on either mutants. This difference may reflect the differences in cell lines as well as reporter genes used.
Furthermore, the ability to repress NFκB mediated gene transcription, which is intact in both R477H and G679S mutant receptors compared to wt GR, has only been addressed in our group.

However, in the group of 12 unrelated patients with primary generalized glucocorticoid resistance described in paper I only two patients were found to have a mutation in the glucocorticoid receptor gene. This indicates that mutations in the GR gene are only one of probably many mechanisms behind the phenotype. As briefly described in this thesis the same applies to the other nuclear hormone receptor resistance syndromes.

Another aspect is that primary generalized glucocorticoid resistance, affecting the effect of a hormone essential for life, so few alteration in the receptor have been found compared to other nuclear receptor resistance syndromes. Most of the mutations described in the GR are heterozygous, thus affecting only one allele which might also contribute a potential under diagnosis of primary generalized glucocorticoid resistance.

Primary generalized glucocorticoid resistance is a rare syndrome although with quite common symptoms such as hirsutism, hypertension and fatigue. One might speculate if the syndrome is more abundant than the literature presents. How often do we perform a dexamethasone suppression test on patients with hypertension if medical treatment works? How many patients with acne and hirsutism do seek healthcare and undergo a Dexamethasone suppression test? The closest estimate is a study by Werner and co workers in which seven of 420 consecutive patients presenting to an adrenal disorders´ clinic had generalized glucocorticoid resistance with functional abnormalities of their glucocorticoid receptor in cultured skin fibroblasts. The predominant symptom among these seven patients was hirsutism [147].

Donner and co workers recently reported a novel two nucleotide deletion in the LBD of the GR causing primary glucocorticoid resistance seen in a patient with hypertension and low plasma renin [162]. In addition, Donner and co workers performed DNA analysis in 51 individuals with hypertension and low plasma rennin activity. No Dexamethasone suppression test was performed prior though. None of the mutations of the GR gene reported in primary generalized glucocorticoid resistance were found among the 51 individuals with hypertension.

The laboratory methods used have since the start of this work developed rapidly and today I would have chosen more efficient screening methods for paper I, II and III such as next generation sequencing, which allows for a rapid sequencing of an entire genome within a week [183].

The promotor region was not sequenced in paper I and the identification of 9 alternative promoters in exon 1 some years ago [73], would indicate that the promoter region should be included when searching for molecular alterations in the genome.

The sensitivity to glucocorticoids varies between individuals but also within tissues. Glucocorticoid resistance can develop in patients on chronic glucocorticoid therapy. Therapeutic benefit of glucocorticoids is also limited by severe adverse side effects.

We studied two subgroups of healthy individuals; high and low secretors of urinary cortisol and the impact of low dose dexamethasone on metabolic parameters and expression of GR mRNA. A low cortisol profile, though still in the normal range, seems to be more sensitive to exogenous cortisol than a high cortisol profile. This could have impact on the response to treatment with exogenous glucocorticoids and the prediction of effect and adverse side effects.

Previous studies in experimental rat models of chronic inflammatory diseases, differences are seen in HPA axis responses to immune stressors. Hyporesponsive rats are associated with increased susceptibility to inflammation as opposed to hyperresponsive rats that are associated with low suceptibility to inflammation [184, 185].

Lekander and co-workers reported low cortisol individuals defined as above, to display a weaker inhibition of IL-6 after a low dose Dexamethasone treatment compared to high cortisol individuals, thus being more sensitive to prolonged glucocorticoid exposure [186]. These results are in accordance to our findings described in paper IV.

The GR mRNA data presented in paper IV represents few observations and accounts only the mRNA levels and not the levels of protein. The possibility of post transcriptional modification of receptor levels cannot be ruled out. Furthermore the solution hybridization analysis used for quantification of GR mRNA would today have been replaced by the more accurate quantitative real-time PCR.

Polymorphisms of the GR gene have been shown to be related to glucocorticoid sensitivity. Whether polymorphisms are present among these individuals and could

account for the differences seen between the two groups of high and low cortisol profiles were not studied.

Glucocorticoids remain the most potent and widely prescribed anti-inflammatory agents worldwide. However, a major factor limiting their clinical use is the wide variation in responsiveness to treatment between individuals, tissues and over time. That fact necessitate high starting doses with slow tapering of the dose in according to clinical response Thus a large proportion of patients are treated with high doses of glucocorticoids over long times with serious side effects as a consequence.

Though more than 30 years of extensive research in the field of molecular mechanism of action of the nuclear receptors, including GR, far from all steps have yet been unveiled. Elucidating the molecular mechanisms may facilitate the development of new glucocorticoids with improved therapeutic indices.

6 CONCLUSION

The investigations presented in this thesis established the following findings:

- Two novel mutations in the human glucocorticoid receptor were identified, R477H and G679S respectively, in two patients with primary generalized glucocorticoid resistance. The R477H mutation is the only reported mutation in the DNA binding domain of the human glucocorticoid receptor.
- *In vitro* characterization of mutant R477H showed an intact ligand binding capacity, severely impaired transactivation capacity and DNA binding, full capacity to repress TNF induced NFκB activity and a dominant negative effect on wild-type GR.
- *In vitro* characterization of mutant G679S showed a reduced transactivation capacity and ligand binding capacity, full capacity to repress TNF induced NFκB activity and a dominant negative effect on wild-type GR.
- *In vitro* characterization of the R477H mutation and G679S mutation correlate to the phenotype of these two patients.
- The mutations were not present when screening a normal population of 80 individuals.
- Healthy individuals with high and low cortisol excretion in urine showed similar metabolic responses prior and after administration of an exogenous glucocorticoid.
- Healthy individuals with low cortisol excretion in urine seem to be more sensitive to exogenous glucocorticoid demonstrated by significant decrease in osteocalcin and increase in leptin and a likely GR mRNA diurnal rhythmicity.

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