Glucocorticoid pharmacogenetics in Addison's disease – The role of the immunophilin FK506-binding protein (FKBP51) for glucocorticoid sensitivity

By

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Summary

There is great variation in the inter-individual sensitivity to glucocorticoids. The immunophilin FK506 binding protein (FKBP51) confers short-loop negative feedback inhibition of the glucocorticoid signalling pathway. FKBP51 keeps the human glucocorticoid receptor (hGR)-protein complex in a state of low hormone binding affinity, and will thus inhibit the effect of glucocorticoids. We investigated the role of FKBP51 for the variation in sensitivity to glucocorticoids in patients with primary adrenal insufficiency (Addison's disease). The specific aim of this study was to evaluate the association between the single nucleotide polymorphism (SNP) rs1360780 in the *FKBP5* gene encoding FKBP51 and the individual glucocorticoid sensitivity in patients with Addison's disease.

Seventeen patients with Addison's disease and 19 controls were genotyped using allelic discrimination assay. In morning blood samples, taken after 18 hours medication fast in the patients, glucocorticoid sensitivity in leukocytes was assessed in an in vitro cell proliferation assay; that is, stimulation with mitogenic lectin phytohemagglutinin (PHA), and incubation with various concentrations of dexamethasone. The *FKBP5* expression and the FKBP51 protein levels in leukocytes was determined before and after intravenous infusion of 100 mg hydrocortisone to the patients; using real-time PCR and Western blot analysis respectively.

The cell proliferation assay points to increased glucocorticoid sensitivity in Addison's patients associated with the rs1360780 variant T-allele (P=0.001). No such association was found for the controls. The *FKBP5* expression, FKBP51 protein levels and ACTH and cortisol levels showed no genotype specific pattern in our study.

Increased understanding of the inter-individual glucocorticoid sensitivity and the mechanisms behind may improve treatment with glucocorticoids and increase the knowledge about the pathogenesis of diseases related to glucocorticoid sensitivity, such as depression and metabolic syndrome. Further research is needed to establish the definitive role of FKBP51 and its isoforms, and the the association rs1360780 with glucocorticoid sensitivity.

Abbreviations

A	
A	Adenine
ACTH	Adrenocortotropic hormone
ADP	Adenosine diphosphate
AF	Activation factor
AIRE	Autoimmune regulator
AP	Alternative promoters
APS	Autoimmune polyendocrine syndrome
ATP	Adenosine triphosphate
B	
BMI	Body mass index
BSA	Bovine serum albumin
BTM	Basal transcription machinery
C	
C	Cytosine
CBG	Corticosteroid binding globulin
CNP	Copy number polymorphism
Cpm	Counts per minute
CRH	Corticotropin releasing hormone
CV	Coefficient of variation
CyP 40	Cyclophilin 40
CYP 450	Cytochrome P450
E	
EDTA	Ethylene diamine tetraacetic acid
D	
DM1	Diabetes mellitus type 1
DM2	Diabetes mellitus type 2
DST	Dexamethasone suppression test
F	
FKBP	FK506-binding protein
G	
G	Guanine
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GRE	glucocorticoid responsive element
H	
HAT	Histone acetyl transferase
HBSS	Hank's balanced salt solution
hGR	human glucocorticoid receptor
HOP	Hsp 90/70 organizing protein
HPA	Hypothalamic-pituitary-adrenal
11β-HSD	Hydroxysteroid dehydrogenase
Hsp	Heat shock proteins
I	
IC50	Inhibitory concentration at 50% inhibition
IDV	Integrated density value
IKK	IκB kinase
IκB	Inhibitor of κB
IL	Interleukin
L	
LD	Linkage disequilibrium
M	
MAF	Minor allele frequency
MAP	Mitogen-activated protein
MKP	MAP kinase phosphatase
N	
NFAT	Nuclear factor of activated T-cell
NFκB	Nuclear factor κB
NLS	Nuclear localisation signal
NO	Nitric oxide
NTC	No template control
P	
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGP	P-glycoprotein
PHA	Phytohemagglutinin
pI	Isoelectric point
PP5	Protein phosphatase 5
PPI-ase	Peptidylpropyl isomerase
PTSD	Post-traumatic stress disorder
PVDF	Polyvinylidene fluoride
R	
Rb-1	Retinoblastoma-1
Rpm	Revolutions per minute
rtPCR	Real time PCR
S	
SEGRA	Selective glucocorticoid-receptor agoist
SNP	Single nucleotide polymorphism
T	
T	Thymine
THE	Tetrahydrocortisol
THF	Tetrahydrocortisone
TNF	Tumor necrosis factor
TPR	Tetratricopeptide repeat
TSS	Transcription start site
U	
UTR	Untranslated region

1 Introduction

1.1 Inter-individual variation in drug response

Individual variation after administration of a drug is seen both in the plasma concentration of the substance and in response to a treatment. The reason for this variance in drug bioavailability and response is thought to be a complex interplay between genetic and environmental factors [1].

1.1.1 Pharmacogenetics

Pharmacogenetics is the study of the genetic variance that gives rise to the different response to drugs. Polymorphisms are variances in the DNA-sequence of a gene, where the less frequent allele is present at a minor allele frequency (MAF) of 1% or greater in a population [1]. Different types of sequence polymorphisms are associated with variation in phenotype, i.e single nucleotide polymorphisms (SNP) and insertion/deletion polymorphisms [1]. The effect of SNPs on protein function is dependent on the localization of the base substitution within a gene, or whether the base change leads to an amino acid substitution or not. A SNP in the coding region of a gene can lead to an altered structure of the protein or even a truncated protein if the substitution produces a stop codon, whereas a SNP in the regulatory regions of a gene (promoter, exon, intron, boundaries and other) can lead to an altered expression of the gene or changes in mRNA-stability [1, 2]. In addition, a SNP can be in linkage disequilibrium (LD) with an unknown allele/SNP. Here, non-random associations exist between Alleles/SNPs at different loci, and the frequency of different haplotypes are therefore not consistent with the haplotype frequency that would be expected from the allele frequency in the population [2]. Polymorphisms are thought to give rise to intra-individual drug response by creating diversity in the proteins involved in the effects of drugs, such as drug transporters, metabolizing enzymes, target receptors and different signal proteins.

Recently, another source of population variance was discovered, that is, variation in number of gene copies [3]. In copy number polymorphisms (CNP), the number of copies of larger segments of the genome is a subject of variation [2]. It is not known to what extent such CNP explains inter-individual variation in pharmacokinetics and pharmacodynamics.

Because polymorphisms are not pathological by themselves, they are inherited from generations to generations. This creates an ethnic diversity, where the polymorphisms differ in their frequencies within human populations [1]. If the population is large, and mating occurs randomly of the polymorphism in question, the allele frequency, and therefore the relative proportion of genotypes will remain constant over time. This is called the Hardy-Weinberg equilibrium [2]. If q is the frequency of allele A and p is the frequency of allele a, the Hardy-Weinberg law states that the frequency of the genotypes AA, Aa and aa is

$$q^2 + 2pq + p^2$$

If the allele frequency in the study population does not comply with the Hardy-Weinberg equilibrium, the study population may not be representative for the whole population. In terms of SNP-studies, this selection bias can cause an incorrect conclusion.

1.1.2 Pharmacokinetics: Drug absorption, distribution, metabolism and elimination

Orally administrated drugs are mainly absorbed in the small intestine, where the drug molecule must transverse the plasma membranes of the epithelial cell layer to reach the systemic circulation. The absorption depends on the chemical properties of the drug, such as molecular size and shape, degree of ionization, and lipid solubility, and whether the drug is absorbed by active or facilitated transport [1].

Drugs absorbed into the epithelical cells in the gastrointestinal tract are subjected to efflux by the p-glycoprotein (PGP). PGP belongs to the superfamily of ATP-binding cassette (ABC)-transporters, and uses energy to function as an efflux pump, transporting drugs back to the intestine. This reduces the intracellular concentration and thereby the bioavailability of drugs. PGP is also present in the liver, pancreas, colon, brain, testis and adrenal glands [4]. Genetic variation in membrane transporters has in recent studies been associated with variation in clinical response [1].

Once absorbed into the epithelial cells in the gastrointestinal tract, the metabolism of drugs by different enzyme systems begin. This involves reactions that convert the hydrophobic drugs into hydrophilic derivates that more easily can be eliminated by conjugation and excretion into the bile or urine [1]. Drug metabolism is grouped into two phases: phase I and phase II

reactions. Phase I reactions involves addition of functional groups, such as -OH, -COOH, -SH, -O- , or -NH_2 to the drug molecule. This is done by several superfamilies of enzymes, among them the cytochrome P450 (CYP)-enzymes, which metabolize the vast majority of drugs. In most cases, the addition of these functional groups leads to an inactivation of the substance [1]. There are great variations in the CYP-enzyme levels between individuals, and this may give rise to inter-individual differences in the capacity of metabolism of drugs [5]. In phase II reactions, a conjugate of the phase I product is formed by the addition of a hydrophilic moiety, for example glutathione, glucuronic acid, sulphate, or an acetyl group. This reaction is performed by different enzyme systems, and leads to improved water solubility and increased molecular weight, which facilitates elimination.

Age, nutrition, liver disease, environmental chemicals and other drugs may influence the concentration and activity of the drug metabolizing enzymes, and can explain some of the inter-individual variation seen in the response to drugs [6].

The enzymes involved in metabolism are found in most tissues, the highest levels however, are present in the liver and the small and large intestine [1]. Orally administered drugs are absorbed by the gut, and transported to the liver by the portal vein. The CYP-enzymes cooperate with PGP in the small intestine to reduce the bioavailability of these drugs [4]. This metabolic processing, together with the first passage trough the liver make up the “first pass effect” seen in the pharmacokinetics of several drugs. Here, the metabolic capacity for the drug may be large, and a great proportion of the drug is metabolised and excreted in the bile before it can enter the systemic circulation and exert its effect [1]. By parenteral administration of drugs, for example intravenous, subcutaneous or inhalational administration, the substances are absorbed directly into the systemic circulation, and the “first pass effect” is bypassed.

Gut micro-organisms are also known to have a metabolic capability for drugs. This includes hydrolysis and reduction transformations in particular, and may influence to which extent the drug is absorbed and which metabolites is formed [7]. Many drugs undergo entero-hepatic circulation, in which the drug is glucuronidated by phase II reactions in the liver and excreted together with the bile in the small intestine. Here, the conjugated drug undergoes cleavage by bacterial glucuronidases, and can be reabsorbed back to the circulation to exert an effect again

if the compound is active [1]. Both the absorption and the entero-hepatic reabsorption is dependent on the individuals gut microbes, and the inter- and intra-individual variation in gut microbe flora can therefore result in different response to drugs.

After absorption of the drug into the systemic circulation, the drug distributes into interstitial and intracellular fluids. This process is dependent on several factors, including regional blood flow and the physiochemical properties of the drug [1]. In the blood stream, many drugs are bound reversibly to plasma proteins. The degree of binding is dependent on the concentration of the drug, its affinity to the binding site and the number of binding sites available on the plasma protein. Only the free fractions of drug escape the blood vessels and reach the target tissue to exert its effect [1]. Albumin is an abundant non specific binding protein in the plasma, which function as a carrier for acidic drugs. In addition, α-acid glycoprotein function a carrier for basic drugs, and several hormone binding globulins bind and carry hormones, such as sex hormone binding globulin (SHBG), thyroxine binding globulin (TBG) and corticosteroid binding globulin (CBG).

Drugs are eliminated from the body either unchanged or as converted metabolites. Whereas the main site of drug metabolisation is the gastrointestinal tract (liver and intestines), the main elimination organ is the kidney, where the drug and its metabolites are cleared from the blood stream and excreted into the urine. Metabolites excreted into the bile are eliminated in the feces, together with orally administered unabsorbed drugs, and drugs that are excreted directly into the intestinal tract and not reabsorbed. Kidney, liver and intestinal diseases can influence the elimination of drugs, and must be considered in pharmacological treatment [1].

1.1.3 Drug-drug interactions

The effect of one drug can be changed by the presence of another drug, both by interfering with the pharmacokinetics and the pharmacodynamics of the drug [8]. Pharmacokinetic interactions are those concerning the absorption, distribution, metabolism and elimination of a drug. Drug-drug interactions affecting the metabolism of a drug are thought to be the most clinically relevant. If two co-administered drugs are metabolized by the same enzyme, for example Tacrolimus (FK506) and glucocorticoids (see Table 2), the competition for the active site can affect the rate of metabolism and thereby increase the plasma concentration of one or both drugs. In addition, some drugs induce the expression of certain enzymes and transport

proteins (PGP) and consequently increase the metabolism and efflux of the substrates, leading to lower plasma levels. Other substances inhibit the action of one or more enzymes independent of being a substrate of the enzyme. This will decrease the metabolism of a substrate, and further result in elevated plasma levels. The transport pump PGP may also be inhibited by several drugs, and this will result in a higher concentration of the substrates of PGP.

Pharmacodynamic interactions involve drugs acting on the same receptors or physiological systems, and may induce additive, synergistic or antagonistic effects of a drug. For example, Tacrolimus (FK506) is known to potentiate the human glucocorticoid receptor (hGR) response to glucocorticoids. This could partly be due to the pharmacokinetic interaction mentioned above, but also a pharmacodynamic mechanism involving FK506 binding protein (FKBP51). FKBP51 renders the hGR complex in a basal inactive state, with low hormone binding affinity and hGR transactivation capacity. It is thought that FK506 increases hormone binding affinity and thus hGR transactivation capacity through displacement of FKBP51 and subsequent recruitment of Protein phosphatase 5 (PP5), an immunophilin known to enhance hGR hormone binding affinity [9].

Interactions may occur in some individuals, but not in others, and the interactions are therefore difficult to predict. Susceptible patients include those using several drugs, patients with renal and hepatic disease, and patients in intensive care or in chronic treatment. Drug-drug interactions are most clinically relevant when drugs with narrow therapeutic range are administered [8].

1.2 Glucocorticoids and glucocorticoid treatment

1.2.1 Glucocorticoids

Glucocorticoids are produced and secreted from the adrenal glands [10]. The adrenal glands are small, pyramidal organs located on the top of each kidney, which consist of an outer cortex and an inner medulla. The inner medulla produces catecholamines i.e. adrenalin and noradrenalin, and releases the hormones upon stimulation from sympathetic nerves. The outer cortex is divided into three layers, each region producing different hormones of steroid nature. The outermost region, zona glomerulosa, produces mineralcorticoids where aldosterone is the principal hormone. Glucocorticoids, of which the most important is cortisol, are produced in the middle region of the adrenal cortex, zona fasiculata. The innermost region, zona reticularis, produces androgen precursor steroids.

The level of cortisol in the blood is regulated by the hypothalamus, the pituitary and the adrenal glands, collectively referred as the hypothalamic-pituitary-adrenal (HPA) axis [1]. The cortisol secretion is dependent on the release of adrenocorticotropic hormone (ACTH) from the pituitary, which is regulated by corticotropin releasing-hormone (CRH) from the hypothalamus. Circulating cortisol in turn acts as a negative suppressor of the CRH- and ACTH-release in the hypothalamus and pituitary, respectively. The level of cortisol follows a diurnal rhythm, maintained by higher neuronal centres, with a peak level in the morning around 08.00. In addition, stress can lead to marked increase in the plasma concentration of cortisol and overrule the negative feedback mechanism.

Subsequently with the circadian rhythm of the cortisol secretion, the sensitivity to glucocorticoids also displays a diurnal variation. In the general population, the glucocorticoid sensitivity is increased in the morning hours compared with that in the evening [11]. In addition, the steroid sensitivity shows great inter-individual variation in healthy subjects, measured by lymphocyte steroid sensitivity, suggesting that up to 30% of the healthy population would fail to respond to steroid therapy [12].

The effects of cortisol are numerous and affect a wide variety of cells and organ systems. The physiological actions of cortisol include adjustment in the metabolism of carbohydrate, protein and fat, which result in increased levels of glucose and fatty acids in the circulation,

and maintenance of normal functions in the immune system, cardiovascular system, the kidney, skeletal muscle, the endocrine system and the nervous system. In addition, cortisol enables the organism to adapt in environmental changes and to resist stressful conditions, such as starvation, trauma, infections and noxious stimuli [1].

In supraphysiological concentrations, as during chronic therapy, the effects of glucocorticoids become more evident. Glucocorticoids have immunosuppressive and anti-inflammatory actions, which are exploited in the treatment of a wide range of conditions (see Table 3). Due to the wide range of target tissues and the non-specific nature of glucocorticoids, they also cause numerous side effects. These effects are further described in section 1.2.4.

Synthetic glucocorticoids, such as prednisolone, metyl-prednisolone, betamethasone and dexamethasone, have enhanced potencies, longer duration and a greater separation of mineralcorticoid and glucocorticoid action, which removes the side effects associated with mineralcorticoid action. The clinical potencies of the various glucocorticoids is dependent on the rate of absorption, the concentration in the target tissues, the affinity for hGR, and the rate of metabolism and clearance [13]. Table 1 lists some of the different glucocorticoids/corticoisteroids available, and their relative potencies and duration of action.

Table 1. Relative potencies and duration of action in different glucocorticoids available. Modified from [1]

Compound	Antiinflammatory potency	Na^+retaining potency (mineralcortidoid effect)	Duration of action
Cortisol/hydrocortisone	1	1	Short
Cortisone	0.8	0.8	Short
Fludrocortisone	10	125	Intermediate
Prednisone	4	0.8	Intermediate
Prednisolone	4	0.8	Intermediate
6α-metylprednisolone	5	0.5	Intermediate
Triamcinolone	5	0	Intermediate
Betamethasone	25	0	Long
Dexamethasone	25	0	Long

Short acting, $t_{1/2}$ = 8-12h; Intermediate, $t_{1/2}$ = 12-36h; Long acting, $t_{1/2}$ = 36-72h

In addition, several glucocorticoids are developed for localized treatment, for example topical, pulmonary and ocular treatment. The advantage of local treatment is that the glucocorticoids are delivered directly to and exert its actions at the side of inflammation, whereas the systemic bioavailability is low. Thus, the side effects associated with systemic accumulation of glucocorticoids are avoided.

Subsequently with its role in treatment, glucocorticoids and dysregulation of the HPA-axis has been associated with the pathogenesis of several diseases, including depression, cognitive disorders (Alzheimer's disease), cancer and metabolic syndrome [14-16]. Both and excess of cortisol and nonsuppression of cortisol production in the dexamethasone suppression test (DST) in depressed individuals have been reported [14, 17]. Normalization of the HPA-axis is also suggested to be the part of the mechanisms of action in antidepressant treatment [18]. In addition, Mifepristone (RU-486), an hGR antagonist used in the treatment of Cushing' syndrome may be used in the treatment of neuropsychiatric disorders, such as depression and Alzheimer's disease [17]. In genetically predisposed individuals, a study/studies suggest that certain environmental triggers could lead to disturbance in the HPA-axis, followed by visceral obesity, insulin resistance and diabetes mellitus type 2 (DM2), typical features of metabolic syndrome [16].

1.2.2 The pharmacokinetics of glucocorticoids

Orally administered glucocorticoids are well absorbed from the gastrointestinal tract, and are classified as class II drugs (low solubility, high permeability) in the biopharmaceutics drug classification system (BCS) [19, 20]. Glucocorticoids are also systematically absorbed from sites of local administration, such as the eye, skin or the lungs. This systemic absorption may lead to systemic effects with prolonged administration, and if occlusive dressings are used or large areas of skin are covered in the case of topical administration [1]. Cortisol binds to CBG, which has a high affinity but low capacity to bind the hormones and to albumin, an abundant, non-specific plasma protein which has high binding capacity, but low affinity for the glucocorticoids. Syntetic glucocorticoids, such as prednisolone also binds to CBG, but others, such as metylprednisolone and dexamethasone bind albumin only [19]. Only free unbound glucocorticoids are biologically active [1, 21].

In order to become biologically active, glucocorticoids with an 11-keto substituent, such as cortisone and prednisone must be enzymatically reduced to an 11β-hydroxy derivate, cortisol and prednisolone, respectively. 11β- hydroxysteroid dehydrogenase type 1 (11β-HSD1) is responsible for this transformation, and is widely expressed in the body. The highest levels are found in the liver, but 11β-HSD1 is also present in lung, adipose tissue, circulatory system, ovary and the central nervous system (CNS) [22]. The opposite reaction, the

conversion from cortisol to cortisone is dependent on 11β- hydroxysteroid dehydrogenase type 2 (11β-HSD2). 11β-HSD2 is mainly expressed in the kidney, but also in other mineralcorticoid target tissues, including colon, salivary glands, and placenta, where its purpose is to prevent the cortisol from binding the mineralcorticoid receptor [19]. As cortisol binds to the mineralocorticoid receptor with similar affinity as aldosterone, the expression of 11β-HSD2 and inactivation of cortisol (and prednisolone) secure the specific action of aldosterone in mineralocorticoid target tissues.

Glucocorticoid metabolism is a two-step process, where hydrogen or oxygen atoms are added in the phase I reaction, and glucuronic acid or sulphate are added in the phase II conjugation reaction [19]. The result is a hydrophilic inactive metabolite, which can be easily eliminated by renal or biliary excretion. The main pathway of cortisol (chemical structure displayed in Figure 1) and cortisone metabolism is the reduction of the C4-C5 double bond to form dihydrocortisol and dihydrocortisone respectively. This is followed by a hydroxylation of the 3-oxo group to form tetrahydrocortisol (THF) from cortisol, and tetrahydrocortisone (THE) from cortisone. THE and THF are rapidly conjugated before they are sercreted in the urine. Furthermore, THE and THF can also be reduced at the 20-oxo group, to yield cortols and cortolones, or be cleaved to the C_{19}-steroids 11-hydroxy and 11-oxo androsterone or etiocholanalone. In the urine, approximately half the secreted cortisol appears as THF and THE, 25% appears as cortols and cortolones, 10% as C_{19}-steroids and 10% as cortolic and cortolonic acid. The remaining metabolites are free, un-conjugated steroids and metabolites from other pathways [23].

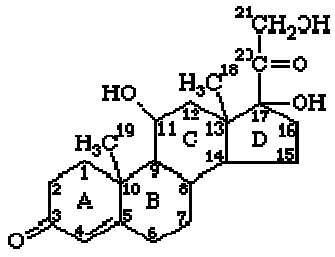

Figure 1. Chemical structure of cortisol/hydrocortisone [24]

Glucocorticoids can also be metabolized by CYP 3A4. Inducers and inhibitors of this enzyme will therefore decrease and increase the plasma concentration of administered glucocorticoids. Table 2 lists some of the substrates, inducers and inhibitors of CYP3A4.

Table 2. Some drug substrates, inducers and inhibitors of CYP3A4, modified from [1]

P450 isoform	Substrate	Inducer	Inhibitor
CYP3A4	Amiodarone	Carbamazepine	Erythromycin
	Terfenadine	Phenytoin	Itraconazole
	Ciclosporin	Barbiturates	Cimetidine
	Oral contraceptives	Dexamethasone	Ketoconacole
	Tacrolimus	Primidone	Fluconazole
	R-warfarin	Rifampicin	Ritonavir
		St John's wort	

1.2.3 Replacement therapy

Glucocorticoids are used in the treatment of adrenal insufficiency, where the adrenal gland is unable to produce adequate amounts of the hormone cortisol [25]. There are two types of adrenal insufficiency, i.e. primary adrenal insufficiency, also called Addison's disease and secondary adrenal insufficiency [26]. Addison's disease has a prevalence of 100-140 per million [27].

In primary adrenal insufficiency, autoimmune inflammation of the adrenal glands (autoimmune adrenalitis) is the cause of about 90-95% of the cases in industrialised countries, with more than 80% of the patients having adrenal cortex autoantibodies or antibodies against 21-hydroxylase [26]. The autoimmune adrenalitis can arise isolated or as a component of autoimmune polyendocrine syndrome (APS) type 1 or 2 [28]. APS-1 is a recessive monogenic disease arising from a mutation in the autoimmune regulator (AIRE) gene, which in addition to Addison's disease characteristically gives rise to autoimmune hypoparathyroidism and chronic mucocutaneous candidiasis. APS-2 is co-appearance of Addison's disease in a cluster of organ-specific autoimmune diseases, most typically diabetes mellitus type 1 and hypothyroidism. APS2 and isolated Addison's disease are caused by a combination of polygenic risk factors and unknown environmental factors. Primary adrenal insufficiency can also be caused by other factors, for example infections such as tuberculosis and human immunodeficiency virus (HIV), single gene mutations, or defects in the steroid synthesis.

Secondary adrenal insufficiency is most commonly caused by chronic therapeutic glucocorticoid administration, where exogenous glucocorticoids induce atrophy of pituitary corticotrophic cells and therefore disrupt the ACTH production. However, this type of adrenal insufficiency is most commonly temporary, and disappears a while after the treatment is

discontinued. Other causes of secondary adrenal insufficiency can be tumours in the pituitary or in the pituitary region, and isolated ACTH deficiency [26].

The symptoms of adrenal insufficiency are rather non-specific, with loss of energy, reduced muscle strength and increased irritability. If the adrenal insufficiency is persistent, it will lead to weight loss, nausea and anorexia or failure to thrive in children [26]
Hyperpigmentation is a specific finding of primary adrenal insufficiency, due to elevation of ACTH, which stimulates the melanocytes. The skin and mucous membranes, especially in areas exposed to sun or friction becomes darker, and typical areas are the hands and mucus membrane in the mouth.

Acute adrenal insufficiency is a life threatening condition characterized by dehydration, hypotension and gastrointestinal symptoms such as nausea, vomiting and abdominal pain. The condition can sometimes follow after rapid withdrawal of glucocorticoids used in high doses or from prolonged periods. The immediate treatment of these patients includes intravenous injection of sodium chloride solution, supplemented with glucose and glucocorticoids.

Chronic adrenal insufficiency is treated with supply of cortisone acetate or hydrocortisone tablets and with hydrocortisone for intramuscular or intravenous administration during intercurrent illnesses. Hydrocortisone is chemically identical to cortisol, but is named hydrocortisone in order to distinguish the endogenous hormone from the substance used in pharmacological treatment [19]. The glucocorticoid replacement is most often given in two or three daily doses, with a half or two-thirds of the dose in the morning to mimic the physiologic pattern of cortisol secretion. Cortisone acetate requires conversion to cortisol by 11β-HSD-1 to become active. The activity of 11β-HSD-1 varies between individuals and cortisone acetate may result in unpredictable effect. However, this is not a common problem when cortisone acetate is used clinically [28]. Both hydrocortisone and cortisone acetate gives high peak concentrations shortly after administration, and the concentration declines rapidly to only just measurable concentrations after only a few hours. Long-acting glucocorticoids, such as prednisolone and dexamethasone can also be used for replacement. However, they are not recommended due to unfavourable high nigh-time glucocorticoid activity, and adverse effects such as insomnia and weight gain [26, 28].

Mineralocorticoid deficiency occurs due to lack of aldosterone production in the zona glomerulosa and is only present in primary adrenal insufficiency. The lack of aldosterone leads to dehydration and hypovolemia, resulting in low blood pressure, postural hypotension and sometimes prerenal failure. 9α-fludrocortisone is a synthetic mineralcorticoid that is used for mineralocorticoid replacement in primary adrenal insufficiency. The need for mineralcorticoid is related to intake and loss of electrolytes, and is usually given in doses of 0.05-0.2 mg once daily.

1.2.4 Pharmacological treatment

Glucocorticoids act anti-inflammatory and immunsuppressive by inhibiting leukocyte functions [1]. The number of lymphocytes is reduced and the immune response is altered in response to glucocorticoids. Thus, glucocorticoids can prevent or suppress inflammation in response to multiple provoking stimuli, such as radiant, mechanical, chemical, infectious and immunological events. In addition, glucocorticoids can be used to treat diseases caused by undesirable immune reactions, for example auto-immune diseases and transplant rejections. This makes glucocorticoids an important and frequently used class of drugs, despite the fact that glucocorticoids do not address the underlying cause of disease. Table 3 list some of the indications, where glucocorticoids are utilized.

Table 3. Indications for glucocorticoid treatment [1]

Systemic treatment	Local treatment available
Organ transplantation	Allergic diseases
Gastrointestinal diseases	Bronchial asthma and other pulmonary diseases
Renal diseases	Ocular diseases
Infectious diseases	Skin diseases
Hepatic diseases	Rheumatic disorders
Malignancies	
Cerebral edema	

The use of glucocorticoids is limited by the wide range of side effects associated with prolonged pharmacological treatment [13]. Side effects can both occur from continuous use of supraphysiological doses of glucocorticoids, and from withdrawal of steroid therapy [1]. Rapid withdrawal of glucocorticoids after prolonged therapy, where the HPA-axis has been suppressed, can lead to acute adrenal insufficiency. A flare up of the initial condition is also a regular problem associated with glucocorticoid withdrawal.

The side effects correlated to therapeutic use of glucocorticoids affect several tissues and organ systems [13]. The most severe effects are seen after systemic treatment; however, side effects can also occur after local treatment.

Prolonged use of glucocorticoids affects the skeleton by decreasing the bone density, leading to an increased risk of osteoporosis. In children, growth retardation and delayed puberty has been seen due to long-lasting glucocorticoid treatment. Muscle atrophy and myopathy, leading to generalized weakness has also been reported as a side effect of glucocorticoids. This can be prevented by physical exercise.

Glucocorticoids also affect the central nervous system, provoking psychiatric symptoms such as mood swings, euphoria, depression and suicide attempts. Use of glucocorticoids also increases the risk of atherosclerosis, coronary artery disease and cardiovascular morbidity and mortality, caused by hypertension, dyslipidemia and reduced fibrinolytic potential.

Metabolism and the endocrine system are disturbed by glucocorticoids. Altered glucose metabolism can lead to hyperglycemia and induce DM2, or worsen the glycemic control in existing diabetes. Furthermore, supraphysiological concentrations of glucocorticoids both result in decreased β-cell insulin production and insulin resistance. Effects on the metabolism and endocrine system lead to the typical Cushinoid characteristics, with moon face, buffalo hump and central obesity. In addition, side effects in the gastrointestinal systems are seen, such as peptic ulcers, upper gastrointestinal bleeding and pancreatitis.

The effects on the immune system can also result in adverse effects. Use of glucocorticoids increase the risk of complicated infections, and the therapy can also mask infection symptoms which in turn prevents clinical recognition.

In both systemic and topical treatment, glucocorticoids can lead to skin atrophy, where the skin becomes thin and fragile. In the eye, therapeutic use of glucocorticoids can lead to the development of cataract and glaucoma, and eye infection especially after topical treatment. In addition, glucocorticoids can lead to disturbed wound healing in skin, and oral candidiasis after inhalation therapy.

1.2.5 New developments

The broad spectrum of side effects seen in the treatment with glucocorticoids demonstrates the need for more optimized anti-inflammatory and immunsuppresive treatment. Targeted delivery of conventional glucocorticoids and the development of new drugs, such as nitrosteroids and selective glucocorticoid-receptor agonists (SEGRA) are different approaches to optimize glucocorticoid therapy [29].

Targeted delivery of conventional glucocorticoids utilizes liposomes as a carrier system for glucocorticoids. Liposomes are small vesicles about 100 nm in size, which will accumulate at the site of inflammation. Glucocorticoids encapsulated into liposomes can therefore give a high local concentration of glucocorticoids directly to their local site of action. Because the glucocorticoid is encapsulated, it is assumed that the occurrence of side effects will be reduced. This targeted delivery of glucocorticoids has shown promising results in experimental animal models of arthritis in rats [30], but is not tried in humans.

The nitrosteroids are new agents composed of conventional glucocorticoids linked to nitric oxide (NO) via an aliphatic or aromatic molecule. NO is slowly released from the glucocorticoid and acts anti-inflammatory in addition to the glucocorticoid. Due to synergy, the anti-inflammatory effect in animal models is up to tenfold compared to the glucocorticoid alone. Therefore, the total dose of glucocorticoids can be reduced, with subsequent reduction in the adverse reactions. The nitrosteroids are not jet tried on humans.

Another group of new agents is the selective GR agonists (SEGRA). It is thought that the anti-inflammatory effects of glucocorticoids are primarily mediated by a transrepression mechanism rather than a transactivation mechanism via DNA-binding (see section 1.3…) [13] SEGRA utilizes this by activating predominantly the desired transrepression mechanism. Thus, adverse effects such as diabetes mellitus and glaucoma, which are mediated through the transactivation mechanism, are avoided. At present, numerous SEGRA are being investigated by cellular in vitro test for hGR-mediated transactivation and repression, followed by various animal models to discover alterations in side-effects [31].

1.3 hGR and intra-cellular glucocorticoid response

1.3.1 Characterisation of the human glucocorticoid receptor (hGR)

Glucocorticoids exert their effect mainly trough the human glucocorticoid receptor (hGR), which unbound to ligand is located in the cytosol. Upon binding of a glucocorticoid receptor agonist, the hGR translocates into the nucleus, and acts as a transcription factor regulating gene expression (section 1.3.3).

There is one known gene for the hGR; it consists of 10 exons, spanning a 110kb genomic region, and it is located at chromosome 5q31-32 [32] [33]. Exon 1 exists in different isoforms (1A, 1B and 1C) [34], and corresponds to the 5' untranslated region of the protein. Exon 2 represents the N-terminus of the receptor, which include the activation factor 1 (AF1) important in transcriptional activation of target genes. Furthermore, exon 3 and 4 separately encode two zink finger motifs involved in binding to the glucocorticoid responsive elements (GRE) in the promoters of glucocorticoid responsive genes. The ligand-binding domain and the ligand-dependent AF2 transactivation domain, together with the 3' untranslated region are encoded by a total of 5 exons (exons 5, 6, 7, 8, 9α or 9β) [32, 35]. The structural organization of the hGRα-protein is shown in Figure 2.

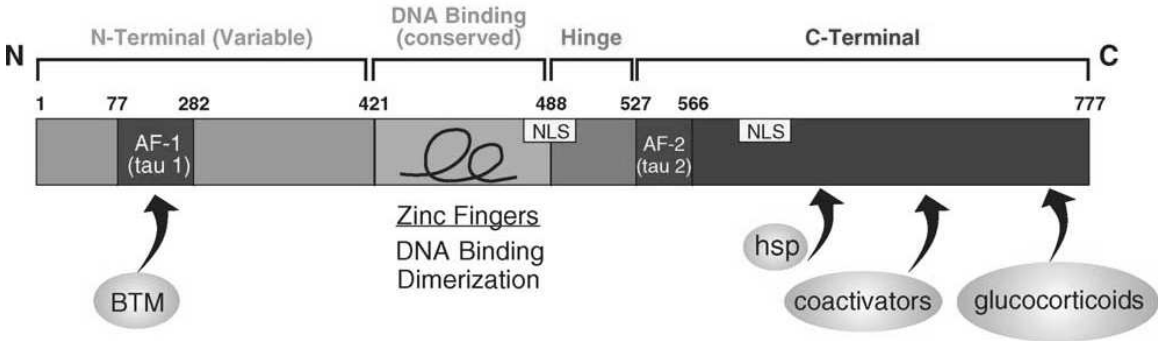

Figure 2. Structural organization of the hGRα protein. AF, activation factor; BTM, Basal transcription machinery; NLS, nuclear localization signal; hsp, heat shock proteins [36]

The hGR-gene is subject to alternative splicing and promoter usage [37], giving rise to multiple mRNA transcripts. Three alternative promoters (promoter 1A, 1B and 1C) each control a unique isotype of exon 1, which despite the missing protein information play an important role in controlling cell-type specific hGR gene expression [37]. Alternative splicing

has been seen in exon 9, resulting in two mRNAs encoding hGRα and hGRβ respectively [32]. The hGRα- isoform binds glucocorticoid ligand and alter transcription of target genes as described in section 1.3.3, whereas hGRβ is thought to work as a dominant inhibitor of hGRα's effect, by making an hGRα-β hetero complex that is not trancriptionally active. This has led to the hypothesis that the cellular ratio of hGRα to hGRβ may determine the cell's sensitivity to glucocorticoids [38]. However, recent work indicates that hGRβ expressed in the absence of hGRα can regulate gene expression. Binding of hGRβ to the glucocorticoid antagonist Mifepristone (RU-486) reduces this gene expression capacity [39].

At the translation level, leaky ribosomal scanning [40] is responsible for the formation of two different isoforms of hGR: hGR-A and hGR-B. These isoforms exhibit similar distribution within the cell and both induce transactivation via ligand. The transrepression activity are similar for the two isoforms, but hGR-B is nearly twice as effective in transactivation compared to hGR-A [37]. In addition, post translational modifications generate further complexity among the different isoforms of hGR. Studies indicate that these modifications have profound effect on the receptor's transcriptional activity and gene specificity, receptor turn over and stability, and the sub-cellular localization of the receptor [37].

In the gene coding for hGR, several polymorphisms has been described [35, 41]. For example, the N363S-polymorphism (rs6195), where the asparagines amino acid is substituted with serine at exon 2, has been shown to correlate with increased sensitivity to glucocorticoids and thus more pronounced glucocorticoid effects [41, 42]. This involves enhanced insulin response to dexamethasone (hypersensitive insulin secretion), more body fat and a tendency to decreased bone mineral density [43]. Obesity and hypersensitive insulin secretion is, together with increased cholesterol levels, hypertension and insulin resistance typical characteristics of metabolic syndrome. Other studies however, do not find an association between the polymorphism and metabolic syndrome [44] or obesity [45]. In addition, the *Bcl*I-polymorphism, which is a restriction fragment length polymorphism (RFLP) located at intron 2 in the gene for hGR, has also been related to metabolic syndrome However, contrasting data exist, and it is unclear whether the polymorphism or other factors such as age are responsible for the metabolic differences and body composition among *Bcl*I carriers [41, 46].

ER22/23EK (rs6189 and rs6190) is another polymorphism in hGR, possible involved in glucocorticoid resistance [42]. The ER22/23EK polymorphism consists of two linked single-nucleotide mutations in exon 2 of the hGR. The second mutation causes an amino acid change from arginine to lysine, while the first mutation does not induce changes in the amino acid sequence. In relation to glucocorticoid sensitivity, this polymorphism seems to be associated with relative glucocorticoid resistence and a healthier metabolic profile, as indicated by the lower cholesterol levels and increased insulin sensitivity. Furthermore, this polymorphism is also associated with other favourable factors, such as a beneficial body composition at young age, and a lower risk of dementia and increased survival in the elderly [41] The ER22/23EK polymorphism have also been related to a faster clinical response to antidepressant treatment [47]. The relative glucocorticoid resistance may be caused by an increased expression of the translational isoform hGR-A, which is less transcriptionally active than the hGR-B [37, 48]

Furthermore, a ATTTA to GTTTA-SNP in the 3'end of exon 9β has been described [49]. This corresponds to a part of the 3' untranslated region (3'UTR) of the GRβ mRNA splice variant, and the polymorphism results in increased stability of the GRβ mRNA and enhanced GRβ protein expression. Increased levels of GRβ may result in greater inhibition of GRα transcriptional activity, and therefore cause glucocorticoid insensitivity. The study of Syed et al. shows that this polymorphism is associated with reduced central adiposity in women and a more favourable lipid profile in men, and suggests that the polymorphism reduces the adverse effect of glucocorticoids on fat distribution and lipid metabolism.

Mutations leading to cortisol resistance have also been described for hGR [35] These mutations are rare, but are leading to more severe cases of glucocorticoid resistance, where the HPA-axis compensate for the resistance by producing more glucocorticoids. This result in condition ranging from completely asymptomatic to severe hyperandgenism in females, fatigue and/or mineralcorticoid excess leading to hypertension and hypokalemic alkalosis [41, 50].

1.3.2 hGR•hsp90 heterocomplex assembly

When hGR is unliganded it resides in the cytosol associated with several other proteins, forming a multi-protein complex [51]. The function of these proteins is to stabilize the protein in an inactive, ligand-activable state, protect the receptor against degradation and to facilitate movement of the complex to the nucleus.

The active hGR protein complex is formed in a dynamic process, involving several ATP- and K^+ dependent steps [52], see Figure 3.

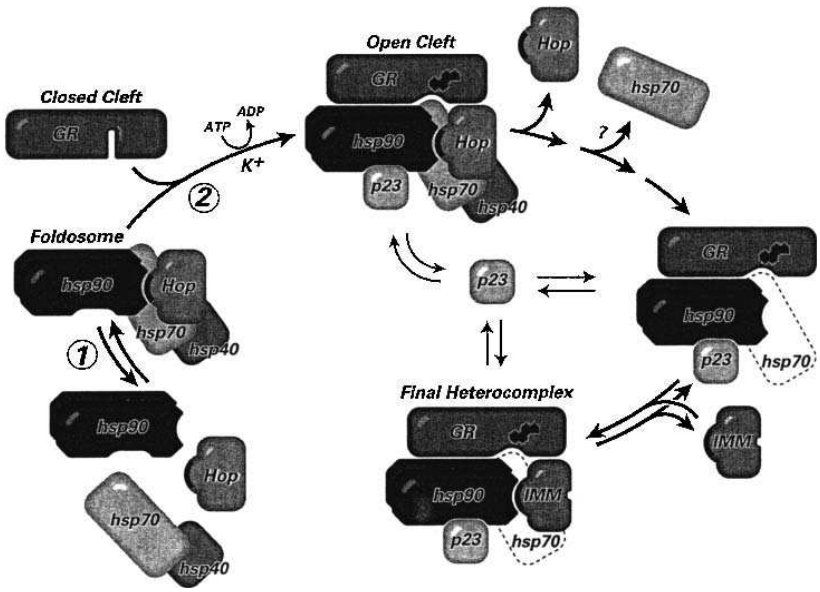

Figure 3. hGR•hsp90 heterocomplex assembly. GR, glucocorticoid receptor; hsp, heat shock proteins; Hop, hsp organizing protein; IMM, immunophilin [52]

First, hGR, hsp40 and hsp70 in ATP-bound state assemble in a complex, and thus preparing the receptor to be activated by an hsp90 homodimer. The heat shock protein 90 is a ubiquitous protein chaperone, regulating over 100 proteins involved in cellular signalling [51]. The bound ATP is hydrolysed to ADP and hsp90 binds to the hGR in its ligand binding domain. In a rate limiting step, ADP on the hsp90 is exchanged for ATP. This exchange induces a conformational change in hsp90, which in turn provoke a conformal change in the hGR, thus allowing glucocorticoid receptor ligand to bind the hGR. Furthermore, p23 is bound to hsp90, which stabilize the glucocorticoid-hGR complex.

Prior to ATP-binding, hsp organizing protein (hop) is attached to the tetratricopeptide repeat (TPR)-acceptor site on the hsp90. However, hop has a higher affinity for ADP-dependent conformation of the hsp90, and will leave the complex when the steroid cleft is opened. This liberates the TPR-acceptor site to other proteins with TPR-domains, for example the immunophilins. Immunophillins are intracellular proteins that bind immunosuppressive drugs, such as cyclosporin, FK506 and rapamycin. Binding of different immunophilins is thought to give diversity in the response mediated by the receptor, albeit the distinct functions of the immunophilins are largely unknown.

The peptidylpropyl isomerase (PPI) domain of the immunophilin is thought to be responsible for the activity by its capacity to isomerize propyl peptide bonds. Binding of an immunophilin to the hsp90 in the protein complex can affect the receptor's ability to bind ligand. In addition, immunuphilins have a variable ability to bind to the motor protein dynein via the PPI-ase domain, and thus inducing transport to the nucleus and modify transcription of target genes, see Figure 4. FKBP52 and cyP-40 are shown to bind dynein, whereas FKBP51 does not bind, or binds it very poorly. Although the immunophillins bind to the motor protein dynein by the PPI-ase domain, it has been shown that the movement of the protein complex is not dependent on the activity of the PPI-ase [51].

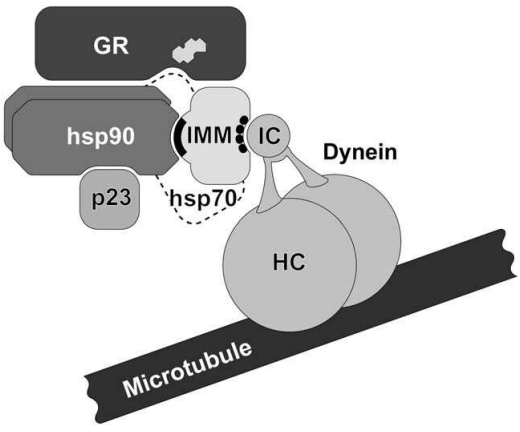

Figure 4. The hGR•hsp90 heterocomplex interacting with the motor protein dynein and microtubule. GR, glucocorticoid receptor, hsp, heat shock proteins; IMM, immunophilin; IC and HC are dynein subunits [51]

1.3.3 Mechanisms of glucocorticoid action

The therapeutic effects of glucocorticoids are thought to be mediated by several mechanisms that involves both genomic and non-genomic pathways [53]. The genomic effects of glucocorticoids are characterized by a slow onset and a prolonged response, because the mRNA transcription and translation is a time-consuming process. Thus, protein levels can be affected after about 30 minutes, and hours or even days are needed to give effects on the tissue or organ level. When it comes to the non-genomic effects however, the response has been detected in less than 15 minutes (Czock, 2005).

The classical genomic glucocorticoid signal pathway involves the cytosolic hGR. Here, binding of a glucocorticoid receptor ligand induces several conformational changes described in section 1.3.2, among them exposal of the nuclear localization signal (NLS). The hGR protein complex is transported along microtubules by the motor protein dynein, and is translocated into the nucleus via importin-α and –β in the nuclear pore [51]. In the nucleus, the chaperones dissociate from the hGR and the hGR homodimerize in order to bind GRE on target DNA. DNA-binding leads to recruitment of several coactivators, which stabilize the hGR-DNA interaction, and initiate assembly of the basal transcription machinery (BTM). In addition, co-activators have histone acetyltransferase (HAT) activity, which opens the DNA-structure for transcription [54]. This mechanism is called transactivation and leads to transcription of anti-inflammatory proteins and regulatory proteins important in metabolism [36, 53].

The hGR can also bind directly to negative GRE's (nGRE) on DNA, inhibiting transcription of inflammatory genes, for example IL-1 and IL-2. The precise role of nGRE's in glucocorticoid effect is however still unclear [53]. Very few glucocorticoid-regulated genes are reported to utilize nGRE, and nGREs may therefore be of minor importance [36].

hGR can influence the transcriptional activity via other transcription factors, both by direct protein-protein binding, and indirectly by modulating the signal pathways involved. The nuclear factor kappa B (NFκB) and activator protein 1 (AP-1) are transcription factors that both are repressed by hGR. NFκB and AP-1 promote expression of several genes involved in the inflammation and in diseases orginating from chronic activation of the immune system, such as asthma, atherosclerosis, inflammatory bowel disease, and autoimmune diseases

including multiple sclerosis and reumatoid arthritis. This involves the expression of several cytokines, chemokines, enzymes and adhesion molecules [55].

Inactive NFκB is localized in the cytoplasm, where the attached protein inhibitor of κB (IκB)-α masks NFκB's NLS, and thus prevents it from translocating into the nucleus and binding to DNA [36, 56]. A wide variety of stimuli can activate NFκB, including proinflammatory cytokines, for example IL-1 and tumor necrosis factor (TNF), by-products from bacterial, fungal and viral infections and some types of radiation (UV, γ). Stimulation from these inducers leads to activation of IκB kinase (IKK), which phosphorylates IκB. Moreover, phosphorylated IκB is ubiquinated by ubiquitin ligase, and this targets the IκB for degradation. Without the IκB attached, NFκB is free to translocate into the nucleus and promote transcription of the inflammatory genes.

Repression of NFκB inhibits immune responses and several mechanisms are involved [36]. First, in the cytoplasm hGR can enhance the activity of IKK; thereby reducing the activity of NFκB. Second, hGR is known to interfere with BTM, interrupting the transcription elongation of NFκB regulated genes. Competition for mutual cofactors is also thought to be a mechanism for hGR's repression of NFκB. Furthermore, hGR can induce histone modifications, making DNA to compact for BTM to bind, and also interfere with proteins associated with NFκB and subsequently prevent NFκB from binding to DNA. In some cell lines, hGR also promotes the production of the NFκB inhibitor IκB, further repressing the activity of NFκB.

The AP-1 complex is activated following a signal cascade, induced by proinflammatory cytokines. This leads to upregulation of the expression of many cytokine genes and tissue destructive enzymes such as collagenase [36]. It is thought that some of the same mechanisms which repress NFκB are involved in repression of AP-1, including direct protein-protein interaction and prevention of binding of AP-1 to DNA. The hGR also induces the transcription of MAP kinase phosphatase 1 (MKP-1), which suppresses the signal cascade needed to activate AP-1, and in addition destabilizes the mRNA of proinflammatory cytokines.

In addition, treatment with glucocorticoids can give rapid outcomes that cannot be explained by the genomic mechanisms [57]. These non-genomic effects are operational in several

tissues, such as muscle, heart, pancreas, adipose tissue, immune system and brain. For example, it has been shown that glucocorticoids induce NO release in the heart, which leads to rapid inhibition of smooth muscle contraction in the trachea. A number of mechanisms for these effects have been proposed [53]. First, cytosolic hGR can inhibit the release of arachidonic acid (AA) from cell membrane-associated phospholipids. AA is an important inducer for cell growth and several metabolic and inflammatory reactions. Furthermore, the release of signalling molecules from the cytosolic hGR protein complex upon ligand binding is also believed to be involved in non-genomic glucocorticoid signalling.

A second hypothesis is that a membrane-associated, G-protein-coupled hGR and intracellular signalling downstream from the receptor can explain some of the rapid effect seen in therapeutic use of glucocorticoids [57, 58]. Membrane associated hGR has been identified in human peripheral blood mononuclear cells (PBMC), and it has been suggested that the membrane hGR is a variant of the cytosolic hGR produced by different splicing, promoter usage or post-translational editing. An up-regulation of the membrane associated hGR is found in cells after immunostimulation and in patients with rheumatoid arthritis [58].

Third, non-specific interaction with cellular membranes, including plasma- and mitochondrial membranes has also been thought to mediate glucocorticoid action. Glucocorticoids at high concentrations have been shown to intercalate into membranes, and in that way changing the physiochemical properties of the membrane and the activity of associated membrane proteins. This physical interaction can affect the immune cells by reducing the calcium and sodium flux across the plasma membrane, which is thought to contribute to immunosuppression and thus reduced inflammatory response. Moreover, direct effect on the mitochondrial membrane can lead to proton leak, which in turn impairs the ATP-production. ATP is essential for the activity of a cell, both in housekeeping activities, and for the specific effector functions of an immune cell, such as migration, cytokine synthesis, phagocytosis and antigen processing and presentation.

1.4 The immunophillin FKBP51

Of the immunophilins, FKBP51 is known to decrease the transcriptional response of hGR, and higher FKBP51 protein levels are associated with partial resistance to glucocorticoids [59, 60]. Its central role as a short-loop feedback inhibitor of glucocorticoid action is evident from microarray studies in leukocytes from Addison's patients before versus two hours after the infusion of hydrocortisone showing that *FKBP5* is the gene most differentially expressed [61]. Furthermore, Woodruff et al. found that *FKBP5* was the most differentially expressed gene in airways epithelial cells; low expression in responders and high expression in non-responders to glucocorticoid treatment in asthma [62].

1.4.1 Structure and function of FKBP51

The FK506-binding protein 1 (FKBP51) is a 51kD immunophilin, which is abundant in many human tissues [63, 64]. FKBP51 resembles the structural organization of FKBP52, where both are composed of four distinct domains [65, 66], see Figure 5.

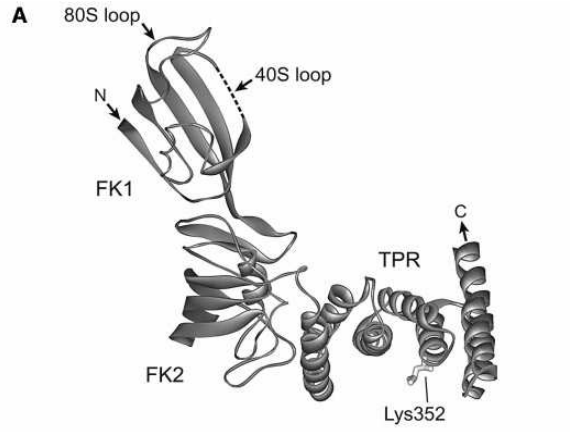

Figure 5. The major structural domains of FKBP51. The FK1-domain includes the PPI-ase. TPR, tetratricopeptide repeat [67]

The first FKBP-domain, named FK1 contains the PPI-ase thought to be involved in hormone binding affinity of the hGR and potential interaction with the motor protein dynein. Moreover, FK1 is also the binding site for the immunosuppressive drug FK506 known as Tacrolimus [68]. The FK2-domain has a similar structure, but in contrast to FK1, FK2 does not exhibit

any PPIase-activity. The TPR-domain is located in the C-terminal of the protein. This domain is made up of three tetratricopeptide repeats involved in protein-protein interactions, including the interaction with hsp90, which links the immunophilins to the hGR protein complex. Although the structural organization of FKBP51 and FKBP52 is similar, the relative orientations of the domains are different, and may explain the differential functions of the FKBPs [65]. In addition, some FKBP-proteins have a calmodulin-binding domain, represented by an amphiphilic α-helix that carries a net positive charge [69].

The FKBPs are named after their ability to bind FK506. FK506 exert its immunosuppressant action by binding to an intracellular FKBP, presumably FKBP12. FK506 and FKBP12 form a complex with Ca^{2+}, calmodulin and calcineurin, which inhibits the phosphatase activity of calcineurin. This prevents dephosphorylation and nuclear translocation of nuclear factor of activated T-cells (NFAT) and inhibits T-cell activation [1]. In addition, FK506 is thought to have a neuroprotective effect, mediated by a calcineurin-independent mechanism [70, 71]. The mechanism is still unclear, but FKBP52 is believed to be involved. In addition, FK506 is known to potentiate hGR-response to glucocorticoids, through displacement of FKBP51 and subsequent recruitment of PP5, as described in section 1.1.3.

Compared to FKBP52, FKBP51 is shown to decrease the hGR affinity to hormone and thus decrease the transcriptional activity of hGR after hormone exposure [59, 60]. This is shown in squirrel monkeys, where high levels of FKBP51 has been suggested to be the origin of compensatory elevated cortisol levels [59, 66], and in mammalian cells, where higher concentrations of cortisol were needed to elicit a hGR response [60].

FKBP51 and FKBP52 compete for binding to the hGR protein complex, and increasing the levels of FKBP52 can mitigate the inhibitory effect of FKBP51. The levels of the respective proteins can therefore determine the response to glucocorticoids [60]. The levels of FKBP51 and FKBP52 do not influence the levels of the hGR, suggesting that the FKBP-proteins does not regulate the degradation (or induction) of hGR [60]. If FKBP51 is attached to the hGR protein complex when a ligand binds, this presumably induces a swapping of the immunophilins, where FKPB51 is replaced with FKBP52 and thus promoting nuclear translocation [72]. However, FKBP51 will keep the hGR-protein complex without hormone in a basal low hormone-binding affinity [67].

In addition to the decreased receptor affinity, the nuclear translocation of hGR is reduced with FKBP51 [60]. This was shown at saturated levels of hormone to ensure that the reduced effect of GR was because of impaired movement, and not a result of the reduced hormone binding affinity. It is thought that the delayed nuclear translocation is a result of FKBP51 not binding the motor protein dynein or binding very poorly [51]. See Figure 4 for the interaction of immunophilins with dynein. Furthermore, studies demonstrate that the expression of FKBP51 is induced by glucocorticoids in a dose-dependent manner and in addition follows a circadian rhythm, with the highest levels of FKBP51 mRNA in the middle of the day. This indicates an autoregulatory mechanism between FKBP51 and glucocorticoids, where FKBP51 may down-regulate the cellular responsiveness to glucocorticoids [62, 73, 74].

1.4.2 Isoforms and single nucleotide polymorphisms (SNP) of *FKBP5*

The gene for FKBP51, *FKBP5* is found on chromosome 6, and consist of 13 exons spanning over a region of 186 kb [75]. Five alternative transcription start sites (TSS) have been identified in *FKBP5*; resulting in five isoforms (in the size range 18-51 kDa) due to alternative promoter usage (AP1-AP5), see Figure 6. The isoform consisting of all 13 exons is wild type 51 kDa FKBP51, whereas the other four isoforms have various truncations in N-terminals. In addition, several other isoforms at the same molecular weight but with different iso-electric points (pI) were identified; some of them found only after cortisol induced augment of the FKBP51 expression [75]. The differences in pI-values indicate posttranslational modifications.

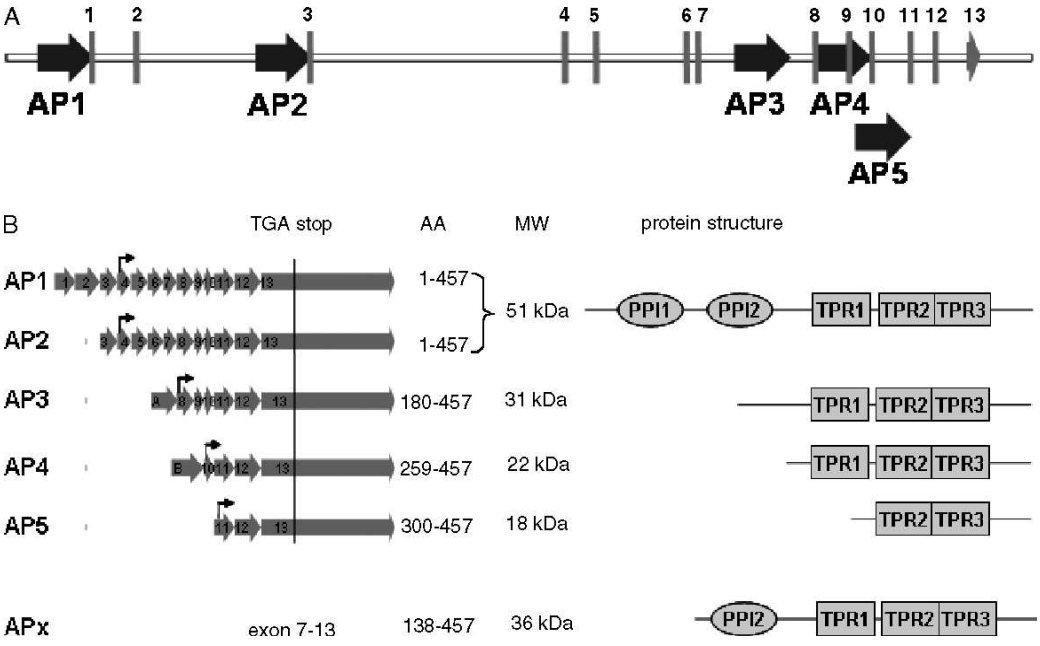

Figure 6. Alternative promoter usage (A) gives rise to different isoforms of FKBP51 (B). The PPI lies within the FK1-domain. AP, alternative promoter; TGA, stop codon; AA, amino acid; MW, molecular weight; PPI, peptidylpropyl isomerase; TPR, tetratricopeptide repeat [76]

Several SNPs in the gene for FKBP51 has been described, and three have been studied in relation to depression, i.e. rs4713916, rs3800373 and rs1360780 [77, 78]. The rs4713916 SNP is located in the promoter region, where the nucleotide change leads to the exchange of guanine (G) for adenine (A). Rs3800373 is found in the 3'UTR, where A is substituted for cytosine (C). We have looked at rs1360780, which is a SNP located in intron 2 in the *FKBP5* gene, and has a MAF of 0.24 in the European population [79]. In rs1360780, the nucleotide change results in a replacement of the base C for thymine (T). Rs1360780 is in strong LD with the rs4713916 SNP [80].

1.4.3 *FKBP5* polymorphisms associated with disease and glucocorticoid sensitivity

The *FKBP5* SNP rs1360780 has been associated with increased relapse of depressive episodes and also rapid response to antidepressant treatment [77]. In this study, the TT variant had more than twice as many depressive episodes compared to the other genotypes, and TT-patients responded earlier on medical treatment, independent on the type of antidepressant used. In addition, the FKBP51 protein levels were significantly higher for the TT genotype,

compared to the CT and the CC-variant. This difference was not shown for the *FKBP5* mRNA-levels. Lekman et al. also found modest evidence for the association of *FKBP5* polymorphisms with treatment response and depression [80]. However, another study was not able to replicate these findings [78]. Nevertheless, FKBP51 might be connected to basic mechanisms of stress related phenomena, as two polymorphisms in *FKBP5*, rs3800373 and rs1360780 were associated with peritraumatic dissociation in medically injured children [81]. Peritraumatic dissociation is a behavioural response to life-threatening stress, characterized by immobilization or freezing, and is a well known risk factor for the development of post traumatic stress disorder (PTSD). In addition, a recent study showed that four SNPs in the *FKBP5* gene, including rs1360780, were predictors of both risk and resilience for PTSD as adult, among survivors of physical and sexual abuse as a child [82].

In a pilot project in our research group, Hammenfors et al found that the expression level of *FKBP5* in leukocytes correlated positively with ACTH levels in seven patients with Addison's disease [83], indicating that this could be an important determinant for individual glucocorticoid sensitivity in these patients. There was also a tendency to both higher levels of ACTH and increased FKBP5 expression in the CC- compared to the CT-variants, indicating a higher sensitivity in CT-subjects. In addition, an association of the rs1360780 SNP with glucocorticoid effects was found in a study of bone metabolism in Addison's disease [84], indicating that carriers of the variant T allele are more sensitive to glucocorticoids than carriers of the wild-type C allele.

1.5 Aim of the study

As reviewed above, there could be many reasons for the great inter-individual variation in glucocorticoid sensitivity. The literature and recent studies indicate that FKBP51 plays a central role in this phenomenon. A better understanding of the factors causing this variation could possibly lead to improved treatment outcomes both in replacement and pharmacological treatment with glucocorticoids. The aim of this study was to evaluate the association between the *FKBP5* SNP rs1360780 and the individual glucocorticoid sensitivity in patients with Addison's disease.

The specific objectives of the study were

- To evaluate whether *FKBP5* genotype is associated with glucocorticoid sensitivity in a cell proliferation assay.
- To evaluate whether the *FKBP5* genotype is associated with *FKBP5* expression and FKBP51 protein levels in leukocytes.
- To determine whether glucocorticoid sensitivity in the cell proliferation assay correlate with the *FKBP5* expression and FKBP51 protein levels.
- To determine whether the ACTH levels in patients is associated with FKBP5 genotype and correlates with *FKBP5* expression and FKBP51 protein levels.

2 Materials

2.1 Chemicals

Table 4. Chemicals

Name	Chemical formula	Manufacturer
Methanol	CH_3OH	Merck
Ethanol	C_2H_5OH	Arcus
Dexamethasone	$C_{22}H_{29}FO_5$	Sigma-Aldrich
Sodium Deoxycholate	$C_{24}H_{39}NaO_4$	Sigma-Aldrich

2.2 Buffers and solutions

BSA-protein standard

Bovine serum albumin (BSA)-protein (Sigma-Aldrich)

Phosphate buffered saline (PBS) (Gibco)

RIPA-lysis buffer

PBS

PBS-tablets (Gibco)

MilliQ-water

RIPA-lysis buffer

1% triton X-100 (Sigma-Aldrich)

0.2% Sodium Deoxycholate (Sigma-Aldrich)

0.15 M NaCl (Ambion)

50 mM Tris base, pH 7.4 (Sigma-Aldrich)

1 µg/ml Aprotinin (Trasylol) (Bayer)

2 mM Ethylene diamine tetraacedic acid (EDTA) (Ambion)

1 mM phenylmethylsulphonyl fluoride (PMSF) (Sigma-Aldrich)

20 U/ml Benzonase nuclease (Sigma-Aldrich)

RPMI medium

RPMI 1640 medium (Lonza)

10 mM Hepes (Lonza)

10% charcoal-absorbed fetal calf serum (FCS) (Lonza)

2 mM glutamine (Lonza)

100 U/ml penicillin (Lonza)

100 mg/ml Streptomycin (Lonza)

1 mM sodium pyruvate (Lonza)

1% non-essential amino acids (Lonza)

Transfer buffer

50 ml NuPage Transfer buffer

100 ml Methanol

1 ml NuPage antioxidant

Milli-Q water to 1 L

Transfer buffer (2 gels)

50 ml NuPage Transfer buffer

200 ml Methanol

1 ml NuPage antioxidant

Milli-Q water to 1 L

2.3 Commercial kit

Table 5. Commercial kits

Reagent kit	Manufacturer	Section/purpose
QIAamp® DNA Mini Kit	Qiagen	DNA extraction
WesternBreeze® Chemiluminicent Western Blot immunodetection kit	Invitrogen	Western blot analysis

Table 6. Buffers and solutions supplied with kit

Name	Description/Contents
QIAamp® DNA Mini Kit	
QIAGEN protease	Protease enzyme
Buffer AL	Lysis buffer
Buffer AW1	Wash buffer
Buffer AW1	Wash buffer
Buffer AE	Eluation buffer
NuPage® SDS Page	
NuPage MOPS running buffer (20×)	Denaturing running buffer for NuPAGE Bis-Tris Gels. 50 mM MOPS, 50 mM Tris base, 0.1% SDS, 1 mM EDTA, pH 7.7
NuPage Antioxidant	For maintaining reducing conditions during electrophoresis and blotting of the NuPAGE gels. N,N-Dimethylformamide, Sodium bisulfite (1:1)
4×NuPage LDS Sample buffer	For preparing protein samples for denaturing gel electrophoresis. 106 mM TrisHCl, 141 mM Tris base, 2% LDS, 10% Glycerol, 0.51 mM EDTA, 0.22 SERVA® Blue G250, 0.175 mM Phenol Red, pH 8.5
10× NuPage Reduction agent	For preparing reduced protein samples for NuPAGE gels.
20× NuPage transfer buffer	For western transfer of NuPAGE gels. 25 mM Bicine, 50 mM Tris base, 0.1 % SDS, pH, 8.4.
WesternBreeze® Chemiluminicent Western Blot immunodetection kit	
Blocker/Diluent A	Concentrated buffered saline solution containing detergent
Blocker/Diluent B	Concentrated Hammersten casein solution
Antibody Wash solution (16×)	Concentrated buffered saline solution containing detergent
Chemiluminiscent substrate	Ready-to-use solution of CDP-star® chemiluminiscent substrate for alkaline phosphatase

Table 7. Other solutions

Name	Contents	Manufacturer	Lot
HBSS	Hanks balanced salt solution	Lonza	
Lymphoprep	9.1 % Sodium Diatrizoate, 5.7 % Polysaccharide	Axis-shield	
Bradford reagent	Brilliant Blue G, Phosphoric acid, methanol	Sigma aldrich	
SeeBlue® prestained Standard (1×)		Invitrogen	LC5625
MagicMark™ XP Western Standard		Invitrogen	LC5602
Microscint™ 0	Scintillation cocktail	Perkin Elmer	

2.4 Enzymes, primers and probes

Table 8. Enzymes

Name	Concentration	Manufacturer	Lot
TaqMan® Universal PCR Master Mix		Applied Biosystem, Roche	J12806
AmpliTaq Gold™	250 Units – 5 U/µl	Applied Biosystem, Roche	HV2860

Table 9. Primers and probes

Name	Concentration	Sequence	Manufacturer	Lot
Allelic discrimination assay				
Fkbp51 probes and primers		c__8852038_10	Applied Biosystems	
Copy number analysis				
Ref.fkbp1 copy forward primer	385.9 µg – 66.6 nmol	CAC-TCC-AGG-TGG-AAC-AAA-C	Eurogentec	794110
Ref.fkbp1 copy reverse primer	306.2 µg – 45.0 nmol	ACT-GAA-ATG-AGC-TGG-ACT-TAA-G	Eurogentec	794111
Ref.fkbp1 copy probe	225.3 µg – 29.6 nmol	CAC-TCC-CTC-ACC-ACA-GTC-A	Eurogentec	794112

2.5 Antibodies

Table 10. Antibodies

Name	Concentration	Manufacturer
Anti FKBP51 mAb[85]	250 µg/ml	Transduction laboratories/BD Biosciences

2.6 Isotopes

Table 11. Isotopes

Isotope	Concentration	Manufacturer
Metyl-[^{3}H]-thymidine	1 mCi/ml	Amersham/ GE Healthcare

2.7 Technical equipment

Table 12. Test tubes

Name	Content	Manufacturer	Section/Purpose
BD Vacutainer® Plus Blood collection tubes	Heparin	BD	Cell assay, Western blot analysis
BD Vacutainer® SST™ II Advance Plus blood collection tubes	Spray coated silica, polymer gel	BD	Serum cortisol
BD Vacutainer® K_2EDTA Plastic Tube	Spray dried K_2EDTA	BD	DNA, ACTH
Tempus™ Blood RNA-tube	6 ml RNA stabilization solution	Applied Biosystems	rtPCR
Salivette	Cotton swab	Sarstedt	Saliva cortisol

Table 13. Gels and membranes

Name	Manufacturer	Cat.nr	Lot
NuPAGE® 10% Bis-Tris Gel 1,5 mm×15 well	Invitrogen	NP0316 BOX	7071373
Immobilon™ PVDF-transfer membranes	Millipore	IPVH07850	KINN9370A

Table 14. Plates

Name	Manufacturer
MicroAmp™ Optical 384-Well Reaction Plate	Applied biosystems
Nunc-96 well transparent plate	Nunc

Table 15. Apparatus

Apparatus	Brand/Manufacturer
Centrifuges	
Eppendorf centrifuge 5417C	Eppendorf
Labofuge 400R	Heraeus
Biofuge Fresco	Heraeus
Other apparatus	
automated harvester	Packard
Vacuum pump RV5	Edwards
Topcount•NXT™	Packard
Tecan infinite 200	Tecan
NuPAGE SDS-page: XCell SureLock™ Mini-Cell and XCell II™ Blot Module	Invitrogen
Hoefer EPS 2A200	Amersham Biosciences
Thermomixer Compact	Eppendorf
FluorChem HD2	Alpha Innotech
ABI 7900HT Genetic Analyzer	Applied Biosystems
Nano Drop® ND Spectrophotometer	NanoDrop technologies

2.8 Computer analysis

Table 16. Programs used

Software	Purpose/section
AlphaEaseFC (FluorChem HD2)	Western blot analysis
Magellan v 6.2	Protein concentration measurement
ND-1000 v 3.3.0	DNA-concentration measurement
TopCount NXT v 1.03	Cell assay
SDS 2.3	SNP and copy number analysis

3 Methods

3.1 Subjects

Seventeen patients verified with Addison's disease were included in the study. The patients were recruited from the National Registry for Addison's disease, and were living in the vicinity of Bergen or Stavanger. Blood samples were collected before and two hours after intravenous administration of 100 mg hydrocortisone, and after having restrained from cortisone treatment for 18 hours. Information about age, diagnosis, weight and treatment were collected from the medical journal.

Nineteen healthy controls were recruited among staff and fellow students at University of Bergen; any disease and use of glucocorticoid treatment being excluding criteria. Blood samples were collected in the morning (8-9 am), and the samples were anonymized. Age and gender was not recorded for the controls due to anonymity reasons.

The subjects, both patients and controls, gave written informed consent and the regional ethics committee approved the study.

3.2 Hormone levels

Venous blood samples were drawn from the cubital vein, into gel and EDTA-tubes (BD) and saliva was collected by Salivette tubes (Sarstedt). At the Hormone laboratory, Haukeland University Hospital salivary cortisol was measured by cortisol enzyme immunoassay for saliva (Diagnostics Systems Labs, Webster TX, USA), and serum cortisol and plasma adrenocorticotropin (ACTH) were measured by immunoassay kits from Diagnostic Products Corp. (Los Angeles, CA, USA). The CV% for the assays are <10%.

3.3 Cell proliferation assay

This assay measures the proliferation of peripheral blood mononuclear cells (PMBC) stimulated by the mitogenic lectin phytohemagglutinin (PHA) by measuring the incorporation of methyl-[^{3}H]-thymidine. Cells are stimulated with PHA and incubated with various concentrations of dexamethasone, which inhibits the proliferation and thus reflects glucocorticoid sensitivity. The protocol was adapted from Vermeer et al. [86]. For the patients, blood drawn before the injection of hydrocortisone was used (0h). Briefly, heparinised whole blood was diluted 1:10 in RPMI-medium, supplemented with 10 mM HEPES, 10 % charcoal-absorbed fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mM sodium pyruvate and 1 % non-essential amino acids, and cultured in a Nunc-96 well transparent plate with PHA and dexamethasone in increasing concentration, ranging from 10^{-10} -10^{-6} M, see Table 17. Each sample was analyzed in triplicates. The cells were incubated at 37ºC in 5% CO_2 and ~90% humidity for 3 days (72 hours).

Table 17. Concentrations of dexamethasone in cell proliferation assay

Well	
1	5 µg/ml PHA
2	10^{-10} M Dexamethasone + 5 µg/ml PHA
3	10^{-9} M Dexamethasone + 5 µg/ml PHA
4	10^{-8} M Dexamethasone + 5 µg/ml PHA
5	10^{-7} M Dexamethasone + 5 µg/ml PHA
6	10^{-6} M Dexamethasone + 5 µg/ml PHA

For the last 6 hours, 25 µl of methyl-[^{3}H]-thymidine diluted in complete RPMI-medium, equivalent to an activity of 0.5 µCi per well was added. The cells were harvested onto 96-well glass fibre filters using an automated harvester and a vacuum pump (RV5, Edwards). After adding 20 µl of scintillation cocktail to each well of the glass fibre filters, the incorporation of methyl-[^{3}H]-thymidine was measured as counts per minute (cpm) using a Topcount•NXT™ scintillation counter (Packard). Values twice as high as the mean of the other parallels, and not consistent with the other concentrations were excluded.

3.4 DNA-analysis

3.4.1 Extraction of DNA from whole blood using QIAamp® DNA Mini Kit

When using QIAamp® DNA Mini Kit, the DNA is extracted in several steps. First, the blood cells are lysed, and the DNA is precipitated and adsorbed to a silica gel membrane. Second, the DNA is washed with several buffers, before the DNA is eluded from the column using elution buffer. The method was conducted according to the manufacturer's manual. In short, 200µl of whole blood, collected in EDTA coated tubes were added to a 1.5 ml microsentrifuge tube, together with 20 µl QIAGEN protease and 200µl Buffer AL. The tube were mixed by pulse-vortexing for 15 seconds and incubated at 56ºC for 10 minutes. 200µl ethanol (100%) was added to the sample, and after mixing, the mixture was carefully added to a QIAamp Spin Column in a 2 ml collection tube. The tube was centrifuged at 8000 rpm for 1 min, and the QIAamp Spin Column was put in a clean 2 ml collecting tube. 500 µl Buffer AW1 and Buffer AW2 were added in turn, each followed by a centrifugation at 8000 rpm for 1 minute and 13000 rpm for 3 minutes respectively. The QIAamp Spin Column was placed in a clean 1.5 ml microcentrifuge tube, and 200µl Buffer AE (elution buffer) was added. After incubation at room temperature for 1 minute, the tube was centrifuged at 8000 rpm for 1 minute, and the filtrate was collected. The DNA yield was analysed using Nano Drop® ND Spectrophotometer (Nano-Drop technology), and the samples were stored at -80ºC.

3.4.2 Detection of SNP using TaqMan® allelic discrimination

In this experiment, genotyping was performed using the 7900HT Fast Real-Time PCR system, the SDS 2.3 software and TaqMan® allelic discrimination assay for the SNP: rs1360780 (*FKBP5*) (Applied Biosystems). In allelic discrimination analysis, PCR amplification is used together with allele-specific probes to determine the presence of one or more SNPs. Two probes are used, one is specific for the wild type allele and the other is specific for the variant allele (assay number: c__8852038_10, Applied Biosystems). Each probe is labelled with its respective fluorescent tag, called the reporter dye, at the 5'end, and a quencher at the 3'end. The wild type probe is labelled to the VIC reporter dye, whereas the variant allele probe is tagged to the FAM reporter dye. The quencher for both the probes is non-fluorescent. As long as the probe is intact, the quencher will remain in close proximity to the reporter dye, and thus eliminate the fluorescent signal. During PCR, the probes will hybridize to its complementary DNA sequence, which are situated between the binding site

for the forward- and reverse primer. The 5'-nuclease activity of AmpliTaq Gold DNA-polymerase cleaves the probe during amplification. This leads to separation of the quencher and reporter dye, and results in a detectable fluorescent signal. The fluorescence from each PCR-cycle is measured and normalized against an internal passive reference (ROX-present in the mastermix), giving a ratio ΔR_n representing a normalized flourescence. The measured fluorescence is plotted against the number of PCR-cycles, giving an amplification plot.

In the PCR-apparatus, the samples were first heated to 50º C for 2 minutes, before denaturation at 95º C for 10 minutes, followed by a PCR-reaction of 40 amplification cycles each at 95º C in 15 seconds and 60º C for 1 minute.

By performing a post-run using the software SDS 2.3, end-point analysis of the signals from the two probe-dyes is performed. The software then generates an allelic discrimination plot (Figure 8) and a list of the SNP identities. If the measured fluorescence from only one of the probes is displayed as a sigmoid curve in the amplification plot, this means that only one of the probes has hybridized to its allele, whereas the other probe has remained intact. Thus, only one allele is present in the sample, and the person is therefore homozygote for this allele. If fluorescence from both the probes is measured and displayed as sigmoid curves however, this indicates that both the probes have hybridized to their respective alleles, and that the person has a heterozygous genotype.

The assay was performed following the recommendations from the supplier, although less volume of the reagents was used. DNA extracted by QIAamp® DNA Mini Kit was diluted to 20 ng/µl and 2 µl was added to a MicroAmp™ Optical 384-Well Reaction Plate using a pipetting robot. Two wells were used as no template control (NTC), containing no DNA. Two µl of Mastermix, containing enzyme, primers and probes (assay name c_8852038_10) was added to the wells, also using a robot.

3.4.3 Copy number analysis

The theory behind the copy number analysis follows almost the same principle as in allelic discrimination although no post-run is performed (section 3.4.2). Here, one probe and its respective fluorescent reporter dye (FAM) are used for the gene of interest (*FKBP5*), and another for a reference gene, retinoblastoma-1 (Rb-1), tagged with HEX, with known copy

number (N=2, i.e. 1 copy in each allele). The determination of copy number is done by comparing the amount of DNA-product from the unknown sample with the amount of DNA-product from the reference, using a standard curve generated from a control sample with known concentrations of DNA, and the C_T-value from the amplification plot. The C_T-value is the cycle number, where the fluorescence from the sample is significantly higher than the fluorescence from NTC, for the first time. If the gene is present in more than one copy in each allele, the result will be proportionally higher level of PCR-product (DNA) compared to the reference.

DNA extracted by QIAamp® DNA Mini Kit was diluted to 15 ng/µl, and 3 µl was dried on a MicroAmp™ Optical 384-Well Reaction Plate. DNA from 196 individuals was analysed, including our subjects. DNA for the standard curve was prepared in five different concentrations ranging from 120 to 7.5 ng/µl using reference DNA. In addition 2 wells were used as NTC, containing no DNA. Eight µl of Mastermix containing enzyme, primers and probes (Sequences forward primer, CAC-TCC-AGG-TGG-AAC-AAA-C; reverse primer, ACT-GAA-ATG-AGC-TGG-ACT-TAA-G; copy number probe, CAC-TCC-CTC-ACC-ACA-GTC-A, see Table 9) was added to the wells, using a pipetting-robot.

3.5 RNA-analysis

Whole blood was collected in Tempus tubes (Applied Biosystems). The RNA extraction and rtPCR procedures were performed by trained personnel at Centre for Genetics and Molecular Medicine, Haukeland University Hospital.

3.5.1 rt-PCR

The *FKBP5* expression before and two hours after 100 mg intravenous hydrocortisone was measured. Results were only available for the 10 first patients. GADPH was used as house-keeper gene control, and the expression was corrected for total serum cortisol: (CTfkbp5/CTgadph)/serum cortisol.

3.6 Protein analysis

3.6.1 Isolation of PBMC from heparinised venous blood

Whole blood was diluted 1:1 in 0.9% NaCl solution (HBSS) and carefully transferred, using a 10 ml pipette, on top of Lymphoprep medium in the proportion of 2:1 into a 15 ml Falcon tube. The tubes were centrifuged at room temperature in a swing out rotor (without brake) at 800 x g for 20 minutes, 30 minutes if the samples were collected more than two hours earlier. After centrifugation, the mononuclear cells could be seen as a distinct band in-between the plasma- and Lymphoprep layers. The cells were removed using a clean Pasteur pipette, and transferred to a clean 15 ml Falcon tube. The harvested cells were diluted in HBSS, and centrifuged at 250 x g for 10 minutes. The supernatant was decanted, and the pellet was resuspended in HBSS, followed by another centrifugation at 250 x g for 10 minutes. This was repeated once, and after the second centrifugation the pellet was dissolved in 1 ml HBSS and transferred to a 1.5 ml microcentrifuge tube. The sample was centrifugated at 20 800 g for 5 minutes, and the supernatant was removed. The cells were resuspended in 15-20 µl RIPA-lysis-buffer and then frozen at -80º C.

3.6.2 Determining the protein concentration in cell-lysate using Bradford Reagent

5 µl of a prepared BSA-protein standard, with concentration ranging from 0.10 to 1.25 µg/µl (see Table 18) was added to a 96 well plate (Nunc), in duplicates for each sample/concentration. Samples with unknown protein concentration were diluted 1:20 in PBS, and 5 µl was added in duplicates to the wells.

Table 18. Concentration of BSA-protein standard

Well	Concentration BSA protein standard (µg/µl)
1	0
2	0.10
3	0.25
4	0.50
5	0.75
6	1.00
7	1.25

250 µl of Bradford reagent equilibrated to room temperature was added to each well being used, and the 96-well plate was mixed on an orbital shaker for approximately 30 seconds. The

samples were incubated at room temperature for 5-10 minutes, before the absorbance at 595 nm was recorded using Tecan infinite 200 (Tecan). A standard curve was prepared plotting the net absorbance versus the protein concentration of each standard, and the protein concentration of the unknown samples by comparing the absorbance values against the standard curve. From the protein concentrations, the amounts of protein solution used in NuPage SDS Page and Western blot analysis were calculated.

3.6.3 NuPage® SDS-Page and Western Blot analysis

In Western blotting, gel electrophoresis is used in order to separate proteins by size, before the proteins are transferred to a membrane. Here the proteins are detected using antibodies specific to the target protein. First, 13 µl of the protein solutions, containing 20 ng protein, together with 5 µl 4x NuPage sample buffer and 2 µl 10x NuPage Reduction agent were added to 1,5 ml microcentrifuge tubes and heated at 70º C for 10 minutes (Thermomixer compact, Eppendorf). The SDS-PAGE apparatus was set up, and the inner chamber was filled with 200 ml NuPage Running buffer containing 500 µl NuPage Antioxidant solution. 15 µl of all the samples were loaded to the NuPAGE® 10% Bis-Tris Gel 1.5 mm×15 well, together with SeeBlue® prestained standard and the MagicMark™ XP Western Standard. The outer chamber was filled with ~600 ml NuPage Running buffer and SDS-PAGE was run at 180 V for 70 minutes.

The polyvinylidene fluoride (PVDF)-membrane (Immobilon™ PVDF-transfer membranes, Millipore) was prepared for blotting by placing the membrane in methanol for 30 seconds, deionized water (milli Q) for 2 minutes, and in transfer buffer for ≥ 5 minutes. Filter papers and blotting pads were soaked in transfer buffer and put in layers into the blotting cassette together with the gel and membrane, as shown in Figure 7. Before the gel, membrane and filter papers was positioned into the blotting cassette, air bobbles were removed using a clean tube. When using two gels, one additional blotting pad were use in order to separate the gels.

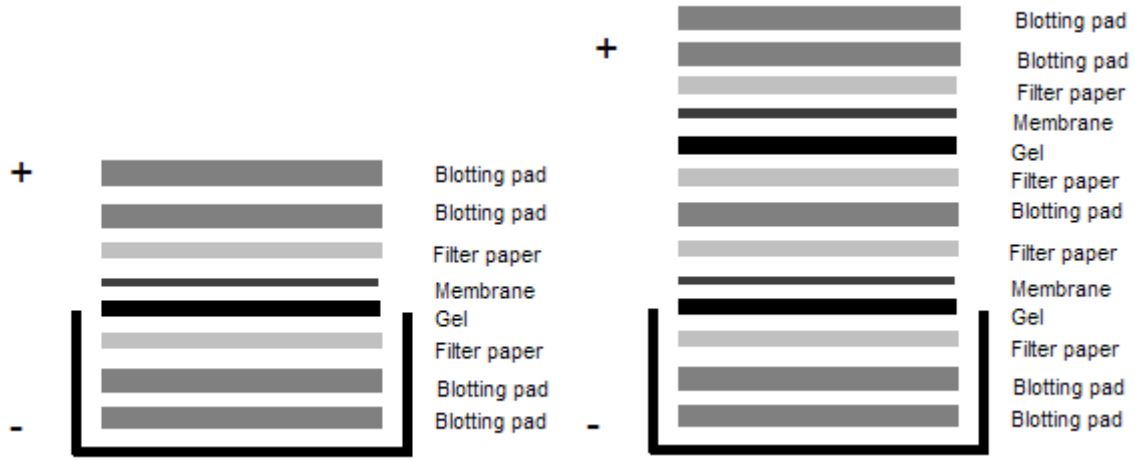

Figure 7. Blotting cassette with one and two gels

The detection of the blot was performed using Western Breeze® Chemiluminescent Western Blot Immunodetection Kit. Here, the blot was run over night, at 10-12 V. After removing the membrane from the blotting cassette, the detection of the protein was performed according to the manufacturer's protocol. In short, the PVDF-membrane was washed twice for 5 minutes in 20 ml pure water. The membrane was incubated in 10 ml of blocking solution on a rotary shaker set for 1 revolution/second, for 30 minutes. After decanting the blocking solution, the membrane was washed twice in 20 ml pure water, and then incubated for 1 hour with 10 ml if the Primary antibody solution (Anti FKBP51 mAb, Transduction laboratories, see Table 10). The membrane was washed in 20 ml Antibody wash four times, each wash for 5 minutes. The antibody wash was decanted, and the membrane was incubated in 10 ml of Secondary Antibody solution for 30 minutes. Again, the membrane was washed four times in 20 ml Antibody wash for 5 minutes, followed by rinsing twice in pure water for 2 minutes.

The membrane was placed in a clean dish, and 2.5 ml Chemiluminescent Substrate was added evenly to the surface. After 5 minutes, the excess Chemiluminescent Substrate was removed from the surface using a filter paper (provided with the kit). Detection of the protein was performed using FluorChem HD2 and the AlphaEaseFC software, according to the manufacturer's manual [87]. The integrated density value (IDV) was determined using Spot Denso and the Autospot function, with the use of automatic background.

The protein concentration for use in Western blotting was optimized by using five different concentrations of protein from the same person, ranging from 5-100 µg/µl (Table 24).

Two independent replications of Western blot analysis were performed for all of the samples, and one of the controls (C1) was included in all of the replications. The IDV-value was measured and adjusted to a % -value of a max response, where the strongest band from the MagicMark™ XP Western Standard was used.

3.7 Statistics

3.7.1 Coefficient of variation (CV%)

The coefficient of variation (CV%) measures variation in a dataset independent of the units used, and can thus compare the variation between different datasets. CV is the standard deviation (σ) divided by the mean (µ) given as percent [88]:

$$CV\ \% = \frac{\sigma}{\mu} \cdot 100$$

3.7.2 Chi-square test

To evaluate the frequency of the genotypes between patient and controls, and between the subjects and the European population, a chi-square test was performed. Here, the subjects are grouped in a table according to their genotype, and the expected number in each group/category (E) is calculated:

$$E = R \cdot \frac{K}{n}$$

A test observer Q was calculated based on the observed number and the expected number (E), and compared with a tabled value.

3.7.3 Mann Whitney U-test

To analyse the results from the cell proliferation assay (section 3.3), a Mann Whitney U-test, which is a non-parametric rank test was performed [88]. For both the patients and controls, two populations were defined, one CC-genotype and one T-allele bearing population (CT and TT-genotype). For the patients and controls respectively, the two populations were pooled and ranked in order according to their position at their inhibitory concentration at 50% proliferation (IC50), in order from the lowest to the highest percent proliferation at 10^{-7} and 10^{-6} M dexamethasone, and at maximum inhibition. A higher ranking number indicate a lower sensitivity to dexamethasone. By using SPSS, the possible difference of the rank-value (W) for each of the group was calculated, and the p-value for the analysis was determined.

3.7.4 Spearman's rank correlation

Like the Mann Whitney U-test, the Spearman's rank correlation is a non-parametric method based on ranks. This involves ranking the values of each parameter independently into order of magnitude, and that the rank rather than the value of outcome is of importance [89]. By using SPSS, the correlation between the different parameters was calculated, and the R- and P-value determined.

3.7.5 Unpaired T-test

In the comparison of different patient characteristics and hormone levels between different groups, an unpaired T-test was performed. Here, the measured values (i.e. hormone levels, daily dose of cortisone) are compared between two groups. Based on the test observer T, the mean values are tested, and defined as similar or dissimilar [88]. In this research, the test was performed using Excel.

4 Results

4.1 Patient characteristics

Patient characteristics, including age, gender, weight, other diseases, the duration of Addison's disease, and the treatment received for Addison's disease are displayed in Table 19. The mean age, duration of Addison' disease and the dosage of cortisone and fludrocortisone are shown for each genotype.

Table 19. Patient characteristics and treatment

Patient no	Gender	Age	Weight (kg)	Duration of AD (years)	Other diagnosis	Treatment	
						Cortisone (mg/day)	Fludrocortisone (mg/day)
CC-patients							
P1	M	78	ND	19		37.5	
P2	M	30	ND	4		50	0.1
P3	F	51	68	14	PA	Cortisol pump	0.1
P4	F	62	ND	16	HT	ND	ND
P5	M	31	ND	5		37.5	0.1
P6	F	51	59	27		25	0.1
P7	F	75	77	1	PA, HT	37.5	0.05
P8	F	62	56	36	HT, V, GF	25	0.1
P9	M	41	57	11	CD, PA	37.5	0.1
Group mean		**53**		**15**		**35.7**	**0.09**
CT-patients							
P10	F	66	68	4		37.5	0.1
P11	F	39	105	4	DM1	37.5	0.15
P12	F	40	62	8	HT	37.5	0.05
P13	F	28	ND	2		20	0.1
P14	M	47	72	8		50	0.15
P15	F	52	61	12	HT	25	0.1
P16	F	43	ND			25	0.1
Group mean		**45**		**6**		**33.2**	**0.11**
TT-patient							
P17	F	88	71	30		43.75	0.05
Group mean CT/TT		**50**		**9**		**34.5**	**0.1**

ND, not detected; AD, Addison's disease; PA, pernicious anemia; HT, hypothyrodism; V, viltigo; GF, gonadal failure; CD, coeliac disease; DM1, diabetes mellitus type 1.

From Table 19, it can be seen that there were more female than male patients (12 females, 5 males) included. The age of the patients ranged from 28 to 88 years (median 51), the oldest being the TT-patient. The CC, CT and TT variants showed no significant variance in the daily dose of cortisone or fludrocortisone (P=0.7 and 0.7 respectively).

4.2 SNP- and copy number analysis

To determine the genotype of the subjects included, an allelic discrimination assay was performed (see section 3.4.2). Most of the patients were genotyped from earlier studies, but the results from the genotyping of the controls and one of the patients are displayed in the allelic discrimination plot in Figure 8. Blue spots indicate the TT-genotype; green spots indicate the heterozygote CT-variant, and red spots point to the CC-variants.

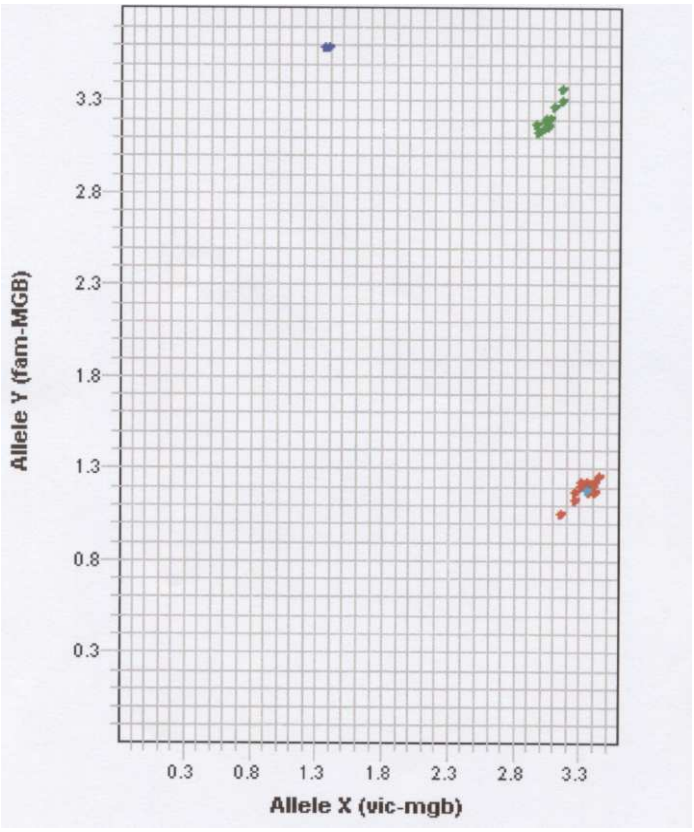

Figure 8. Allelic discrimination plot. TT, blue spot; CT, green spots; CC, red spots.

The patients are sorted and numbered according to their genotype in Table 19. The T-allele frequency found was 26.4%. The controls are sorted and numbered according to their genotype and the results are displayed in Table 23. The T-allele frequency for the controls was calculated to 34.2%. Since the TT genotype was rare in the patient and control populations, all T-allele carriers (CT and TT genotypes) were analysed as one group in the statistical analyses. When performing a chi-square test, see Table 20, no difference was found

in the genotype frequency between patients and controls (P>0.1). E indicates expected number.

Table 20. Chi-square test of the genotype frequency of patients and controls. E, expected number

	CC-subjects	CT/TT-subjects	Total (R)
Patients	9, E=8	8, E=9	17
Controls	8, E=9	11, E=10	19
Total (K)	17	19	n=36

By using a chi-square test, see Table 21, the SNP was found to be in Hardy-Weinberg equilibrium (P >0.1) when compared to a study mapping 120 individuals in the European population [79].

Table 21. Chi-square test of the Hardy-Weinberg equilibrium. E, expected number

	CC-subjects	CT/TT-subjects	Total (R)
Our study	17, E=19	19, E=17	36
European population	64, E=62	56, E=58	120
Total (K)	81	75	n=156

To exclude variation in the number of gene copies of *FKBP5* as a cause of inter-individual variation in glucocorticoid sensitivity, a copy number analysis was performed (see section 3.4.3). Figure 9 displays the DNA-yield (ΔR_n) from each PCR-cycle for the standard concentrations of DNA, which further gives rise to the standard curves seen in Figure 10.

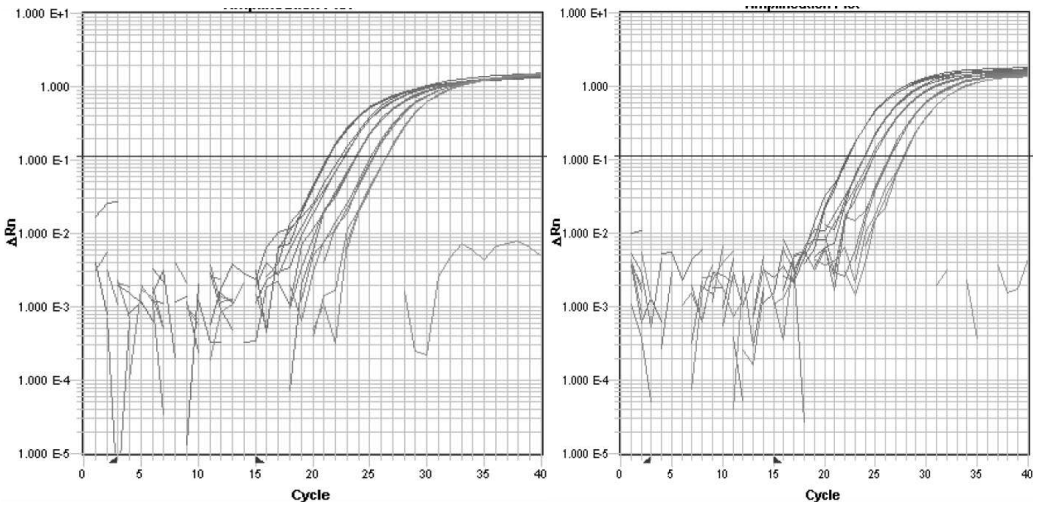

Figure 9. Amplification plot for standard concentrations of DNA for FAM-mgb (*FKBP5*) and HEX (Rb-1) respectively

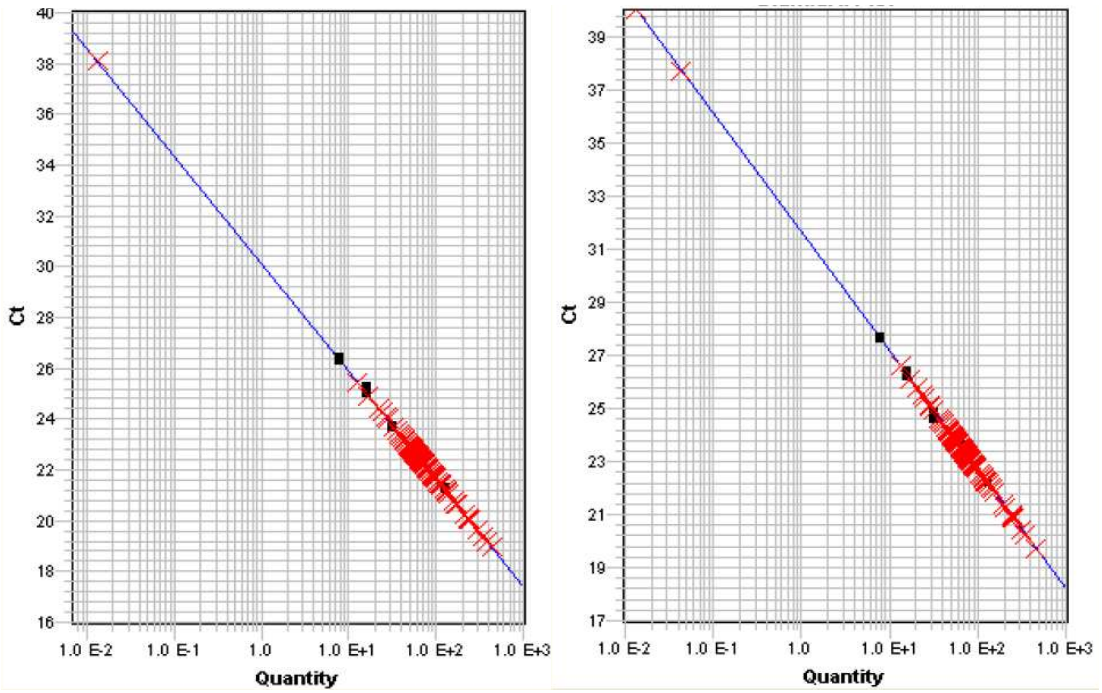

Figure 10. The standard curves of FAM-mgb (FKBP5) and HEX (Rb-1) respectively, with unknowns displayed in red.

Nearly all of the subjects lie within the range of the standard curve (see Figure 10), the ones beyond the range were excluded. When comparing the DNA-yield of the unknown DNA for the FKBP5 (FAM-mgb) and Rb-1 (HEX), the copy number analysis shows a ratio of ~1 between the PCR-yield for FKBP51 and Rb-1. This confirms that only one copy of the *FKBP5* gene exists.

4.3 Serum cortisol, salivary cortisol and ACTH levels

Serum and salivary cortisol were measured to evaluate if the patients were cortisol deplete at baseline, and to assess the inter-individual variation cortisol levels two hours after infusion of 100 mg hydrocortisone. In addition, the ACTH levels before and two hours after hydrocortisone infusion were measured as an indicator of glucocorticoid sensitivity in the HPA axis, as the ACTH secretion is feed-back inhibited by cortisol. The results from the assays are described in Table 22. No differences in hormone levels were seen between the genotypes neither at 0h nor 2h.

Table 22. Hormone levels in patients before and 2 hours after intravenous injection of 100mg hydrocortisone

		0h			2h		
Patient no	**Gender**	**Serum cortisol (nmol/l)**	**Salivary cortisol (nmol/l)**	**ACTH (pmol/l)**	**Serum cortisol (nmol/l)**	**Salivary cortisol (nmol/l)**	**ACTH**
CC-patients							
P1	M	<28	ND	>278	1862	>280	24,2
P2	M	<28	ND	>278	1672	>280	35,7
P3	F	<28	46,1	39,1	2621	>280	3,5
P4	F	<28	ND	15,3	2687	>280	4,1
P5	M	<28	ND	178	1333	>280	11,4
P6	F	<28	<3	278	2072	582	17,3
P7	F	<28	<3	53,5	2246	549	6,6
P8	F	<28	<3	ND	3256	870	ND
P9	M	<28	<3	ND	2097	744	ND
Group mean CC					**2205**		**14.7**
CT-patients							
P10	F	<28	ND	>280	1879	ND	>280
P11	F	<28	ND	>280	1490	ND	104,6
P12	F	109	ND	198	1708	>280	15,1
P13	F	<28	ND	<1,1	2759	>280	5,5
P14	M	<28	ND	<1,1	1873	>280	<1,1
P15	F	<28	ND	196	2925	ND	10,1
P16	F	<28	<3	ND	1998	654	ND
Group mean CT					**2253**		**33.8**[1]
TT-patient							
P17	F	46	3.1	195	2431	672	10,7
Mean CT and TT					**2133**		**29.2**[1]

Salivary cortisol samples >280 nmol/l and ACTH >278 pmol/l are not diluted
[1]mean of exact values

All, except two of the patients had undetectable serum cortisol levels (<28 nmol/l) before 100 mg hydrocortisone was injected. Two hours after intravenous injection of 100 mg hydrocortisone, the serum cortisol levels were highly variable between the patients. In later

analysis, this variation is corrected for by dividing the results on the serum cortisol levels. In the female patients, the mean serum cortisol levels after intravenous injection of 100 mg hydrocortisone were significantly higher than the males' serum cortisol levels (men= 1767 nmol/l, women=2339 nmol/l, P = 0.02).

Salivary cortisol before intravenous injection of hydrocortisone were <28 nmol/l in all the patients, except two patients, where the salivary cortisol levels were over the detectable limit. Two hours after intravenous injection of hydrocortisone, the salivary cortisol levels were all >280 nmol/l. The samples denoted >280 nmol/l were not diluted.

The ACTH levels in the patients measured both before and 2 hours after intravenous injection of hydrocortisone range from > 278 pmol/l, to < 1.1 pmol/l. The average two hour ACTH levels are higher for the CT/TT genotype compared to the CC. However, the difference is not significant (P = 0.3). The samples denoted >278 and >280 are not diluted. Figure 11 shows the ACTH levels in fourteen patients before and two hours after intravenous injection of hydrocortisone.

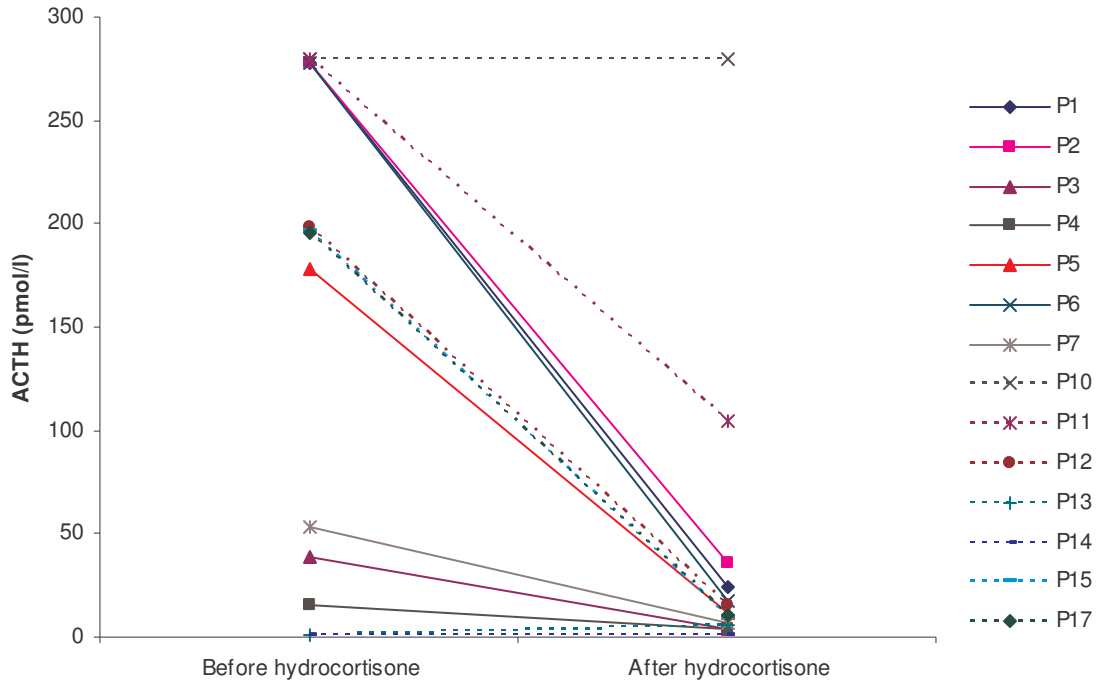

Figure 11. ACTH levels before and 2 hours after hydrocortisone. CT/TT-variants are displayed with dotted line

As seen from the figure, there is great variation both in the levels of ACTH before and after hydrocortisone, and also in the extent of reduction in the ACTH levels as a response to hydrocortisone. However, this variation is not genotype specific.

The correlation between ACTH and cortisol levels two hours after hydrocortisone infusion was analysed (see correlation plot, Figure 12) to evaluate if the great variation in ACTH levels relate to variation in serum cortisol levels to a greater extent than the SNP. Calculated by Spearman' rank correlation test, a weak negative correlation (R=-0.59) exists between the ACTH levels and cortisol levels (P= 0.027). Performing the same test when the outlier (P10) is removed, gives a stronger negative correlation (R=-0.65, P=0.017).

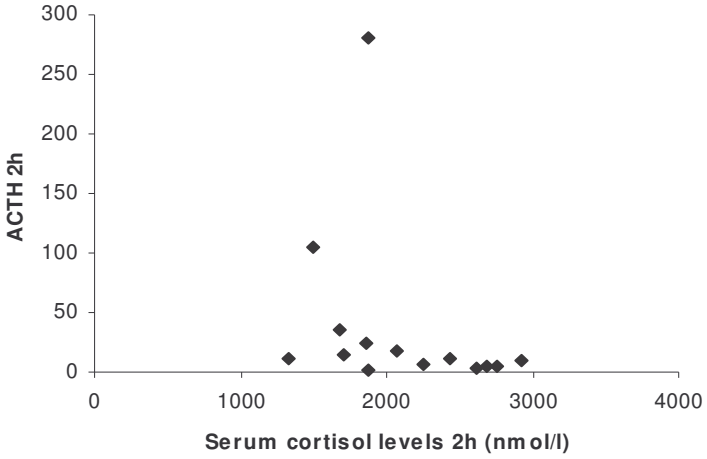

Figure 12. Correlation plot of ACTH and serum cortisol levels after two hours

Serum cortisol was measured in the controls to assess the variation in glucocorticoid sensitivity in the HPA axis, and to evaluate the possible impact the cortisol levels on FKBP51 at baseline. The mean and median serum cortisol levels for the controls are shown in Table 23. The serum cortisol levels for the controls varies widely and no differences are seen between the CC and T-carrying genotypes (P = 0.9).

Table 23. Serum cortisol levels in controls

Controls	Mean serum cortisol (nmol/L)	Standard deviation	Median	Range
CC-controls n= 8	555	193	516	309-938
CT-controls n= 9	566	202	560	270-836
TT-controls n= 2	593	313		372-814

4.4 Cell proliferation assay

The cell proliferation assay was performed to directly measure the glucocorticoid sensitivity in the leukocytes of the patients and controls, and to see if the FKBP5 SNP plays a significant role in glucocorticoid sensitivity. The results from the cell proliferation assay (section 3.3) are displayed in Figure 13 and Figure 14. The figures show the percentage proliferation of PBMC inhibited by different concentrations of dexamethasone, compared to the proliferation of PHA stimulated cells, for patients and controls respectively. CV% for the assay was 24%. Solid lines indicate the CC-genotype, whereas the CT- and TT-genotype is shown with dotted and dashed lines, respectively. In addition, the line indicating the TT genotype is thicker.

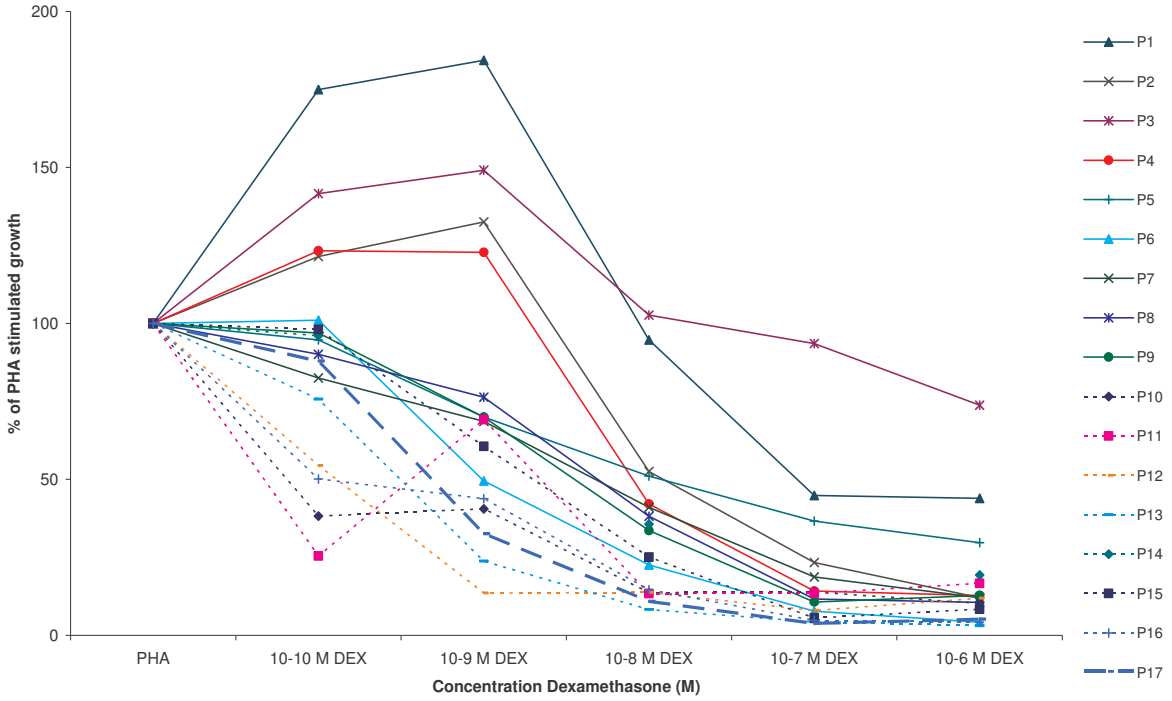

Figure 13. % proliferation of PHA-stimulated growth in patient cells after dexamethasone inhibition. CC, solid lines (P1-P9); CT, dotted lines (P10-P16); TT, thicker dashed line (P17)

The Mann Whitney test (section 3.7) showed a significant difference in proliferation upon dexamethasone inhibition between the CC and CT/TT-patients ranked at IC50 and 10^{-7} M dexamethasone (P=0.001 and P=0.027), in which the T-allele was associated with greater dexamethasone sensitivity. The difference at 10^{-6} M dexamethasone and maximum inhibition were not significant (P=0.068 and P=0.082).

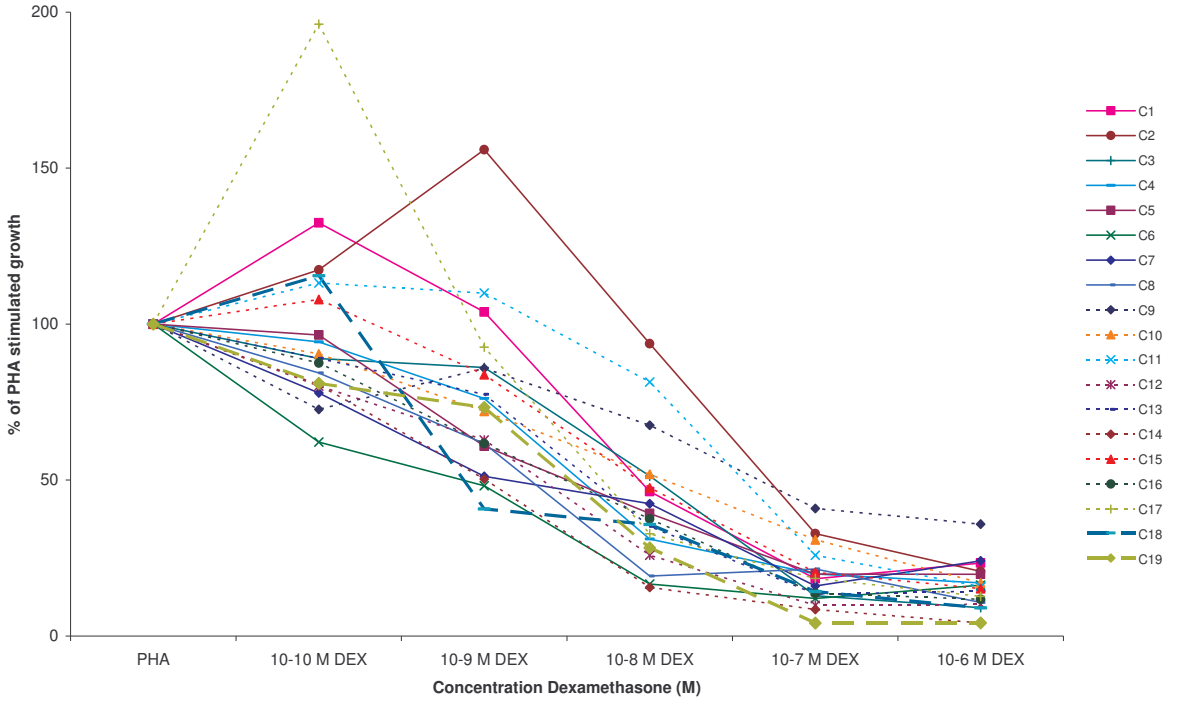

Figure 14. % proliferation of PHA-stimulated growth in cells from controls after dexamethasone inhibition. CC, solid lines (C1-C8); CT, dotted lines (C9-C17); TT, thicker dashed lines (C18-C19)

The association between the T-allele and glucocorticoid sensitivity was not found in the control group at IC50 (P=0.74). Neither at 10^{-7} and 10^{-6} M dexamethasone, nor at maximum inhibition, were the differences between the genotypes significant (P=0.59, P=0.083 and P=0.21).

By studying Figure 13 and Figure 14, we can see that most of the proliferation of the control samples shows a more intermediate proliferation inhibition than the patient samples, which tend to be severely or mildly inhibited by dexamethasone. In general, the controls show an intermediate sensitivity to dexamethasone, whereas the patients tend to be more extreme in their sensitivity.

4.5 *FKBP5* expression

Real time-PCR (rtPCR) was performed in order to investigate whether the *FKBP5* SNP could lead to alterations in the expression of *FKBP5*, and whether increased expression of *FKBP5* correlate with lower levels of ACTH as a response to hydrocortisone, or a lower sensitivity to glucocorticoids in the cell proliferation assay. The *FKBP5* expression relative to the *GADPH* was only available for 10 patients (only CC and CT-variants included) two hours after intravenous injection of hydrocortisone. The result for each of the patients is shown in Figure 15, and displays the relative *FKBP5* expression corrected for serum cortisol levels. No differences are seen between the genotypes, CC (P1-P5, white) and CT (P10-P14, dark gray) in the FKBP5 expression relative to the GADPH expression (P= 0.9) or when corrected for serum cortisol levels (P = 0.7).

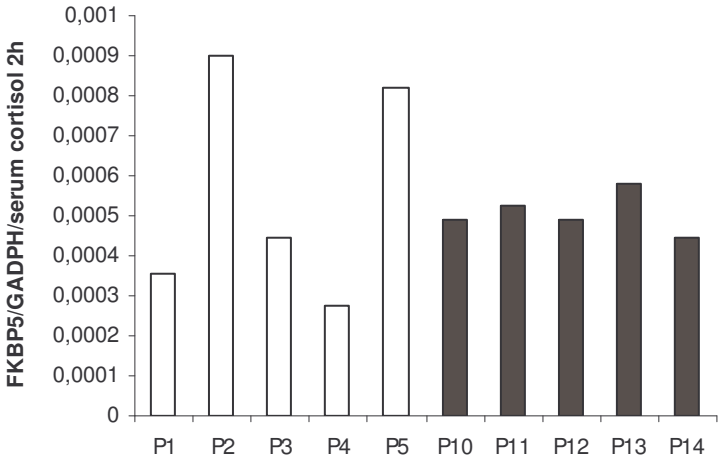

Figure 15. FKBP5 expression relative to GADPH expression two hours after hydrocortisone infusion

No correlation was found between the ACTH-levels two hours after hydrocortisone injection and *FKBP5* expression, corrected for serum cortisol levels (R=0.38, P=0.28). No correlation was found between ranking number in the cell proliferation assay and the *FKBP5* expression, independent of SNP, when serum cortisol levels were corrected for (R= -0.16, P=0.65).

4.6 Western blot analysis

Western blot analysis was chosen to investigate whether the *FKBP5* SNP determines the FKBP51 protein levels both in patients and controls. Further, we wanted to find a possible correlation between FKBP51 protein levels and sensitivity to glucocorticoids in the cell proliferation assay, and ACTH levels after infusion of hydrocortisone. The patient samples analysed in Western blot analysis was taken before the infusion of hydrocortisone.

4.6.1 Results from optimization of the protein concentration in Western blot analysis

Optimization of the protein concentration used in Western blot analysis is allowing us to find a protein concentration which is detectable on the blot, and also to determine if the protein concentration is proportional to the IDV-value calculated. Figure 16 displays the bands from the blot (AlphaEaseFC software) in increasing concentration from left to the right. The FKBP51 band using a protein concentration of 20 µg/µl (third from the left), gives a weak, but detectable band.

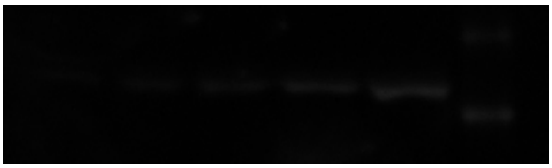

Figure 16. Bands from the optimization of protein concentration

The IDV-value from the optimization of Western blotting is shown in
Table 24. From a protein concentration of 20 µg/µl, the IDV per ng protein are stabilised to a value around 12-13 IDV per ng protein.

Table 24. Protein concentrations used in the optimization of Western blotting

[protein] µg/µl	IDV	IDV per ng protein
5	6944	1,4
10	219144	21,9
20	255852	12,8
50	622832	12,5
100	1374931	13,7

Preparing a standard curve (Figure 17) from the known protein concentration and the IDV values gives a linear correlation, allowing us to use IDV as a measure of the relative amount of FKBP51. A concentration of 20 µg/µl protein used later in the Western blot analysis, ends up in the linear region.

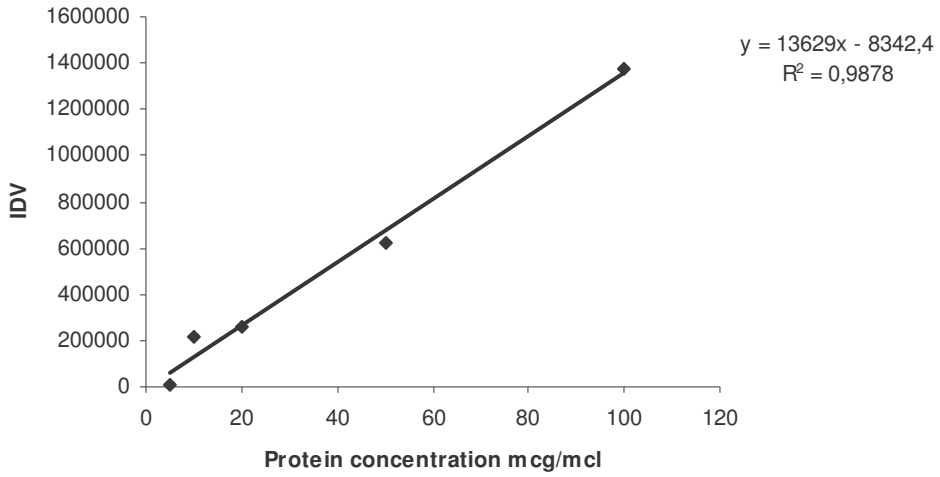

Figure 17. Optimization of the protein concentration

4.6.2 FKBP51 protein levels measured in Western blot analysis

Figure 18 displays the bands seen on the blot after Western blot analysis. Both patients and controls for each genotype (CC and CT/TT) are included in each blot.

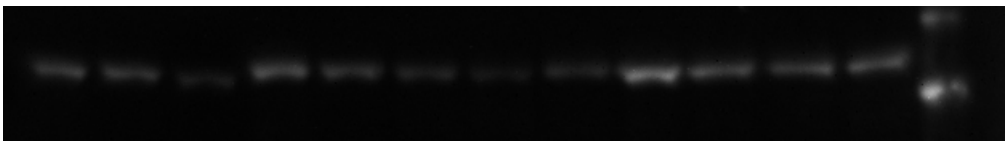

Figure 18. Bands from one Western blot analysis with standards seen furthest to the right

Figure 19 displays the mean FKBP51 protein levels for the CC, CT and TT genotype, presented as the percent of max IDV-value in the blot. The Western blot analysis has a CV% of 42%.

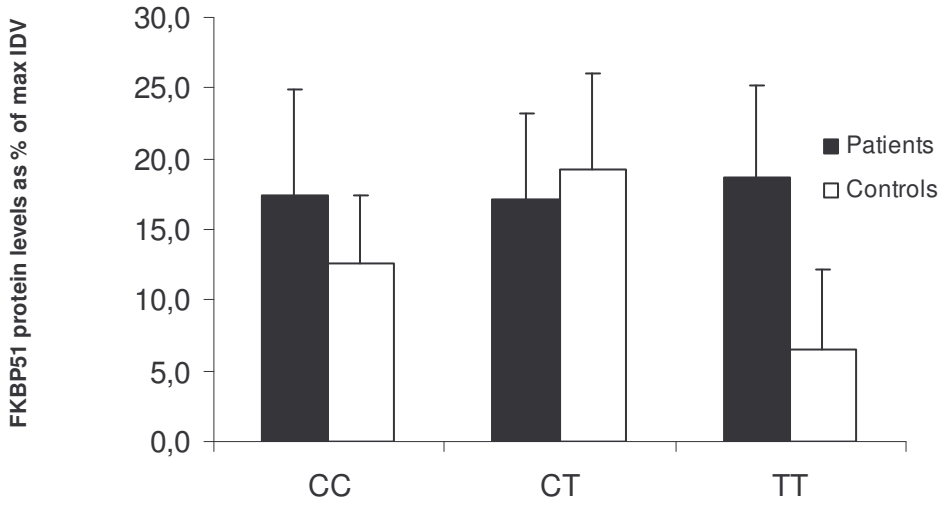

Figure 19. FKBP51 protein levels for the CC, CT and TT-genotype

As can be seen from the figure, no differences are seen between the CC, CT and TT-variants among the patients (P=1.0). The figure indicates a greater variance in the FKBP51 protein levels among the controls, but no significant genotype specific pattern is observed.

No correlation was found between the ACTH levels measured two hours after hydrocortisone infusion and FKBP51 protein levels using Spearman's rank correlation test (R=-0.099, P=0.75).

A correlation plot of FKBP51 protein levels (% of max IDV) and ranking at IC50 in cell proliferation assay is shown in Figure 20. A low ranking in the cell proliferation indicate a higher sensitivity. As seen from the figure, a weak positive correlation exists, indicating that high protein levels give a decreased sensitivity to glucocorticoids. This correlation is however not significant for patients (P=0.9) nor controls (P=0.5).

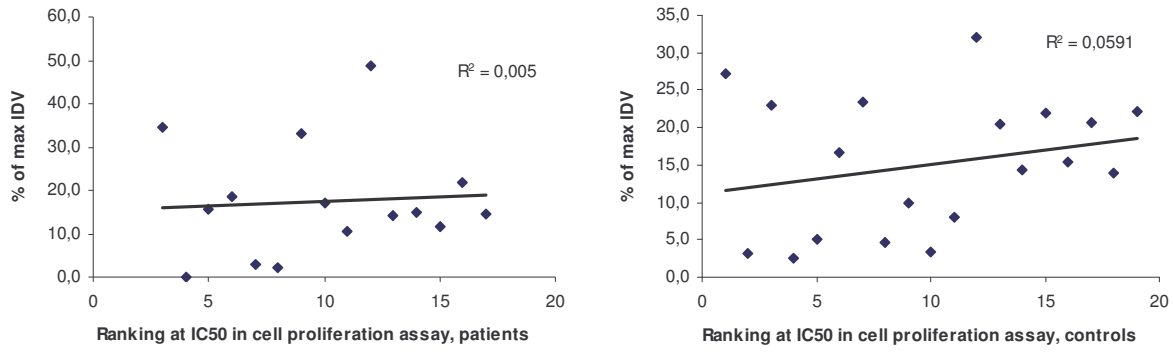

Figure 20. Correlation between FKBP51 protein levels (% of max IDV) and sensitivity in the cell proliferation assay for patients and controls. Low ranking indicate a higher sensitivity

5 Discussion

5.1 Polymorphisms in *FKBP5* and glucocorticoid sensitivity

Several studies points to the involvement of FKBP51 in glucocorticoid sensitivity. FKBP51 is known to participate in a short negative feedback loop to limit hGR-signalling within cells, both by reducing the hormone binding affinity to the hGR, and impairing the nuclear translocation of the receptor complex [62, 73]. In squirrel monkeys, constitutively high levels of FKBP51, in connection with elevated cortisol levels also indicate a role of FKBP51 in the inhibition of glucocorticoid signalling, as the elevated cortisol levels are needed to compensate for the reduced glucocorticoid effects. In addition, the increased effect of glucocorticoids on hGR-signalling seen when FKBP51 is displaced from the hGR-complex and bound to FK506 further support that the FKBP51 might be involved in the sensitivity to glucocortioids [9]. This also means that variations in the *FKBP5* gene, for example polymorphisms (SNP, CNP) could play a role in the inter-individual variation in sensitivity to glucocorticoids. We found however no indication that CNP exists for *FKBP5.*

Our results from the cell proliferation assay suggest, together with findings in previous studies [77, 80, 82, 84] that the *FKBP5* SNP rs1360780 is indeed associated with glucocorticoid sensitivity. Further studies are however needed, as most of our experiment did not establish this association. Our results are outlined below, referring to the specific objectives of the study (section 1.5).

- Evaluation of whether *FKBP5* genotype is associated with glucocorticoid sensitivity in a cell proliferation assay.

The cell proliferation assay indicates that the patients carrying the T-allele are more sensitive to glucocorticoids compared to the homozygous CC-variants. The CT/TT-variants shows inhibition of the proliferation at lower dexamethasone concentrations, and also displays generally lower rates of proliferation at all dexamethasone concentrations in comparison with the CC-variants. This difference in glucocorticoid sensitivity between the genotypes was not found in the controls, who display intermediate inhibition by dexamethasone as compared to the variation in the patients. However, whereas the patients were cortisol deplete after having restrained their cortisone treatment for 24 hours, the controls have high levels of endogenous

cortisol in their blood during the night before leukocyte sampling. Even if this is corrected for by using the percent proliferation of the PHA-stimulated growth for each of the subjects rather than the actual value, the endogenous cortisol or level of FKBP51 at baseline could affect the interpretation of the assay.

- Evaluation of whether the *FKBP5* genotype is associated with *FKBP5* expression and FKBP51 protein levels in leukocytes.

Glucocorticoids are known to induce the expression of *FKBP5* gene [62, 73] and its encoded protein FKBP51 including different isoforms (splice variants) with unknown functional capacities [76]. We did not, however, detect significant differences in the expression of *FKBP5* or in the FKBP51 protein levels in leukocytes from subjects with the different genotypes. The poor reproducibility of the Western blot analysis (discussed below) and the limited number of subjects included make it impossible to establish a link between the SNP and FKBP51 protein levels. It is also evident that the cortisol levels obtained in the individuals after a standard intravenous hydrocortisone dose vary considerably. The observed difference in serum cortisol levels between men and women could be due to body size or amount of body fat. Women tend to have smaller bodies and a larger portion of fat compared to men. Thus, the in vivo assessment of *FKBP5* gene expression and FKBP51 protein levels may be more influenced by cortisol levels than the SNP. In this way the in vitro cell proliferation assay is more standardised and the effects of the SNP more readily interpreted.

Binder et al also found no significant correlation between *FKBP5* expression in healthy controls and the rs1360780 SNP [77]. However, they found significantly higher FKBP51 protein levels in the TT-variant compared to the CT and CC-variants in their Western blot analysis [77]. However, their analysis included few subjects, a mixture of depressed and healthy individuals, and showed the lowest values for the heterozygous subjects, which render their conclusion questionable. The association between FKBP51 levels and glucocorticoid sensitivity may however be far more complicated than previously thought, as several isoforms of the protein have now been identified [76].

- Determination of whether glucocorticoid sensitivity in the cell proliferation assay correlates with the *FKBP5* expression and FKBP51 protein levels.

We did not establish a correlation between the individual *FKBP5* expression level and the sensitivity to dexamethasone in the cell proliferation assay in the patients. Due to the limited number of subjects and the problems with the standardisation of the hydrocortisone infusion this analysis could by no means exclude that such a correlation may exist. Furthermore, no significant correlation was seen between the sensitivity in the cell proliferation assay and the FKBP51 protein levels, independently of SNP in neither patients nor controls (Figure 20). Because of the uncertainty in the Western blot results, the value of this correlation test is limited.

- Determination of whether the ACTH levels in patients is associated with FKBP5 genotype and correlates with *FKBP5* expression and FKBP51 protein levels.

The levels of ACTH two hours after intravenous injection of 100 mg hydrocortisone were intended as an in vivo measure of glucocorticoid sensitivity of the HPA-axis. It can be seen in Table 22 that two individuals (P10, P11) are mainly responsible for the higher (non-significant) average ACTH-levels in the CT/TT patients than in those with the CC genotype. However, in the cell proliferation assay the sensitivity to dexamethasone in PBMC is quite high in both of these patients (Figure 13). A mix-up of the samples of ACTH is possible, or there might be another explanation to these opposite findings. This could for example be different tissue specific sensitivity in PBMC compared to hypothalamus/pituitary, in other words that the *FKBP5* SNP exhibit tissue specific effects. Furthermore, no correlation was found between ACTH levels and *FKBP5* expression independent of the SNP; unlike the association found in the pilot project [83]. As discussed above, the hydrocortisone infusion study turned out not to be appropriate for assessment of the possible effects of the SNP, which are likely to be overruled by the variation in cortisol levels. This is illustrated by the significant negative correlation between the ACTH and cortisol levels in patients after two hours (Figure 12). No correlation was found between the ACTH levels and FKBP51 protein levels, indicating that the FKBP51 protein levels do not influence the secretion of ACTH as a response to hormone. However, due to the uncertainty in the results from the Western blot analysis mentioned above, this correlation test is of limited value.

Previous studies have shown that the T-allele in the *FKBP5* SNP rs1360780 is associated with increased recurrence of depressive episodes [77, 80] and the risk of PTSD [81, 82]. Both depression and PTSD are conditions in which the HPA-axis and glucocorticoid sensitivity are thought to be involved [47, 82]. However, is the T-allele associated with increased sensitivity or resistance to glucocorticoids? Depression has been associated with glucocorticoid resistance causing impaired negative feedback regulation and a hyperactivity of the HPA-axis, but it has also been associated with increased hGR-activation in the limbic regions of the brain [47]. SNPs associated with either increased sensitivity or resistance to glucocorticoids could therefore explain this association to depression. Binder et al. found higher protein levels of FKBP51 in TT-subjects, compared to CT and CC-variants, and therefore suggested that the T-allele is associated with *resistance* to glucocorticoids. However, only a narrow selection of the study population was included in the Western blot analysis, and the subjects included were a mixture of depressed and healthy individuals. In addition, Western blotting is at best a semiquantitative method that is not very good for quantification of protein levels. In the same study, the TT-variants showed lower levels of ACTH and cortisol levels in the combined dexamethasone-suppression/CRH-stimulation (Dex-CRH) test, consistent with *increased* glucocorticoid sensitivity. This test is used to evaluate the HPA-axis hyperactivity in depressed patients, where the depressed subjects are associated with glucocorticoid resistance and thus get higher levels of ACTH and cortisol upon dexamethasone inhibition and CRH stimulation than healthy individuals. The low levels of ACTH and cortisol seen in the TT-variants therefore indicate that depressed individuals carrying the T-allele are *less* resistant to glucocorticoid (the dexamethasone inhibition) than the other depressed patients, and further that the T-allele are associated with a higher sensitivity to glucocorticoids.

Increased risk of osteoporosis is a well known side effect of glucocorticoids. The trend towards lower bone mineral density and reduced levels of bone markers seen in *FKBP5* T-allele carriers and homozygote TT-carriers demonstrated in Addison's patients by Løvås et al [84], indicates that the T-allele carriers are more susceptible to this side effect. These results points to an association between the T-allele and increased glucocorticoid sensitivity in bone.

The lower levels of ACTH and *FKBP5* expression seen in the CT/TT-variants in the study by Hammenfors et al. [83], indicates that the lower expression of *FKBP5* makes the CT/TT-subjects more sensitive to the injected hydrocortisone, and therefore inhibits the ACTH

response at to a greater extent via the negative feedback mechanism than in CC-subjects. This indicates that the T-allele is associated with enhanced sensitivity to glucocorticoids. However, only seven Addison's patients were included in the pilot project, and we were not able to replicate the findings in our sample of Addison's patients.

Despite the increased glucocorticoid sensitivity that we found in the cell proliferation assay, the CC and CT/TT-patients do not differ in the doses of cortisone acetate received in their replacement treatment. If the CT/TT-patients are more sensitive to glucocorticoids, the optimal treatment dose would be expected to be lower than in the CC-patients. However, the means for determining the appropriate dosage is crude, and we do not know whether the current treatment is optimal for these patients. Moreover, the increased glucocorticoid sensitivity seen in the T-allele carriers are perhaps too small in terms of the cortisone acetate dosage required in the patients. Most likely, other pharmacogenetic variation in several genes that govern cortisol metabolism (pharmacokinetics) and action (pharmacodynamics) contribute to the total picture of cortisone requirement.

There are numerous ways in which the *FKBP5* SNP rs1360780 could be associated with inter-individual sensitivity to glucocorticoids. The T-allele can affect the glucocorticoid sensitivity by directly influencing the expression of *FKBP5*, and thereby also the protein levels of FKBP51. The rs1360780 SNP is located in an intron and could therefore affect the splicing of the *FKBP5* mRNA, possibly by discrimination between the different AP giving rise to alternative splice variants of *FKBP5* mRNA; resulting in different FKBP51 isoforms [76]. The polymorphism could also directly cause a decreased efficiency of the transcription of *FKBP5*, leading to a decreased level of *FKBP5* mRNA and eventually lower level of FKBP51 protein. This would comply with increased glucocorticoid effect due to the absent FKBP51 inhibition. The opposite is seen in squirrel monkeys, where increased expression of *FKBP5* is thought to give rise to glucocorticoid resistance [59].

The isoforms might have altered characteristics or efficacy in inhibiting the hGR-signalling compared to the full-length FKBP51, and higher (or lower) levels of certain isoforms might therefore affect the sensitivity to glucocorticoids. If the SNP is associated with the expression of a variant isoform of FKBP51, this may not be detectable in Western blotting or in rtPCR. This will depend on the binding site to the FKBP51 protein for the antibodies used in Western

blotting and on the binding sites on *FKBP5* mRNA for the primers used in rtPCR. The antibodies used in our Western blot analysis binds to amino acids 4-199 in the C-terminal of the FKBP51 protein [85]. The isoform utilizing AP1 and AP2 (Figure 6) will therefore both be detected by the antibody, but since the proteins have the same size, they will appear as one band in the blot, and the presence of the two different isotypes will not be discriminated. It is possible that the antibody used in the Western blot analysis will bind to the isoform at 31 kDa, since this isoform consists of amino acids 180-457. However, no band corresponding to 31 kDa was identified on the blot in our experiment. The shorter isoforms described by Billing et al. [76], where AP4 and AP5 are utilized, are not detected by the antibody used in this experiment.

Still many questions about the function of the introns remain unresolved, and the introns may have other purposes than are currently known. The rs1360780 SNP may therefore impact on the stability, and perhaps also alter the post translational modification of the FKBP51 protein. This can influence the level, but also the activity or efficacy of the protein. Post translational modifications can also affect the structure of the protein; hence it is possible that the polymorphism can alter for example the FK1-domain of the FKBP51. This domain is thought to be involved in the hormone binding affinity of hGR and the potential interaction with the motor protein dynein. An alteration in this domain is likely to affect these properties, which is important in hGR-signalling and thus glucocorticoid sensitivity.

Linkage disequilibrium can produce doubt about an association between a SNP and the studied characteristic. The *FKBP5* SNP rs1360780 is shown to be in strong linkage disequilibrium with a the SNP rs4713916 in the promoter region of *FKBP5* [80]. It may therefore be the SNP in linkage disequilibrium/the promoter region causing the increased sensitivity seen with the T-allele in the SNP rs1360780, and not the SNP studied. The rs1360780 SNP may possibly also be in linkage disequilibrium with other SNPs in *FKBP5*, which can cause altered glucocorticoid sensitivity.

5.2 Evaluation of methods

In our study, both in vivo and in vitro experiments were performed, and all methods used have both advantages and disadvantages. The cell proliferation assay is an in vitro standardized test, where equal amounts of glucocorticoids (dexamethasone) are added to blood from the patients and controls. The advantage of this is that the levels of steroids that actually reach the cells are equal in each individual, regardless of weight/body size and different levels of binding proteins. On the other hand, since the cell proliferation assay is performed by stimulating only blood cells, this does not necessary say anything about the glucocorticoid sensitivity in other tissues. The tissue specificity of FKBP51 is still unknown, and even if we find that the T-allele is associated with increased glucocorticoid sensitivity in blood cells, we cannot claim that the *FKBP5* SNP is associated with increased glucocorticoid sensitivity in for example the brain.

The ability to detect differences in glucocorticoid sensitivity by using whole blood instead of isolated PBMC has been discussed [86]. Vermeer et al. found that whole blood based assays may be less able to distinguish between different levels of glucocorticoid sensitivity. In addition, the glucocorticoid sensitivity in the PBMC may be altered by the stimulation from the mitogen (PHA), and such stimuli can also alter the transcription and translation pattern profoundly [86]. These factors might interfere with our results, especially if these alterations affect some samples more than others. The use of dexamethasone instead of the natural glucocorticoid hydrocortisone (cortisol) in the cell proliferation assay can also be discussed. Dexamethasone is a more potent and longer lasting glucocorticoid compared to hydrocortisone, and may therefore produce a different glucocorticoid sensitivity compared to the in vivo situation (Table 1). However, dexamethasone is frequently used in standardized *FKBP5* in vitro bioassays [86], and can therefore be used to compare results in different studies.

The results from the cell proliferation assay could also be affected by different experimental errors and uncertainties, such as pipetting inaccuracy, incomplete collection of the cells after incubation and uncertainties in the measurement of the incorporation of metyl-[^{3}H]-thymidine by the scintillation counter or mix-up of the samples.

Real time PCR as performed here measures the levels of *FKBP5* mRNA in vivo. The advantages of this method are that it measures the actual level of *FKBP5* expression in each patient after they have received the same amount of hydrocortisone, and therefore displays a true picture of the *FKBP5* expression in their leukocytes. However, the hormone levels two hours after hydrocortisone vary greatly between the patients, and even if we are correcting for serum cortisol levels in the calculation of *FKBP5* expression, this in vivo test will not be perfectly controlled. In addition, the serum cortisol level is not a very precise measure of active hormone. Serum cortisol is a measure of both unbound and bound hormone, whereas the active hormone is found only as free unbound cortisol [21]. Therefore, differences in body weight/size and different levels of binding proteins among other factors, will lead to variation in hormone levels in the patients.

The Western blot analysis in this study also reflects the situation in vivo, which could indicate a tendency towards less or greater levels of the FKBP51 protein. However, the disadvantage is that the method does not offer great possibilities for quantifying the protein levels. The many steps in preparation of the blot give rise to many experimental uncertainties. Irregular blocking of the membrane or uneven transfer of the proteins during blotting could possibly lead to an erroneous level of protein on the blot both on the same blot and between different blots. In addition, imprecision in the detection of protein concentration and IDV values, and pipetting inaccuracy may also lead to false protein levels.

5.3 Further work / perspectives

The results in this study indicate that the *FKBP5* SNP rs1360780 might be associated with *increased* glucocorticoid sensitivity, not glucocorticoid *resistance* as the protein levels in the study of Binder et al could indicate [77]. However, further work is needed to establish this association. For later projects regarding polymorphisms in the *FKBP5* gene, there are several possibilities for improvement. First of all, the experiments need to be done with higher number of subjects included, both patients and controls. By doing this, an assumption of associations can be made with more certainty, and false positive associations can be avoided. Furthermore, the use of a more quantitative method than Western blotting in order to detect

the protein levels of FKBP51 could give a more reliable result. This could possibly be achieved by the use of (indirect) ELISA or mass spectrometry.

In addition, further investigation needs to be performed concerning the functional role of the *FKBP5* SNP rs1360780 for glucocorticoid sensitivity. The SNPs in linkage disequilibrium with rs1360780, for example the rs4713916 [80] located in the promoter region of *FKBP5*, must be further examined. Moreover, the isotypes found in the proteomic study of FKBP51 [76] must be studied with regard to their role in hGR signalling, and the potential influence on the levels of the different isoforms by the *FKBP5* SNPs must be determined.

A better knowledge about the variation in the glucocorticoid sensitivity among individuals could first and foremost improve the treatment with glucocorticoids. This applies both to the replacement therapy in patients with Addison's disease, but also in the pharmacological treatment with glucocorticoids in other diseases such as asthma, rheumatoid arthritis and malignant diseases/cancer. By understanding the mechanisms behind variable glucocorticoid sensitivity, the treatment outcomes could be optimized by a reduction of the side effects, and a decreased incidence of iatrogenic Cushing's disease.

It is possible that the *FKBP5* SNP may influence the increased glucocorticoid effects seen with concurrent use of Tacrolimus (FK506). Tacrolimus is known to potentiate glucocorticoid action by binding FKBP51 and replacing it with PP5 [9] and by competitive binding to the metabolizing enzyme CYP3A4 (Table 2). If the *FKBP5* SNP rs1360780 decreases the levels of FKBP51, the inhibition form FKBP51 will be reduced and less Tacrolimus will be needed to potentiate the effect of glucocorticoids. This could possible be utilized after organ transplantation, where a life-long treatment of Tacrolimus and glucocorticoids are needed. By identifying individuals more exposed to this interaction, the dose of glucocorticoids could possibly be reduced, together with the susceptibility to side-effects.

Improved understanding of factors influencing the inter-individual glucocorticoid sensitivity could advance the knowledge about the pathogenesis of diseases associated with increased sensitivity to glucocorticoids or glucocorticoid resistance. This includes diseases such as depression, metabolic syndrome, and cancer [14-16]. The *FKBP5* SNP could be one of such factors, which may affect the susceptibility to disease. Indeed, the study of Binder et al. finds

an association between the *FKBP5* SNP rs1360780 and the number of lifetime episodes of depression and response to antidepressant treatment [77]. Subsequently, a polymorphism in the hGR associated with glucocorticoid resistance (ER22/23EK) has also been linked to a faster response to antidepressive treatment [47].

Prolonged stress, both physical and psychological, such as a depression is known to disturb the HPA-axis [47]. Through HPA-axis activation, mediators are released which suppress some parts of the immune system and impair the immune response. It is speculated that this might promote the initiation and progression of some types of cancer [15]. Furthermore, DNA-damage, accumulation of somatic mutations, alteration in DNA-repair and inhibition of apoptosis are also affected by prolonged stress, and might be involved in the onset and outcome of some types of cancer. Inter-individual variation in the glucocorticoid sensitivity, for example by *FKBP5* SNP rs1360780 may therefore predict the susceptibility of an individual to cancer.

Polymorphisms in hGR are shown to lead to increased glucocorticoid sensitivity, and further to increased risk of metabolic syndrome [41, 43]. Other polymorphisms in the hGR are associated to glucocorticoid resistence and a more healthy metabolic profile [41, 42]. There is a possibility that the *FKBP51* SNP rs1360780, which we find to be in association with increased glucocorticoid sensitivity, could be a risk factor for metabolic syndrome as well. However, to date no studies are performed on this subject.

In addition to the knowledge of the mechanisms behind this diseases, and how polymorphisms affect the susceptibility to disease, the understanding of glucocorticoid sensitivity could reveal new molecular targets for treatment and prevention of depression, cancer and metabolic syndrome. However, the individual glucocorticoid sensitivity is most probably a characteristic in which several factors are at play. This may include both genetic factors, including polymorphisms in genes involved in glucocorticoid signalling and pharmacokinetics, but also environmental factors such as gut microflora and drug interactions.

5.4 Conclusion

Our result from the cell proliferation assay points to increased glucocorticoid sensitivity associated with the T-allele in *FKBP5* SNP rs1360780 in patients. No such association was found for the controls, but this could be due to less standardized experimental conditions in controls. No association was found in the in vivo studies in patients two hours after a standardized intravenous infusion of hydrocortisone between the studied SNP and *FKBP5* expression or FKBP51 protein levels in lymphocytes and no correlation was found between glucocorticoid sensitivity in the cell proliferation assay and *FKBP5* expression or FKBP51 protein levels. The ACTH levels did not vary between the different genotypes, and no correlation was found between the ACTH-levels and *FKBP5* expression.

Further studies are needed to confirm the association of *FKBP5* SNP rs1360780 in glucocorticoid sensitivity; which would require a larger number of subjects and refined methodology. Research is also needed to establish the definitive role of FKBP51 and its isoforms in the regulation of glucocorticoid sensitivity.

The growing understanding of inter individual variation in glucocorticoid sensitivity and the mechanisms behind may ultimately lead to improved glucocorticoid therapy and increase the knowledge about the pathogenesis common diseases, such as depression, cancer and metabolic syndrome.

6 References

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