BASIC AND CLINICAL ASPECTS OF THE MEDICAL TREATMENT OF CUSHING'S DISEASE

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Basic and Clinical Aspects of the Medical Treatment of Cushing's Disease

Basale en klinische aspecten van de medicamenteuze behandeling van de ziekte van Cushing

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CHAPTER 1

GENERAL INTRODUCTION AND AIMS OF THIS THESIS.

Based on:

New developments in the medical therapy of Cushing's syndrome

R. van der Pas, W.W. de Herder, L.J. Hofland and R.A. Feelders

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GENERAL INTRODUCTION

1. The hypothalamus-pituitary-adrenal axis

Under physiological conditions, the production of cortisol is tightly regulated by the so-called hypothalamus-pituitary-adrenal (HPA) axis (Figure 1a). Adrenocorticotropin (ACTH) producing cells in the anterior pituitary lobe respond to the hypothalamic corticotropin-releasing hormone (CRH) by increasing the transcription of the pro-opiomelanocortin (POMC) gene. POMC is further processed by prohormone convertases that cleave the POMC molecule into various molecules, e.g. ACTH and α-melanocyte stimulating hormone [1-3]. By binding to its receptor (the melanocortin type 2 receptor; MC2R), ACTH induces the expression of several steroidogenic enzymes in the zona fasciculata of the adrenal cortex that are required for the biosynthesis of cortisol, including *CYP17A1* and *CYP11B1* [4]. MC2R activation also triggers phosphorylation of the steroidogenic acute regulatory protein (STAR), which facilitates cholesterol transport over the mitochondrial inner membrane. Consequently, the production rate of cortisol will increase.

Figure 1. The hypothalamus-pituitary-adrenal axis

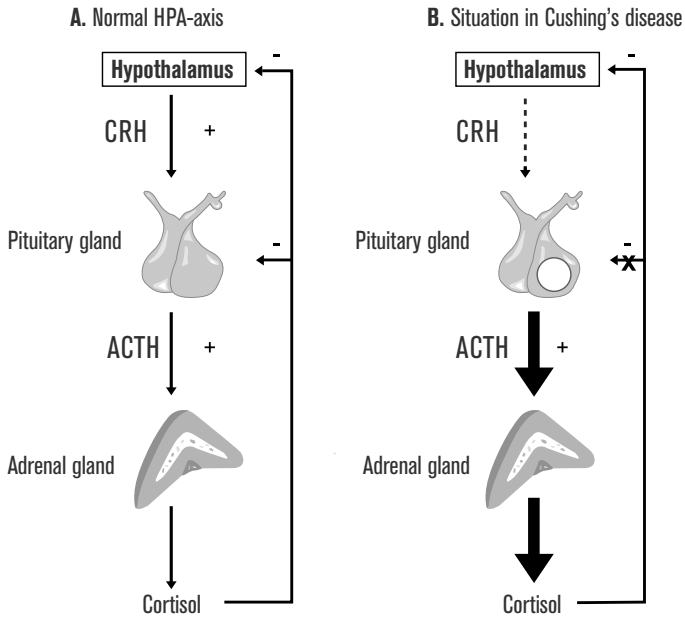
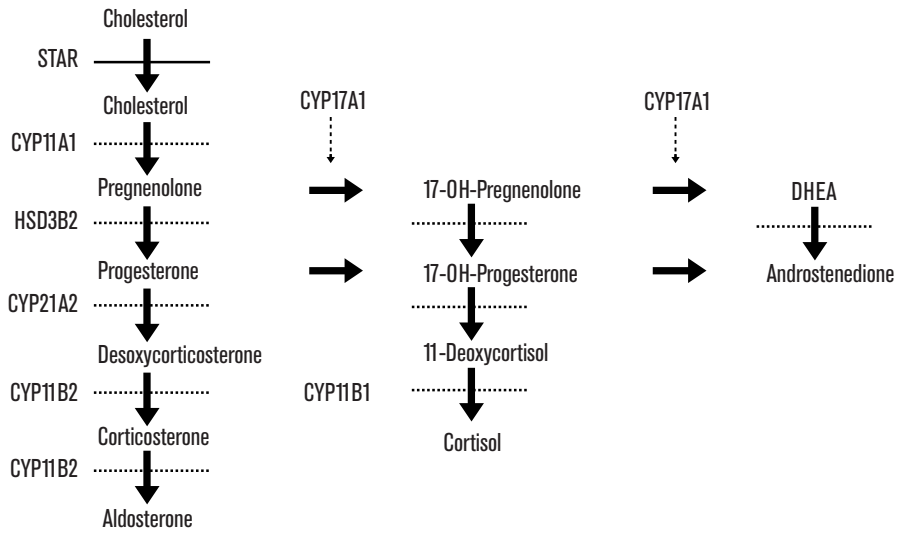


Figure 2 shows the pathways of adrenocortical steroidogenesis. This cascade follows a specific diurnal rhythm, with peak serum concentrations of ACTH and cortisol in the morning that gradually decline throughout the day [5]. Cortisol exerts its effects by binding to the glucocorticoid receptor (GR), which is widely expressed throughout the body. Important effects of cortisol in physiology include its role in glucose- and lipid metabolism. Furthermore, cortisol has anti-inflammatory effects and is an important factor in the regulation of vascular responsiveness to catecholamines and, therefore, in maintaining blood pressure. Because of their immunosuppressive effects, glucocorticoids are often used to treat patients with auto-immune disorders or inflammatory diseases. A fine-tuned balance exists between CRH, ACTH and cortisol in the HPA axis. By binding to the GR on CRH- and ACTH-producing cells in the hypothalamus and the pituitary gland, cortisol decreases the production and

secretion of these hormones and, consequently, its own production [6, 7]. Disruption of this system can either lead to cortisol overproduction (Cushing's syndrome) or glucocorticoid deficiency (Addison's disease).

Figure 2. Simplified scheme of the adrenocortical steroidogenic pathway.



Cholesterol is the common precursor of the steroid molecules that are produced in the adrenal cortex. It is transported across the mitochondrial membrane and undergoes several enzymatic reactions before it is ultimately converted into the biologically active steroid hormones with either mineralocorticoid, glucocorticoid or androgenic properties.

2. Cushing's syndrome and Cushing's disease

Cushing's syndrome (CS) is characterized by a chronic overproduction of cortisol resulting in significant morbidity and, when left untreated, an increased mortality [8-12]. Traditionally, CS is divided into ACTH-dependent CS and ACTH-independent CS. ACTH-dependent CS, which represents approximately 80% of cases, can be caused by a corticotroph pituitary adenoma (Figure 1b) or, more rarely, by ectopic ACTH production. ACTH-independent CS is usually caused by a unilateral adrenal adenoma and less frequently by an adrenocortical carcinoma or bilateral micro- or macronodular adrenal hyperplasia [10, 13].

Chronic cortisol excess leads to a typical clinical phenotype with truncal and facial fat deposition, plethoric facial appearance, easy bruisability and muscle and skin atrophy (Table 1), although the prevalence of these symptoms can be highly variable between patients, which is in part related to the underlying cause of CS [10,14]. Clinical symptoms develop gradually which often delays the diagnosis for years. In addition, chronic hypercortisolism is associated with serious morbidity including an increased cardiovascular risk due to clustering of risk factors (obesity, diabetes mellitus, hypertension and dyslipidemia), an increased risk of venous thromboembolism, osteoporosis and psychological and cognitive disturbances [10, 15, 16]. This multitude of signs, symptoms and morbidity severely impairs quality of life in patients with CS [17, 18]. If CS is not or suboptimally treated, continuous cortisol excess will lead to an increased mortality in particular due to cardiovascular disease [8, 9, 11, 12, 19]. Importantly, the morbidity that contributes to the increased risk of cardiovascular disease has been reported to be only partially reversible. It generally takes a long period of time for obesity, hypertension, glucose intolerance and dyslipidemia to normalize following successful treatment of CD and in some patients, these

symptoms did not disappear at all during the follow-up period [20].
Several diagnostic tests are used to establish endogenous hypercortisolism including urinary free cortisol (UFC) excretion, midnight serum- and salivary cortisol concentrations and dexamethasone suppression test(s) [14].

Table 1. Most frequently occurring clinical features observed in Cushing's syndrome. (Adapted from [10, 13, 162, 163])

Feature	Frequency (% of patients)
Obesity	39-96
Facial plethora	82-90
Decreased libido	24-90
Muscle weakness	60-82
Menstrual irregularity	74-80
Glucose intolerance	50-80
Hypertension	62-78
Hirsutism	72-75
Osteoporosis	38-75
Psychiatric disorders	53-70
Easy bruising	46-65

Pituitary-dependent CS or Cushing's disease (CD) is caused by an ACTH-secreting pituitary tumor and accounts for approximately 70% of all cases of endogenous CS [10, 13, 21, 22]. It is generally accepted that these tumors originate from corticotroph cells in the anterior pituitary. As already described, in the pituitary gland, POMC is cleaved by prohormone convertases (PC) 1 and 2, which are members of the family of subtilisin-like proprotein convertases [1-3, 23, 24]. PC1 is believed to be expressed in both the anterior and intermediate pituitary lobe, whereas PC2 expression is restricted to the intermediate lobe, which is believed to regress soon after birth and, therefore, to be absent in adults [1, 2, 23, 25]. However, various groups reported biochemical remission from CD after transsphenoidal surgery without any histological evidence of a true pituitary adenoma [26, 27]. In contrast, these patients had hyperplasia of corticotroph cells at histological examination [26, 28]. Another study showed neural tissue in pituitary tumors from a subgroup of patients with CD [29]. These tumors were found to be located in the region between the anterior and posterior pituitary lobes and consisted of basophilic adenomatous tissue or multiple microadenomas accompanied by hyperplastic cell nests. The tumors localized in the region between the anterior and posterior pituitary lobe were less successfully removed by transsphenoidal surgery and were more often responsive to dopamine 2 receptor (D_2R) agonists [29]. Observations that clusters of cells in the region between the anterior and posterior human adult pituitary lobe stain positive for β-endorphin further support the existence of PC2-expressing cells in the intermediate lobe in adults [30]. Together, these data led to the hypothesis that CD may not only originate from corticotroph adenomas of the anterior lobe, but also from an adenoma or adenomatous hyperplasia of ACTH-producing cells of the intermediate zone [29].

Therefore, one of the aims of this thesis is to determine the molecular characteristics of ACTH-producing pituitary tumors with respect to the expression levels of PC1 and PC2, as well as the expression levels of D2R, somatostatin receptor subtypes and neurofilaments (NF). These data will be correlated with radiological findings and histology. In this way, we aim to demonstrate that there are different entities of CD. The results of this study are described in Chapter 2 of this thesis.

3. Morbidity in Cushing's disease

As described above, chronic hypercortisolism is associated with serious morbidity including an increased cardiovascular risk due to clustering of risk factors (obesity, diabetes mellitus, hypertension and dyslipidemia), an increased risk of venous thromboembolism, osteoporosis and psychological and cognitive disturbances [10, 15, 16]. This multitude of signs, symptoms and morbidity severely impairs quality of life in patients with CS [17, 18].
Several studies report that the incidence of venous thromboembolism (VTE) is increased, both in untreated CS and following successful surgery [16, 31-35]. A recent retrospective cohort study among more than 450 Dutch CS patients showed that the incidence of VTE before surgery was 14.6/1000 person years in these patients, which is considerably higher than the estimated VTE incidence of 1-2/1000 person years in the general population [16]. The risk for postoperative VTE in patients with CD was 3.4% (12/350 cases) vs. 0% after transsphenoidal surgery for nonfunctioning pituitary adenomas. This percentage is comparable to the postoperative VTE risk after total hip- or knee replacement. An important observation is that VTE after transsphenoidal adenomectomy for CD not only occurred in the first week but also up to two months postoperatively [16].
The underlying mechanism of the hypercoagulable state in CS has not been fully clarifed [36, 37]. Several studies have evaluated the coagulation pathway in patients with CS [35]. Most of them report shortening of activated partial thromboplastin time (aPTT), indicating functional hypercoagulability, and increased levels or activity of fibrinogen, Factor VIII, Factor IX and Von Willebrand Factor (vWF) [32, 36-45]. Impaired fibrinolysis probably also plays an important role in the hypercoagulability in patients with CS. Only a limited number of studies has been carried out that evaluated the fibrinolytic potential, but the observed increase of plasminogen activator inhibitor type 1 (PAI-1) in CS suggests that fibrinolysis might be modulated by hypercortisolism as well [32, 37, 41, 43, 46].
Few studies have been published that evaluated the effects of surgical cure of CS/CD on hemostasis. A recent Italian study showed improvement, but not normalization of the hypercoagulable state in 27 patients with CS one year following successful surgery [47]. In these patients, the aPTT significantly increased and levels of vWF antigen and thrombin-antithrombin complexes decreased. Moreover, fibrinolysis also improved in patients that were cured, as suggested by significant decreases in plasma levels of PAI-1 and α2-antiplasmin [47]. In contrast, none of these ameliorations occurred in the group of 13 patients with persistent hypercortisolism after surgery. No study has been published thus far in which the effects of medical therapy of CD on the coagulation cascade were investigated.

As described above, cortisol has marked effects on the human brain. Psychological disturbances such as anxiety, irritability, psychosis, emotional instability and even suicidal behavior have been described among patients with CS [48, 49]. Depression is the most frequently observed psychiatric problem in patients with CS, occurring in 32-62% of the patients [50-52]. Furthermore, CS is associated with physical, social and emotional impairment [20, 53]. Patients report fatigue, feel weak, their social life is interfered with because of their disease and they experience impaired cognitive functioning and sleeping problems [54]. Taken together, these severe symptoms lead to a marked decrease in quality of life in patients with CS [13, 17, 18, 20, 55, 56].
A number of studies has assessed the effects of surgical treatment of CS on quality of life. Santos et al. found that quality of life only begins to improve after 6 months following successful surgery [57]. Another study reported improved mental and physical health after 3.2 years (mean) post-surgery for CD, but quality of life remained impaired after 11.8 years (mean) following surgical cure of CS (adrenocortical CS, pituitary and ectopic ACTH overproduction combined) in this study [56]. Improved quality of life in children with adrenal or pituitary dependent CS was reported one year post-surgery compared to baseline, but in this study as well, quality of life remained worse than in healthy controls according to multiple subscales [58]. Both the impairment in quality of life and decreased cognitive functions and psychological functioning appear to be partially irreversible in patients with CS even after long-term cure [53, 56, 59]. This irreversibility might partially be caused by the occurrence of hypopituitarism after successful transsphenoidal surgery. In a cross-sectional study, it was observed that the lack of improvement in quality of life after 13 years following surgical cure from CD was predominantly explained by the presence of hypopituitarism after pituitary surgery [53]. The effects of medical therapy of CD on quality of life have not been studied as yet. The physiological cortisol diurnal rhythm, which is characterized

by a cortisol peak in the early morning followed by a gradual decrease during the day to low midnight-levels, is usually absent in patients with CS [5]. An abnormal cortisol day-night profile might also contribute to impaired quality of life in CS, e.g. by inducing sleep disturbances [53, 60]. It has been reported that patients that had been surgically cured from CS for a mean of 8.2 years had a normal cortisol diurnal rhythm [61], but as for quality of life, the effects of medical therapy on the cortisol circadian rhythm are unknown so far.

Hypertension is a very important hallmark of CS with a prevalence of approximately 75% [10]. Mechanisms by which glucocorticoids are believed to induce hypertension include increased mineralocorticoid activity, enhanced reactivity to vasoconstrictors such as angiotensin II and endothelin-1 and increased activity of the renin-angiotensin-aldosterone system (RAAS) [62]. The pressor responses to infusion of both norepinephrine and angiotensin II were reported to be increased in patients with CS [63]. An explanation for this finding was provided by a study showing that dexamethasone stimulated the mRNA expression levels of the angiotensin II type 1 receptor in vascular smooth muscle cells from the aorta of Wistar Kyoto rats, an effect that was counteracted by the addition of the glucocorticoid receptor antagonist mifepristone [64]. Similarly, it was reported that an increased cardiac output induced by enhanced cardiac sensitivity to catecholamines contributes to the pathogenesis of hypertension in CS [65]. Another study showed that, compared to controls, patients with CD exhibit significantly higher ET-1 increments following insulin injection [66]. Plasma concentrations of renin and aldosterone have not extensively been investigated in patients with CD. Although blood pressure normalization occurs in 44-75% of the patients, a considerable percentage of patients (24-56%) remains hypertensive despite surgical cure [20]. The effects of drug therapy on blood pressure and the RAAS are unknown so far.

One of the aims of this thesis is to explore the effects of medical combination therapy of Cushing's disease on the morbidity in these patients. The various complications that are studied are discussed in detail below.

4. Treatment of Cushing's disease

The primary treatment of CD is a transsphenoidal resection of the adenoma. Long-term remission rates after surgery vary between 50-90% [22, 67, 68], but a recurrence risk of up to 26% has been observed during 10 years of follow-up [67, 68]. The outcome of surgery is highly dependent on the size of the adenoma, since macroadenomas and non-visible adenomas have a relatively poor success rate compared to microadenomas [69, 70]. Since repeat pituitary surgery has a relatively disappointing success rate and is often complicated by hypopituitarism, there is a clear need for alternative treatment modalities [22, 71, 72]. In case of disease recurrence or persistent hypercortisolism after surgery, radiotherapy can be applied to induce definitive remission. However, it can take years for radiotherapy to become effective, leaving the patients exposed to the toxic effects of cortisol excess. In addition, 30-40% of patients develop pituitary insufficiency after pituitary irradiation [13, 21, 22, 67, 73-75]. Medical treatment for patients with persistent or recurrent CD has the advantage over radiotherapy of a direct onset of action and preserving pituitary function.

Rapid normalization of cortisol production is important because of the reversibility of complications seems to be inversely related to the duration of hypercortisolism [20]. There are several treatment aims for medical therapy in CS (Table 2) [76, 77]. Medical treatment can be indicated because of acute complications of CS like acute psychosis, severe hypertension and opportunistic infections. These potentially life-threatening conditions are mainly associated with the ectopic ACTH syndrome and require rapid reversal of cortisol excess. Furthermore, cortisol-lowering medical therapy is given in some centers as pre-treatment before pituitary surgery with the aim to improve a patient's condition, i.e. to decrease catabolism and improve regulation of blood pressure and glucose homeostasis, in order to reduce perioperative morbidity. In addition, the lowering of cortisol production may decrease bleeding tendency in the surgical area. Although this may be a rational concept, no studies have been performed that prove efficacy of preoperative medical treatment [76]. Medical therapy can further

be considered in patients with CD with a low a priori chance of surgical cure, i.e. in case of an adenoma with an unfavorable localization, e.g. in the parasellar region. Finally, medical therapy is indicated for treatment of hypercortisolism: (1) after unsuccessful surgery for pituitary-dependent CS or the ectopic ACTH syndrome; (2) in patients with metastasized disease, i.e. ACTH-producing neuroendocrine tumors and cortisol producing adrenocortical carcinoma and (3) in patients with any cause of CS with a high operation risk due to e.g. co-morbidities or high age. Quantitatively, patients with persistent or recurrent pituitary-dependent CS represent the largest group that needs medical therapy to control hypercortisolism. As outlined above, pituitary surgery is not successful in 10 to 50% of patients [22, 67, 68] and recurrences occur in up to 26% of patients [67, 68]. In these patients it is essential to induce long-term normalization of cortisol production in order to reverse co-morbidity, improve quality of life and to normalize life expectancy [22]. For this purpose, medical therapy can be applied either as chronic treatment or as bridging therapy during the period after which radiotherapy becomes effective.

Table 2.

Possible indications for medical therapy of Cushing's syndrome.
Acute complications of (severe) hypercortisolism
Pretreatment before pituitary surgery
After unsuccessful surgery
Bridging therapy after pituitary irradiation
Primary medical therapy in Cushing's disease • invisible adenomas • adenoma with unfavorable localization
Inoperability • metastatic disease • high operation risk

5. Targets for the medical treatment of Cushing's disease

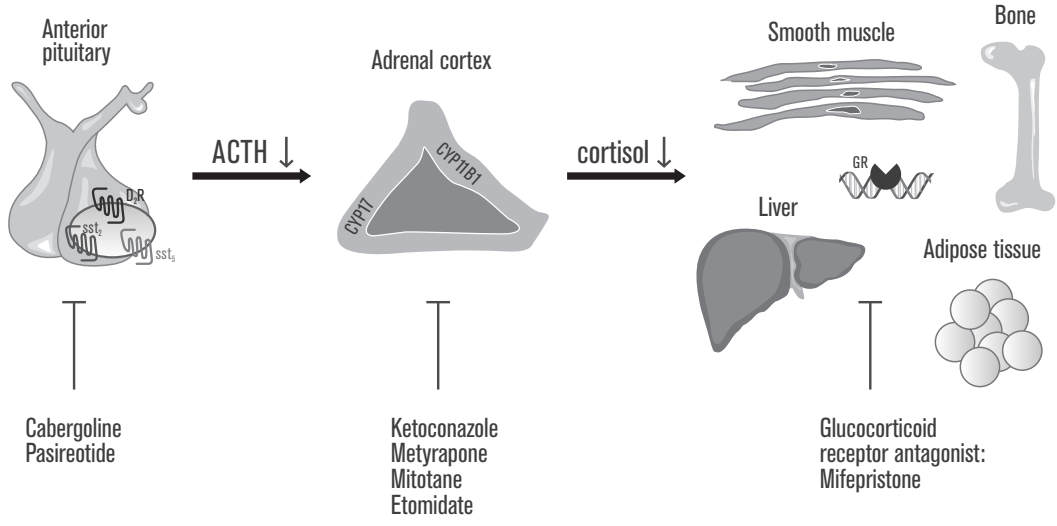
The cause of CD is located in the pituitary gland so ideally, drug therapy would target the corticotroph adenoma. Alternatively, adrenal blocking drugs or glucocorticoid receptor antagonists can be used in order to reverse the effects that are caused by long-term exposure to high levels of cortisol. Optimal medical therapy for CD would normalize ACTH and cortisol production without escape, stabilize or reduce tumor growth, reverse morbidity and mortality and improve quality of life [22]. Unfortunately, to date no long-term efficacy and safety data are available of any available drug. The possible targets for drug therapy in CD are illustrated in Figure 3 [78] (see also Table 3 for an overview of drugs).

5.1. Pituitary-directed medical therapy: dopamine agonists and somatostatin analogs

5.1.1. Somatostatin and dopamine receptor expression in corticotroph pituitary tumors

Dopamine is the main catecholamine neurotransmitter in the human brain and is involved in the regulation of various functions, e.g. locomotor activity, food intake and endocrine function [79]. In the periphery, dopamine has multiple roles as well, including the regulation of catecholamine release, gastrointestinal motility and vascular tone [79].

Figure 3. Drug targets in Cushing's Disease. Reproduced with permission (reference 78).



Drugs that are used to treat patients with Cushing's can either target the corticotroph pituitary adenoma in order to inhibit ACTH secretion (left), directly decrease adrenocortical cortisol secretion (middle) or prevent cortisol from exerting its effects by antagonizing the glucocorticoid receptor at tissue level (right).

Table 3. Dosages and most important side-effects of drugs used to treat Cushing's syndrome.

Group	Drug		
Pituitary-targeted drugs	Cabergoline	Up to 7 mg/week	Headache, dizziness, gastrointestinal discomfort, cardiac valve fibrosis
	Pasireotide	750-2400 µg/day	Hyperglycemia, GH-deficiency, gastrointestinal complaints
Inhibitors of adrenocortical steroidogenesis	Ketoconazole	400 to 1600 mg/day	Hepatotoxicity, gynecomastia, gastrointestinal complaints
	Metyrapone	0.5-4.5 g/day	Dizziness, rash, gastrointestinal discomfort, worsening of hypertension, acne and hirsutism
	Mitotane	3-5 g/day	Gynecomastia, hepatotoxicity, hypercholesterolemia, prolonged bleeding time, gastrointestinal complaints, dizziness, ataxia, confusion, dysarthria, memory problems
	Etomidate	0.1-0,3 mg/kg/h	Nephrotoxicity
Glucocorticoid receptor antagonists	Mifepristone	300 to 1200 mg/day	Hypokalemia, worsening of hypertension, clinical adrenal insufficiency, endometrial hyperplasia, gastrointestinal complaints

Dopamine exerts its functions through binding to five specific receptors belonging to the G protein-coupled receptor (GPCR) family. These receptors can be divided into D1-like receptors (D_1R and D_5R), which are believed to have stimulatory functions and D2-like receptors (D_2R, D_3R and D_4R) which are associated with inhibitory functions [79, 80].
Originally discovered by Brazeau et al. in 1973 [81], somatostatin is a cyclic peptide that consists of either 14 or 28 amino acids [82]. Somatostatin originates from pre-pro-somatostatin and apart from being a neurotransmitter, it mainly has inhibitory effects on both endocrine and exocrine secretion processes [83, 84]. Upon somatostatin or dopamine binding, somatostatin- or D2-like dopamine receptors exert their respective functions by inhibiting the adenylyl-cyclase - cAMP pathway, as well as by decreasing the calcium influx [79, 85, 86]. To date, 5 somatostatin receptor subtypes (sst) have been identified [86]. They are also members of the so-called GPCR family and have been code-named $sst_{1\text{-}5}$ [87].

Corticotroph pituitary adenomas predominantly express the D_2R and the sst_5 [88]. It was found in a previous study by our group that 60% of these adenomas co-express these receptors at the mRNA level [88].
D_2R agonists are frequently used to treat patients with Parkinson's disease, but are also very efficacious in the treatment of prolactinomas [89]. Cabergoline, which has a higher D_2R binding affinity and a longer plasma half-life than bromocriptine, is currently the most widely used agonist of this receptor. The binding affinities of dopamine and D_2R agonists for the receptor are shown in Table 4.

Table 4. IC_{50} values (nM) indicating the binding affinities of somatostatin (analogs) and dopamine (agonists) for their respective receptors. Adapted from [93, 164].

Compound					
Dopamine agonist	**D_2R_{short}**	**D_2R_{long}**	**D_3R**	**D_4R**	
Dopamine	350	320	11	100	
Bromocriptine	4.50	3.90	4.2	>1000	
Cabergoline	0.53	0.41	0.78	81	
SRIF-analog	**sst_1**	**sst_2**	**sst_3**	**sst_4**	**sst_5**
SRIF-14	0.93	0.15	0.56	1.50	0.29
Octreotide	280	0.38	7.10	>1000	6.30
Pasireotide	9.30	1.00	1.50	>100	0.16

The results of both preclinical and clinical studies that investigated the effects of cabergoline on corticotroph adenoma cells are discussed in the next paragraphs. Somatostatin was first isolated from ovine hypothalami and shown to inhibit growth hormone release from the pituitary gland [81]. However, native somatostatin has a short plasma half-life of only approximately three minutes [90] and therefore, somatostatin analogs with a longer half-life were developed in order to clinically benefit from the effects of somatostatin. The sst_2-preferring analogs lanreotide and octreotide were the first clinically available somatostatin analogs and are often successfully used in the treatment of acromegaly [91]. However, in contrast to growth hormone producing pituitary adenomas which have a high degree of sst_2 expression, sst_2 expression on corticotroph adenomas is low [88]. The sst_5, on the other hand, is often highly expressed in CD adenomas [88, 92]. Pasireotide is another somatostatin analog that was developed more recently [93]. The binding affinities of somatostatin and its analogs for $sst_{1\text{-}5}$ are shown in Table 4. The results of *in vitro* and *in vivo* studies evaluating the effects of pasireotide are discussed in the next paragraph.

The low sst_2 expression on corticotroph adenoma cells likely explains why the sst_2-targeting somatostatin analogs octreotide and lanreotide are generally ineffective in the treatment of CD [94, 95]. The low sst_2 expression is thought to result from down-regulating effects of high circulating cortisol levels. Indeed, several *in vitro* studies suggested that exposure to glucocorticoids diminishes sst_2 expression both in GH- and ACTH secreting cell lines [95-99].

Therefore, another aim of the thesis is to compare somatostatin- and dopamine receptor expression in corticotroph adenomas from patients with elevated preoperative UFC excretion to those from patients with normalized preoperative UFC excretion after medical pre-treatment. In this way, we could obtain more insight in the tumor biology of corticotroph pituitary adenomas with respect to the regulation of somatostatin receptor expression in vivo. In addition, this study could provide further information regarding the efficacy of octreotide in CD in relation to sst_2 expression. The results of this study are outlined in Chapter 3.

5.1.2. In vitro efficacy of somatostatin analogs and dopamine agonists on tumoral ACTH secretion

Approximately 80% of ACTH secreting pituitary adenomas expresses the D_2R, although expression levels show vary considerably between adenomas [88, 100]. Cabergoline is an ergot-derived dopamine agonist with particular high affinity for the D_2R. In an *in vitro* setting, Pivonello et al. found that dopamine agonist treatment with either bromocriptine or cabergoline inhibited ACTH secretion in 100% of D_2R positive human corticotroph adenomas [100].
Pasireotide is a somatostatin analog which binds somatostatin receptor subtypes 1, 2, 3 and 5 with high affinity [93]. In particular, it has subnanomolar affinity for the sst_5, the predominant somatostatin receptor subtype in corticotroph pituitary adenomas [92, 93]. Early in *vitro* studies showed that pasireotide (10 nmol/L) significantly inhibited ACTH secretion by 30-40% in 3/5 primary cultures of human corticotroph pituitary adenomas, whereas octreotide had much less pronounced effects [92].

5.1.3. Clinical efficacy of somatostatin analogs and dopamine agonists in patients with Cushing's disease

Recently, three studies were published in which the effects of cabergoline monotherapy in patients with CD were evaluated [101-103]. The first study was performed in 20 patients with persistent CD after surgery. After 3 months of treatment, ACTH concentrations had decreased by more than 25% in 15 of these patients. After 2 years of treatment, 7/20 patients had escaped from cabergoline therapy and 8/20 (40%) were in sustained remission with a median cabergoline dosage of 3.5 mg/week [102]. A retrospective analysis of 30 patients treated with cabergoline monotherapy showed a 50% response rate (either complete or partial) after 6 months of treatment with cabergoline in a mean dose of 1.5 mg/week [101]. Long-term analysis showed a sustained normalization of UFC excretion in 9/30 (30%) patients after a mean of 37 months treated with, on average, 2.1 mg/week (range 0.5-6 mg/week). However, in the group of non-responders, the mean cabergoline dose was only 2 mg/week (range 1-4.5 mg/week) for an average period of 4 months (range 1-9 months). The relatively low dose of cabergoline that was used in most patients in this study might have underestimated the maximal inhibitory effect [101]. Another recently published study prospectively evaluated the effects of low-dose cabergoline (up to 3 mg/week) in 12 patients with persistent CD after unsuccessful surgery. After 6 months, UFC excretion had normalized in 3 patients (25%) [103]. In the other patients, cabergoline decreased UFC excretion by 15-48%. Again, the modest cabergoline dosage might have caused underestimation of the maximal inhibitory effect [103]. In this study, low-dose ketoconazole (200-400 mg daily) was added to cabergoline in case of persistent hypercortisolism after 6 months of cabergoline monotherapy. Interestingly, UFC excretion normalized in 6/9 patients with cabergoline and low-dose ketoconazole combination therapy after an additional 6 months of treatment [103]. Frequently occurring, but mostly transient side-effects of cabergoline include nausea, headache, dizziness and abdominal discomfort [101, 102]. A point of concern, although controversial, is the possible association between chronic use of cabergoline and the development of valvular heart disease. In different

studies in which patients with CD were treated with cabergoline, dosages varying from 0.5 mg up to 7 mg per week were used [101-103]. Patients with Parkinson's disease are treated with much higher dosages of cabergoline, which can cause cardiac valve fibrosis via serotonin receptor 2B-mediated activation of valvular fibroblasts [104, 105]. In cabergoline-treated patients with prolactinomas, an increased prevalence of valvular calcification was found but this was not accompanied by valvular dysfunction [106].

The efficacy of pasireotide in a clinical setting was first evaluated by Boscaro et al. [107]. In this study, 29 patients with pituitary-dependent CS were treated with pasireotide 600 µg s.c. twice daily during 15 days. On average, UFC excretion decreased by 44.5% compared to baseline. Overall, UFC decreased in 22/29 patients and 5 patients (17%) reached normal UFC excretion after 15 days of treatment [107]. The results of a large, multicenter phase III study, in which the long-term efficacy of pasireotide was examined, have recently been published [108]. In this study, 162 patients were included and randomized to treatment with pasireotide s.c. in a dose of either 600 µg or 900 µg twice daily. After three months of treatment, these dosages were increased by 300 µg (to either 900 µg or 1200 µg twice daily) in patients in whom UFC excretion did not fall below two times the upper limit of normal (ULN) or, in case baseline UFC excretion did not exceed 2 times ULN, in whom UFC had increased after three months compared to baseline. On average, UFC excretion decreased by 48% after 6 months of treatment [108]. Without needing up-titration of the dose of pasireotide, 33 patients (20.4%; 14.6% in the 600 µg group, 26.3% in the 900 µg group) reached normal UFC excretion levels after 6 months [108]. Parallel with the decrease in UFC, improvements were observed in blood pressure, weight and quality of life [18, 108]. Hyperglycemia was the most important adverse event in this study, since 73% of the patients experienced a hyperglycemia-related adverse event. Moreover, glycated hemoglobin levels increased from 5.8% to 7.2 or 7.4%, depending on the dosage of pasireotide that was used. Incidental cases have been described showing that pasireotide may even control hypercortisolism for up to 7 years [109].

In healthy volunteers, pasireotide-induced hyperglycaemia was shown to be due to inhibition of incretin secretion with a concomitant decreased insulin secretion [110]. Glucose levels were most effectively lowered with glucagon-like peptide 1 (GLP-1) agonists or dipeptidyl peptidase 4 (DPP-4; an enzyme that inactivates the incretins) inhibitors [110]. Another major drawback of pasireotide is the possible development of GH- and IGF-1 deficiency. Pasireotide has been shown to potently decrease serum levels of these hormones in both rats [93] and humans [111, 112]. Other than hyperglycaemia and GH-deficiency, pasireotide might cause gastro-intestinal side-effects [107, 108]. Pasireotide was recently approved in the EU for treatment of CD after unsuccessful surgery.

As described above, the effects of pasireotide and cabergoline on corticotroph tumor cells have been extensively investigated *in vitro* and *in vivo*. Both agents have been shown to have beneficial effects in patients with CD, i.e. they are able to reduce UFC excretion levels in these patients. Whether the reduction in cortisol concentrations is solely due to the effects of pasireotide and cabergoline on the pituitary tumor is as yet unclear. The adrenocortical expression of somatostatin and dopamine receptors has thus far not been well investigated. Few attempts have been made to assess expression levels of somatostatin and dopamine receptors in the adrenal gland, but with obvious limitations. In some studies, mRNA expression levels were measured [113-115], a method that does not allow for discrimination between adrenal cortex and medulla or between the three zonae of the adrenal cortex. Other studies used polyclonal antibodies to demonstrate receptor expression at protein level [113, 116] or used autoradiography with radiolabelled somatostatin, an indirect method to investigate somatostatin receptor expression [117, 118]. Moreover, it is unclear whether agonists of sst_5 and D_2R receptors can inhibit the adrenocortical steroid production and cortisol production in particular.

In this thesis, we aim to analyze the protein expression levels of the sst_2, sst_5 and D_2R in the human adrenal gland. By incubating dispersed primary adrenocortical cells with pasireotide and cabergoline, we hope to answer the question as to whether or not these agents, apart from their effect on the pituitary gland, also influence steroidogenesis. The results of this study are described in Chapter 4 of this thesis.

5.2. Drugs that target the adrenal cortex

Ketoconazole is an imidazole derivative that was originally developed to treat fungal infections. However, ketoconazole has also been shown to inhibit adrenocortical steroidogenesis when administered in high dosages, i.e. between 400 and 1600 mg/day [119]. Its main mechanism of action is inhibition of the steroidogenic enzymes 17-hydroxylase and 17,20-lyase and it is one of the most frequently used cortisol-lowering drugs [22, 119-126]. In a retrospective study, ketoconazole treatment, in a dose between 200 and 1000 mg/day, resulted in control of hypercortisolism in 17 out of 33 patients within 3 months with a concomitant clinical improvement. In 5 other included patients, ketoconazole was stopped within the first week of treatment because of intolerance [119]. One older study with 28 patients showed that ketoconazole treatment was successful in 93% of patients but this result was biased as patients had been treated before with radiotherapy [126]. Ketoconazole is hepatotoxic, can cause gynecomastia and has serious, mainly gastrointestinal side-effects which together can limit its long-term use [119, 125-128]. Fluconazole is another antimycotic drug that is considered less toxic compared to ketoconazole [129] and may also inhibit adrenocortical steroidogenesis [127, 129-133]. The potency of fluconazole with respect to *in vitro* inhibition of human adrenocortical steroid production has not been compared yet to that of ketoconazole.

Therefore, one of the aims of this thesis is to compare the effects of fluconazole and ketoconazole on human adrenocortical steroidogenesis. The results of this study could give an answer to the question whether fluconazole might be an alternative to ketoconazole to treat patients with CS. Moreover, we aim to determine the mechanism of inhibition of steroidogenesis of both drugs. The results of this comparative study are presented in Chapter 5.

Ketoconazole may also have inhibitory effects on ACTH secretion by corticotroph tumor cells [76, 134]. This might explain the absence of a rise in ACTH levels during ketoconazole treatment in response to a decrease in cortisol levels. Although an increase in ACTH production upon ketoconazole treatment was found in rats [135], several studies show no rise in ACTH concentrations in patients with CD following treatment with ketoconazole [120, 126, 136]. In recent clinical trials, ketoconazole has been combined with either pituitary-targeted medical therapy or other adrenal-blocking drugs [103, 111, 137]. Metyrapone is another frequently used inhibitor of steroidogenesis. Its main site of action is 11β-hydroxylase, as Verhelst et al. found a concomitant decrease in cortisol concentrations and increase in 11-deoxycortisol levels in 91 CS patients treated with metyrapone [138]. Metyrapone, at dosages between 750 and 6000 mg/day, can effectively control cortisol overproduction in approximately 75% of patients during short-term treatment, i.e. up to 16 weeks, whereas long-term treatment resulted in biochemical remission in about 80% of patients although these patients were also treated with radiotherapy [138]. Side-effects that occur during metyrapone therapy include dizziness, rash and gastrointestinal complaints [67]. In addition, concentrations of mineralocorticoid precursors and adrenal androgens can increase due to the 11β-hydroxylase block and the subsequent increase in ACTH production which stimulates steroidogenesis [67, 128]. As a result, metyrapone administration might be accompanied by worsening of hypertension, acne and hirsutism [76, 128, 138]. In contrast to ketoconazole, metyrapone does not cause gynecomastia. Hence, metyrapone might preferably be used in males, whereas ketoconazole may be more appropriate to treat female patients with CS [139]. However, although metyrapone treatment often has a good short-term response, long-term efficacy data are scarce. Similar to ketoconazole, metyrapone has been used in combination with other adrenocortical-targeted drugs [137, 140]. Mitotane or o,p'-DDD is a derivative of DDT and is mainly used in the treatment of adrenocortical carcinomas because of its adrenolytic properties [141, 142]. It has been shown to inhibit cortisol production via inhibition of multiple steroidogenic enzymes and to be efficacious in the treatment of CD [143, 144]. Schteingart et al. reported clinical and biochemical remission of CD in 80% of patients treated with mitotane and pituitary irradiation [145]. Another study showed control of hypercortisolemia using mitotane monotherapy in 38/46 patients, whereas mitotane and pituitary irradiation combination therapy was successful in 100% of patients [144]. Mitotane has a slow onset of action and based on its lipophilic nature, it is stored in adipose tissue, ensuring a long half-life of 18-159 days [128]. Consequently, mitotane remains present in the body, even up to 2 years after withdrawal of the drug [67, 128]. Especially when administered in high dosages, mitotane has many

side-effects including gastrointestinal (anorexia, nausea, vomiting, diarrhea) and neurological (dizziness, ataxia, confusion, dysarthria, memory problems) complaints [128, 132, 146] that require close monitoring of the patient and plasma mitotane concentrations. Other frequently reported adverse events are gynecomastia, hepatotoxicity, hypercholesterolemia and prolonged bleeding time. Due to its adrenolytic nature, mitotane often causes adrenal insufficiency necessitating concomitant hydrocortisone substitution [146].

Recently, a clinical trial was performed in which patients with severe CS were treated with medical combination therapy [137]. In this series, 11 patients were included, 4 of which had CD and 7 had EAS. Patients had severe (e.g. pulmonary, cardiovascular or infectious) complications that needed immediate intervention. Treatment was initiated with mitotane (3 g/24h) and, since mitotane has a slow onset of action, ketoconazole (800 mg/24h) and metyrapone (2.25 g/24h). These dosages were adjusted based on clinical signs. Patients were reported to experience clear improvement of their Cushingoid features. Interestingly, UFC (near-)normalized in all patients within the first 3 days [137]. This treatment regimen was generally well tolerated, but main side effects were gastrointestinal complaints, hypokalemia and increases in cholesterol and liver enzyme values. Considering its efficacy, this approach might be an alternative to emergency bilateral adrenalectomy, in particular for critically ill patients in whom surgery is contraindicated. Similar to ketoconazole, etomidate is an imidazole derivative [147]. Originally used as an anesthetic agent, etomidate, which is administered intravenously, was soon reported to cause adrenocortical insufficiency in critically ill patients [148]. Subsequent studies showed that etomidate can be used to induce eucortisolemia in patients with CS [149, 150]. Its suggested mechanism of action is inhibition of the 11β-hydroxylase and cholesterol side-chain cleavage enzymes [121, 132]. Currently, etomidate infusion is used to obtain rapid control of hypercortisolism in severe cases of CS (e.g. in case of glucocorticoid-induced psychosis), in which oral cortisol-lowering agents are ineffective or oral therapy is impossible [22, 151].

5.3. Glucocorticoid receptor antagonists

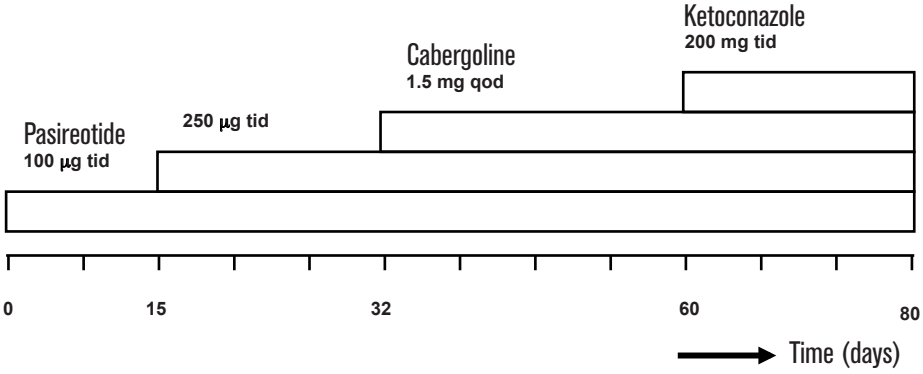
Mifepristone is the only glucorticoid receptor (GR) antagonist that is currently available [152] and counteracts the effect of cortisol at tissue level. It was initially developed as a compound with antiprogestin activity by blocking the progestin receptor. Subsequently, antiglucocorticoid effects were recognized because mifepristone binds to the GR with an 18-fold higher affinity than cortisol [153]. As a consequence, cortisol cannot exert its negative feedback effects at the hypothalamic and pituitary level, which can lead to an increase in ACTH secretion with a concomitant increased cortisol production. Clinical improvement during mifepristone treatment has been observed in all forms of CS [152, 153]. A recently published study showed beneficial effects of treatment with mifepristone. In this study, 50 patients with CS (43 with CD) were treated for 24 weeks with mifepristone at dosages between 300 and 1200 mg daily [153]. Clear metabolic improvements were achieved with this treatment, as reflected by decreased glucose levels after oral glucose tolerance tests, decreased glycated hemoglobin levels and improved insulin sensitivity. Moreover, body weight significantly decreased and quality of life significantly improved after 24 weeks of treatment. As was expected, plasma concentrations of ACTH and cortisol increased at least 2-fold in 72% of the patients with CD. This illustrates the most important drawback of this treatment, since therapy with mifepristone does not target the pituitary adenoma. In this study, only one patient experienced an increase in adenoma size after 24 weeks of treatment, but as the authors emphasize in their paper, it is uncertain whether the risk of tumor growth increases with longer treatment duration. Frequently encountered adverse effects included fatigue, headache, abdominal discomfort, decreased serum potassium and peripheral edema [153]. Mifepristone was recently approved in the U.S. for the treatment of hyperglycemia in patients with CS and type II diabetes if surgery is not an option or unsuccessful. Mifepristone might also be used to treat patients with CS that have acute psychosis, in order to rapidly counteract the GR-mediated effects [154]. A disadvantage of mifepristone is that there is no biochemical parameter available according to which the mifepristone dose can be adjusted. Consequently, clinical adrenal insufficiency can develop after overtreatment, which may require dose interruption and corticosteroid replacement. In female patients, mifepristone can cause endometrial hyperplasia that requires ultrasonic monitoring. In addition, increasing cortisol levels during mifepristone treatment can cause mineralocorticoid effects with induction or worsening of hypokalemia and hypertension [67, 152, 155]. Theoretically, long-term use of mifepristone could provoke Nelson's

syndrome in patients with CD. Dose titration of mifepristone should be performed according to clinical signs and serum potassium levels [152].

6. A prospective trial with pasireotide, cabergoline and ketoconazole combination therapy

As described earlier, monotherapy with either cabergoline or pasireotide induces complete biochemical remission in about 20-40% of patients with CD. Previously, our group performed a prospective open-label trial in which 17 patients with either *de novo* or persistent/recurrent CD were treated in a stepwise manner with pasireotide mono- or combination therapy with cabergoline and low-dose ketoconazole during 80 days [111]. The rationale of this approach was that the majority of corticotroph adenomas do co-express sst_5 and D_2R [88] and that combined treatment with sst_5 and D_2R targeting compounds may have additive or even synergistic effects [156, 157].

Figure 4. Treatment regimen used in reference 93.

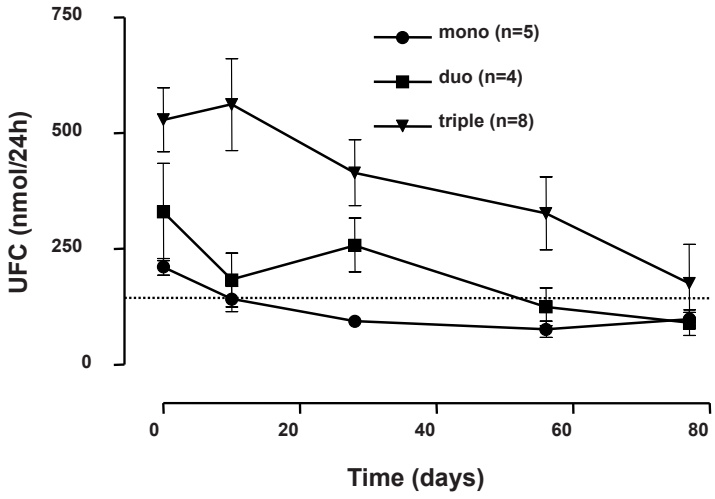


After inclusion, patients started with pasireotide monotherapy (100 µg three times daily (tid)). In case of persistent hypercortisolism after 10 days of treatment, this dosage was increased to 250 µg tid. Patients who reached normal cortisol excretion levels after 4 weeks remained on pasireotide monotherapy, while patients who were not biochemically cured received cabergoline (1.5 mg every other day (qod)) on top of pasireotide. Finally, ketoconazole (200 mg tid) was added to these two drugs in case of persistently elevated urinary free cortisol excretion levels after 8 weeks of treatment. The last evaluation took place after 11 weeks (77 days) of treatment.

All patients started with pasireotide monotherapy in a dosage of 100 µg s.c. thrice daily. After 10 days, this dosage was increased to 250 µg s.c. thrice daily in case UFC excretion remained elevated.
UFC excretion was measured again at day 28, after which, in case of persistent hypercortisolism, cabergoline (1.5 mg every other day) was added to pasireotide. If UFC remained elevated at day 56, ketoconazole (200 mg thrice daily) was added to pasireotide and cabergoline (Figure 4). After 80 days, patients could choose to participate in an extension study with drug therapy or to proceed to transsphenoidal surgery. In patients with normalized UFC after mono- or duo therapy, the drug regimen was continued. In patients in whom hypercortisolism was corrected after triple therapy, an attempt was made to stop ketoconazole continuing with duo therapy with pasireotide and cabergoline from day 80 onwards. Using this treatment regimen, 15/17 patients (88%) achieved biochemical remission after 80 days. Five patients already normalized within one month of pasireotide monotherapy. An additional four patients reached normal UFC excretion values after

the addition of cabergoline to pasireotide. Six out of the remaining eight patients ultimately normalized with triple therapy, as ketoconazole was added after 8 weeks. Of the 2 remaining patients, UFC excretion levels had almost normalized in 1 patient, whereas 1 patient did not respond to triple therapy. He had an invasive adenoma and postoperative analysis of tumor tissue showed low expression levels of sst_5 and D_2R. As can be seen in Figure 5, patients with more severe hypercortisolism at baseline needed more drugs to reach normal UFC excretion levels [111].

Figure 5.



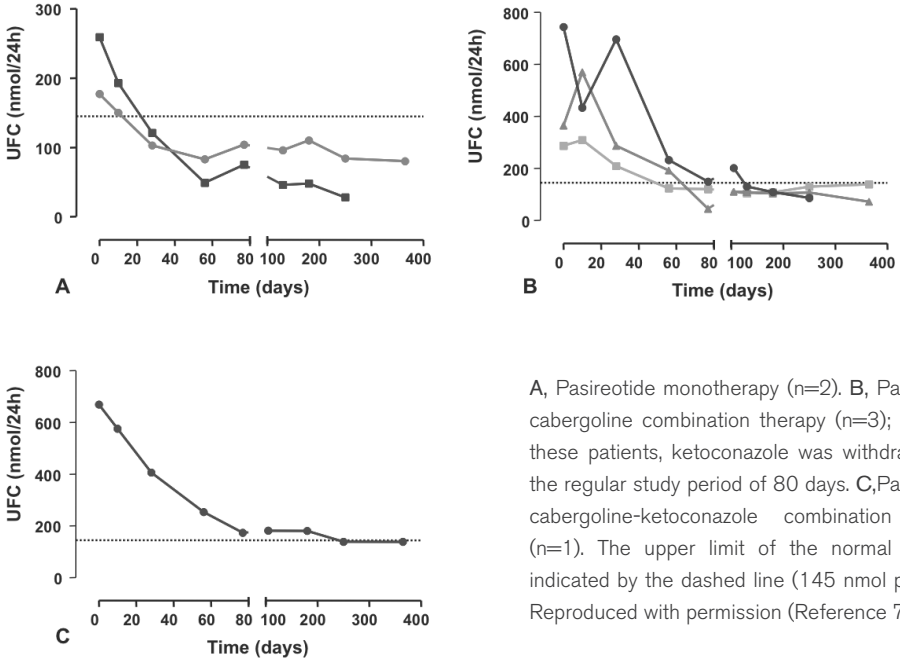
Mean levels of urinary free cortisol in five patients treated with pasireotide monotherapy, four patients treated with combination therapy with pasireotide plus cabergoline, and eight patients treated with pasireotide plus cabergoline and ketoconazole. Cabergoline was added to pasireotide if the level of urinary free cortisol had not normalized at day 28. Ketoconazole was added to pasireotide and cabergoline at day 60 if the patient had persistent hypercortisolism. The upper limit of the normal range is indicated by the dashed line. Bars indicate the standard errors. Adapted from ref. 93.

Besides biochemical remission, patients showed some clear improvements of Cushingoid features. These included: (1) decrease in facial fat deposition and plethora (n=12), hirsutism (n=6), acne (n=3), easy bruisability (n=6), skin atrophy (n=3), tiredness (n=6), muscle weakness (n=3), edema (n=1) and depression (n=1); (2) return of menstrual cycle (n=3); (3) a mean decrease in body weight and waist circumference by 2.4±0.9 kg and 4.2±1.3 cm, respectively; (4) a mean decrease in systolic and diastolic blood pressure by 12±4 mmHg and 8±3 mmHg. Antihypertensive drugs could be stopped in 4 patients and lowered in 2 patients.

The most prevalent clinical adverse events included anorexia, nausea, dizziness, myalgia and arthralgia, occurring in all three medication groups and presumably indicating steroid withdrawal. Symptomatic hypocortisolism developed in 1 patient under pasireotide-cabergoline combination therapy and was treated temporarily with hydrocortisone. Two patients presented with bilateral pulmonary embolism after respectively 3 and 10 days of treatment with pasireotide 100 µg thrice daily, which had induced an acute, transient decrease in UFC excretion levels by 76 and 77 % from baseline. A transient increase in liver enzymes, mostly 1-3 times the upper limit of normal, was observed in 9 patients, in particular during the first 28 days (n=5). At baseline, 2 patients had diabetes mellitus treated with oral antidiabetic drugs. At day 77, an increase was found in mean HbA1c, fasting glucose and oral glucose tolerance test (OGTT) 2h glucose values. According to the OGTT 2h glucose value, 8/10 patients with diabetes mellitus at day 77 had an impaired glucose tolerance at baseline. Treatment with

oral antidiabetic drugs was initiated in 3 patients and adapted in 2 patients of whom 1 ultimately needed insulin therapy to achieve glucose control (this patient had a HbA1c value at baseline of 8.4 %). IGF-I levels decreased in all patients to a variable degree. Two patients had decreased IGF-I levels at baseline. In 9 patients IGF-I levels decreased below the lower limit of the age- and gender-adjusted normal range. Six patients participated in the extension study: 2 patients continued with pasireotide monotherapy that maintained UFC excretion levels within the normal range up to 1 year. Three patients continued with pasireotide and cabergoline, in 2 of these 3 patients ketoconazole was withdrawn after day 80, sustaining biochemical remission up to 1 year. Finally, 1 patient continued with triple therapy resulting in normal UFC levels (Figure 6).

Figure 6. Individual UFC profiles in 6 patients who participated in the extension study of the pasireotide monotherapy and combination therapy trial.



A, Pasireotide monotherapy (n=2). B, Pasireotide-cabergoline combination therapy (n=3); in two of these patients, ketoconazole was withdrawn after the regular study period of 80 days. C,Pasireotide-cabergoline-ketoconazole combination therapy (n=1). The upper limit of the normal range is indicated by the dashed line (145 nmol per 24 h). Reproduced with permission (Reference 78).

This study was the first to investigate the effects of medical combination therapy with these three agents in patients with CD. Although the primary endpoint of the study was normalization of UFC excretion, it is of extreme importance to also evaluate other parameters in these patients. They were biochemically cured after 80 days, but what happened to the complications of CD as described in paragraph 3? To answer these questions, a number of additional studies were performed. The results of these studies are presented in this thesis.

With respect to the hypercoagulability that is observed in CD, this thesis aims to evaluate the coagulation pathway in the 17 patients with CD described above [111] *and to determine the effects of medical combination therapy with pasireotide, cabergoline and ketoconazole on parameters of coagulation and fibrinolysis. The results of this study could further contribute to the understanding of disorders of the hemostatic system and the reversibility of these abnormalities in patients with CD. These results are described in Chapter 6 of this thesis.*

In the same cohort of 17 patients with CD, we aim to study the quality of life and cortisol diurnal rhythm as well as the effects of medical combination therapy on these parameters. These results are outlined in Chapter 7.

To address the pathogenesis of glucocorticoid-mediated hypertension, another aim of this thesis is to evaluate parameters of the RAAS in these patients with CD [111] *and to determine the effects of combination therapy with pasireotide, cabergoline and ketoconazole on blood pressure and the RAAS. Studying the RAAS in patients with CD could provide more insight in the pathogenesis of glucocorticoid-induced hypertension. The results of this study are described in Chapter 8.*

7. A prospective trial with ketoconazole and octreotide combination therapy for treatment of Cushing's disease.

As described above, it was found that pasireotide monotherapy resulted in normalization of UFC excretion levels in only 20% of the patients after 6 months of treatment, especially in those patients with mildly elevated cortisol concentrations [108]. Apart from this modest success rate, pasireotide treatment has been reported to cause severe hyperglycemia, especially in patients with pre-existent impaired glucose tolerance, [107, 108, 111] and lowering of IGF-I levels, in some patients even below the lower limit f normal [111]. Together, these data show that pasireotide might play a role in the treatment of a subset of patients with CD, but more drugs are needed in case of moderate to severe hypercortisolism.
The sst_2, which is highly expressed in somatotroph pituitary adenomas that cause acromegaly [158], is expressed to a much lesser extent in corticotroph pituitary adenomas [88]. As described earlier, this most likely results from down-regulating effects of high circulating cortisol levels on sst_2 expression by corticotroph adenoma cells [95, 96, 159], to which the sst_5 and D_2R seem less sensitive [97]. Again, these observations might explain why octreotide is generally ineffective in CD with respect to suppression of ACTH production [94, 95]. In contrast, native somatostatin as well as octreotide do inhibit ACTH production in patients with Nelson's syndrome, especially when glucocorticoid substitution therapy is temporarily withheld [94, 160]. Similarly, native somatostatin has been shown to inhibit ACTH-release in patients with Addison's disease after cessation of replacement therapy for 24h prior to somatostatin infusion [161].

From these observations, it was hypothesized that cortisol-lowering therapy may induce up-regulation of sst_2 *expression in corticotroph adenomas of patients with CD. Indeed, it is described in Chapter 3 of this thesis that after achieving normocortisolism induced by medical therapy, cortisol-mediated* sst_2 *down-regulation on corticotroph adenomas appears to be a reversible process at mRNA level. Although the increase in* sst_2 *mRNA expression was not followed by an increase in protein expression in this study, higher* sst_2 *levels on corticotroph tumor cells could potentially increase the efficacy of* sst_2*-preferring somatostatin analogs and pasireotide in lowering ACTH production by these cells* [82]*. Therefore, the final aim of this thesis is to prospectively evaluate the efficacy of successive combination therapy with ketoconazole and octreotide. This proof-of-concept study will contribute to the understanding of the possible role of sst2-targeted therapy in CD. Preliminary results of this study are discussed in Chapter 9.*

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2 CHAPTER

DIFFERENT ENTITITIES OF CORTICOTROPH PITUITARY TUMORS ON THE BASIS OF PROHORMONE CONVERTASE EXPRESSION AND THE RELATIONSHIP WITH DOPAMINE D2 AND SOMATOSTATIN RECEPTOR SUBTYPE 2 AND 5 EXPRESSION.

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Submitted.

CHAPTER 3

PREOPERATIVE NORMALIZATION OF CORTISOL LEVELS IN CUSHING'S DISEASE AFTER MEDICAL TREATMENT: CONSEQUENCES FOR SOMATOSTATIN AND DOPAMINE RECEPTOR SUBTYPE EXPRESSION AND IN VITRO RESPONSE TO SOMATOSTATIN ANALOGS AND DOPAMINE AGONISTS.

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Submitted.

ABSTRACT

Context

Corticotroph pituitary adenomas often highly express the dopamine 2 receptor (D_2R) and somatostatin receptor subtype (sst) 5. The sst_2 expression is relatively low, likely resulting from down-regulating effects of high cortisol levels. This may explain why the sst_2 preferring somatostatin analog octreotide, compared to the multi-receptor targeting somatostatin analog pasireotide, is generally ineffective in Cushing's disease.

Objective

To compare sst and D_2R expression levels between adenomas from patients with elevated and normalized preoperative UFC excretion.

Patients and Design

Corticotroph adenoma tissue was examined from patients from group 1 (n=22; elevated preoperative UFC) and group 2 (n=11; mean duration of preoperative normocortisolism 10 weeks). Somatotroph adenoma tissue from 10 acromegalic patients was examined to compare receptor expression profiles.

Main Outcome Measures

Receptor mRNA and protein expression levels and effects of octreotide, pasireotide and cabergoline on ACTH secretion by cultured human corticotroph adenoma cells.

Results

Sst_2 mRNA expression in group 2 was 10-fold higher than in group 1 ($p<0.01$), even comparable to that in somatotroph adenomas. There were no statistically significant differences in sst_5 and D_2R mRNA expression or in sst_2, sst_5 and D_2R protein expression between both groups of corticotroph adenomas. Octreotide (-18.9 ± 9.1% (n=4)) was less potent than pasireotide (-36.3 ± 11.2% (n=6)) and cabergoline (-31.4 ± 12.9% (n=4)) with respect to inhibition of ACTH secretion by adenomas from group 2.

Conclusions

After achieving normocortisolism induced by medical therapy, cortisol-mediated sst_2 down-regulation on corticotroph adenomas appears to be a reversible process at mRNA, but not at protein level. Octreotide remains less potent than pasireotide and cabergoline with respect to *in vitro* inhibition of ACTH secretion. Whether sustained normocortisolism induced by medical therapy induces re-expression of functional sst_2 protein in corticotroph adenomas and whether this increases the ACTH-lowering potency of octreotide remains to be established.

INTRODUCTION

Transsphenoidal surgery is the primary treatment for Cushing's disease (CD), a condition that is caused by an adrenocorticotropin (ACTH) producing pituitary adenoma [1]. However, the long-term remission rate is only approximately 50-70% [2, 3], which clearly indicates the need for alternative treatment modalities. It has been shown in previous studies that the somatostatin receptor subtype 5 (sst_5) and the dopamine 2 receptor (D_2R) are the most widely expressed receptors of their respective families in ACTH-producing pituitary adenomas [4-6]. Indeed, these receptors have been used as molecular targets to treat patients with CD with the sst_5-preferring agonist pasireotide and with the D_2R agonist cabergoline [7-9]. The expression level of the sst_2, which is widely expressed in somatotroph pituitary adenomas that cause acromegaly [10], is relatively low in corticotroph adenomas [4]. This presumably results from down-regulating effects of high endogenous cortisol levels on sst_2 expression by corticotroph tumor cells [11-13]. In contrast, the sst_5 and D_2R seem less sensitive to down-regulating effects of glucocorticoids [14]. These observations might explain why octreotide, a somatostatin analog with high binding affinity for the sst_2, is generally ineffective in CD with respect to suppression of ACTH production [12, 15]. In contrast, native somatostatin and octreotide do inhibit ACTH production in patients with Nelson's syndrome, especially when replacement therapy with glucocorticoids is temporarily withheld [15, 16]. Similarly, native somatostatin has been shown to inhibit ACTH-release in patients with Addison's disease after cessation of replacement therapy for 24h prior to somatostatin infusion [17].

From these observations, it can be hypothesized that cortisol-lowering therapy with drugs that suppress adrenocortical steroidogenesis may induce up-regulation of sst_2 expression in corticotroph adenomas of patients with CD. This could potentially increase the efficacy of sst_2-preferring somatostatin analogs and pasireotide in lowering ACTH production by corticotroph tumor cells [18].

The first aim of this study was to compare receptor expression profiles of corticotroph pituitary adenomas from patients with elevated preoperative cortisol levels with those of corticotroph adenomas from patients in whom cortisol levels have normalized after preoperative cortisol-lowering therapy. Furthermore, the *in vitro* effects of octreotide and pasireotide were compared with respect to their ACTH-lowering potential in primary cultures of human corticotroph adenomas from patients with normalized preoperative cortisol levels.

Patients and methods

Adenoma tissue

Corticotroph and somatotroph pituitary adenoma tissue from patients with CD (n=33) or acromegaly (n=10) was available after transsphenoidal surgery. 32/33 corticotroph adenomas were ACTH-immunopositive. The diagnosis of CD was based on clinical features, elevated 24h urinary free cortisol (UFC) levels, inadequate suppression of plasma cortisol levels after administration of 1 mg dexamethasone and/or the absence of a physiological circadian cortisol rhythm. The pituitary origin of ACTH overproduction was confirmed by MR imaging or, when MRI was inconclusive, by bilateral inferior petrosal sinus sampling. Patients received various pre-treatments as described in Table 1. In our center, all patients are medically pre-treated with ketoconazole for 3 months prior to surgery on a routine basis. Some of the patients included in this study participated in a clinical trial in which stepwise medical therapy with pasireotide, cabergoline and ketoconazole was applied in patients with Cushing's disease [8].

Approval from the Medical Ethical Committee of the Erasmus MC, as well as informed consent to use the tumor tissues for research purposes, was obtained. To check for purity of corticotroph adenoma tissue, GH/POMC mRNA expression ratios were calculated. Adenomas with GH/POMC ratios <10% compared with normal pituitary tissue were regarded as pure corticotroph adenoma tissues. Adenomas with GH/POMC ratios >10% were considered significantly contaminated with normal pituitary tissue and were excluded from the mRNA analysis [4].

Quantitative PCR

Quantitative PCR was performed as described previously [19]. Briefly, poly A+ mRNA was isolated using Dynabeads Oligo (dT)25 (Dynal AS, Oslo, Norway) from isolated pituitary adenoma cell pellets (200.000 cells) or cultured AtT20 cells. cDNA was synthesized using the poly A+ mRNA, which was eluted from the beads in 40 µL H_2O twice for 2 min at 65° C, using Oligo $(dT)_{12-18}$ primer (Invitrogen, Breda, The Netherlands). The assay for sst_2 and sst_5 was performed using 15µL TaqMan Universal PCR master mix (Applied Biosystems, Capelle aan de IJssel, The Netherlands), 500nM forward primer, 500nM reverse primer, 100nM probe and 5µL cDNA template, in a total reaction volume of 12.5µL. For D_2R, the forward and reverse primers were used in a concentration of 300nM, the probe was used in a concentration of 100nM. The reactions were carried out in an ABI 7900 sequence detector (Perkin-Elmer, Groningen, The Netherlands). After an initial heating at 95° C for 8 min, samples were subjected to 40 cycles of denaturation at 95° C for 15 sec and annealing for 1 min at 60° C. The primer and probe sequences that were used have been published elsewhere [4, 13]. The sequences for the D_4R are: GCTCTTCGTCTACTCCGAGGT (forward), CGCACAGGTTGAAGATGGA (reverse) and the probe was obtained from Roche Diagnostics, The Netherlands (catalog number 04688651001). The detection of hypoxanthine-phosphoribosyl-transferase (HPRT) mRNA served as a control and was used for normalization of the ssts and D_2R mRNA levels.

Immunohistochemistry

Five-micrometer sections were deparaffinized, dehydrated, exposed to microwave heating (in TRIS/EDTA (pH 9.0), 20 min at 100° C), rinsed in tap water and blocked in hydrogen peroxide 3% (final concentration) in phosphate buffered saline (PBS). The sections were incubated with the primary antibodies for ACTH, D_2R (both 1h at room temperature), sst_2 and sst_5 (both overnight at 4° C) respectively. Thereafter, sections were washed with TRIS/Tween 0.5% (pH 8.0) and incubated with a second antibody (Real EnVision Detection System, Dako Denmark, Glostrup, Denmark). Negative controls for IHC included omission of the primary antibody. The normal

Table 1. Baseline characteristics of both groups of patients with corticotroph adenomas.

Patient	Gender	Age	MRI	Pre-treatment	Pre-op UFC (xULN)	Duration of normocortisolism (weeks)
1	F	27	Micro	Keto	3,49	-
2	F	23	Macro	Keto	1,28	-
3	F	38	Micro	Keto	2,80	-
4	F	22	Micro	Keto	3,77	-
5	F	23	Macro	Keto	9,27	-
6	F	22	Macro	Keto	3,94	-
7	F	25	Macro	Keto	6,36	-
8	F	38	Macro	Metyrapone	4,32	-
9	M	68	Macro	Keto	7,75	-
10	M	67	Macro	Keto	7,91	-
11	F	46	Micro	Keto	7,24	-
12	M	14	Macro	-	18,7	-
13	F	61	Non-visible	Keto	3,35	-
14	M	24	Micro	Keto	4,55	-
15	F	39	Non-visible	Keto	1,17	-
16	F	52	Macro	Metyrapone	1,89	-

17	F	40	Micro	Keto	3,09	-
18	F	27	Micro	Rosiglitazone	2,94	-
19	F	48	Macro	-	1,17	-
20	F	67	Micro	-	2,41	-
21	M	67	Micro	Pas, Cab, Keto	7,25	-
22	F	41	Macro	Keto	1.06	-
23	F	37	Micro	Keto	0.74	8
24	F	42	Micro	Pas, Cab, Keto	0,78	24
25	M	47	Micro	Pas, Cab, Keto	0,27	8
26	F	23	Micro	Pas, Cab	0,77	6
27	F	55	Micro	Pas	0,88	12
28	F	56	Micro	Pas, Cab, Keto	0,59	5
29	M	56	Micro	Pas, Cab	0,30	14
30	F	51	Micro	Keto	0,78	7
31	M	36	Macro	Keto	0,47	8
32	M	29	Macro	Keto	0,79	9
33	F	45	Micro	Keto	0,59	12

Keto: ketoconazole; Pas: pasireotide; Cab: cabergoline. Pre-op: preoperative; UFC: urinary free cortisol excretion. ULN: upper limit of normal. Ketoconazole was administered in dosages up to 1200mg daily when used as monotherapy. In combination with pasireotide (250 µg s.c. three times daily) and cabergoline (1.5 mg every other day), ketoconazole was administered in a dosage of 200 mg three times daily. Patients that received pasireotide treatment (either monotherapy or in combination with cabergoline and ketoconazole) were included in a clinical trial [8]. Metyrapone was used in dosages up to 1500 mg daily. Rosiglitazone was used in a dosage of 8 mg three times daily.

human pancreas was used as a positive control for sst_2 and sst_5 staining, whereas the normal pituitary gland was used as a positive control for D_2R staining.

Sst_2 immunohistochemical staining on AtT20 cells were performed using poly-L lysine-coated (10 µg/mL) chamber slides (Thermo Fisher Scientific, Rochester, NY, USA). After removal of culture medium, cells were fixed with paraformaldehyde 4% followed by 50% and 100% methanol, respectively. The cells were then blocked in hydrogen-peroxide and further processed as described above. The mouse monoclonal anti-ACTH antibody (Lab Vision Corporation, CA, USA) was used at a dilution of 1:400. The rabbit monoclonal anti-sst_2 antibody (BioTrend, Köln, Germany; validated in [20-22] was used at a dilution of 1:50, while the rabbit monoclonal anti-sst_5 antibody (kind gift from Dr. S. Schulz, Friedrich-Schiller University Jena, Germany [23]) was used at a dilution of 1:10. Finally, the mouse monoclonal anti-D_2R antibody (Tebu-bio, Heerhugowaard, The Netherlands) was used at a dilution of 1:400. The Immunoreactivity Score (IRS) was calculated to compare stainings from different adenomas [24]. The IRS is calculated by the product of the percentage of positive cells (>80%: 4; 51-80%: 3; 10-50%: 2; <10%: 1 and 0%: 0) and the intensity of the staining (strong: 3; moderate: 2; mild: 1 and no staining: 0), which results in IRS scores between 0 (no staining) and 12 (maximum staining). The IRS is based on the evaluation of two independent observers.

Corticotroph tissue isolation and cell culture

Cell suspensions were prepared from corticotroph adenoma tissue by enzymatic dissociation with dispase [25]. Subsequently, cells were seeded in 48-well plates (Corning costar, Schiphol-Oost, The Netherlands) in Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 10% fetal calf serum, L-glutamine 2mmol/L,

penicillin 1x10^5 U/L and fungizone 0.5 mg/L and cultured at 37°C in a 5% CO_2 incubator. Murine corticotroph AtT20-D16v cells (provided by Dr. J. Tooze, European Organization for Molecular Biology) were cultured in 75cm^2 flasks (Corning costar, Schiphol-Oost, The Netherlands) under the same conditions. Once a week medium was refreshed and cells were harvested with trypsin (0.05%)-EDTA (0,53mM) as described previously [26]. Culture media and supplements were obtained from Invitrogen (Breda, The Netherlands). Cells were incubated with pasireotide, octreotide (both from Novartis Pharma A.G., Basel, Switzerland) or cabergoline (Pfizer, Capelle aan den IJssel, The Netherlands) for 72h in a concentration of 10 nmol/L. After the incubation period, media were collected and stored at -20° C until ACTH measurement after the addition of aprotin 4x10^5 IU/mL (Bayer, Mijdrecht, The Netherlands) to prevent degradation of ACTH.

Hormone measurements

Patients were included in 2 institutions. UFC excretion was measured using the routinely used assays in the respective hospitals (enzyme immunoassay (Immulite, Siemens Healthcare, UK) and radioimmunoassay (GammaCoat, Diasorin, Stillwater, MN, USA), respectively). Values are expressed as times upper limit of normal (xULN). Concentrations of ACTH in culture media from cultured corticotroph cells were measured using a commercially available, non-isotopic automatic chemiluminescence system (DPC Immulite, Los Angeles, USA).

Statistical analysis

To compare receptor expression levels between groups, unpaired t-tests were used. To perform subanalyses after exclusion of the adenomas from patients that were pre-treated with pasireotide, the Mann-Whitney-U test was used. One-way analysis of variance (ANOVA) followed by post-hoc Newman-Keuls test was used to analyze differences in ACTH concentrations between treatment groups *in vitro*. Spearman's rho was calculated for correlation analysis. Statistical significance was accepted at the 0.05 level of probability.

Results

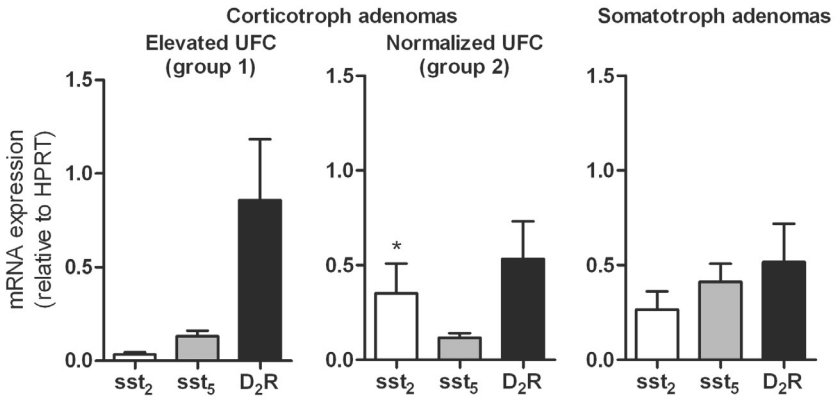
Treatment and characteristics

Patient characteristics are outlined in Table 1. 18 patients had a microadenoma, 13 had a macroadenoma and in 2 cases, no adenoma was identified on MRI. 22/33 patients (group 1; mean age 40.0 ± 17.4 yr; 17 females) with CD had elevated preoperative UFC excretion levels, despite the fact that 19/22 patients received cortisol-lowering therapy (ketoconazol: 15 patients; metyrapone: 2 patients; pasireotide/cabergoline/ketoconazole combination treatment: 1 patient; rosiglitazone: 1 patient). The remaining 11 patients (group 2; mean age 43.4 ± 11.2 yr; 7 females) reached normal UFC excretion levels under cortisol-lowering therapy preceding their surgery. Mean UFC excretion differed significantly between both groups (4.81 ± 3.96 vs. 0.63 ± 0.21 times the upper limit of normal (ULN); $p<0.001$). The mean duration of normocortisolism in group 2 was 10 ± 5 weeks (range 5-24 weeks). Of these patients, one patient was treated with pasireotide monotherapy, 2 patients reached normal UFC excretion with pasireotide and cabergoline combination therapy, while three patients were administered triple therapy, as ketoconazole was added to pasireotide and cabergoline. Finally, 4 patients reached the normal range for UFC excretion using ketoconazole monotherapy.

Receptor expression profiles

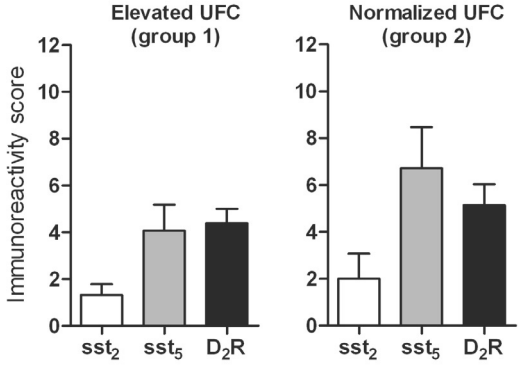
Compared to corticotroph adenomas from group 1 (n=22), adenomas from group 2 (n=10) had a significant 10-fold higher sst_2 mRNA expression level (ratio over HPRT 0.35 ± 0.50 vs. 0.03 ± 0.05; $p<0.01$; Figure 1). In fact, sst_2 mRNA expression levels in the latter group were even comparable to those in somatotroph adenomas (n=10; ratio over HPRT 0.27 ± 0.30). There were no statistically significant differences in sst_5 and D_2R mRNA expression levels between both groups of corticotroph adenomas (Figure 1).

Figure 1.



mRNA expression levels of sst2, sst5 and D2R, relative to the housekeeping gene HPRT, in somatotroph adenomas (n=10) and corticotroph adenomas from patients with elevated (n=22) and normalized (n=10; in one case, no tissue was available for mRNA measurement) preoperative UFC excretion. * $p<0.01$ vs sst2 mRNA in adenomas from patients with elevated preoperative UFC excretion (group 1).

Figure 2.



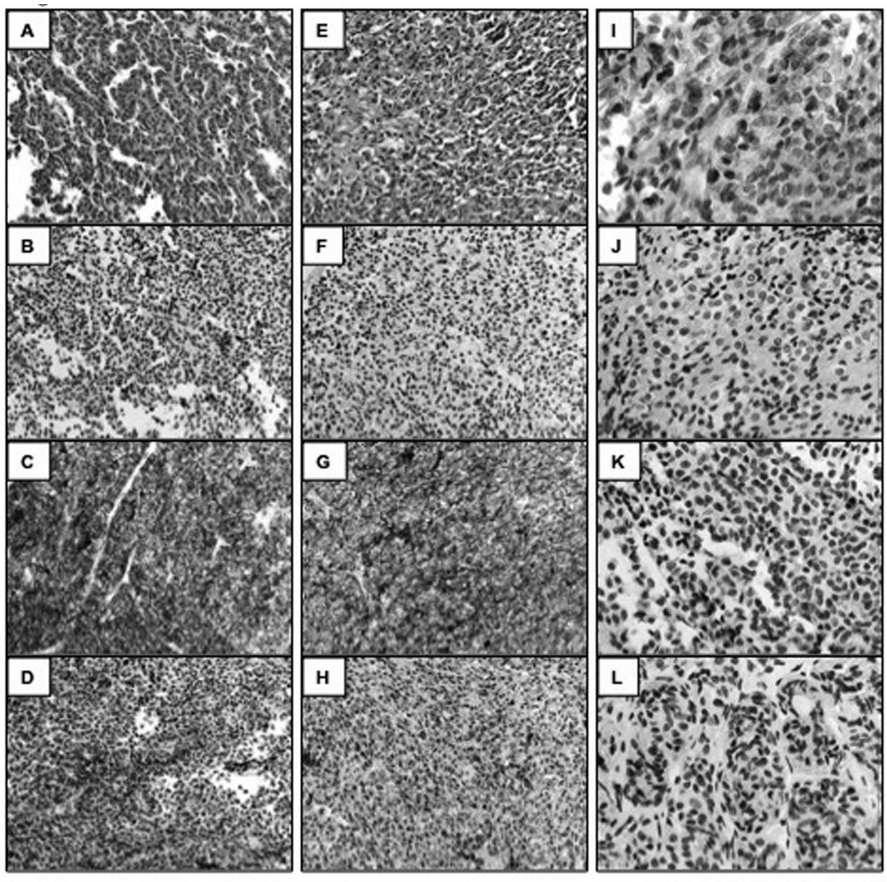
Immunoreactivity score (IRS) of sst2, sst5 and D2R in corticotroph adenomas from patients with elevated (n=16) and normalized (n=7) preoperative UFC excretion. The method of IRS calculation is outlined in the methods section.

Paraffin-embedded tissue from 16 adenomas from group 1 and 7 adenomas from group 2 was available for immunohistochemistry. At the protein level, no statistically significant differences in receptor expression between groups were found for sst2, sst5 or D2R (Figures 2 + 3).

The mean IRS for sst2 was 1.31 ± 1.89 in adenomas from group 1 vs. 2.00 ± 2.83 in adenomas from group 2 (p=0.50). Similarly, there were no differences in IRS for the sst5 (4.06 ± 4.50 vs. 6.71 ± 4.65; p=0.21) and the D2R (4.38 ± 2.53 vs. 5.14 ± 2.34; p=0.50). A remarkable heterogeneity in sst2 protein expression was

observed, both between and within corticotroph adenomas (Figure 3, panels I-L). Of note, the results of our analysis did not change after exclusion of the patients with preoperative UFC-excretion levels of 1-2x ULN.

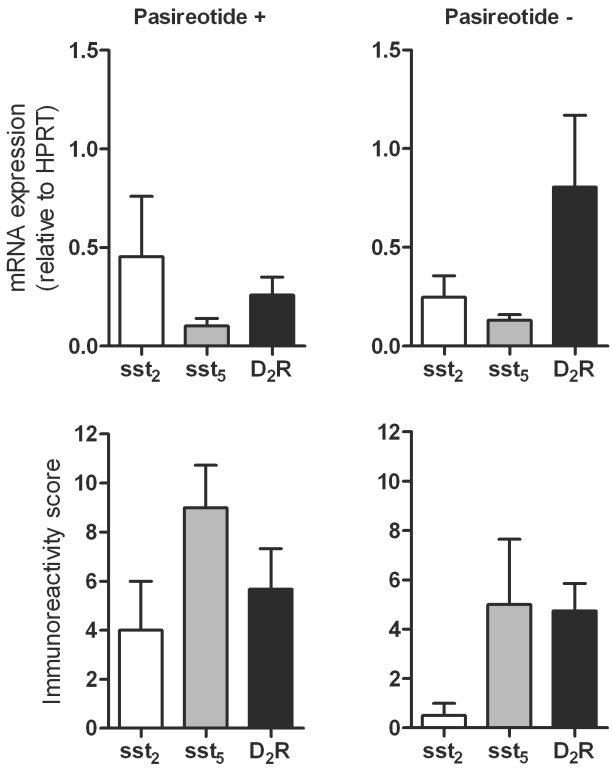
Figure 3.



Immunohistochemical stainings of two representative adenomas from patients with normalized preoperative UFC excretion. Left: adenoma positive for sst_2 after normalization of UFC excretion. Right: adenoma negative for sst_2 despite normalization of UFC excretion. A+E: hematoxylin eosin staining. B+F: sst_2 staining. C+G: sst_5 staining. D+H: D_2R staining. Magnification 200x. I-L: sst_2 staining from adenomas of 2 patients from group 1 (I&J) and 2 patients from group 2 (K&L). Magnification 400x.

Because theoretically pasireotide treatment might modulate sst expression, a subanalysis was performed after exclusion of adenomas from patients that were pre-treated with pasireotide to assess differences in receptor expression profiles between both groups. Again, the sst_2 mRNA expression was significantly higher in group 2 (ratio over HPRT 0.25 ± 0.24 (n=5) vs. 0.03 ± 0.05 (n=21); $p<0.05$; data not shown). There were no differences in sst_5 and D_2R mRNA expression levels or in protein expression levels of either receptor between the two groups of corticotroph adenomas (n=4 vs. n=15, respectively). Finally, in group 2, no statistically significant differences were found in either mRNA or protein receptor expression levels between patients that were treated with pasireotide, cabergoline or the combination of both drugs and patients that were treated with ketoconazole only (Figure 4).

Figure 4.



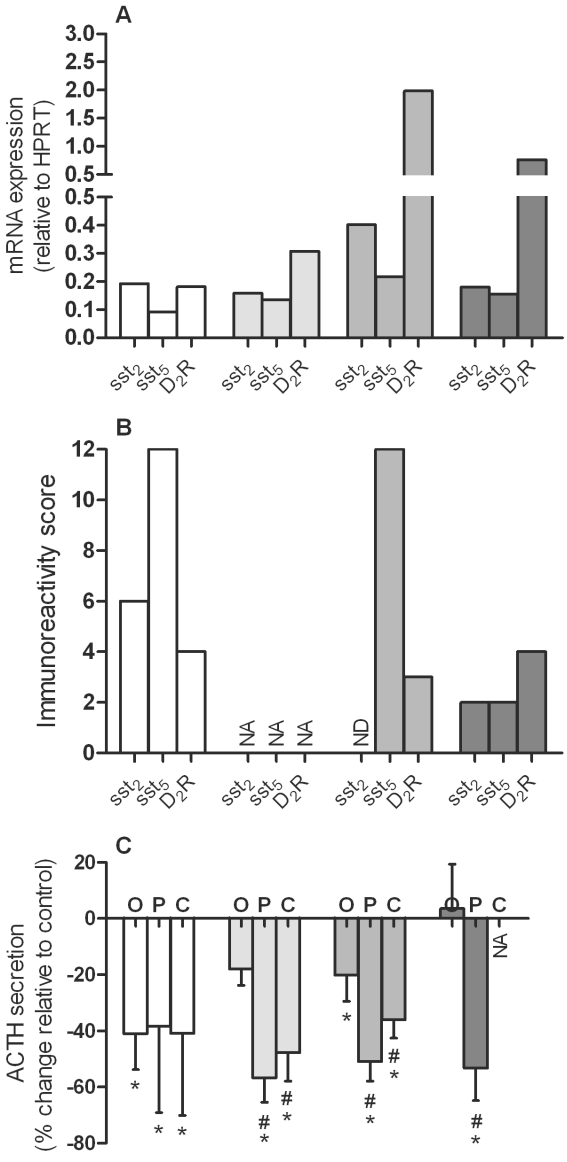
mRNA expression levels relative to the housekeeping gene HPRT (A and B) and immunoreactivity score (C and D) of sst2, sst5 and D2R in corticotroph adenomas from patients with normalized preoperative UFC excretion (group 2). Figures A and C depict receptor expression profiles of adenomas from patients that were pre-treated with pasireotide alone or in combination with cabergoline. Figures B and D depict receptor expression profiles of adenomas from patients that were only pre-treated with ketoconazole.

Corticotroph cell culture: effects on ACTH production

Of 6 adenomas from group 2, enough tissue was available for cell culture. ACTH production was significantly inhibited in 2/4 adenomas that were incubated with octreotide. The mean inhibition of ACTH production compared to control was 18.9 ± 9.1% (mean ± SEM). In contrast, pasireotide significantly suppressed ACTH production in 5/6 cultures with a mean of 36.3 ± 11.2%. Cabergoline induced a mean decrease in ACTH production of 31.4 ± 12.9%, which was statistically significant in 3/4 cultures that were incubated with cabergoline. The effects of octreotide and cabergoline were only tested in 4 cultures, because not enough cells were available for culture in the remaining cases. Figure 5 depicts individual data from 4 adenomas from group 2. In all cases, sst2 mRNA expression was higher than sst5 mRNA expression. In contrast, sst5 protein expression was higher than the sst2 protein expression in 2 out of 3 adenomas in which protein expression could be measured. With respect to inhibition of ACTH secretion, pasireotide and cabergoline were significantly more potent than octreotide in 3/4 and 2/3 primary cultures, respectively. The remaining 2 cases in which the *in vitro* effects of pasireotide were examined are not shown here because no concomitant data on octreotide treatment were available. In three primary cultures of adenomas from group 2, the effects of all three drugs were tested. Overall, pasireotide inhibited the *in vitro* ACTH secretion by 48.7 ± 5.4 % in these 3 cultures,

whereas octreotide induced a 26.3 ± 7.3 % inhibition. The mean inhibitory effect of cabergoline was 41.9 ± 3.1 % (data not shown).

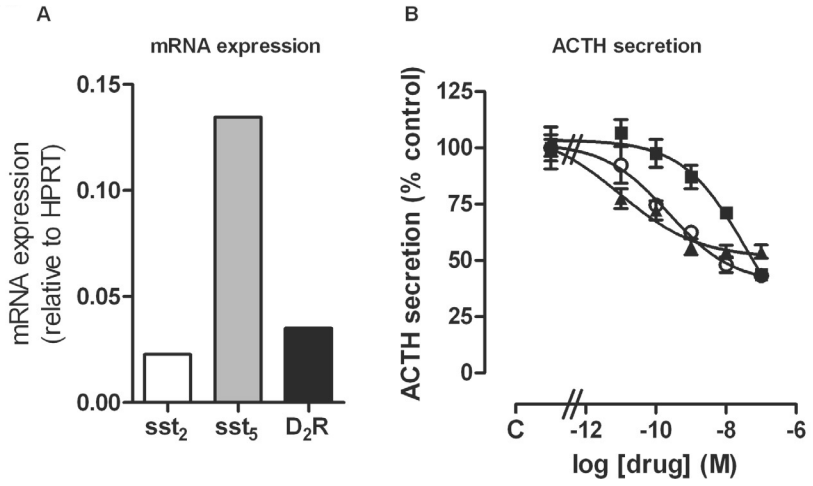
Figure 5.



Individual data from 4 adenomas from patients with normalized preoperative UFC excretion. A: mRNA expression levels of sst2, sst5 and D2R, relative to the housekeeping gene HPRT. B: immunoreactivity scores. C: Mean percentage of inhibition of ACTH secretion *in vitro*. The percentage of inhibition is outlined relative to control. One-way analysis of variance (ANOVA) followed by post-hoc Newman-Keuls test was used to analyze differences in ACTH concentrations between treatment groups. O: octreotide; P: pasireotide; C: cabergoline. NA: not available. ND: not detectable. * $p<0.001$ vs. control. # $p<0.001$ vs. octreotide.

Finally, we tested a full dose-responsiveness of octreotide, pasireotide and cabergoline in one adenoma from group 1 (Figure 6). In this patient, UFC excretion decreased from 1.47 to 1.06 times the upper limit of normal after ketoconazole pre-treatment. In this adenoma, sst2 mRNA expression was very low, as compared to a 6-fold higher sst5 mRNA expression. Remarkably, the D2R mRNA expression was very low in this adenoma. We also determined D4R mRNA expression in this adenoma, which was undetectable (data not shown). After 144h of incubation, pasireotide showed a dose-dependent inhibition of ACTH-secretion with an EC50 of 0.2 nmol/L and a maximum decrease of 56.7% (Figure 5B). In contrast, octreotide only induced a statistically significant decrease in ACTH-secretion when used in a concentration of 10 and 100 nmol/L (EC50 of 39 nmol/L). Similar to pasireotide, cabergoline dose-dependently inhibited the in vitro ACTH-secretion in this primary culture with an EC50 of 0.01 nmol/L and a maximum inhibitory effect of 46.1%. Unfortunately, we were not able to determine protein receptor expression levels in this case.

Figure 6.



Case of an adenoma from a patient with elevated preoperative UFC excretion. After pretreatment with ketoconazole, UFC excretion had decreased to 1.06 x ULN. A: mRNA expression levels of sst2, sst5 and D2R, relative to the housekeeping gene HPRT. B: dose-response curves of pasireotide (open circles), octreotide (solid squares) and cabergoline (solid triangles) on *in vitro* ACTH secretion after 144h of incubation. EC50's: pasireotide: 0.2 nmol/L, octreotide: 39 nmol/L, cabergoline: 0.01 nmol/L.

Correlations

The preoperative UFC excretion level was significantly correlated with the sst2 mRNA ($r=-0.44$; $p<0.05$), but not with the sst2 protein expression level ($r=-0.001$; $p=1.00$). The duration of normocortisolism was not correlated with either the sst2 mRNA or protein expression level. The sst2 mRNA expression level was significantly correlated with both sst5 mRNA ($r=0.40$; $p<0.05$) and protein ($r=0.43$; $p<0.05$) expression levels and with D2R mRNA expression ($r=0.51$; $p<0.01$), but not with the sst2 protein expression level ($r=-0.07$; $p=0.76$). The sst5 mRNA expression level was significantly correlated with D2R mRNA expression levels ($r=0.77$; $p<0.001$) and protein expression levels of both sst2 ($r=-0.45$; $p<0.05$) and sst5 ($r=0.45$; $p<0.05$).
There was no significant correlation between mRNA and protein expression levels of the D2R ($r=0.27$; $p=0.22$).

Effects of pasireotide on receptor expression in AtT20 cells

To exclude that the difference in sst2 expression levels between both groups of corticotroph adenomas might

have been caused by pasireotide pre-treatment, additional experiments were carried out in which AtT20 cells (a murine corticotroph tumor cell line that expresses sst_2 and sst_5) were incubated with pasireotide. The sst_{2A} (ratio over HPRT 0.38 ± 0.04 vs. 0.40 ± 0.01) and sst_5 (0.51 ± 0.09 vs. 0.58 ± 0.10) mRNA expression of these cells was not influenced by a 72h incubation with pasireotide (10 nmol/L). Similarly, no major changes occurred in sst_2 protein expression after pasireotide treatment (data not shown).

Discussion

The aim of this study was to compare somatostatin and dopamine receptor expression profiles of corticotroph adenomas from CD patients with preoperatively uncontrolled hypercortisolism with those from patients with normalized preoperative UFC excretion. We found that after an average period of 10 weeks of normocortisolism induced by various cortisol-lowering therapies, adenomas from the latter group had significantly higher sst_2 mRNA expression levels, even comparable to levels in somatotroph adenomas. However, this difference in sst_2 mRNA expression could not be confirmed at the protein level. In contrast, sst_5 and D_2R levels remained unchanged, both at the mRNA and protein level. Accordingly, pasireotide remained significantly more potent with respect to *in vitro* inhibition of ACTH secretion in primary cultures of these corticotroph adenomas.

Although transsphenoidal surgery is still the primary treatment option for patients with CD, the long-term remission rate of approximately 50-70% indicates an obvious need for alternative treatment modalities [1, 2]. The D_2R has been reported to be expressed in 80% of ACTH-secreting pituitary adenomas [4] and the efficacy of cabergoline has been evaluated in recent studies. After 6-37 months of treatment, cabergoline monotherapy induced sustained normocortisolism in 25-40% of the patients [9, 27, 28]. The sst_5 is the predominantly expressed somatostatin receptor in corticotroph pituitary adenomas [4]. A recently completed phase III trial prospectively investigated the efficacy of high-dose pasireotide treatment in 162 patients with CD. Pasireotide is a somatostatin analog with high binding affinity for multiple somatostatin receptor subtypes, in particular the sst_5. Although the mean UFC excretion decreased by 48% after 6 months of treatment, normalization was achieved in only 20% of patients. Moreover, hyperglycemia-related adverse events were observed in 73% of the patients [29].

The sst_2, which is highly expressed in GH-secreting pituitary adenomas, is expressed to a much lesser extent in corticotroph adenomas [4]. This might result from down-regulating effects of high circulating cortisol levels. Indeed, several *in vitro* studies suggested that exposure to glucocorticoids diminishes sst_2 expression both in GH- and ACTH secreting cell lines [11, 12, 14, 30, 31]. Moreover, a study by van der Hoek et al. showed that in the presence of dexamethasone, the maximum binding capacity of the sst_2-preferring radioligand [^{125}I-Tyr^3]-octreotide was dramatically decreased in AtT20 cells, whereas the maximum binding capacity of native somatostatin was hardly affected by dexamethasone, pointing towards an sst_2-specific effect. In the same study, it was shown that pre-treatment with dexamethasone abolished the ACTH-lowering potency of octreotide in AtT20 cells [13]. These observations may explain why octreotide, a sst_2-preferring somatostatin analog, is generally ineffective in patients with untreated CD [12, 15, 32], while it has been shown to lower ACTH concentrations in patients with Nelson's syndrome [15]. Moreover, native somatostatin inhibited ACTH secretion in patients with adrenal insufficiency [17]. In the present study, we show that at mRNA level, it is possible to reverse the glucocorticoid-induced sst_2 down-regulation by treating patients with CD with cortisol-lowering therapy. Adenomas from patients with normalized preoperative UFC excretion had a 10-fold higher sst_2 mRNA expression compared to adenomas from patients with elevated preoperative UFC excretion. Moreover, the fact that the latter group of patients also received cortisol-lowering therapy prior to surgery might cause an underestimation of this difference in sst_2 mRNA expression. An obvious limitation of our study is that a comparison could only been made between groups of patients instead of within individual patients. Therefore, the receptor expression profiles of the adenomas prior to initiation of medical pre-treatment are unknown.

In the subset of adenomas in which protein expression could be measured, it was found that the sst_2 mRNA up-regulation was not followed by an up-regulation of sst_2 protein expression. In addition, whereas a significant correlation was found between sst_5 mRNA and protein expression, such a correlation was not found between

sst_2 mRNA and protein expression. Although speculative, this might result from changes in translational efficiency of sst_2 mRNA. Therefore, it needs to be established whether sustained normocortisolism restores expression of biologically active sst_2 protein in ACTH-secreting pituitary adenomas. Interestingly, it has previously been described that transfecting AtT20 cells with sst_2 receptors increased the octreotide-mediated cAMP inhibition. Moreover, sst_2 overexpression prevented octreotide desensitization in these cells [33]. Thus, it may be an interesting concept to treat CD patients with cortisol-lowering therapy which, in case of reappearing sst_2 protein expression, is followed by treatment with a sst_2-preferring somatostatin analog [34, 35]. In support of this hypothesis, the primary culture in which octreotide showed the largest ACTH-lowering effect was the one in which sst_2 protein expression was highest, with an immunoreactivity score of 6. Moreover, a recently published study by de Bruin et al. showed that glucocorticoid receptor (GR) antagonizing therapy with mifepristone was able to induce sst_2 expression in ACTH-secreting neuroendocrine tumors *in vivo* that were previously found to be undetectable with somatostatin receptor scintigraphy [36].

In contrast to the sst_2, the expression levels of sst_5 and D_2R have been reported to be relatively resistant to down-regulating effects of glucocorticoids [14]. Whereas these observations were obtained in neuroendocrine cell lines, the present study is the first to describe the inability of glucocorticoids to down-regulate expression levels of sst_5 and D_2R in human corticotroph adenoma tissue.

In adenomas from patients with normalized UFC excretion in which positive sst_2 staining was found, this staining was generally very heterogeneous. At most, the percentage of cells that stained positive for sst_2 was 50%. This is an important issue, since a significant correlation has been described between the GH-decreasing effect of octreotide and sst_2 protein expression in somatotroph adenomas [37-40].

The relatively scarce sst_2 protein expression does not necessarily rule out profound sst_2-mediated intracellular effects in response to, in this case, octreotide. However, in agreement with the lack of up-regulation of sst_2 protein expression after normalization of UFC excretion in patients from group 2, octreotide was significantly less potent compared to pasireotide with respect to *in vitro* inhibition of ACTH secretion by cultured corticotroph adenoma cells.

In a previously reported series of adenomas from patients with elevated preoperative cortisol concentrations, the degree of octreotide-induced decrease of ACTH-secretion was comparable to that in the current series of adenomas from patients with normalized preoperative cortisol concentrations [41]. In the present study, only one primary culture of an adenoma from a patient with elevated preoperative UFC excretion was available. Pre-treatment with ketoconazole induced a decrease, but not a normalization of UFC excretion in this patient. In agreement with the elevated UFC excretion level, the sst_2 mRNA expression was very low in this adenoma. Consequently, pasireotide inhibited the *in vitro* ACTH-secretion in a much more potent manner compared to octreotide, which was only effective when administered in the highest dosages. The EC_{50} values of inhibition of ACTH secretion by octreotide and pasireotide (39 and 0.2 nmol/L, respectively) are well in agreement with previously reported EC_{50} values of both somatostatin analogs on ACTH secretion in murine corticotroph tumor AtT20 cells [41]. Remarkably, while D_2R mRNA expression was very low in this adenoma, cabergoline induced a firm ACTH-lowering effect in this particular primary culture. Unfortunately, no paraffin-embedded tissue was available to determine the D_2R protein expression level. However, considering the lack of correlation between mRNA and protein expression levels of this receptor in our series of adenomas, it cannot completely be ruled out that compared to the mRNA expression, there is a relatively high D_2R protein expression in this adenoma. A D_4R-mediated cabergoline effect is unlikely in this case, since this receptor was not detectable at mRNA level.

Some of the patients included in this study were treated with pasireotide, either alone or in combination with cabergoline. Elevations in ACTH concentrations have been observed in both sst_2 and sst_5-knockout mice, highlighting the importance of these receptors in murine corticotrophs [42]. The (post-)receptor effects of somatostatin receptor ligands in pituitary cells have been extensively reviewed [43]. In some studies, it has been shown that octreotide, but not pasireotide can induce sst_2 receptor internalization, e.g. in human neuroendocrine tumor cells [44] and in human embryonic kidney cells [45]. In contrast, treatment with pasireotide was associated with rapid recycling of the sst_2 to the cell membrane. It has been suggested that sst_5 activation attenuates internalization of the sst_2 [42].

Based on these studies, it seems unlikely that pasireotide pre-treatment may have down-regulated the sst_2 expression in the adenomas from patients that received this drug. However, to the best of our knowledge, it is currently unknown whether somatostatin analogs and dopamine agonists affect the receptor expression profile of corticotroph pituitary adenomas. Although our series of adenomas was relatively small, no differences were found either in receptor mRNA or protein expression levels between adenomas from patients that were treated with pasireotide and adenomas from patients that were not. The same was found for cabergoline treatment. Moreover, no changes in either sst_2 mRNA and protein expression were observed in AtT20 cells that were treated with pasireotide. Thus, our results suggest that the observed up-regulation of sst_2 mRNA expression should be attributed to the normalization of UFC excretion rather than to a possible drug-induced effect. At the mRNA level, the sst_5 and D_2R expression levels were significantly correlated, a finding that has also been reported in the study by De Bruin et al. [4]. Due to the co-expression of both receptors that frequently occurs in corticotroph pituitary adenomas, there seems to be a rationale to treat patients with CD with a combination of drugs that target these receptors [4, 8]. In particular, although speculative, targeting both receptors might lead to synergistic effects, as somatostatin and dopamine receptors have been shown to be able to heterodimerize [46]. In this respect, evaluation of the possible role of sst_5/D_2R-preferring chimeric compounds may be of interest [47]. In conclusion, after 10 weeks of normocortisolism induced by medical therapy, cortisol-mediated sst_2-downregulation on corticotroph adenomas appears to be a reversible process at mRNA, but not at protein level. Octreotide remains less potent than pasireotide with respect to the *in vitro* inhibition of ACTH secretion. It remains to be elucidated whether sustained normocortisolism induced by medical therapy induces re-expression of biologically active sst_2 protein in corticotroph adenomas and if so, whether this increases the ACTH-lowering potential of sst_2-preferring somatostatin analogs or pasireotide.

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CHAPTER 4

EXPRESSION OF DOPAMINE AND SOMATOSTATIN RECEPTORS IN THE HUMAN ADRENAL GLAND AND EFFECTS OF THEIR AGONISTS ON IN VITRO CORTISOL PRODUCTION.

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CHAPTER 5

FLUCONAZOLE INHIBITS HUMAN ADRENOCORTICAL STEROIDOGENESIS IN VITRO.

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ABSTRACT

The antifungal agent ketoconazole is often used to suppress cortisol production in patients with Cushing's syndrome. However, ketoconazole has serious side-effects and is hepatotoxic. Here, the *in vitro* effects of ketoconazole and fluconazole, which might be less toxic, on human adrenocortical steroidogenesis were compared.
The effects on steroidogenesis were examined in primary cultures of nine human adrenocortical tissues and two human adrenocortical carcinoma cell lines. Moreover, the effects on mRNA expression levels of steroidogenic enzymes and cell growth were assessed.

Ketoconazole significantly inhibited 11-deoxycortisol- (H295R cells; maximum inhibition 99%;EC_{50} 0.73μM) and cortisol production (HAC15 cells;81%;EC_{50} 0.26μM and primary cultures (mean EC_{50} 0.75μM)). In cultures of normal adrenal cells, ketoconazole increased pregnenolone, progesterone and deoxycorticosterone levels, while concentrations of 17-hydroxypregnenolone, 17-hydroxyprogesterone, 11-deoxycortisol, DHEA and androstenedione decreased. Fluconazole also inhibited 11-deoxycortisol production in H295R cells (47%;only at 1mM) and cortisol production in HAC15 cells (maximum inhibition 55%;EC_{50} 35μM) and primary cultures (mean EC_{50} 67.7μM). In the cultures of normal adrenals, fluconazole suppressed corticosterone, 17-hydroxypregnenolone and androstenedione levels, whereas concentrations of progesterone, deoxycorticosterone and 11-deoxycortisol increased. Fluconazole(1mM) slightly increased *STAR* mRNA expression in both cell-lines.Neither compound affected mRNA levels of other steroidogenic enzymes or cell number.

In conclusion, by inhibiting 11β-hydroxylase and 17-hydroxylase activity, pharmacological concentrations of fluconazole dose-dependently inhibit cortisol production in human adrenocortical cells *in vitro*. Although fluconazole seems less potent than ketoconazole, it might become an alternative for ketoconazole to control hypercortisolism in Cushing's syndrome. Furthermore, patients receiving fluconazole because of mycosis might be at risk for developing adrenocortical insufficiency.

INTRODUCTION

Cushing's syndrome (CS) is characterized by chronic glucocorticoid excess and can be caused by an adrenocorticotropin (ACTH) producing pituitary adenoma (Cushing's disease, CD), ectopic ACTH production by neuroendocrine tumors or autonomous cortisol production by adrenal neoplasia [1-3]. CS is associated with significant morbidity and, when uncontrolled, an increased mortality [3-5]. In most cases of CS, the primary choice of treatment is surgery. Medical therapy is currently applied in CS to treat acute complications (e.g. psychosis), as pretreatment before surgery, after unsuccessful surgery and in patients with inoperable neuroendocrine and adrenocortical tumors [6]. The spectrum of medical therapy includes adrenal blocking drugs, neuromodulatory agents that aim to inhibit ACTH production and glucocorticoid receptor antagonists that counteract the effects of cortisol at tissue level [6, 7]. Drugs blocking adrenal steroidogenic enzymes, like ketoconazole, etomidate, mitotane and metyrapone directly suppress production of cortisol [7-10]. The antifungal agent ketoconazole, an imidazole derivative, is one of the most widely used drugs to lower cortisol concentrations in patients with CS [7, 11-19]. When administered at relatively high dosages, ketoconazole inhibits adrenocortical steroidogenesis by blocking steroidogenic enzymes, e.g. 17-hydroxylase and 11β-hydroxylase [9, 15, 20-25]. However, ketoconazole has serious, mainly gastrointestinal side-effects and is hepatotoxic [12, 16, 17, 26, 27]. On the other hand, fluconazole, another antifungal drug, has fewer side-effects than ketoconazole [26, 28, 29] and may also inhibit adrenocortical steroidogenesis, as has been suggested in some case reports [7, 26, 28, 30-32]. The potency of fluconazole, which is a triazole derivative, has, however, not been directly compared to that of ketoconazole with respect to *in vitro* inhibition of human adrenocortical steroid production.

The aim of this study was, therefore, to compare the effects of fluconazole on adrenocortical steroid production to those of ketoconazole. We carried out in vitro studies with primary cultures of human adrenocortical tissue, as well as with two human adrenocortical carcinoma cell lines, with measurement of supernatant steroid concentrations and levels of mRNA encoding for steroidogenic enzymes. Finally, the effects of ketoconazole and fluconazole on cell growth and apoptosis were examined.

Methods

Cell culture

Human adrenocortical carcinoma H295R cells (ATCC, Manassas, VA, USA) and its clone HAC15 (kindly provided by Dr. W. Rainey, Medical College of Georgia, Georgia, USA; described in [33]) were cultured in 75cm^2 flasks (Corning Costar, Schiphol-Oost, The Netherlands) in Dulbecco's Modified Eagle Medium F12 (D-MEM/F12; GIBCO Biocult Europe, Invitrogen, Breda, The Netherlands) containing 5% fetal calf serum, L-glutamine and penicillin 10^5 U/L (Bristol-Myers Squibb, Woerden, The Netherlands) at 37°C in a 5% CO2 incubator. Once a week medium was refreshed and cells of both cell lines were harvested with trypsin (0.05%)-EDTA (0.53mM) as described previously [34]. All incubations were performed in quadruplicate and the cell line experiments were performed at least twice.

Human adrenal glands were collected during surgery of patients with cortisol excess due to cortisol-producing adrenocortical adenoma, ACTH-independent macronodular adrenal hyperplasia (AIMAH) and patients with normal adrenals undergoing surgery for renal cell carcinoma. Written informed consent was obtained from all patients before surgery. The study was approved by the medical ethics committee of the Erasmus Medical Centre. Diagnoses were histologically confirmed in all cases. Adrenal tissues were divided in pieces for different experiments. A part was taken up in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands), frozen and stored at –80°C for later analysis. The remaining tissue was cut into small fragments of about 2-5 mm^3. The fragments were washed in culture medium and centrifuged for 5 minutes at 600 x g. Medium was then refreshed and the tissue was stored overnight at 4°C. Cells were centrifuged again and the supernatant was removed. Collagenase type-I (Sigma Aldrich, Zwijndrecht, The Netherlands; 2 mg/mL) in culture medium was added and the resulting suspension was incubated in a 37°C water bath for up to two hours. Subsequently,

the suspension was filtered through a sterile surgical gauze (single layer) to remove any remaining large tissue fragments, after which it was centrifuged for 5 minutes at 600 x g. 10 mL of the cell suspension was brought on 15 mL ficoll (GE Healthcare, Uppsala, Sweden) and centrifuged for 20 minutes at 500 x g. The interphase was then collected in culture medium, viable cells were counted and ultimately seeded in 24-well plates at a density of 100.000 cells/well.

Measurement of steroid hormone concentrations

After 72h of incubation, media were stored at -20 °C until further analysis. Cortisol concentrations were measured using a non isotopic, automatic chemiluminescence immunoassay system (Immulite; Siemens DPC Inc., Los Angeles, CA, USA). 11-deoxycortisol levels were estimated using a previously described radioimmunoassay (RIA) [20].

In addition, we carried out multi-steroid analysis by liquid chromatography/mass spectrometry (LC/MS) for accurate identification and quantification of pregnenolone, progesterone, deoxycorticosterone, corticosterone, 17-hydroxypregnenolone, 17-hydroxyprogesterone, 11-deoxycortisol, cortisol, cortisone, dehydroepiandrosterone (DHEA), androstenedione and testosterone concentrations (Table 1). Steroids were extracted from 0.5 mL of cell media via liquid/liquid extraction using 2.5mL of tert-butyl-methyl-ether (MTBE). The mixture was frozen and the MTBE layer removed and evaporated under nitrogen at 55˚C. The sample was then reconstituted in 100µL of 50% of LC/MS grade methanol prior to LC/MS analysis. A Waters Xevo mass spectrometer with Acquity uPLC system was used fitted with a HSS T3, 1.8µm, 1.2x50mm column. The column temperature was maintained at 60°C throughout the experiments. The following settings were used: an electrospray source in positive ionization mode, capillary voltage 2.0kV, cone voltage 12-32V, collision energy 8-30eV (depending on the mass transition), a source temperature of 150°C, and a desolvation temperature of 600°C. A gradient system of (A) water with 0.1% formic acid and (B) methanol with 0.1% formic acid was optimized for resolution of the steroids. Steroids were quantified with respect to a calibration series, with appropriate internal standards, ranging from 0.25 to 500ng/mL. Each steroid was identified by a matching retention time and 2 mass transitions in comparison to a reference compound.

Levels of mRNA encoding for steroidogenic enzymes

RNA was isolated from plated cells and homogenized adrenal tissues with Trizol reagent (Invitrogen) according to manufacturer's protocol. RNA measurement, reverse transcriptase reaction and quantitative polymerase chain reaction (qPCR) were performed as described previously [35]. The qPCR was performed in a 12.5 µl volume for the housekeeping gene HPRT1 and steroidogenic enzymes *STAR, CYP11A1, HSD3B2, CYP17A1, CYP21A2* and *CYP11B1* (primer sequences have been reported in [35]). After 72h, vehicle-controlled mRNA expression levels of steroidogenic enzymes were calculated relative to those of the housekeeping gene *HPRT1* using the delta-Ct method. Experiments were carried out three times in each cell line.

Cell number/cell growth and apoptosis

HAC15 cells were seeded in 24-well plates at a density of 50.000 cells/well. H295R cells were seeded at a density of 100.000 cells/well. Cells from primary cultures and both cell lines were allowed to attach overnight and were incubated with either ketoconazole (0.05-50 µM) or fluconazole (5-1000 µM). Both agents were obtained from Sigma Aldrich, Zwijndrecht, The Netherlands. After 72 hours of incubation, DNA concentration per well (as a measure of cell number) was measured using the fluorescent dye Hoechst 33258 as previously described [36]. Apoptosis was measured using a commercially available ELISA kit (Cell Death Detection ELISA^{PLUS,} Roche Diagnostics GmbH, Penzberg, Germany). This assay detects the amount of DNA fragmentation as a measure of apoptosis. Measurements were performed using a Wallac Victor 2 multiplate reader.

Statistical analysis

We used one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test to analyze the results on hormone production and cell growth in the dose-response experiments. Unpaired t-tests were used to analyze differences in steroid hormone concentrations between control and treated cells of the two primary cultures of

normal adrenal glands described in Table 1. The same test was used to compare the effects of ketoconazole to those of fluconazole in the cultures of Table 1. Significance was accepted at the 0.05 level of probability. Data on mRNA expression of steroidogenic enzymes in primary cultures and cell lines were analyzed using paired t-tests with Bonferroni correction.

Table 1.

Group	Preg	Prog	DOC	Corticosterone	17-OHpreg	17-OHprog	11-deoxycortisol	Cortisol	Cortisone	DHEA	Androstene-dione	Testosterone
HAC15	18.8 ± 0.9	0.72 ± 0.16	77.3 ± 14.4	0.0 ± 0.0	64.4 ± 3.9	28.8 ±1.4	598.6 ± 21.0	1.4 ± 0.4	0.0 ± 0.0	36.8 ± 2.9	456.2 ± 36.2	4.08 ± 0.52
Control	3.4 ± 0.7	7.4 ± 0.8	15.1 ± 1.6	0.0 ± 0.0	1.6 ± 1.8	0.0 ± 0.0	1.1 ± 1.2	0.7 ± 1.3	0.0 ± 0.0	0.2 ± 0.4	1.2 ± 1.5	0.00 ± 0.00
Keto	30.1 ± 3.0	4.9 ± 0.4	129.8 ± 9.0	0.0 ± 0.0	65.5 ± 3.8	34.7 ± 3.6	392.1 ± 26.9	0.4 ± 0.4	0.0 ± 0.0	27.0 ± 1.8	286.0 ± 21.6	2.26 ± 0.45
Control	15.3 ± 1.6	3.1 ± 0.2 §	69.7 ± 4.1	0.0 ± 0.0	25.2 ± 5.0 *	26.2 ± 1.1 *	177.2 ± 4.0 §	0.2 ± 0.4	0.0 ± 0.0	8.0 ± 1.2 *	80.2 ± 4.6 §	0.17 ± 0.35 *
Fluco												
H295R												
Control	23.0 ± 1.0	0.0 ± 0.0	17.0 ± 2.5	0.0 ± 0.0	182.1 ± 10.7	16.4 ± 1.0	324.4 ± 9.4	0.0 ± 0.0	0.0 ± 0.0	241.6 ± 5.8	389.4 ± 17.5	11.89 ± 0.47
Keto	7.0 ± 0.7	0.0 ± 0.0	2.9 ± 0.3	0.0 ± 0.0	2.1 ± 0.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.2	0.00 ± 0.00
Control	31.6 ± 2.6	0.0 ± 0.0	13.2 ± 1.5	0.0 ± 0.0	166.8 ± 11.0	30.2 ± 2.5	160.9 ± 26.2	0.0 ± 0.0	0.0 ± 0.0	162.0 ± 6.3	255.6 ± 41.1	3.39 ± 1.40
Fluco	15.8 ± 0.5	0.0 ± 0.0	5.8 ± 0.4 *	0.0 ± 0.0	108.3 ± 10.3 §	33.9 ± 1.3 §	90.2 ± 7.7 §	0.0 ± 0.0	0.0 ± 0.0	73.1 ± 7.4 §	95.1 ± 8.3 §	0.00 ± 0.00 *
Normal adrenal culture 1												
Control	3.2 ± 1.2	0.0 ± 0.0	118.1 ± 31.9	1396.3 ± 35.9	11.1 ± 1.2	17.3 ± 2.2	362.7 ± 35.3	2543.7 ± 152.7	452.0 ± 35.2	0.3 ± 0.4	86.2 ± 12.1	0.52 ± 0.66
Keto	16.4 ± 2.9	12.0 ± 2.7	915.2 ± 270.8	6270.3 ± 323.1	7.2 ± 0.5	9.5 ± 1.9	305.7 ± 69.3	1195.0 ± 339.0	83.9 ± 16.3	0.0 ± 0.0	5.7 ± 1.2	0.00 ± 0.00
Control	9.2 ± 1.6	2.0 ± 0.4	738.1 ± 390.1	2640.4 ± 247.4	21.0 ± 3.9	24.3 ± 2.3	892.1 ± 142.5	2994.8 ± 305.5	447.9 ± 68.2	0.3 ± 0.5	255.9 ± 8.3	6.77 ± 1.08
Fluco	6.0 ± 1.0 *	4.9 ± 1.0 *	4508.5 ± 1028.3 *	278.6 ± 52.5 §	7.5 ± 3.8 *	22.2 ± 5.2	1518.7 ± 53.9 §	140.4 ± 32.3 §	23.4 ± 4.3	0.0 ± 0.0	118.1 ± 5.8 *	4.34 ± 0.20 ‡
Normal adrenal culture 2												
Control	1.2 ± 1.2	0.0 ± 0.0	52.1 ± 14.6	854.3 ± 71.1	6.7 ± 7.4	14.5 ± 5.6	175.6 ± 8.5	1935.6 ± 560.4	705.5 ± 77.7	0.0 ± 0.0	40.0 ± 4.3	0.480 ± 0.70
Keto	4.5 ± 0.4	50.6 ± 5.3	3941.3 ± 539.7	197.9 ± 45.0	3.7 ± 2.9	0.9 ± 0.9	73.9 ± 7.2	14.9 ± 2.6	2.0 ± 0.9	0.0 ± 0.0	0.2 ± 0.4	0.00 ± 0.00
Control	3.4 ± 2.4	0.0 ± 0.0	232.6 ± 146.0	1081.6 ± 65.2	9.4 ± 3.5	9.4 ± 1.4	406.7 ± 98.4	1159.2 ± 340.8	254.9 ± 59.6	0.0 ± 0.0	80.9 ± 5.8	2.24 ± 1.18
Fluco	4.2 ± 2.4	3.2 ± 0.6 §	2289.8 ± 252.9 *	166.6 ± 21.2 *	3.0 ± 2.0	14.8 ± 1.8 *	1144.6 ± 55.1 *	70.3 ± 14.4	12.8 ± 1.9 §	0.0 ± 0.0	51.0 ± 6.5	1.52 ± 0.25

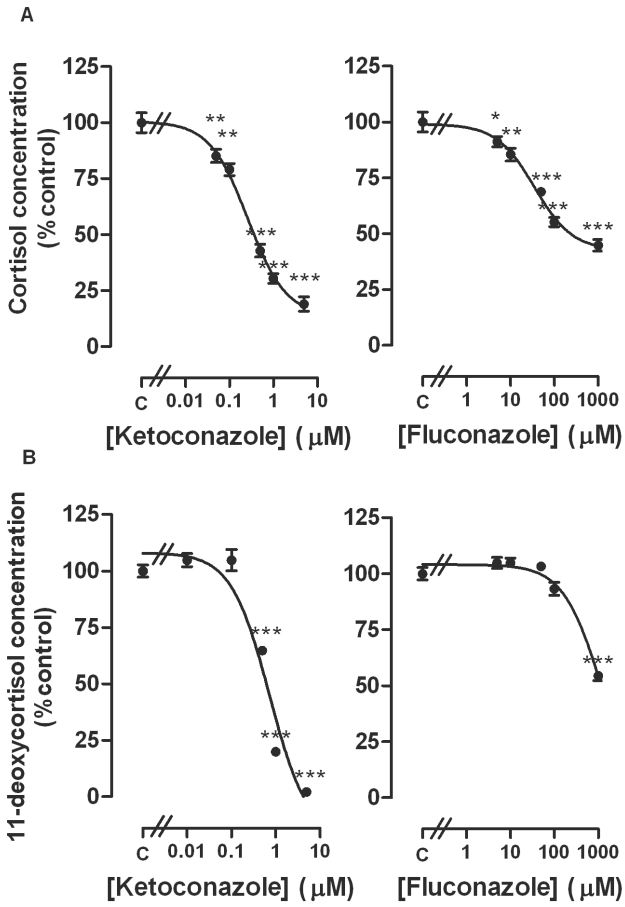
Effects of ketoconazole and fluconazole on levels of pregnenolone (Preg), progesterone (Prog), deoxycorticosterone (DOC), corticosterone, 17-hydroxypregnenolone (17-OHpreg), 17-hydroxyprogesterone (17-OHprog), 11-deoxycortisol, cortisol, cortisone, dehydroepiandrosterone (DHEA), androstenedione and testosterone in culture media of HAC15 cells, H295R cells and 2 primary cultures of normal adrenal glands (cultures 1 and 2). Steroid concentrations were measured using LC/MS. Figures represent means (all: nmol/L) of 4 measurements ± SD. Keto: ketoconazole (5 µM); Fluco: fluconazole (1 mM); grey cells indicate statistically significant decreases/increases in hormone concentrations compared to control, respectively; black cells indicate no statistically significant difference between treatment and control groups. ‡ indicates $p<0.05$, * indicates $p<0.01$ and § indicates $p<0.001$ compared to the effect of ketoconazole.

Results

Effects on corticosteroid production

After 72h of incubation, ketoconazole and fluconazole significantly inhibited cortisol production in HAC15 cells in a dose-dependent fashion with EC_{50}'s of 0.26 ± 1.13 µM (mean ± SEM) and 35 ± 1.22 µM, respectively (Figure 1a).

Figure 1.

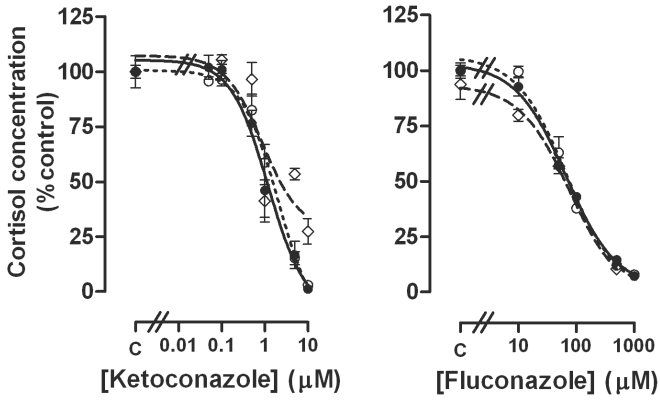


Dose-dependent effects of 72h incubation with ketoconazole or fluconazole on **A.** cortisol production by HAC15 cells and **B.** 11-deoxycortisol production by H295R cells. Values are depicted as % of vehicle control and represent means ± SEM. C control, *** $p< 0.001$, ** $p<0.01$, * $p<0.05$ compared to control.

Maximum inhibition was approximately 81% for ketoconazole ($p<0.001$) and 55% for fluconazole ($p<0.001$). In H295R cells, 11-deoxycortisol was measured instead of cortisol, because these cells show a block in CYP11B1 expression and therefore 11-deoxycortisol is produced in much larger quantities. In these cells, fluconazole inhibited 11-deoxycortisol production by 47% only at a concentration of 1 mM ($p<0.001$), while ketoconazole induced a profound dose-dependent decrease (EC_{50} 0.73 ± 1.74 µM, maximal inhibition 99%;

p<0.001). The effects of ketoconazole and fluconazole on cortisol production were also assessed in eight primary cultures of human adrenocortical tissue, respectively: two normal adrenal glands, three AIMAHs and three cortisol producing adrenocortical adenomas (Figure 2).

Figure 2.



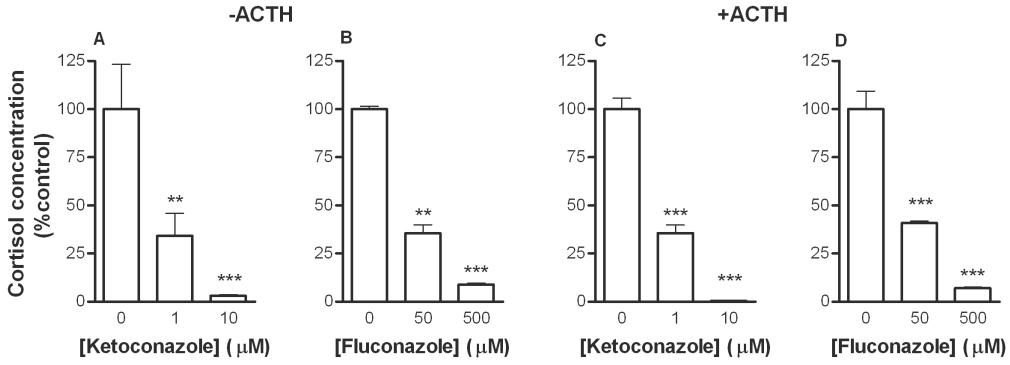
Effects of 72h incubation with ketoconazole (left) or fluconazole (right) on basal unstimulated cortisol production by primary cultures, expressed as % of vehicle control. Data represent means ± SEM. Closed circles and solid line: normal adrenal cortices (n=2); open diamonds and thick broken line: ACTH-independent macronodular adrenal hyperplasias (AIMAH; n=3); open circles and thin broken line: cortisol producing adrenocortical adenomas (n=3).

The effects of both drugs were evaluated in the same primary cultures of human adrenal glands. Both ketoconazole and fluconazole induced a significant dose-dependent decrease in cortisol production in all cultures. EC_{50} values of ketoconazole-induced inhibition of cortisol production in cultures of normal, hyperplastic and adenomatous adrenals were 0.82 μM, 0.53 μM and 1.31 μM, respectively (overall EC_{50} 0.75 μM). For fluconazole, these values were 37.9 μM, 88.2 μM and 57.5 μM, respectively (overall EC_{50} 67.7 μM). Next to basal conditions, the effects of both compounds were examined in the presence of ACTH in one case. Figure 3 depicts a primary culture of the adrenocortical adenoma in which the effects of 72h of incubation with either ketoconazole or fluconazole in the absence or presence of ACTH (250 pg/mL) were measured. In terms of relative inhibition of cortisol production, both compounds had similar effects in the presence and absence of ACTH. In the presence of ACTH, cortisol production was inhibited by 64 and 100% by 1μM and 10 μM ketoconazole, respectively (p<0.001). Without ACTH, these percentages were 66% (p<0.01) and 97% (p<0.001). In comparison, 50 and 500 μM fluconazole inhibited cortisol production by 59 and 93% in the presence of ACTH (p<0.001), while these percentages were 65% (p<0.01) and 92% (p<0.001) in the absence of ACTH.

To determine the mechanism by which ketoconazole and fluconazole inhibit corticosteroid production, multi-steroid analysis was carried out using LC/MS. This included measuring the concentrations of pregnenolone, progesterone, deoxycorticosterone, corticosterone, 17-hydroxypregnenolone, 17-hydroxyprogesterone, 11-deoxycortisol, cortisol, cortisone, dehydroepiandrosterone (DHEA), androstenedione and testosterone in culture media of both cell lines and the two primary cultures of normal adrenal glands (Figure 4; Table 1) after incubation with ketoconazole (5 μM) or fluconazole (1 mM). Compared to control, ketoconazole induced an increase in progesterone production by HAC15 cells (p<0.001), while the amounts of pregnenolone (p<0.001), deoxycorticosterone (p<0.01), 17-hydroxypregnenolone, 17-hydroxyprogesterone, 11-deoxycortisol, DHEA,

androstenedione and testosterone (all: p<0.001) were strongly decreased (Table 1). Fluconazole, on the other hand, decreased the amounts of all measurable steroids in HAC15 culture media, albeit in a less potent manner than ketoconazole. In H295R cell culture media, ketoconazole significantly suppressed the concentrations of all steroids. Fluconazole induced an increase in 17-hydroxyprogesterone concentrations (p<0.05), but as in HAC15 cell culture media, it decreased the concentrations of all other steroids in H295R cells.

Figure 3.



Effects of 72h incubation with ketoconazole or fluconazole in the absence (A and B) or presence (C and D) of ACTH (250 pg/mL) on cortisol production in a primary culture of an adrenocortical adenoma. Results are depicted as % compared to vehicle control and represent means ± SEM. Cortisol values in control groups: 1023±238 (mean±SEM) and 1142±16.4 nM (without ACTH), 5095±285 and 4250±393 nM (with ACTH). *** p<0.001; ** p<0.01 compared to vehicle control.

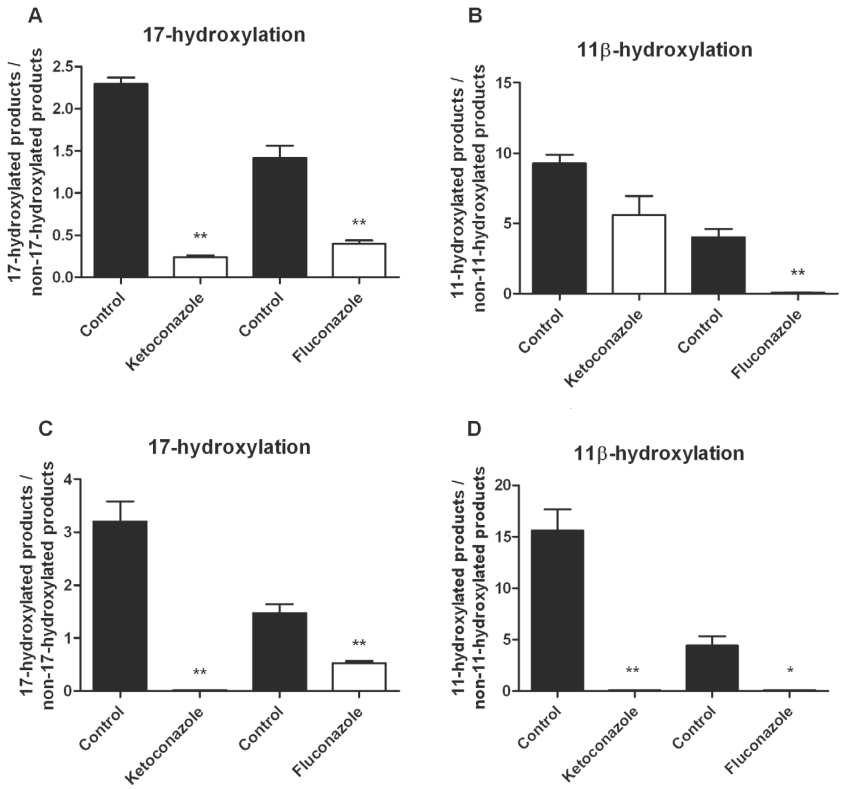
In culture media of normal adrenal no. 1, concentrations of progesterone (p<0.01), deoxycorticosterone and 11-deoxycortisol (both p<0.001) were significantly elevated compared to control after treatment with fluconazole. Concentrations of pregnenolone (p<0.05), corticosterone (p<0.001), 17-hydroxypregnenolone (p<0.01), cortisol (p<0.001), cortisone (p<0.01), androstenedione (p<0.001) and testosterone (p<0.01), on the other hand, were significantly decreased by treatment with fluconazole. Ketoconazole treatment increased concentrations of pregnenolone, progesterone, deoxycorticosterone and corticosterone in culture media of this primary culture (all: p<0.001), whereas concentrations of 17-hydroxypregnenolone, 17-hydroxyprogesterone, (both: p<0.01) cortisol, cortisone and androstenedione (all: p<0.001) decreased significantly under ketoconazole treatment.

This pattern was also recognized in culture media of culture no. 2, with ketoconazole increasing the concentrations of pregnenolone, progesterone and deoxycorticosterone (p<0.01) and suppressing concentrations of 17-hydroxyprogesterone (p<0.0.5), 11-deoxycortisol (p<0.001), cortisol (p<0.01), cortisone and androstenedione (both: p<0.001). In sharp contrast to the primary culture of the previous normal adrenal gland, ketoconazole significantly decreased corticosterone levels in media of this culture (p<0.001). In the same culture, fluconazole suppressed the concentrations of corticosterone (p<0.05), 17-hydroxypregnenolone (p<0.001), cortisol, cortisone and androstenedione (all: p<0.01), while it induced increases in concentrations of progesterone, deoxycorticosterone and 11-deoxycortisol (all: p<0.001).

When calculating the ratio between 17-hydroxylated and non-17-hydroxylated steroids, it was found that both ketoconazole and fluconazole significantly inhibited 17-hydroxylase activity in both patients, with ketoconazole having the most profound effect (Figure 4). Fluconazole significantly inhibited 11-hydroxylation in both normal adrenal primary cultures as well, but ketoconazole only inhibited the activity of this enzyme in culture no. 2. As already mentioned, ketoconazole induced an up-regulation of corticosterone in primary culture no. 1, but a statistically significant down-regulation of this hormone in culture no. 2, which suggests a differential regulation of 11-hydroxylase activity by

ketoconazole. In accordance with this finding, cortisol concentrations were more potently suppressed in patient 2 compared to patient 1 after treatment with ketoconazole (Table 1). In both cell lines, the ratio between 17-hydroxylated steroid concentrations and non-17-hydroxylated steroid levels indicates that ketoconazole induces a firm decrease of 17-hydroxylase (data not shown).

Figure 4.



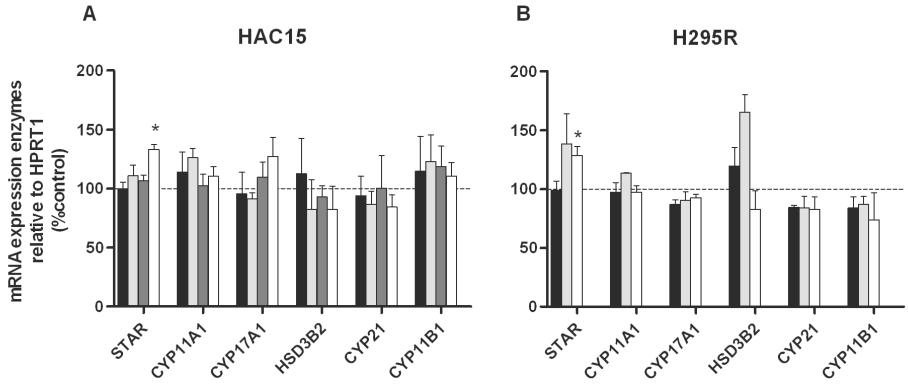
Effects of ketoconazole (5 μM) and fluconazole (1 mM) on 17-hydroxylation and 11-hydroxylation in 2 primary cultures of normal adrenal glands. A and B: culture 1; C and D: culture 2. 17-hydroxylase activity was calculated by dividing the sum of concentrations of 17-hydroxylated products (i.e. 17-hydroxypregnenolone, 17-hydroxyprogesterone, 11-deoxycortisol, cortisol, cortisone, dehydroepiandrosterone (DHEA), androstenedione and testosterone) by the sum of concentrations of non-17-hydroxylated products (i.e. pregnenolone, progesterone, corticosterone and deoxycorticosterone). 11-hydroxylation was calculated by dividing the sum of concentrations of 11-hydroxylated products (i.e. cortisol, cortisone and corticosterone) by the sum of concentrations of non-11-hydroxylated products (i.e. deoxycorticosterone and 11-deoxycortisol). ** $p<0.01$; * $p<0.05$.

mRNA expression levels of steroidogenic enzymes

The effects of ketoconazole and fluconazole on mRNA expression levels of steroidogenic enzymes following 72h of incubation are depicted in Figure 5. In HAC15 cells, ketoconazole (0.5 or 5 μM) did not affect the mRNA expression level of any enzyme, whereas it significantly decreased cortisol production in these concentrations (see above). Fluconazole (50 μM or 1 mM) did not suppress levels of mRNA encoding for steroidogenic enzymes either. In fact, in a

concentration of 1 mM it upregulated the mRNA expression of steroidogenic acute regulatory protein (STAR; p=0.01), the protein that facilitates cholesterol transport into the mitochondria of steroidogenic cells. In H295R cells, mRNA expression levels of none of the steroidogenic enzymes were altered by ketoconazole. As in HAC15 cells, fluconazole increased the expression level of STAR (p=0.01). We only used fluconazole in a concentration of 1 mM because lower concentrations did not inhibit 11-deoxycortisol production in H295R cells. In three primary cultures of AIMAHs treated with either ketoconazole or fluconazole, no significant effects on the expression of any of these mRNAs were found (data not shown).

Figure 5.



mRNA expression levels of steroidogenic enzymes in **A.** HAC15 cells and **B.** H295R cells following 72h incubation with ketoconazole or fluconazole. Each graph represents the mean of three experiments. Data are normalized against the housekeeping gene HPRT1 and depicted relative to vehicle control. Data represent means ± SEM. Black bars: ketoconazole 0.5 μM; light grey bars: ketoconazole 5 μM; dark grey bars: fluconazole 50 μM; white bars: fluconazole 1 mM. * p=0.01.

Cell growth and apoptosis in human adrenocortical carcinoma cell lines

Finally, we examined the effects of ketoconazole and fluconazole on cell growth and apoptosis in both cell lines. Under the same experimental conditions as described in the experiments on hormone production, we observed no effect on cell growth, nor was there any induction of apoptosis in either cell line (data not shown).

DNA concentrations were measured in 6 out of 9 cultures in which the effects on cortisol production were measured. In none of these 6 cultures (2 normal adrenals, 2 AIMAHs and 2 adrenocortical adenomas), we found an effect of either ketoconazole or fluconazole on DNA concentrations after 72h (data not shown).

Discussion

Ketoconazole is frequently used to suppress cortisol production in patients with Cushing's syndrome [6, 7]. It inhibits adrenocortical steroidogenesis by interacting with cytochrome P450 enzymes [20, 23]. However, ketoconazole has serious, mainly gastrointestinal side effects that limit prolonged treatment and often lead to discontinuation of drug therapy [12, 17]. Several case reports described the effects of fluconazole, a triazole antifungal agent with less side effects [26, 28, 29], on adrenocortical steroidogenesis. While two studies reported that fluconazole in a dose of 400 mg daily did not alter cortisol levels [37, 38], other studies showed that fluconazole can already induce adrenocortical insufficiency even at lower concentrations, particularly in critically ill patients [30-32]. Riedl et al. reported decreased cortisol levels in a patient with CS who was treated with 200 mg fluconazole daily because of sepsis. This is the only study that also examined the effects

of fluconazole in vitro, although a rat adrenal adenoma cell line was used [28].

In the present study, we show that fluconazole can suppress human cortisol production in vitro, both in two human adrenocortical carcinoma cell lines and in primary cultures of human adrenocortical cells. In HAC15 cells, fluconazole dose-dependently suppressed cortisol production, whereas it inhibited 11-deoxycortisol production in H295R cells only when added in the highest concentration (1mM). The observation that concentrations of virtually all measured steroids decreased in culture media of both cell lines suggests that fluconazole exerts its effect at a level upstream of the 17-hydroxylase enzyme in these cell lines, e.g. by inhibiting StAR, the cytochrome P450 side chain cleavage enzyme or HSD3B2. Compared to fluconazole, ketoconazole inhibited cortisol and 11-deoxycortisol levels in a more potent manner in our cell lines, which is in agreement with what was found in a rat adrenocortical adenoma cell line [28]. The ratio between 17-hydroxylated steroid concentrations and non-17-hydroxylated steroid levels indicates that ketoconazole induces a firm decrease of 17-hydroxylase activity in both cell lines (data not shown), a mechanism that has already been described by Lamberts et al. [20]. Since cortisol, cortisone and corticosterone concentrations were at or below the detection limit in media of both control and treated cells, the effects of ketoconazole and fluconazole on 11-hydroxylase activity could not be determined in HAC15 and H295R cells.

As a proof of concept we carried out the same experiment in nine primary cultures of human adrenocortical tissue. In these primary cultures, fluconazole dose-dependently decreased cortisol production with an overall EC_{50} of 67.7 μM, while the overall EC_{50} of ketoconazole-induced inhibition of cortisol production was 0.80 μM. There was no difference between the inhibitory effects of ketoconazole and fluconazole in the three groups of adrenal tissues used. Similar to the pattern that was found in both cell lines, ketoconazole induced a block at the 17-hydroxylase level in both primary cultures of normal adrenal glands. A remarkable difference was found between the effects of ketoconazole on 11-hydroxylase activity of these normal adrenocortical cells. Whereas ketoconazole significantly inhibited the activity of this enzyme in the normal adrenal primary culture no. 2, no effect was found in culture no. 1. Accordingly, cortisol concentrations in culture no. 1 were decreased in a less potent manner compared to culture no. 2 after treatment of the cells with ketoconazole. Moreover, fluconazole (although used in a higher concentration) more potently inhibited cortisol concentrations compared to ketoconazole in this culture, a finding that contrasts with what was found in our cell lines and other primary cultures. Our observation in culture no. 2 is in accordance with what Engelhardt et al. reported, since they also found a ketoconazole-induced inhibition of 11β-hydroxylase activity [22]. The inhibitory effect of ketoconazole on both 11β-hydroxylase and 17-hydroxylase enzyme activity illustrates that this drug can also decrease androgen and aldosterone production. Ketoconazole has indeed been reported to have beneficial effects in castration-resistant prostate cancer [39]. Moreover, it has also been shown to decrease serum concentrations of aldosterone in patients with Cushing's disease [11]. However, in our cultures of normal adrenal cells, treatment with ketoconazole strongly increased concentrations of deoxycorticosterone, which also has mineralocorticoid effects. It is therefore unknown how treatment with ketoconazole would influence the net mineralocorticoid activity. In agreement with what we found for ketoconazole in normal adrenal culture no. 2, fluconazole not only inhibited 17-hydroxylase activity, but was also found to completely attenuate the 11-hydroxylation in both primary cultures, an observation that was supported by the combination of decreased cortisol concentrations and increased 11-deoxycortisol levels.

In one primary culture of a cortisol-producing adrenocortical adenoma, enough cells were available to study the effects of ketoconazole and fluconazole both in the absence and in the presence of ACTH. It was found that both drugs inhibited the cortisol secretion to the same extent in the presence of ACTH as they did in the absence of ACTH. Thus, in this particular case, ACTH did not influence the cortisol-lowering effect of either drug. However, it has to be emphasized that this comparison has only been made in one culture, so it is hard to extrapolate the effects of ACTH on the cortisol-lowering potency of both drugs to other situations.

The marked differences in steroid production between adrenocortical carcinoma cell lines and primary cultures of normal adrenal glands indicate that these cell lines do not provide a good model to investigate steroid biosynthesis. Although the effects of inhibitors of steroid production can be examined in HAC15 and H295R cells, these cells do not mimic the physiological situation.

To the best of our knowledge, this is the first study to report the mechanism of action of fluconazole on human steroidogenesis. Despite the fact that ketoconazole and fluconazole are antifungal agents from different classes, they appear to have similar properties regarding their adrenal-blocking action. In order to determine to what extent each steroidogenic enzyme is exactly inhibited by these agents, future studies could be performed in which cell lines

transfected with the respective enzymes are treated with ketoconazole and fluconazole. This would provide a more pure situation, since the effects on only one enzyme at a time could be assessed, without the obscuring effects of steroids being further converted. Obviously, the drawback of such studies would be that they do not resemble the *in vivo* situation.

It has been reported previously that ketoconazole inhibits adrenocortical steroidogenesis by direct binding to cytochrome P450 enzymes, thereby impairing their activity [24]. However, the effects of ketoconazole and fluconazole in the concentrations that we used on mRNA expression levels of genes encoding steroidogenic enzymes have not been described before. In other words, these compounds might also decrease steroidogenesis by inhibiting the mRNA expression of cytochrome P450 enzymes. Ohlsson et al. examined the effects of ketoconazole on levels of mRNA of genes encoding for these enzymes in H295R cells. Although these authors observed a general down-regulation of mRNA expression levels by very low dosages of ketoconazole (0.03 μM), no significant inhibition was found [24]. In the present study, neither ketoconazole nor fluconazole significantly inhibited mRNA expression levels of steroidogenic enzymes in HAC15 and H295R cells. In fact, at a concentration of 1 mM, fluconazole increased the expression of mRNA encoding StAR in both cell lines. In 3 AIMAH cultures, no significant effects of both drugs on the mRNA expression levels of steroidogenic enzymes were observed. These results suggest that the decrease in hormone production cannot be explained by alterations in mRNA expression levels of adrenocortical steroidogenic enzymes. Therefore, given our results and the earlier reported direct inhibition of enzyme activity by ketoconazole [24], direct inhibition of enzyme activity is the most likely mechanism by which both ketoconazole and fluconazole inhibit steroidogenesis.

Previously reported serum concentrations of fluconazole range from 23 μM in healthy volunteers receiving 400 mg daily to 243 μM in cancer patients suffering from mycosis receiving 1600 mg daily [40-42]. However, it is important to realize that these values represent serum concentrations and not tissue concentrations of fluconazole. The EC_{50} values that we obtained for fluconazole-induced inhibition of cortisol levels in primary cultures and HAC15 cells are within the range of the previously reported therapeutic *in vivo* serum concentrations, implicating that fluconazole might be a valuable alternative for ketoconazole to control cortisol overproduction in Cushing's syndrome.

Because of this effect, the adrenal function of patients treated with fluconazole because of fungal infections should be closely monitored. In particular in patients on intensive care units, in whom optimal adrenal function is of vital importance, treatment with fluconazole can induce adrenal insufficiency [28, 30, 31]. Therefore, clinicians who treat these patients with fluconazole should be aware of this side effect. The effects of other imidazole or triazole derivatives in this context have not been extensively investigated yet, but regarding the effects of fluconazole, it is not unlikely that other antifungal agents might also affect adrenocortical steroidogenesis [20].

In conclusion, we show that pharmacological concentrations of fluconazole inhibit corticosteroid production in two human adrenocortical carcinoma cell lines and in primary cultures of human adrenocortical tissue. Based on our data in primary cultures of normal adrenal glands, fluconazole seems to block the activity of the 11-hydroxylase and 17-hydroxylase enzymes. Although fluconazole seems less potent than ketoconazole, our results indicate that fluconazole, while less toxic, might be an alternative for ketoconazole to reduce cortisol levels in patients with Cushing's syndrome. Future studies should examine the efficacy and optimal dose of fluconazole in this context. Finally, patients treated with fluconazole because of mycoses should be carefully monitored, because they might be at risk for developing adrenocortical insufficiency.

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CHAPTER 6

THE HYPERCOAGULABLE STATE IN CUSHING'S DISEASE IS ASSOCIATED WITH INCREASED LEVELS OF PROCOAGULANT FACTORS AND IMPAIRED FIBRINOLYSIS, BUT IS NOT REVERSIBLE AFTER SHORT-TERM BIOCHEMICAL REMISSION INDUCED BY MEDICAL THERAPY.

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ABSTRACT

Context

Cushing's disease (CD) is accompanied by an increased risk of venous thromboembolism. Surgery is the primary treatment of CD.

Objective

To compare haemostatic parameters between patients with CD and controls and to evaluate the effect of medical treatment of CD on haemostasis.

Design

During 80 days, stepwise medical treatment was applied with the somatostatin analog pasireotide, the dopamine agonist cabergoline and ketoconazole, which suppresses adrenocortical steroidogenesis.

Setting

Four university medical centers in The Netherlands.

Patients

Seventeen patients with *de novo*, residual or recurrent CD were included.

Main outcome measures

Urinary free cortisol (UFC) and parameters of coagulation and fibrinolysis.

Results

Patients with CD had significantly higher BMI ($p<0.001$), shortened aPTT ($p<0.01$) and higher levels of fibrinogen, Factor VIII and protein S activity ($p<0.05$) compared to healthy control subjects. In addition, fibrinolytic capacity was impaired in patients with CD as reflected by prolonged clot lysis time ($p<0.001$) and higher levels of PAI-1, TAFI and α2-antiplasmin ($p<0.01$). There were no statistically significant differences in vWF:Ag, antithrombin and protein C activity. After 80 days, 15/17 patients had normalized UFC excretion. Despite biochemical remission, only slight decreases in antithrombin ($p<0.01$) and TAFI ($p<0.05$) levels were observed. Other parameters of coagulation and fibrinolysis did not change significantly.

Conclusions

The hypercoagulable state in patients with CD, which is explained by both increased production of procoagulant factors and impaired fibrinolysis, is not reversible upon short-term biochemical remission following successful medical therapy. This may have implications for the duration of anticoagulant prophylaxis in patients with (cured) CD.

INTRODUCTION

Cushing's syndrome (CS) is associated with an increased cardiovascular risk [1, 2]. This is mainly due to the clustering of obesity, hypertension, glucose intolerance and hyperlipidemia caused by the chronic glucocorticoid excess [1, 3]. However, several studies report that the incidence of venous thromboembolism (VTE) is also increased, both in untreated CS and following successful surgery [2, 4-8]. In a systematic review, a risk between 1.9-2.5% was reported for VTE not related to surgery and a risk varying between 0-5.6% for postoperative thrombosis in patients with CS [2]. In addition, VTE was indicated as the cause of death in 0-1.9% of the patients [2]. Recently, a large retrospective cohort study among 473 Dutch patients with CS showed that the incidence of VTE prior to treatment was 14.6/1000 person years, which is clearly increased compared to the estimated VTE incidence of 1-2/1000 person years in the general population [4].

The underlying mechanism of the hypercoagulable state in CS has not been fully elucidated [9, 10]. Several studies have evaluated haemostatic parameters in CS [2]. The most consistent findings are shortening of activated partial thromboplastin time (aPTT), indicating functional hypercoagulability, and increased levels or activity of fibrinogen, Factor VIII, Factor IX and Von Willebrand Factor (vWF) [6, 9-18]. Impaired fibrinolytic capacity may also contribute to the hypercoagulable state in CS. Parameters of fibrinolysis have not been extensively investigated in CS, but the observed increase of plasminogen activator inhibitor type 1 (PAI-1) in CS suggests that fibrinolysis might be modulated by hypercortisolism as well [6, 10, 14, 16, 19].

Pituitary dependent CS or Cushing's disease (CD) is primarily treated by transsphenoidal surgery. Surgery results in remission rates of approximately 60-70%, but up to 20% of the patients develops recurrent CD during follow-up [1, 20, 21]. Radiotherapy can be applied in patients with recurrent disease or incompletely removed adenomas. However, it can take years for radiotherapy to become effective [22]. Considering the increased morbidity and mortality associated with uncontrolled hypercortisolism [1], there is a clear need for effective medical treatment for CD. Recent studies have shown that corticotroph adenomas often co-express the somatostatin receptor subtype 5 (sst_5) and dopamine receptor subtype 2 which may therefore be potential therapeutic targets for medical therapy [23-25]. Recently we performed a prospective open-label trial in which stepwise treatment for CD was applied with the somatostatin-analog pasireotide, which has high affinity for the sst_5, the dopamine agonist cabergoline and the antifungal agent ketoconazole [26]. After 80 days, biochemical remission of CD was achieved in almost 90% of the patients [26]. The effects of medical therapy of CD on haemostatic parameters are currently unknown. The first aim of the present study was to compare parameters of coagulation and fibrinolysis between patients with active CD and healthy volunteers. Moreover, the effects of short-term biochemical remission of CD on haemostasis after this medical treatment regimen were examined.

Methods

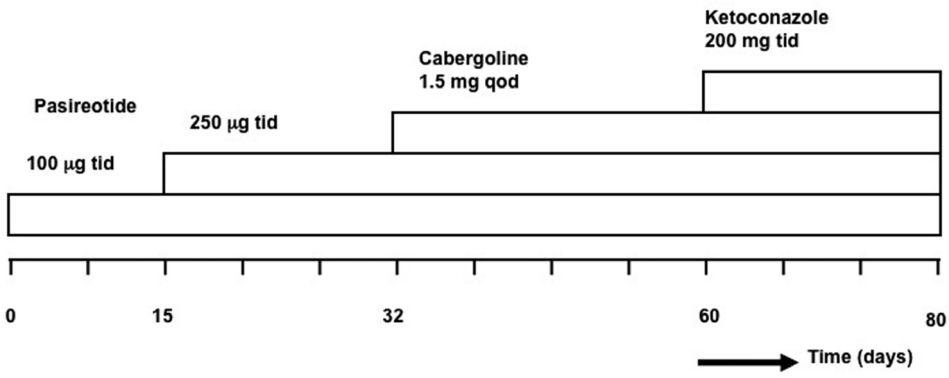
Study protocol

The study design has been described previously [26]. Briefly, 17 patients (mean age 45.7 years, range 22-67 years, 13 females) with either *de novo* (n=12), residual (n=2) or recurrent (n=3) CD were included in four university medical centers. The total duration of the study was 80 days (Figure 1). Treatment was initiated with pasireotide 100µg s.c. three times daily (tid). If after 10 days urinary free cortisol excretion (UFC, mean of 2 collections) had not normalized, this dose was increased to 250µg s.c. tid at day 15. If UFC (mean of 3 collections at day 26 to 28) had normalized at day 28, patients continued treatment with pasireotide 250µg tid. In case of persistent hypercortisolism, cabergoline was added to pasireotide at day 32 in a dose of 0.5mg every other day (qod), which was increased to 1mg qod and 1.5mg qod after 5 and 10 days, respectively. If UFC (mean of 3 collections at day 54 to 56) remained elevated, ketoconazole was added to pasireotide and cabergoline at day 60 in a dosage of 600mg/day (200mg tid). The last evaluation started at day 77. Citrated blood was collected at baseline, day 28 and day 77 and plasma was stored until further analysis.

The protocol was approved by the Ethics Committees of all participating centers. A total of 40 age-matched

healthy volunteers (26 females) were included as a control group. Each participant of the study gave written informed consent.

Figure 1.



Schematic overview of the treatment schedule. The total duration of the study was 80 days. Treatment was initiated with pasireotide 100µg subcutaneously (s.c.) three times daily (tid). If after 10 days urinary free cortisol excretion (UFC, mean of 2 collections) had not normalized, this dose was increased to 250µg s.c. tid at day 15. If UFC (mean of 3 collections at day 26 to 28) had normalized at day 28, patients continued treatment with pasireotide 250µg tid. In case of persistent hypercortisolism, cabergoline was added to pasireotide at day 32 in a dose of 0.5mg every other day (qod), which was increased to 1mg qod and 1.5mg qod after 5 and 10 days, respectively. If UFC (mean of 3 collections at day 54 to 56) remained elevated, ketoconazole was added to pasireotide and cabergoline at day 60 in a dosage of 600mg/day (200mg tid). The last evaluation started at day 77.

Measurement of urinary free cortisol (UFC)

UFC levels were measured in a central laboratory by HPLC as described previously [27] (ULN: 145 nmol/24h).

Measurements of coagulation and fibrinolysis variables

aPTT was measured using Triniclot aPTT-HS (Kordia, Leiden, the Netherlands). Fibrinogen was measured using the clotting rate assay described in [28] with Thrombin Reagent (Siemens, Breda, the Netherlands). D-dimer levels were measured using the AutoDimer (Biopool International, Sweden). Antithrombin activity levels were determined using the Coamatic® Antithrombin kit (Chromogenix, Intrumentation Laboratory, Breda, the Netherlands). Factor VIII clotting activity was measured by a one stage clotting assay using Triniclot aPTT HS (Kordia, Leiden, the Netherlands) and Factor VIII deficient plasma (Biopool, Ventura, USA). Protein C activity was determined using the Siemens Protein C-reagent kit (Siemens, Breda, the Netherlands). Protein S activity was measured using the Staclot® Protein S-kit (Diagnostica Stago, Roche Diagnostics, Almere, the Netherlands). All tests mentioned above were performed using the Sysmex CA1500 coagulation analyzer (Siemens, Breda, the Netherlands). vWF:Antigen (vWF:Ag) was measured using an in-house sandwich enzyme-linked immunosorbent assay (ELISA) using rabbit anti-human vWF and horseradish peroxidase conjugated anti-human vWF.

Fibrinolysis was assessed by measurement of plasma clot lysis time (CLT) using a previously described method [29]. The following factors that influence fibrinolysis were measured: plasminogen activator inhibitor type 1 (PAI-1) activity using the Trinilize PAI-1 activity assay (Trinity Biotech, Ireland); thrombin-activatable fibrinolysis inhibitor (TAFI) activity using the Actichrome TAFI activity kit (American Diagnostica Inc., Stamford, CT, USA) and α2-antiplasmin using the Coamatic® Plasmin-Inhibitor testkit (Chromogenix, Breda, the Netherlands).

Statistical analysis

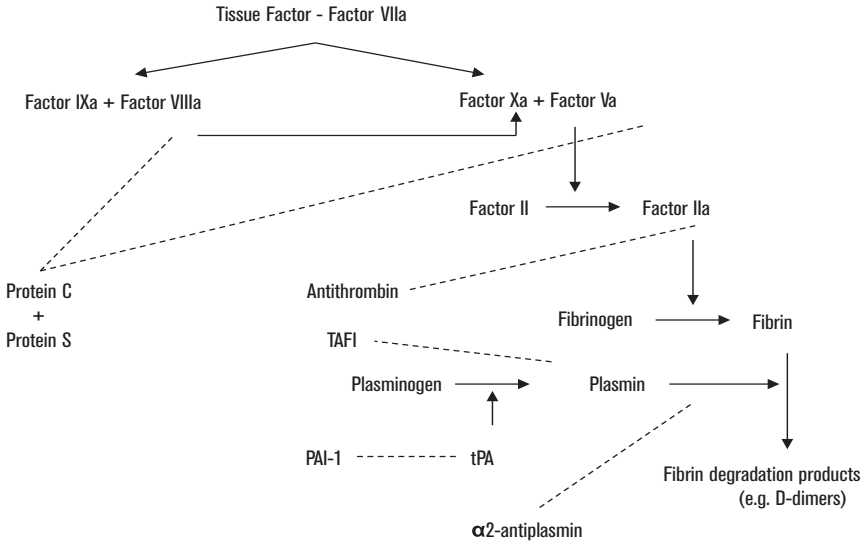
Data were analyzed using SPSS 15.0 for Windows. The Mann-Whitney U test was used to compare baseline scores in the patients to controls. To compare scores at baseline to those at day 28 and 77, the Wilcoxon signed ranks test was used. To adjust for BMI, ANCOVA was used to determine differences between patients and controls. Pearson correlation was calculated for correlation analysis. Statistical significance was accepted at the 0.05 level of probability.

Results

Comparison CD and healthy controls

The baseline characteristics of patients and healthy controls are outlined in Table 1. Patients had a mean age of 45.5 yrs vs. 41.2 yrs for controls (NS). Mean BMI was 29.9 kg/m^2 in patients, whereas controls had a mean BMI of 22.6 kg/m^2 ($p<0.001$). The coagulation and fibrinolysis pathways are depicted in Figure 2.

Figure 2.



The coagulation and fibrinolysis pathways depicted in a simplified manner. Factors involved in coagulation (either stimulatory or inhibitory) are depicted in red; factors involved in fibrinolysis (either stimulatory or inhibitory) are depicted in blue. Upon tissue damage, tissue factor (TF) binds Factor VII. The TF-Factor VII complex activates both Factor IX to Factor IXa and X to Xa. The Factor IXa-VIIIa complex also activates Factor X to Xa. The Factor Xa-Va complex activates Factor II to IIa (thrombin). Thrombin facilitates the conversion of fibrinogen into fibrin. Besides its important role in primary haemostasis, Von Willebrand Factor (vWF) binds Factor VIII and thereby decreases Factor VIII clearance. Factors that inhibit fibrin formation include antithrombin, Protein C and Protein S. Antithrombin binds and inactivates thrombin and, to a lesser extent, inhibits TF-bound Factor VIIa and Factors Xa and IXa. Protein S is a cofactor to protein C. Activated protein C inactivates Factors Va and VIIIa. Fibrinolysis is initiated by Tissue Plasminogen Activator (tPA), which stimulates the formation of plasmin out of plasminogen. Plasmin in turn dissolves the fibrin complex. Proteins that inhibit fibrinolysis include Plasminogen Activator Inihibitor type 1 (PAI-1), thrombin-activatable fibrinolysis inhibitor (TAFI) and α2-antiplasmin. PAI-1 inhibits tPA induced plasmin formation. TAFI is a carboxypeptidase that inhibits fibrinolysis by reducing tPA and plasminogen binding to fibrin. α2-antiplasmin inhibits plasmin-induced fibrin breakdown. Black arrows indicate activation of a next step in the pathway. Dashed lines indicate inhibitory actions.

Table 1. Baseline characteristics patients and controls.

	Controls (n=40)	Patients (day 0) (n=17)	P-value	P-value after correction for BMI
Age (yrs)	41.2 ± 2.43	45.5 ± 3.4	0.34	
BMI (kg/m^2)	22.6 ± 0.32	29.9 ± 1.63	<0.001	
aPTT (seconds)	36.2 ± 0.62	31.1 ± 1.74	<0.01	<0.05
Fibrinogen (g/L)	2.92 ± 0.10	3.35 ± 0.21	<0.05	0.23
Factor VIII (U/mL)	0.96 ± 0.04	1.18 ± 0.11	<0.05	0.24
vWF:Ag (U/mL)				
(n=40/17)	1.10 ± 0.06	1.38 ± 0.13	0.10	0.56
Blood group 0				
(n=15/4)	0.87 ± 0.08	1.11 ± 0.30	0.40	0.49
Blood group non-0				
(n=25/13)	1.24 ± 0.06	1.46 ± 0.14	0.30	0.91
Antithrombin				
(U/mL)	1.10 ± 0.01	1.03 ± 0.04	0.11	0.19
Protein C (U/mL)	1.23 ± 0.04	1.41 ± 0.11	0.24	0.65
Protein S (U/mL)	0.89 ± 0.04	1.07 ± 0.07	<0.05	0.28
CLT (minutes)	81.9 ± 1.53	132.1 ± 14.5	<0.001	<0.05
TAFI (µg/mL)	18.1 ± 0.44	20.3 ± 0.57	<0.01	0.19
PAI-1 (IU/mL)	1.05 ± 0.26	7.18 ± 3.07	<0.01	0.81
α2-antiplasmin				
(U/mL)	1.13 ± 0.02	1.25 ± 0.03	<0.01	0.22

Characteristics of healthy control subjects and untreated patients with CD. BMI: body mass index. aPTT: activated partial thromboplastin time. vWF:Ag: von Willebrand Factor antigen. CLT: plasma clot lysis time. TAFI: thrombin-activatable fibrinolysis inhibitor. PAI-1: plasminogen activator inhibitor type 1. vWF:Ag concentrations were analysed separately for subjects with blood group 0 and subjects with blood group non-0, since blood group 0 is associated with lower levels of vWF:Ag [40]. Figures represent mean ± SEM.

Compared to healthy volunteers, CD patients had significantly shortened aPTT ($p<0.01$) and higher levels of fibrinogen ($p<0.05$), Factor VIII activity ($p<0.05$) and protein S activity ($p<0.05$). Antithrombin and vWF:Ag concentrations and protein C activity did not differ significantly from controls in patients with CD. With respect to fibrinolysis, plasma CLT was significantly prolonged in patients compared to control subjects ($p<0.001$). Furthermore, patients had higher levels of PAI-1, thrombin-activatable fibrinolysis inhibitor (TAFI) and α2-antiplasmin ($p<0.01$ for all three parameters). After adjustment for BMI, aPTT and CLT were still significantly

different between patients and controls ($p<0.05$ in both cases), while all other differences lost their significance (Table 1). At baseline, 2 patients had diabetes mellitus and 13 had hypertension. There were no significant differences in haemostatic parameters between patients with or without diabetes mellitus or hypertension.

Correlations

At baseline, BMI was significantly correlated with aPTT ($r=-0.33$), Factor VIII ($r=0.27$), protein C ($r=0.32$), protein S ($r=0.32$; $p<0.05$ for all parameters), vWF:Ag (only in patients with blood group non-0; $r=0.49$; $p<0.01$), CLT ($r=0.63$), PAI-1 ($r=0.55$), α2-antiplasmin ($r=0.52$; $p<0.001$ for all 3 parameters) and TAFI ($r=0.36$; $p<0.01$). CLT was significantly correlated with parameters of fibrinolysis, in particular: PAI-1 ($r=0.89$), α2-antiplasmin ($r=0.47$) and TAFI ($r=0.51$; $p<0.001$ in all cases). There were no statistically significant correlations between the level of UFC and any parameter of coagulation or fibrinolysis. This correlation analysis was performed among both patients and healthy control subjects.

At the end of the study period, BMI was significantly correlated with Factor VIII ($r=0.47$), antithrombin ($r=-0.49$; $p<0.001$ for both parameters) and protein C ($r=0.41$; $p<0.01$). The correlation with aPTT was borderline significant at this time point ($r=-0.26$; $p=0.06$). Finally, BMI was significantly correlated with parameters of fibrinolysis at day 77: CLT ($r=0.49$), PAI-1 ($r=0.52$; $p<0.001$ for both parameters) and α2-antiplasmin ($r=0.29$; $p<0.05$).

Treatment effect

The results with respect to UFC excretion are outlined in [26]. Briefly, pasireotide monotherapy induced sustained normalization of UFC excretion in 5 of 17 patients (29%) at day 28. The addition of cabergoline normalized UFC excretion in an additional 4 of 17 patients (24%) after a month of combination treatment. At day 60, low-dose ketoconazole was initiated in 8/17 patients that still had elevated UFC levels despite pasireotide-cabergoline combination therapy. Addition of ketoconazole induced biochemical remission in six of these eight patients at day 80, which increased the percentage of patients with a complete response to 88%. The most prevalent side-effects were signs of steroid withdrawal. Moreover, HbA1c and fasting glucose levels increased throughout the study period (further detailed in [26]).

Effects of medical therapy of CD on coagulation and fibrinolysis

Table 2 shows the effects of the treatment regimen on haemostatic parameters. The 2 patients that developed pulmonary embolism were excluded from the analysis, because of effects of VTE and the use of acenocoumarol on haemostatic parameters. Throughout the study period, a slight decrease in antithrombin levels was observed ($p<0.01$ at day 28 and 77 compared to baseline). PAI-1 levels slightly increased during pasireotide monotherapy ($p<0.05$). No significant changes in aPTT, D-dimers, protein C and S activity, vWF:Ag, Factor VIII activity, CLT, TAFI and α2-antiplasmin were observed during pasireotide monotherapy. At day 77, plasma levels of TAFI had decreased compared to baseline ($p<0.05$). After an initial increase, mean PAI-1 concentrations returned to baseline levels at day 77. Again, aPTT, D-dimers, protein C and S activity, vWF:Ag, Factor VIII activity, CLT and α2-antiplasmin did not significantly change at day 77 compared to baseline values. Compared to controls, patients still had shortened aPTT at day 77 ($p<0.001$).

Furthermore, they had higher levels of fibrinogen ($p<0.05$), Factor VIII activity ($p<0.05$), lower levels of antithrombin ($p<0.001$), prolonged CLT ($p<0.001$) and higher levels of PAI-1 ($p<0.001$) and α2-antiplasmin ($p<0.05$). vWF:Ag concentrations, Protein C and S activity and TAFI levels did not significantly differ between patients and controls at the end of the study period.

The effects of the treatment regimen were also analyzed separately in the group of patients that reached normalized UFC excretion, but these results did not differ from the total group analysis. In addition, subanalyses were performed to compare treatment effects between the treatment groups (mono-, duo- or triple therapy, respectively), but no differences were found.

Table 2. Haemostatic parameters throughout the study period.

	Day 0	Day 28	Day 77	P-value Day 0-28	Day 0-77
aPTT (seconds)	31.5 ± 1.97	31.1 ± 1.77	30.1 ± 1.65	0.88	0.91
D-dimers (µg/mL)	0.10 ± 0.02	0.21 ± 0.08	0.13 ± 0.02	0.05	0.48
Fibrinogen (g/L)	3.19 ± 0.20	3.05 ± 0.21	3.34 ± 0.18	0.16	0.97
Factor VIII (U/mL)	1.11 ± 0.12	1.26 ± 0.17	1.29 ± 0.13	0.47	0.22
vWF:Ag (U/mL)	1.35 ± 0.14	1.35 ± 0.15	1.49 ± 0.14	0.53	0.11
Blood group 0	1.11 ± 0.30	1.03 ± 0.28	1.20 ± 0.30	0.47	0.11
Blood group non-0	1.46 ± 0.14	1.50 ± 0.15	1.54 ± 0.13	0.27	0.25
Antithrombin (U/mL)	1.04 ± 0.04	0.95 ± 0.04	0.94 ± 0.03	<0.01	<0.01
Protein C (U/mL)	1.41 ± 0.12	1.41 ± 0.12	1.33 ± 0.11	0.91	0.10
Protein S (U/mL)	1.04 ± 0.08	1.04 ± 0.09	0.99 ± 0.07	0.59	0.13
CLT (minutes)	134.5 ± 16.4	145.8 ± 19.2	140.6 ± 21.3	0.43	0.94
TAFI (µg/mL)	20.5 ± 0.61	19.8 ± 0.88	18.8 ± 0.78	0.19	<0.05
PAI-1 (IU/mL)	7.70 ± 3.46	10.7 ± 3.97	7.65 ± 2.46	<0.05	0.38
α2-antiplasmin (U/mL)	1.25 ± 0.04	1.21 ± 0.05	1.24 ± 0.04	0.21	0.33

Patient characteristics at baseline, day 28 and day 77 of the study period. Both patients that developed pulmonary embolism during the first month of the study were excluded from this analysis, because they were using acenocoumarol both at day 28 and day 77. aPTT: activated partial thromboplastin time. vWF:Ag: von Willebrand Factor antigen. CLT: plasma clot lysis time. TAFI: thrombin-activatable fibrinolysis inhibitor. PAI-1: plasminogen activator inhibitor type 1. vWF:Ag concentrations were analysed separately for subjects with blood group 0 and subjects with blood group non-0, since blood group 0 is associated with lower levels of vWF:Ag [40]. Figures represent mean ± SEM.

Patients with thromboembolic events

During the first month (at day 15 and day 4, respectively) of the study period, 2 patients presented with pulmonary embolism during pasireotide monotherapy. Both had *de novo* CD and had never had venous thromboembolism before. Neither patient had an inherited thrombophilic factor such as Factor V Leiden mutation, prothrombin gene variant or antithrombin or ProteinC/S deficiency. At baseline, both patients had relatively short aPTT values (28 and 29 seconds, respectively) and high levels of fibrinogen (4.9 and 4.3 g/L) and Factor III activity (1.60 and 1.69 U/mL) compared to the other patients. In addition, patient 2 had relatively low levels of antithrombin (0.76 U/mL) and very high levels of vWF:Ag (2.05 U/mL) and D-dimers (1.20 µg/mL; normal reference value <0.25 µg/mL). Considering the increased baseline D-dimer concentrations and the (in retrospect) presence of mild dyspnoea, pulmonary embolism might already have been present at baseline in patient 2, a 22-year old female that also used oral contraceptives. Remarkably, in both patients an acute strong decrease in UFC excretion was observed after the initiation of pasireotide therapy and before the diagnosis of pulmonary embolism was made (Fig. 3). Patient 1 (a 47-year old male) had a baseline

UFC excretion of 4.2 times the upper limit of normal, which decreased to 2.0 and 0.5 times the upper limit of normal within the first three days after the start of pasireotide treatment. The baseline UFC excretion in the second patient was 1.6 times the upper limit of normal. At days 1, 2 and 3 after pasireotide initiation the UFC excretion rates were 0.4, 0.4 and 0.7 times the upper limit of normal, respectively. Subsequently, UFC excretion returned to initial values, possibly due to tachyphylaxis. Pulmonary embolism was treated with low molecular weight heparin and oral anticoagulants.

Discussion

CS is associated with a hypercoagulable state resulting in an increased risk for VTE [2, 4]. In physiological conditions, a fine-tuned balance exists between coagulation and fibrinolysis. Our data show that this balance is disturbed in CD due to both increased production of coagulation proteins and impaired fibrinolytic capacity. Short-term biochemical remission induced by medical therapy is, however, not accompanied by recovery of the haemostatic balance.

Several studies have shown increased levels of components of the coagulation cascade in CS, including Factor VIII, Factor IX, vWF:Ag and fibrinogen [6, 10, 14, 16, 19]. Accordingly, we found significantly higher levels of Factor VIII activity and fibrinogen and a trend towards increased vWF:Ag levels in our CD patients compared to healthy controls. Activation of the coagulation pathway was functionally reflected by a significantly shortened aPTT. Increased production of clotting factors in CD may at least partially be mediated by direct effects of glucocorticoid excess, as dexamethasone has been shown to increase plasma concentrations of Factor VIII, Factor IX and fibrinogen in healthy volunteers [30]. With respect to anticoagulant factors, patients had higher protein S levels than controls. The clinical significance of this finding is, however, unclear. In the present study, no differences between patients and controls were observed in protein C activity and antithrombin levels. In contrast, antithrombin has previously been found to be elevated in CS, which was hypothesized to reflect a compensatory mechanism to the hypercoagulable state [14].

We show that an impaired fibrinolysis also contributes to the hypercoagulable state in CD, as reflected by a significantly prolonged plasma CLT in untreated patients compared to healthy controls. Several factors were examined that are known to inhibit fibrinolysis, including PAI-1, TAFI and α2-antiplasmin (Figure 2). Compared to controls, our patients had higher levels of these parameters, which may explain the hypofibrinolysis in CD. *In vitro*, glucocorticoids induce PAI-1 and TAFI mRNA expression by human hepatoma cells and glucocorticoid responsive elements have been identified in the promoter of both these genes [2]. In addition, glucocorticoids stimulate PAI-1 production by human visceral adipose tissue [2]. Associations have been found between levels of CLT, PAI-1 and TAFI and the risk of cardiovascular disease [31, 32]. Hypofibrinolysis may therefore contribute to the increased cardiovascular risk in CD [33, 34].

An important difference between patients and controls is the observation that on average, patients had a significantly higher BMI. Whereas some studies did not correct for BMI when comparing haemostatic variables between patients with CS and controls [6, 10, 16], others did not find correlations between BMI and these variables [14, 19]. Abdominal obesity, an important hallmark of CD, is associated with increased plasma levels of fibrinogen, Factor VII, vWF and PAI-I [35, 36]. In agreement, we found statistically significant correlations between BMI and parameters of coagulation, both at baseline and at the end of the study period. This suggests that the disturbances in coagulation and fibrinolytic parameters observed in our CD patients might partially be explained by their increased abdominal fat mass. However, aPTT and CLT significantly differed between patients and controls even after adjustment for BMI, indicating that the increased BMI in CD patients is not the only explanation for their hypercoagulable state. Accordingly, a relative risk of 2.5 for VTE has been reported among the obese population [37], while compared to the general population, patients with CS may have a more than tenfold increased risk for VTE [4]. It seems, therefore, that the incidence of VTE among obese subjects without hypercortisolism is not as high as in patients with CS.

Surgery is the primary treatment for CD, but only scarce data are available evaluating the effect of surgical

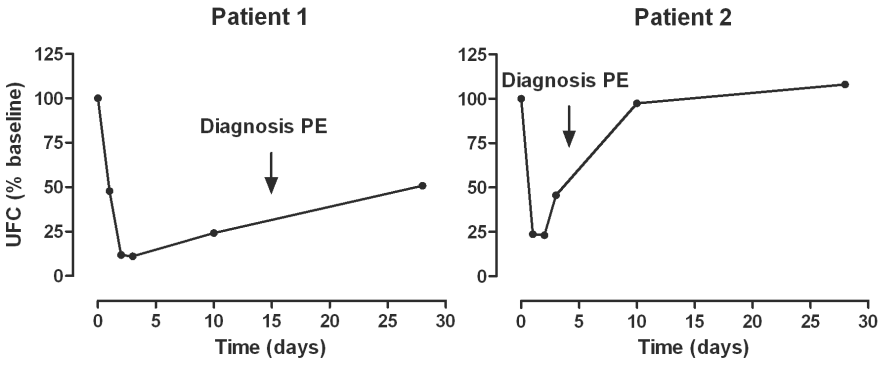
cure of CD on haemostasis. Casonato et al. described 20 patients with CS with shortened aPTT and increased Factor VIII activity and vWF:Ag levels that underwent surgery. In the cured patients, Factor VIII activity and vWF:Ag levels decreased from the third month after surgery onwards and normalized within one year [12]. A recent study showed an improvement in the hypercoagulable state as suggested by decreased levels of vWF:Ag, PAI-1 Ag, antithrombin III and α2-antiplasmin one year after successful surgery. Moreover, aPTT significantly increased after surgical cure. However, despite the observation that these parameters improved within one year, haemostasis did not fully normalize [19].

To the best of our knowledge, our study is the first to describe the effects of medical treatment of CD on coagulation and fibrinolysis. In this study, we observed a slight decrease in antithrombin levels after three months of successful medical therapy. However, it is questionable whether this decrease is clinically relevant, since antithrombin levels were already in the normal range to begin with. No changes in fibrinogen, D-dimer levels, Factor VIII activity and protein C and S activity were observed, which is in agreement with the study by Manetti et al. [19]. In contrast to their results, no significant changes in aPTT and vWF:Ag levels were found in the present study. In addition, hypofibrinolysis persisted as reflected by prolonged CLT and elevated levels of PAI-1 and α2-antiplasmin throughout the study period. Manetti et al., on the other hand, reported significantly decreased levels of PAI-1 and α2-antiplasmin, suggesting that fibrinolytic capacity improves after surgical cure of CS. An explanation for the differences between both studies could be the different follow-up period (12 months vs. less than 3 months, respectively). This suggests that a minimal period of sustained biochemical remission is required to reverse the hypercoagulable state in patients with CD. The observation that the hypercoagulable state persists in both studies, even after normalization of cortisol levels, indicates that the increased risk for thrombosis in patients with CD might in part be due to the persistence of abdominal obesity rather than to the presence of hypercortisolism. This would explain why apparently it takes a long time to reverse the hypercoagulable state in patients cured from CD. Therefore, it would be interesting to assess the long-term effects of medical therapy on coagulation and fibrinolysis.

Currently, there are no guidelines on type and duration of thromboprophylaxis for patients with CS before and after (surgical) treatment. Previously, it has been proposed to routinely administer anticoagulant prophylaxis to patients with CS [6]. Indeed, the incidence of VTE in CS was recently reported to be comparable to the incidence following total hip- or knee replacement, suggesting that thromboprophylaxis is indicated [4]. In CD, the postoperative risk on VTE is 3.4% compared to 0% in patients with non-functional pituitary adenomas. Interestingly, most VTE occurred between 1 week and 2 months after surgery [4]. These and our observations may indicate that the duration of thromboprophylaxis should be prolonged after treatment, although the exact period has not been established yet.

As mentioned above, two patients developed pulmonary embolism when using pasireotide monotherapy. Several possible explanations exist for this observation. First, hypercortisolism causes a hypercoagulable state and given the high baseline levels of D-dimers and the presence of dyspnoea in patient 2, her pulmonary embolism was most likely due to the presence of CD, with the use of oral contraceptives as additional risk factor. Moreover, procoagulant factors like Factor VIII activity and fibrinogen were relatively high at baseline in these two patients compared to the other patients. Second, Boden et al. found a stimulatory effect of somatostatin on Tissue Factor (TF) procoagulant activity and TF antigen levels [38] and it has been reported that somatostatin increased the activity of factors II, VII and X in rats [39]. Thus, a direct effect of pasireotide on the coagulation pathway cannot be fully excluded. However, considering the lack of significant changes in procoagulant factors at day 28 compared to baseline, a causal relationship between the administration of pasireotide and the development of pulmonary embolism seems unlikely. A third possibility might be the acute decrease (even normalization) of UFC after initiation of pasireotide treatment. The rebound inflammatory response following such rapid suppression of cortisol levels may also have contributed to the development of pulmonary embolism [36]. This phenomenon, i.e. the development of VTE following an acute decrease in cortisol levels, was recently also suggested by Stuijver et al. [4], as 3.4% of the patients with CD developed VTE, mostly within 1-2 months after surgery. In support of this hypothesis, patients that were treated with cortisol lowering therapy before undergoing transsphenoidal surgery had a lower risk of postoperative VTE than patients that were not [4].

Figure 3.



Concentrations of urinary free cortisol (depicted as % compared to baseline) during the first month in the 2 patients that developed pulmonary embolism. The arrows indicate the day at which the diagnosis was confirmed. Baseline UFC values were 4.2 and 1.6 times the upper limit of normal, respectively.

In conclusion, this study shows that the hypercoagulable state in CD results from both increased production of procoagulant factors and impaired fibrinolytic capacity. Successful medical treatment did not improve this hypercoagulable state after 80 days. This might partially be explained by persistence of the metabolic syndrome, in particular abdominal obesity, which is known to modulate haemostatic parameters. Therefore, sustained control of hypercortisolism seems required to reverse the hypercoagulable state in CD. This may have implications for the duration of thromboprophylaxis in patients with (cured) CD. Future studies should further assess the reversibility of the hypercoagulable state in CD and examine when and to what extent the changes in haemostasis normalize in order to determine the optimal duration of thromboprophylaxis.

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CHAPTER 7

CORTISOL DIURNAL RHYTHM AND QUALITY OF LIFE AFTER SUCCESSFUL MEDICAL TREATMENT OF CUSHING'S DISEASE

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ABSTRACT

Purpose

Cushing's disease (CD) is associated with severely impaired quality of life (QoL). Moreover, the physiological cortisol diurnal rhythm (CDR) is disturbed in CD. QoL can improve after successful surgery, the primary treatment for CD. We evaluated the effects of medical treatment on QoL and CDR.

Methods

In 17 patients, stepwise medical treatment was applied with the somatostatin analog pasireotide, the dopamine agonist cabergoline and the adrenal-blocking agent ketoconazole. After 80 days, 15/17 (88%) patients had reached normal urinary free cortisol excretion (UFC). Subsequently, patients continued medical therapy or underwent surgery. UFC, plasma and salivary CDR and QoL-related parameters (assessed using 5 questionnaires: Nottingham Health Profile, Hospital Anxiety and Depression Scale, Multidimensional Fatigue Index-20, RAND-36, CushingQoL) were measured.

Results

At baseline, 5/17 patients had preserved CDR. In 6/12 patients with disturbed baseline CDR, recovery was observed, but without any correlation with QoL.

QoL was significantly impaired according to 18/20 subscales in CD patients compared to literature-derived controls. According to the RAND-36 questionnaire, patients reported more pain at day 80 ($p<0.05$), which might reflect steroid-withdrawal. Generally, QoL did not improve or deteriorate after 80 days. CushingQoL scores seemed to improve after 1 year of remission in 3 patients that continued medical therapy ($p=0.11$).

Conclusions

CDR can recover during successful pituitary- and adrenal-targeted medical therapy. Patients with CD have impaired QoL compared to controls. Despite the occurrence of side-effects, QoL does not deteriorate after short-term biochemical remission induced by medical therapy, but might improve after sustained control of hypercortisolism.

INTRODUCTION

Cushing's disease (CD) is caused by an adrenocorticotropin (ACTH) producing pituitary adenoma that chronically stimulates adrenocortical cortisol production. This chronic state of hypercortisolism leads to various symptoms, e.g. obesity, muscle weakness and fatigue [1]. Moreover, cortisol has marked effects on the human brain. Psychological disturbances such as anxiety, irritability, psychosis, emotional instability and even suicidal behavior have been described among patients with Cushing's syndrome (CS) [2, 3]. Depression is the most frequently observed psychiatric disorder in CS, with reported incidence numbers varying from 32-62% [4-6]. Additionally, CD has been reported to be accompanied by physical and emotional impairment [7]. Taken together, these severe symptoms unambiguously lead to an impaired quality of life (QoL) in patients with CS [8-12], even after long-lasting biochemical remission [10, 13]. Recently, a disease-specific questionnaire (CushingQoL) has been validated to measure QoL in CS. Hypercortisolemic patients were shown to have worse CushingQoL scores compared to patients in remission. [11].

Patients with CS usually lack the physiological cortisol diurnal rhythm (CDR), which is characterized by a cortisol peak in the early morning followed by a gradual decrease during the day to low midnight-levels [14]. A flat cortisol day-night profile might also contribute to worsened QoL in CS, e.g. by inducing sleep disturbances [7, 15]. CDR usually recovers after successful surgical treatment [16], but the effects of medical treatment on CDR are currently unknown.

CD is primarily treated by transsphenoidal surgery, resulting in remission rates of approximately 60-70% [17]. Radiotherapy can be applied in patients with recurrent disease or incompletely removed adenomas. However, it can take years for radiotherapy to become effective [18]. Considering this, there is growing interest for effective medical treatment options for CD. Recent studies show that corticotroph adenomas often co-express the somatostatin receptor subtype 5 and dopamine receptor subtype 2 and it is has been suggested that these receptors are potential therapeutic targets [19, 20]. Previously [20], we carried out a prospective open-label trial in which stepwise treatment for CD was applied with the somatostatin-analog pasireotide, the dopamine agonist cabergoline and ketoconazole, which suppresses adrenocortical steroidogenesis [21]. After 80 days of treatment, biochemical remission was achieved in 88% of patients. The main adverse events were anorexia, nausea, dizziness, myalgia, arthralgia and hyperglycemia [20]. QoL in CD can improve after surgical treatment [7, 22]. The effects of medical therapy on QoL in patients with CD are currently unknown, but it is conceivable that beneficial effects of medical therapy on hypercortisolism can be attenuated or even counterbalanced by side effects. The aim of the present, longitudinal study was to examine the effects of medical therapy on CDR and QoL in patients with CD during the induction phase of biochemical remission. In addition, QoL after sustained control of cortisol excess was evaluated.

Patients and methods

Study design

The study design has been previously described [20]. In short, 17 patients (mean age 45.5 years, range 22-67 years, 13 females) with either ***de novo*** (n=12), residual (n=2) or recurrent (n=3) CD were included in four university medical centers. Patients were treated in a stepwise manner with the universal somatostatin analog pasireotide, the dopamine agonist cabergoline and with ketoconazole, which directly suppresses the adrenocortical cortisol production. The complete study period was 80 days, after which patients could choose to continue medical therapy or to proceed to transsphenoidal adenomectomy or radiotherapy. The protocol was approved by the Ethics Committees of all participating centers. Each participant of the study gave written informed consent.

The results with respect to UFC are outlined in [20]. In short, pasireotide monotherapy induced sustained normalization of UFC in 5/17 patients at day 28 and the addition of cabergoline normalized UFC in an

additional 4/17 patients after a month of combination therapy. After the addition of ketoconazole at day 60, biochemical remission was induced in six of the remaining eight patients at day 80, increasing the percentage of patients with a complete response to 88%. After 80 days of medical treatment, several clinical features had improved in our patients [20]. Four patients had developed signs of steroid withdrawal after 80 days, including dizziness (3 patients), myalgia (3), arthralgia (1) and nausea (1). Side-effects associated with the treatment included hyperglycemia, gastrointestinal discomfort and transient increases in liver enzymes [20]. Moreover, 5/17 patients had become IGF-1 deficient (defined as total IGF-1 Z-scores <-2SD) after 80 days of pasireotide treatment. Two patients were already IGF-1 deficient before the start of the study.

Hormone measurements

UFC was measured in a central laboratory by HPLC as described previously [23] (ULN: 145 nmol/24h). Plasma cortisol was determined when patients were in a resting state at different time points (09.00h, 17.00h, 22.00h and 0.00h) at baseline and at day 77 and measured by a chemiluminescence-based immunoassay (reference value, 200–800nM; Siemens-DPC, Los Angeles, CA, USA). Salivary cortisol (ULN 10.8nM) was examined using enzyme-linked immunoassay (DRG, Marburg, Germany).

Questionnaires

To assess QoL, patients completed 5 different questionnaires at baseline, day 80 and in the extension period (after 761±174 days on average). Questionnaire-outcomes before and after treatment were compared and related to the treatment schedule and the presence or absence of normalization of UFC. QoL scores were also compared to West-European controls, for which we used age-adjusted literature reference values, a method previously described in [7].

The Nottingham Health Profile (NHP)

The NHP has been developed to provide an indication of a patient's perceived health problems [24]. It consists of 38 questions (answered by yes or no) divided in 6 subscales: energy level (three items), pain (eight), emotional reaction (nine), sleep (five), social isolation (five) and physical abilities (eight). Subscale scores are calculated by adding the weight of each positively answered question, which results in a score between 0 (best QoL) and 100 (worst QoL). Reference values from the general population were derived from [25].

Hospital anxiety and depression scale (HADS)

To measure anxiety and depression, the HADS contains 14 questions, 7 for each parameter. Answers correspond to scores varying from 0 to 3, adding up to a possible total score of 21 per parameter and an overall score of 42. Higher scores indicate more anxiety and depression. To compare our data to the general population, data were retrieved from [26].

Multidimensional fatigue index-20 (MFI-20)

The MFI-20 contains 20 items and measures 5 different dimensions of fatigue (4 items each): general, physical and mental fatigue, as well as reduced activity and motivation. A maximum of 20 points per dimension can be scored and higher scores are associated with more profound fatigue [27]. Reference values were derived from [28].

RAND 36-item Health Survey

The RAND-36 is subdivided in 9 scales and measures functional status and general well-being in 36 questions [29]. Assessed parameters include physical and social functioning, role limitations due to physical and emotional problems, mental health, vitality, pain, general health and health change. The particular version of the questionnaire that we used lacked the questions that comprise vitality and mental health, so these parameters were not measured. Subscale-scores are expressed on a 0-100 scale. Higher scores indicate better quality of life. Reference values were derived from [30].

CushingQoL

The CushingQoL is a recently developed questionnaire meant to assess health-related QoL in patients with CS [11]. It comprises twelve questions that specifically regard problems frequently reported by these patients. Each question is answered on a 5-point scale. Total scores on a 12-60 scale were transformed into a 0-100% scale, higher scores corresponding to better QoL. Since CushingQoL is specifically designed for CS, no reference values from the normal population can be obtained.

Statistical analysis

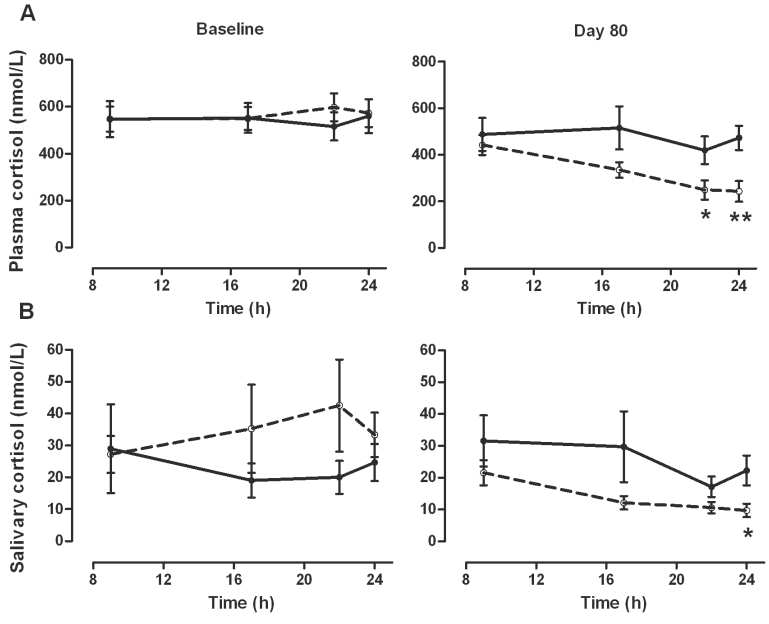
Data were analyzed using SPSS 15.0 for Windows. To compare scores at baseline to those at day 80 and in the extension period, the Wilcoxon signed ranks test was used. Unpaired t-tests were used to compare age and baseline QoL scores to the general population. To compare the gender distribution between the groups, the Chi-squared test was used. Statistical significance was accepted at the 0.05 level of probability.

Results

Cortisol diurnal rhythm (CDR)

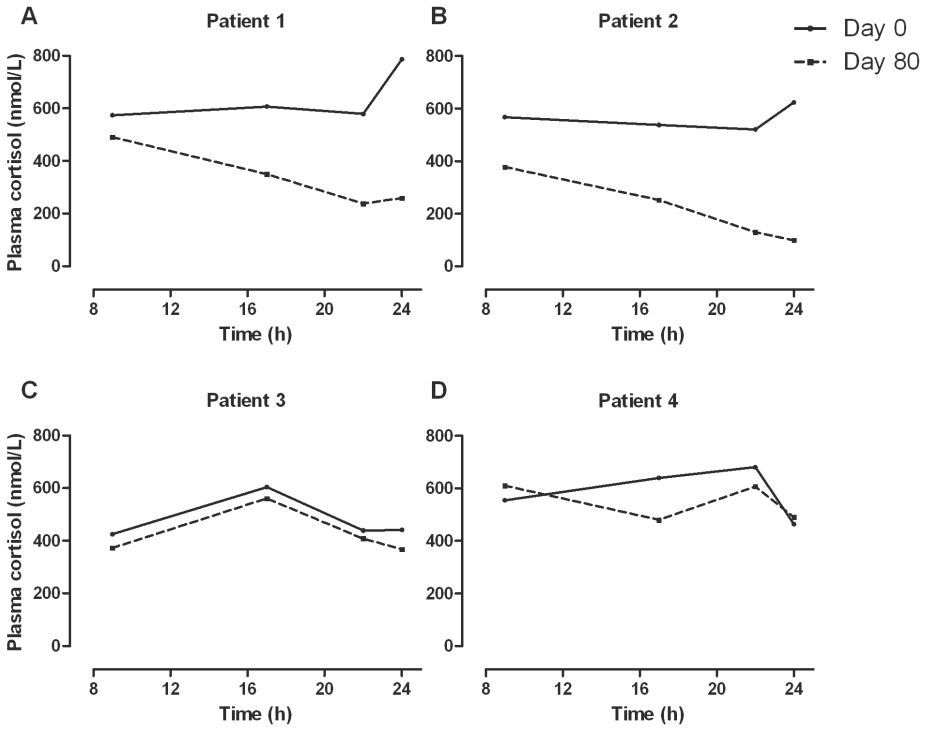
Recovery of CDR was defined by midnight serum and salivary cortisol level of less than 75% of the 09.00h value [31]. According to this criterion, CDR at baseline was disturbed in 12 patients, but still preserved in 5 patients. There was no difference in baseline UFC excretion between both groups. In 6 of these 12 patients (1 mono-, 1 duo- and 4 triple therapy), recovery of serum- and salivary CDR was observed after 80 days. In the remaining 6 patients (3 mono-, 1 duo- and 2 triple therapy), the circadian rhythm of serum- and salivary cortisol did not (yet) recover, despite normalization of UFC excretion in five of these patients (Figures 1+2). Serum cortisol levels at 22.00h ($p<0.05$) and 0.00h ($p<0.01$) and salivary cortisol levels at 0.00h ($p<0.05$) at day 80 were significantly lower in patients in whom diurnal rhythm recovered.

Figure 1.



Circadian rhythm of plasma (A) and salivary (B) cortisol in patients in whom diurnal rhythm recovered (dashed lines; n=6) and in whom it did not (solid lines; n=6). Left: baseline; right: day 80. Data represent mean ± SEM. **$p< 0.01$; *$p<0.05$.

Figure 2.



Representative examples of circadian plasma cortisol levels in 2 patients in whom cortisol diurnal rhythm recovered (A and B, both treated with triple therapy) and 2 patients in whom, despite biochemical remission, it did not (C (monotherapy) and D (duotherapy)). Solid lines: baseline cortisol levels; dashed lines: cortisol levels at day 80.

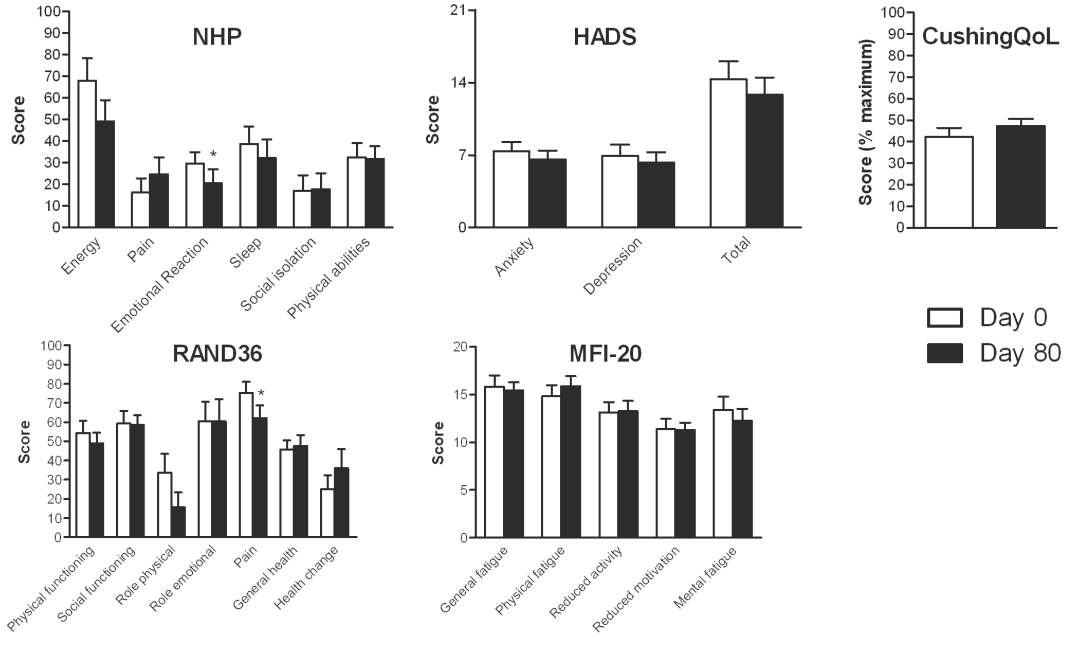
Baseline QoL in patients compared to controls

QoL analysis could be performed in 16 patients (12 females). At baseline, patients had reduced QoL compared to healthy controls as measured by 4 questionnaires (Table 1). There were no statistically significant differences in age and gender distribution between patients and controls. As assessed by the NHP, patients with active CD scored significantly worse for emotional reactions and experienced less energy than controls. Moreover, patients reported more sleep disturbances, social isolation and impaired physical abilities. The HADS indicated that patients scored significantly higher for anxiety and depression. According to the MFI-20, patients had higher fatigue scores on the general, physical and mental fatigue scales and were less active and motivated. Patients with CD experienced impairment in their usual role activities due to both physical and emotional problems as measured with the RAND-36 questionnaire. Furthermore, physical and social functioning was impaired and patients with untreated CD reported lower general health scores. Patients did not report more pain at baseline than control subjects, according to both the NHP and RAND-36 questionnaires. Baseline CushingQoL scores were slightly lower in our population (42.3%) than in the population described by Webb et al. (49%11). There were no differences in QoL scores between patients with preserved and patients with disturbed baseline CDR. Finally, there was no correlation between UFC excretion and any QoL-score.

Effects of medical therapy of CD on QoL

After 3 months of treatment, several symptoms of CD improved including decreases in body weight, waist circumference and blood pressure [20]. QoL at baseline was compared to QoL at the end of the study period (Figure 3). As represented by the NHP, emotional reaction significantly improved ($p<0.05$); all other parameters did not improve or deteriorate over the study period. There was a trend towards experiencing more pain at day 80 compared to baseline ($p=0.051$). As for the HADS, MFI-20 and RAND-36 questionnaires, no significant improvement was observed over time for any parameter. Pain (RAND-36) was reported more frequently after the study period compared to baseline ($p<0.05$). Although slightly higher, there was no significant improvement in QoL according to the CushingQoL score ($p=0.12$). Neither subanalysis of the group of patients that had normalized UFC excretion after the treatment period ($n=14$), nor subanalysis after correction for the duration of biochemical remission (i.e. within 4 weeks, after 56 or 80 days) showed significant changes in QoL (data not shown).

Figure 3.



Effects of 80 days medical treatment with pasireotide monotherapy, pasireotide and cabergoline or triple therapy with ketoconazole on quality of life parameters in 16 patients with CD. Meaning of the scores is outlined in the methods section. Data represent mean ± SEM. White bars: baseline; black bars: day 80. *$p<0.05$ compared to baseline.

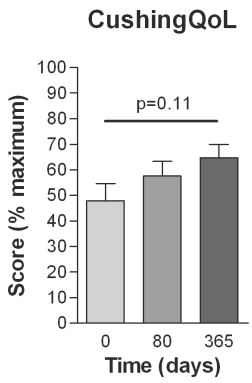
When a subanalysis was performed to assess improvement of QoL at day 80 compared to baseline between the group of patients with recovered CDR and the group of patients in whom CDR did not recover, there were no significant differences. In particular, patients in whom CDR recovered did not report more improvement in sleep ($p=0.41$; not shown). After the initial study period, 3 patients continued pasireotide-cabergoline combination therapy for 1 year. Although not statistically significant ($p=0.11$), there was a trend towards improvement of QoL according to the CushingQoL after 1 year (Figure 4).

Table 1. Baseline quality of life scores.

Questionnaire	Patients (Day 0)		Literature reference		P-value
	(Mean)	(SD)	(Mean)	(SD)	
NHP					
Energy	68.0	41.6	12.1	25.5	<0.001
Pain	16.3	25.8	7.5	18.4	NS
Emotional reaction	29.5	21.5	8.1	16.2	<0.001
Sleep	38.7	32.6	14.8	25.0	<0.001
Social isolation	17.1	28.1	5.3	16.0	<0.01
Physical abilities	32.5	26.3	8.5	13.9	<0.001
HADS					
Anxiety	7.4	3.6	5.1	3.6	<0.05
Depression	6.9	4.3	3.4	3.3	<0.001
Total	14.3	7.0	8.4	6.3	<0.001
MFI-20					
General fatigue	15.8	4.7	9.9	5.2	<0.001
Physical fatigue	14.8	4.7	8.8	4.9	<0.001
Reduced activity	13.1	4.3	8.7	4.6	<0.001
Reduced motivation	11.4	4.3	8.2	4.0	<0.01
Mental fatigue	13.4	5.6	8.3	4.8	<0.001
RAND36					
Physical functioning	54.4	25.1	81.9	23.2	<0.001
Social functioning	59.4	26.4	86.9	20.5	<0.001
Role physical	33.6	39.5	79.4	35.5	<0.001
Role emotional	60.4	40.8	84.1	32.3	<0.01
Pain	75.3	23.6	79.5	25.6	NS
General health	45.6	19.7	72.7	22.7	<0.001
Health change	25.0	28.9	NA	NA	NA
CushingQoL	42.3	16.3	NA	NA	NA

Comparison of health related quality of life parameters per questionnaire between untreated patients with CD and literature-derived control subjects. Control values were retrieved from references 25 (NHP; n=1996), 26 (HADS; n=199), 28 (MFI-20; n=139) and 30 (RAND-36; n=1063). Figures indicate mean ± SD. Differences are calculated using unpaired t-tests. NS: not significant. NA: not available.

Figure 4.



CushingQoL scores in three patients with CD that continued medical combination therapy with pasireotide and cabergoline for one year. Meaning of the scores is outlined in the methods section. Data represent mean ± SEM.

Discussion

In this study we assessed the effects of medical (combination) therapy on CDR and QoL in CD. After three months of successful treatment with pasireotide mono- or combination therapy with cabergoline and ketoconazole, CDR recovered in 50% of the patients with a disturbed baseline CDR. Furthermore, patients improved clinically but, as can be anticipated, short-term biochemical remission did not yet improve QoL. Importantly, despite the occurrence of several side effects of the administered drugs, QoL did not deteriorate after 3 months of medical therapy. An interesting observation is the apparent improvement of CushingQoL scores in the 3 patients that continued medical combination therapy for a total period of one year. We realize that this is a statistically non-significant effect only obtained in a subset of patients, but it asks for assessment of long-term effects of medical therapy for CD on QoL.

The physiological circadian rhythm of cortisol levels is usually disturbed in CS, but is preserved in a subset of patients [14, 16, 32]. We observed a midnight decrease of serum and salivary cortisol levels to less than 75% of morning values in 5/17 patients at baseline, supporting the latter observation. There was no difference in baseline UFC excretion between patients with preserved and those with disturbed CDR. In a cross-sectional study, Veldman et al. found a normal CDR in patients that had been surgically cured from CS for a mean of 8.2 years [16]. The present, longitudinal study, is the first to describe recovery of CDR within 3 months after successful medical treatment in 6/12 patients. This suggests that normalization of cortisol production by medical therapy allows for recovery of hypothalamic control of normal corticotroph cell function in patients with CD. Of note, it is not known whether any of the drugs that were used, in particular the centrally acting agents pasireotide and cabergoline, might influence CDR.

It could be hypothesized that QoL improves after reinstitution of a normal circadian rhythm. However, we did not find differences in QoL scores in patients with or without a restored CDR. In particular no improvement of sleep disturbances was (yet) found in patients with recovered circadian cortisol levels. In 6 of 12 patients with an absent CDR at baseline, no recovery of CDR was found after three months of treatment. It might be speculated that recovery of CDR requires prolonged remission in a subset of patients, which should be verified in future studies.

Patients with CS have an impaired health related QoL compared to healthy controls and, in case of pituitary dependent CS, compared to patients with other pituitary adenomas [10-12]. In our cohort of 16 patients, QoL at

baseline was decreased compared to controls according to virtually all subscales of 4 different questionnaires. This impairment in QoL is multifactorial and in accordance with previous studies [7, 12, 15, 33]. Thus, patients with untreated CD experience less energy and score worse for physical, mental, emotional and social functioning compared to control subjects. HADS scores higher than eight points on the scales for anxiety and depression are indicative of a psychiatric condition. Although our patients scored 7.4 and 6.9 points (mean) for these subscales, 5 and 7 patients scored more than eight points for anxiety and depression, respectively, confirming the presence of considerable psychopathology in these patients [2]. Pain was the only parameter that did not differ between patients with active CD and controls. This could be due to an improvement of pain tolerance induced by elevated cortisol levels. An important limitation of this analysis is the comparison with controls derived from existing literature instead of using an age- and gender matched control group, a method previously used by Van Aken et al. [7]. However, considering the major differences in scores in the various parameters, comparable with previous studies [7, 10, 11], we believe that indeed QoL is severely impaired in our patients compared to the general population.It should be emphasized that patients in our cohort received medical therapy. This is an important difference compared to transsphenoidal surgery: surgery implicates perioperative risks and the risk of hypopituitarism, while medical therapy can be accompanied by side-effects. In general, when analyzing the effects of medical treatment on QoL, it can be very difficult or even impossible to distinguish whether certain effects result from decreased cortisol levels or are a consequence or adverse effect of any of the drugs that were used. Cabergoline can cause nausea and dizziness, while ketoconazole often has gastrointestinal side-effects. Pasireotide had to be autoinjected three times daily, has gastrointestinal side-effects and can induce hyperglycemia. Additionally, treatment with pasireotide rendered 5 patients IGF-1 deficient [20]. These adverse effects might also have prevented any improvement in the quality of life of our patients. A subanalysis of the effects of the three different treatment regimens was performed, but neither group showed improvement or deterioration of QoL during the study period.

Furthermore, the fact that this was an open-label study is a limitation for the evaluation of QoL at the end of the study period. Awareness of patients of (successful) treatment effects and knowledge on the drugs used throughout the study period may have influenced their QoL perception, although this cannot be proven from our data. In the present study, patients reported more pain after the study period compared to baseline according to the RAND-36 questionnaire. Although drug-induced pain cannot be excluded, the increase in reported pain after 80 days most likely reflects steroid withdrawal, since most patients had normalized UFC excretion after 3 months. Steroid withdrawal could also be a possible explanation for the lack of improvement in QoL after three months. In this case, one would expect that QoL improves after the initial steroid withdrawal phase. Indeed, although only tested in three patients that continued medical therapy, we observed a trend towards improvement of CushingQoL scores after a year of biochemical remission. The improvement in QoL after sustained biochemical remission is in agreement with other studies evaluating QoL in CS before and after surgical treatment [7, 22, 34-37]. Santos et al. found that QoL only begins to improve after 6 months following successful surgery [37]. Another study reported improved mental and physical health after 3.2 years (mean) post-surgery for CD, but QoL remained impaired after 11.8 years (mean) following surgical cure of CS (adrenocortical CS, pituitary and ectopic ACTH overproduction combined) in this study [10]. Keil et al. reported improved QoL in children with adrenal or pituitary dependent CS one year post-surgery compared to baseline, but in this study as well, QoL remained worse than in healthy controls according to multiple subscales [13]. Therefore, both the impairment in QoL and cognitive functions and psychological functioning appear to be partially irreversible in patients with CS even after long-term cure [7, 10, 38]. This phenomenon may in part be explained by the development of hypopituitarism in patients with CD following successful surgery. In a cross-sectional study, Van Aken et al. found that the lack of improvement in QoL after a mean of 13.4 years following surgical cure from CD was mainly due to the presence of hypopituitarism after pituitary surgery [7]. Future studies should evaluate whether different treatment modalities have a different outcome with respect to long-term quality of life.

In conclusion, successful medical treatment of CD with pituitary- and adrenal targeting drugs can be accompanied by recovery of CDR. This suggests that normalization of cortisol production by medical therapy allows for recovery of hypothalamic control of normal corticotroph cell function in patients with CD. QoL in

patients with untreated CD is significantly worse compared to controls from literature references according to many subscales of the questionnaires used. After three months of medical combination therapy for CD with pasireotide, cabergoline and ketoconazole, QoL might have been expected to become even worse due to the occurrence of side effects. However, QoL neither deteriorated nor improved as measured with 5 different health-related QoL questionnaires, including the disease-specific CushingQoL and was not related to the presence or absence of CDR. Although only tested in a small number of patients, CushingQoL scores seem to improve after one year of successful medical combination therapy. Finally, future longitudinal studies should determine whether QoL improves after a prolonged period of medical treatment of CD and if so, whether this improvement at least equalizes the amelioration that can be accomplished by surgical cure.

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8 CHAPTER

CUSHING'S DISEASE AND HYPERTENSION: ROLE OF THE RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM AND EFFECTS OF MEDICAL THERAPY.

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CHAPTER 9

PRELIMINARY RESULTS OF A PROSPECTIVE TRIAL WITH KETOCONAZOLE AND OCTREOTIDE COMBINATION THERAPY FOR TREATMENT OF CUSHING'S DISEASE.

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CHAPTER 10

GENERAL DISCUSSION

GENERAL DISCUSSION

1. The medical treatment of Cushing's disease

Despite the fact that transsphenoidal surgery is still the primary treatment for Cushing's disease (CD) [1], both preclinical and clinical observations from recent years have provided significant insights in the medical therapy of CD. In particular, the somatostatin receptor subtype (sst) 5 and the dopamine receptor subtype 2 (D_2R) have been identified as molecular targets for medical therapy 2 and both *in vitro* [3-5] and *in vivo* [6-11] investigations have shown the potential of pituitary-targeting drugs that bind these receptors. When used as monotherapy, pasireotide and cabergoline can induce normalization of cortisol concentrations in approximately 20-40% of the patients, thus leaving the majority of patients exposed to the risks of persisting hypercortisolism [12]. Several studies have shown higher remission rates when using combination therapy [8, 11, 13] and moreover, preclinical studies have shown promising results of retinoic acid and epidermal growth factor receptor (EGFR) antagonizing drugs [14-17]. Despite all these efforts, to date there are no long-term efficacy and safety data of any available drug. For this reason, the search for new targets for drug therapy in CD, as well as for the optimal (combination of) medical therapy continues. This thesis focused on three main subjects. First, the biology of CD with respect to the origin of corticotroph pituitary tumors and their somatostatin and dopamine receptor expression was investigated. The adrenal gland is the most important topic in the second part of the thesis. The expression of somatostatin receptors and the D_2R in the adrenal gland is reported and additionally, the effects of the antimycotic agents ketoconazole and fluconazole on adrenal steroidogenesis have been compared. Finally, the effects of medical combination therapy with pasireotide, cabergoline and ketoconazole on complications of CD have been studied and are detailed in part three of this thesis.

2. Different entities of corticotroph pituitary tumors?

CD is caused by an ACTH-producing pituitary tumor and is primarily treated with transsphenoidal adenomectomy. The long-term remission rate after this procedure is approximately 60-70% [18] and is partially dependent on the size of the adenoma [19, 20]. Apart from the adenoma size, the localization of the tumor may also play an important role. Tumors localized in the region close or adjacent to the posterior pituitary lobe have been reported to be removed less successfully by transsphenoidal surgery [21]. Although it is generally accepted that CD originates from corticotroph adenomas in the anterior pituitary lobe, the results that are described in Chapter 2 of this thesis suggest that a subset of patients might develop CD because of a hyperplasia or adenomatous hyperplasia of ACTH-producing cells of the intermediate zone of the pituitary gland. These findings are well in agreement with studies that reported the absence of a well-described adenoma in the anterior pituitary in patients with CD. Histological examination in these cases showed hyperplasia of corticotroph cells in the presence of neural tissue or even no evident microadenoma or corticotroph hyperplasia [21-24]. Again, these tumors were located in the region between the anterior and posterior pituitary lobes [21]. In our study, we showed that compared to corticotroph pituitary tumors that were histologically classified as pure adenoma, tumors identified as adenomatous hyperplasia or hyperplasia of ACTH-producing cells have higher prohormone convertase 2 (PC2) and, although not statistically significant, neurofilament (NF) protein expression. Inversely, tumors with high PC2 protein expression were more often classified as adenomatous hyperplasia or corticotroph hyperplasia, rather than as pure adenoma compared to tumors with low PC2 immunopositivity. These data suggest a possible intermediate lobe origin, because the PC2 enzyme is predominantly expressed in this region of the pituitary gland [25-27]. Moreover, although the NF protein expression was generally very low, the higher NF expression in the group of (adenomatous) hyperplasias suggests close proximity of these lesions to the posterior pituitary lobe.

Whereas the existence of the human intermediate pituitary lobe is disputed, the presence of clusters of cells in the region between the anterior and posterior human adult pituitary lobe that stain positive for β–endorphin

pleads for the existence of PC2-expressing cells in the human intermediate lobe [28]. Moreover, it has been reported that α-melanocyte stimulating hormone (α-MSH) levels in the inferior petrosal sinus were higher than the peripheral serum concentrations in patients with CD, but not in patients with other pituitary diseases [29]. This result points toward the possible secretion of α-MSH and, therefore, the expression of PC2 by ACTH-producing pituitary tumors. However, it was stressed in the discussion of this paper that it cannot be ruled out that increased serum α-MSH levels have resulted from peripheral α-MSH production or ACTH-processing [29]. Very recently, Pax7 was identified as a protein the expression of which is restricted to the melanotroph lineage of the intermediate pituitary lobe and is therefore not expressed in the anterior lobe corticotroph cells [30]. In intermediate lobe cells of rat pituitary glands lacking PAX7, melanotroph-specific genes (e.g. POMC, PC2 and D_2R) were down-regulated and these cells switched towards the corticotroph lineage. Similar observations were made in studies with AtT20 cells [30]. Together, these data show that PAX7 drives POMC-positive pituitary cells towards melanotroph differentiation. To corroborate our observations in Chapter 2 of this thesis, future studies might determine the expression of both PC2 and Pax7 in pituitary tumors from patients with CD.

Somewhat surprisingly, in our study no difference was found in the percentage of patients that reached early postoperative remission between the group of patients with pure corticotroph adenomas and the group of patients with adenomatous hyperplasia or corticotroph hyperplasia. This observation might in part be explained by the availability of better surgical techniques in recent years compared to the time this hypothesis was first postulated [21]. Moreover, there was a relatively high number of macroadenomas among this group, which are known to be more difficult to remove compared to microadenomas.

The PC2 immunopositivity within hyperplasias and adenomatous hyperplasias was very heterogeneous, which is well illustrated by the observation that in the majority of cases, less than 50% of the cells expressed PC2. Additionally, most adenomas also expressed PC2 at the protein level, albeit to a more variable extent. Together, these data again underline the complex etiology of CD.

As is discussed in detail below, the sst_5 and D_2R are often highly expressed in corticotroph pituitary tumors. The expression levels of these receptors did not differ between adenomas on the one hand and (adenomatous) hyperplasias on the other hand. However, the sst_5 expression in the latter group appeared to be lower than in the adenoma group and an inverse correlation was observed between the protein expression levels of the sst_5 and PC2. Thus generally, the sst_5 seems to be expressed to a higher degree in corticotroph pituitary adenomas than it is in adenomatous hyperplasias and corticotroph hyperplasias. Whether this indeed means that treatment with pasireotide would be less efficacious in the latter group is a question that remains to be answered.

The D_2R is highly expressed in the majority of corticotroph tumors [2, 5] and the D_2R agonist cabergoline is efficacious in a considerable percentage of patients with CD [9-11]. Interestingly, although no differences were found in D_2R expression between adenomas and (adenomatous) hyperplasias, the expression of this receptor was higher in tumors with high PC2 immunopositivity compared to tumors with low expression of this enzyme.

3. Molecular targets for the medical treatment of Cushing's disease

Drug therapy for CD can be directed at three possible levels. First and most importantly, the ACTH-secreting pituitary adenoma can be targeted with neuromodulatory drugs. Secondly, medical therapy can be used to directly suppress cortisol production in the adrenocortical zona fasciculata and finally, the effects of excess serum concentrations of cortisol can be counteracted by antagonizing the glucocorticoid receptor at the tissue level.

3.1. Somatostatin receptors and the dopamine subtype 2 receptor

The expression level of the sst_2, which is highly expressed in growth hormone (GH) secreting pituitary adenomas, is generally very low in corticotroph adenomas [2, 4]. As discussed in detail below, this is probably caused by down-regulating effects of high circulating cortisol levels in patients with CD [31, 32] and explains why sst_2-preferring somatostatin analogs are generally not efficacious in the treatment of CD [32, 33].

On the other hand, ACTH-secreting pituitary adenomas express large numbers of sst_5 receptors and therefore, the use of somatostatin analogs that target this receptor would be more rational [2, 4, 34]. After it had been shown that pasireotide, which has a subnanomolar binding affinity for the sst_5, inhibited ACTH secretion in primary cultures of human corticotroph adenomas [3, 4], beneficial effects were also observed in a phase II study with 39 patients with CD [35]. In this study, 5/29 patients that completed the 15-day study period reached normal urinary free cortisol (UFC) excretion levels and the mean decrease in UFC levels was 44.5% after these 15 days. Subsequently, a large, multicenter phase III trial was initiated in which the long-term effects of pasireotide were studied. After 6 months of treatment, 33/162 patients (20.4%) reached normal UFC excretion levels. A major drawback of this therapy was the occurrence of hyperglycemia in a high percentage of patients, especially in those who already had impaired glucose tolerance at baseline. In fact, 73% experienced a hyperglycemia-related adverse event and 6% even discontinued pasireotide treatment because of hyperglycemia [7]. Unfortunately, the effects on serum concentrations of GH and insulin-like growth factor-I (IGF-1) were not reported in this publication. However, considering the convincing effects of pasireotide on secretion of these hormones in both rats [36] and humans [37], it is very likely that IGF-1 levels decreased in a considerable percentage of patients.

The observation that pasireotide treatment induced normalization of UFC excretion levels in only 20% of the patients after 6 months [7] is in part related to the severity of hypercortisolism in this patient group because the mean UFC level at baseline was approximately 6.5 times the upper limit of normal. In addition, variations in the somatostatin receptor subtype pattern among corticotroph adenomas may explain different responses to pasireotide treatment. Although evaluated at the mRNA level, it has been reported that up to 40% of the ACTH-secreting pituitary adenomas does not express the sst_5 [2]. Compared to the sst_5, the sst_2, to which pasireotide also binds with high affinity, is expressed to a much lesser extent and in only 30% of the adenomas [2]. Moreover, in the studies that are presented in this thesis, considerable heterogeneity in sst_5 protein expression was observed within corticotroph pituitary adenomas: some adenomas highly express the receptor in virtually all cells while others only show immunopositivity in a subpopulation of the adenoma or do not express the sst_5 at all. This might explain why some patients do exhibit decreases in UFC excretion levels following treatment with pasireotide but do not fully normalize. In the phase III trial, in which the long-term efficacy of pasireotide was studied, it was found that the percentage of patients that reached normal UFC excretion after 6 months of treatment was highest in the group of patients with mildly elevated baseline UFC excretion levels. Moreover, the effects of pasireotide were already visible within 2-3 months of treatment and patients that had not normalized in this initial phase were unlikely to reach normal cortisoluria after 6 or 12 months of treatment [7]. In responders, pasireotide was able to maintain biochemical control up to 2 years [38], which contrasts to long-term cabergoline therapy during which a subset of patients shows a treatment-escape [10]. An interesting observation that warrants further investigation is the reduction in tumor volume after 12 months in patients treated with high-dose pasireotide (900 µg twice daily) [7].

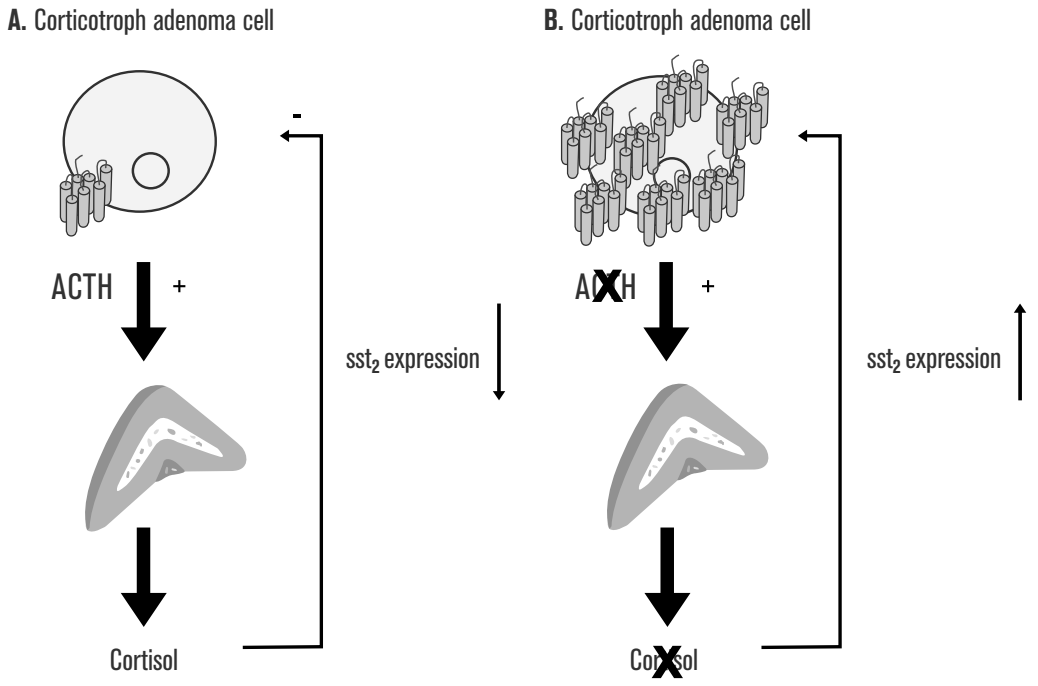
It is well known that the clinical response to octreotide correlates with the expression level of the sst_2 on somatotroph adenoma cells [39]. It would be intriguing to investigate whether or not similar correlations exist between the degree of $sst_{2/5}$ receptor expression and the *in vivo* response to pasireotide in patients with CD. In 2012, pasireotide was approved in the European Union for the treatment of patients with CD only after unsuccessful surgery. This means that, provided that the tumor has at least partially been removed, pituitary adenoma tissue is available from these patients to study the sst_5 protein or mRNA expression levels. Since high-quality sst_5 (and sst_2) antibodies are available [40, 41], it would be recommendable to evaluate the sst expression pattern prior to initiating treatment with somatostatin analogs [39], in particular pasireotide, in order to determine whether there is indeed a correlation between receptor expression and biochemical response.

Although 6 months of pasireotide treatment resulted in biochemical remission in only 20% of the patients, a mean decrease in UFC levels of 48% was observed, also including patients with severe hypercortisolism. This shows that this drug might still be beneficial in a subset of patients with CD. In general, medical therapy for CD

should be given with a tailor-made approach, involving relevant patient characteristics, in which potential clinical efficacy of a drug should be carefully weighed against its potential side effects. In the case of pasireotide, this would mean that this drug is particularly interesting to use in patients with mild hypercortisolism since they are more likely to reach normal cortisol levels than patients with severe hypercortisolism. The latter group of patients might preferably be treated with a combination of drugs (see paragraph 4, 'Effects of medical combination therapy on complications of Cushing's disease), since higher success rates have been obtained by using medical combination therapy than by using single drugs [8, 11, 13]. Furthermore, patients that are already insulin resistant are preferentially treated by other means than pasireotide, since their glucose metabolism could deteriorate further with pasireotide treatment. In addition, it is important that future clinical trials in which the efficacy of pasireotide in patients with CD is examined also address the possible induction of GH- and IGF-1 deficiency.

Due to the low sst_2 expression levels in corticotroph adenomas, presumably caused by down-regulating effects of high circulating cortisol levels, sst_2-preferring somatostatin analogs currently do not have a role in the treatment of CD [1]. An important aim of this thesis was to evaluate the reversibility of sst_2 down-regulation on corticotroph adenoma cells. Although several *in vitro* studies reported that glucocorticoids decrease the sst_2 expression by GH- or ACTH-secreting cell lines, it was not yet known whether these effects could be abrogated in human corticotroph adenomas. The results that we obtained in the study that is described in Chapter 3 show that treatment with cortisol-lowering drugs may counteract the inhibitory effects of cortisol on sst_2 expression (Figure 1).

Figure 1. Dynamic sst_2 expression on corticotroph adenoma cells.



It was shown that at mRNA level, it is possible to reverse the glucocorticoid-induced sst_2 down-regulation. Adenomas from patients with normalized preoperative UFC excretion had a 10-fold higher sst_2 mRNA expression compared to adenomas from patients with elevated preoperative UFC excretion. Unexpectedly, the sst_2 protein expression did not differ between both groups of corticotroph adenomas. Although it could not be proven from our data, this discrepancy might be caused by persistent (cortisol-induced) disturbed translation of sst_2 mRNA. The average period that patients were normocortisolemic prior to surgery was 10 weeks. Although there is no good explanation why sst_2 mRNA translation would not recover after this period, it cannot be entirely excluded that cortisol-lowering therapy ultimately results in higher sst_2 protein expression levels in these corticotroph adenomas. Obviously, the efficacy of sst_2-preferring somatostatin analogs is unlikely to improve without higher expression levels of the receptor.
Importantly, octreotide had less profound ACTH-lowering effects than pasireotide even in primary cultures of adenomas with high sst_2 mRNA expression. An important issue in this study was the fact that half of the patients that had normalized preoperative UFC excretion levels were pre-treated with pasireotide. Although the effects of somatostatin analogs, including pasireotide, on somatostatin receptor internalization have been evaluated in multiple cell systems, these effects on corticotroph tumor cells are unknown. In some studies, it has been shown that octreotide can induce sst_2 receptor internalization, e.g. in human neuroendocrine tumor cells [42] and in human embryonic kidney cells, whereas pasireotide did not [43]. In contrast, treatment with pasireotide was associated with rapid recycling of the sst_2 to the cell membrane, thereby increasing the receptor availability. It has been suggested that activation of the sst_5 attenuates internalization of the sst_2 [44]. Thus, it seems unlikely that the sst_2 expression was down-regulated by pasireotide pre-treatment. In agreement, no differences were found either in receptor mRNA or protein expression levels between adenomas from patients that were treated with pasireotide and adenomas from patients that were treated with other drugs. In fact, the adenomas from patients that were pre-treated with pasireotide had higher sst_2 protein expression, although this difference was not statistically significant. Moreover, we found similar results in our experiments with AtT20 cells. Taken together, these results plead for the hypothesis that the observed up-regulation of sst_2 mRNA expression should be attributed to the normalization of UFC excretion rather than to a possible drug-mediated effect. To really clarify this issue, larger groups of adenomas of patients that are pre-treated with pasireotide should be compared to adenomas of patients in whom preoperative biochemical remission is induced by other drugs. Alternatively, especially considering the rarity of the disease, the effects of pasireotide on sst expression could be investigated in primary cultures of human corticotroph adenoma cells.

The sst_2 up-regulation in corticotroph adenomas that was observed after normalization of hypercortisolism is in line with a recent observation by our group that 2 ACTH-producing neuroendocrine tumors that were not detected with somatostatin receptor scintigraphy became apparent on the scintigram after treatment with the glucocorticoid receptor antagonist mifepristone [45]. Together, these results show that the glucocorticoid-mediated sst_2 down-regulation might be a dynamic process that is abrogated by cortisol-lowering or GR-antagonizing therapy. Whether higher sst_2 expression levels would also improve the ACTH-lowering effect of sst_2-preferring somatostatin analogs or pasireotide remains to be elucidated. However, in this respect, octreotide and native somatostatin have been shown to have ACTH-lowering ability in patients with Nelson's syndrome and in patients with primary adrenal insufficiency [33, 46, 47].
The study that is described in Chapter 9 was conducted to investigate whether octreotide has ACTH-lowering potential in patients with CD who have normalized UFC excretion levels. Despite normalization of UFC excretion after 1 month of ketoconazole pre-treatment in 3/4 patients and sustained control of hypercortisolism during ketoconazole/octreotide combination therapy in 2 of these 3 patients, subsequent monotherapy with octreotide proved to be inefficacious. These preliminary results obviously raise the question whether normalization of UFC excretion indeed results in increased sst_2 protein (in addition to mRNA) expression levels on the cell membrane of these patients' corticotroph tumor cells. Since no adenoma tissue was available to study receptor expression levels after cortisol normalization, we can only speculate about the cause of the lack of efficacy of this combination treatment. If an increase in sst_2 protein expression occurred, this would imply that the receptor was not functional because octreotide treatment did not maintain control of cortisol excess. As

described above, preoperative normalization of UFC excretion levels induced an up-regulation of sst_2 mRNA, but not protein expression levels after an average period of 10 weeks of normocortisolism. Accordingly, no functional effects of sst_2 activation with octreotide were observed in primary cultures of adenoma cells with increased sst_2 mRNA expression. For this reason, we hypothesize that the duration of biochemical control under ketoconazole/octreotide combination therapy may have been insufficient to realize an increase in functional sst_2 protein expression on the corticotroph cell surface. Alternatively, especially since we observed a direct inverse correlation between UFC excretion and sst_2 mRNA expression levels (Chapter 3), the cortisol levels in these 3 patients might not have been decreased far enough. This would suggest that the GR on the adenoma cells was still exposed to sufficiently high levels of circulating cortisol to suppress the transcription/translation of the sst_2 gene. Another approach to test the efficacy of octreotide after sst_2 up-regulation might be to pre-treat patients with mifepristone, especially taking the results from the study by De Bruin et al. into consideration [45]. This approach would ensure that the GR on corticotroph adenoma cells is not exposed to cortisol and, consequently, would not mediate sst_2 down-regulation [45]. This would, however, be a challenging study to carry out because of the difficulties in titrating the mifepristone dosages and determining whether the GR is sufficiently antagonized. Still, it would be an interesting concept to study the ACTH-lowering potential of octreotide following sst_2 up-regulation on corticotroph tumor cells.

Another aim of this thesis was to study the expression of somatostatin receptors and the dopamine 2 receptor at the level of the adrenal cortex. The sst and D_2R expression on corticotroph pituitary adenoma cells, as well as the effects of somatostatin analogs and dopamine agonists on both *in vitro* and *in vivo* ACTH secretion have been well investigated. In contrast, the expression levels of these receptors in the adrenal gland had not been extensively studied before we started the experiments that are described in Chapter 4. It was found that the expression levels of the sst_2, sst_5 and D_2R were very low in the zona fasciculata of both normal human adrenal cortices and human ACTH-dependent adrenocortical hyperplasias. This is well in agreement with the observation that neither pasireotide, nor octreotide or cabergoline inhibited the *in vitro* cortisol release in primary cultures of these human adrenal glands. It was therefore concluded that the beneficial effects of pasireotide and cabergoline in patients with CD should be attributed to their ACTH-lowering potential.
Previous studies that evaluated the somatostatin receptor expression in the adrenal gland were not without limitations, as was discussed in Chapter 4. The strength of the study that we conducted is the fact that for the first time, monoclonal antibodies were used in paraffin-sections of normal human adrenal glands to evaluate protein receptor expression levels of sst_2, sst_5 and D_2R.
The lack of effect of either agonist on *in vitro* cortisol secretion by human adrenocortical cells is in accordance to what was observed in rat studies [48, 49]. In contrast, a recently published study described that in primary cultures of human normal adrenal tissue, 24h of incubation with pasireotide (at 10 nmol/L) induced a slight increase in cortisol secretion [50]. Together, the studies described here do not show a direct cortisol-lowering effect of cabergoline and pasireotide. Again, this means that these drugs can have beneficial effects in patients with CD because of their effects on ACTH-secretion and not likely because of direct adrenal-mediated actions.
The zona reticularis cells appeared to have abundant expression of both the sst_5 and the D_2R. The D_2R agonists bromocriptine and cabergoline do not decrease the ACTH-stimulated androstenedione or cortisol secretion in primary cultures of ACTH-dependent adrenocortical hyperplasias [51]. However, *in vivo*, serum levels of DHEAS increased by 25% after dopamine withdrawal in critically ill patients, which was accompanied by a concomitant increase of prolactin serum levels. Concentrations of cortisol were unchanged after dopamine withdrawal [52], again suggesting that cortisol secretion is not under dopaminergic control. The effects of somatostatin analogs and cabergoline on the *in vitro* secretion of adrenal androgens were not investigated in our study and should be further explored.
In our series of adrenal glands, the ZG showed focal positivity for sst_2, which seemed to be mainly restricted to endothelial cells. The role of somatostatin and dopamine on the renin-angiotensin-aldosterone system (RAAS) has extensively been studied. In six normal subjects, somatostatin infusions inhibited increases in serum concentrations of aldosterone induced by exogenous angiotensin II and orthostasis [53]. In Wistar rats, treatment with octreotide or native somatostatin resulted in significant decreases in both cellular volume of ZG cells and

serum concentrations of aldosterone [48, 54].
The D_2R was not expressed in the ZG of most adrenal glands that we examined. Using a polyclonal antibody, strong D_2R immunopositivity in the ZG and ZR of both three normal adrenal glands and five ACTH-dependent adrenal hyperplasias was found in a previous study [51]. Other studies showed the presence of dopamine-binding sites in the human ZG. Dopamine and bromocriptine, as well as domperidone and metoclopramide were able to displace [3H]spiperone from membranes of human adrenocortical cells [55]. The D_2R appears to play an important role in the regulation of aldosterone secretion by ZG-cells. Native dopamine and the D_2R-preferring dopamine agonist bromocriptine did not affect basal aldosterone concentrations in healthy men [56]. In contrast, the D_2R antagonist metoclopramide has been repeatedly shown to induce an increase in plasma aldosterone, but not cortisol concentrations [56-58]. Concluding, these results suggest that aldosterone secretion is under tonic dopaminergic control [51, 56-58].

3.2. Adrenal cortex-directed drug therapy with ketoconazole and fluconazole

In Chapter 5 of this thesis, it is described how the effects of fluconazole on *in vitro* steroidogenesis compare to those of ketoconazole. Fluconazole strongly and dose-dependently decreased cortisol secretion in cultured HAC-15 cells and in primary cultures of human adrenal glands. In the primary cultures, evidence was found that fluconazole, similar to ketoconazole, inhibits the activity of both the 17-hydroxylase and 11β-hydroxylase enzymes. Despite the fact that ketoconazole and fluconazole are antifungal agents from different classes, they appear to have similar properties regarding their adrenal-blocking action. In order to determine to what extent these agents exactly inhibit each steroidogenic enzyme, future studies could be performed in which cell lines transfected with the respective enzymes are treated with ketoconazole and fluconazole. This would provide a more pure situation, since the effects on only one enzyme at a time could be assessed, without the obscuring effects of steroids being further converted. Obviously, the drawback of such studies would be that they do not resemble the *in vivo* situation.
Importantly, whereas the EC_{50} for fluconazole-mediated inhibition of cortisol secretion by primary cultures of normal adrenal glands was approximately 80-fold higher compared to the EC_{50} of ketoconazole, the fluconazole concentrations that were used are in the range of previously reported fluconazole serum concentrations [59-61]. This suggests that this drug, which is much less toxic than ketoconazole [62, 63,] can be used to control cortisol hypersecretion in patients with Cushing's syndrome (CS), which indeed has already been hypothesized by several authors that treated critically ill patients with fluconazole [64-67]. Therefore, in particular in patients on intensive care units, in whom optimal adrenal function is of vital importance, treatment with fluconazole can induce adrenal insufficiency [64-66]. Clinicians who treat these patients with fluconazole should be aware of this side effect. The effects of other imidazole or triazole derivatives in this context have not been extensively investigated yet, but regarding the effects of fluconazole, it is not unlikely that other antifungal agents might also affect adrenocortical steroidogenesis [68].
Taken together, it is concluded that fluconazole, which is less toxic but also less potent, might become an alternative to ketoconazole to reduce cortisol levels in patients with CS. Further investigation is required to determine the clinical efficacy and optimal dosage of fluconazole treatment in patients with CS.

4. Effects of medical combination therapy on complications of Cushing's disease

The clinical efficacy of pasireotide- or cabergoline monotherapy in the treatment of CD has extensively been described in the introduction of this thesis. When used as monotherapy, pasireotide and cabergoline can induce normalization of cortisol concentrations in approximately 20-40% of the patients [69], thus leaving the majority of patients exposed to the risks of persisting hypercortisolism [12]. Several studies have shown higher remission rates when using combination therapy [8, 11, 13].
Due to the co-expression of sst_5 and D_2R that frequently occurs in corticotroph pituitary adenomas, there seems to be a rationale to treat patients with CD with a combination of drugs that target these receptors [2, 8]. In particular, although speculative, targeting both receptors might lead to synergistic effects, as somatostatin and

dopamine receptors have been shown to be able to heterodimerize [8, 70]. In this respect, the possible role of sst5/D2R-preferring chimeric compounds would be of interest. Although to data no studies have been published that evaluated the effects of such compounds on either *in vitro* or *in vivo* ACTH secretion, the additive effects of cabergoline and pasireotide compared to pasireotide alone in a subset of patients with CD [8] raise the question as to whether or not chimeric compounds may indeed have synergistic effects [71].
An important study that was carried out by our group showed that 80 days of stepwise combination therapy with pasireotide, cabergoline and ketoconazole induced normalization of UFC excretion levels in 15/17 patients [8]. Of these 15 patients, 5 already normalized within one month of pasireotide monotherapy, an additional 4 patients reached biochemical remission after the addition of cabergoline and the remaining 6 patients needed triple therapy in order to reach normal UFC excretion levels. Six patients participated in an extension study and remained biochemically controlled with pasireotide (n=2), pasireotide and cabergoline (n=3) or triple therapy (n=1) [72]. Simultaneously with the UFC excretion levels, other clinical parameters were monitored throughout the study period. The effects of medical combination therapy with these drugs on the coagulation cascade, quality of life and blood pressure-related parameters are described in Chapters 6-8 of this thesis, respectively. As a general remark, when analyzing the effects of medical treatment on these parameters, it can be very difficult or even impossible to distinguish whether certain effects result from decreased cortisol levels or are a consequence or adverse effect of any of the drugs that were used. For instance, it is described in Chapter 7 that despite biochemical remission, quality of life did not improve after 80 days. This might well be explained by persisting effects of long-term exposure to supraphysiological concentrations of cortisol [73, 74], but could also be due to the fact that pasireotide for instance had to be autoinjected three times per day and is often accompanied by gastrointestinal discomfort and hyperglycemia [7, 35]. These adverse effects might also have prevented any improvement in the quality of life of these patients.

In Chapter 6, it was described that patients with CD are in a hypercoagulable state with higher levels of Factor VIII activity and fibrinogen and a trend towards increased Von Willebrand Factor antigen levels compared to healthy controls. Activation of the coagulation pathway was functionally reflected by a shortened activated partial thromboplastin time (aPTT). These results are well in agreement with previous studies that showed increased plasma concentrations of components of the coagulation cascade in CS [75-79]. Increased production of clotting factors in patients with CD may at least partially be mediated by direct effects of glucocorticoid excess, as dexamethasone has been shown to increase plasma concentrations of Factor VIII, Factor IX and fibrinogen in healthy volunteers [80]. It was also reported in Chapter 6 that an impaired fibrinolytic capacity also contributes to the hypercoagulable state in CD, as reflected by a prolonged plasma clot lysis time (CLT) in untreated patients compared to healthy controls. Several factors were examined that are known to inhibit fibrinolysis, including plasminogen activator inhibitor type 1 (PAI-1), thrombin-activatable fibrinolysis inhibitor (TAFI) and α2-antiplasmin. Compared to controls, our patients had higher levels of these parameters, which may explain the hypofibrinolysis in CD. *In vitro*, glucocorticoids have previously been shown to increase PAI-1 and TAFI mRNA expression by human hepatoma cells and glucocorticoid-responsive elements have been identified in the promoter of both these genes [81]. Glucocorticoids also stimulate PAI-1 production by human visceral adipose tissue [81]. Associations have been found between levels of CLT, PAI-1 and TAFI and the risk of cardiovascular disease [82, 83] and therefore, hypofibrinolysis may contribute to the increased cardiovascular risk in patients with CD [84, 85]. Although the mean BMI differed between patients and controls and abdominal obesity has been associated with increased levels of prothrombotic proteins [86, 87], aPTT and CLT differed between patients and controls even after adjustment for BMI. This suggests that the increased BMI in CD patients is not the only explanation for their hypercoagulable state. Accordingly, a relative risk of 2.5 for venous thromboembolism (VTE) has been reported among the obese population [88], while compared to the general population, patients with CS may have a more than tenfold increased risk for VTE [89]. It seems, therefore, that the incidence of VTE among obese subjects without hypercortisolism is not as high as in patients with CS.
Our study was the first to investigate the effects of medical combination therapy on the coagulation and fibrinolysis pathways. In contrast to previous studies that evaluated the effects of successful surgery for CD on these parameters [90, 91], the abnormalities in plasma concentrations of proteins involved in both pathways

remained present. I.e., no significant changes in aPTT were found and hypofibrinolysis persisted as reflected by prolonged CLT and elevated levels of PAI-1 and α2-antiplasmin throughout the study period. A possible explanation for the lack of improvement might be the relatively short follow-up period of 80 days. Amelioration in the hemostatic profile was observed in the aforementioned studies after 1 year following surgical cure [90, 91]. This suggests that a minimal period of sustained biochemical remission is required to reverse the hypercoagulable state in patients with CD. However, even after 1 year post-surgery, the abnormalities had not completely disappeared [91], which suggests that the increased risk for thrombosis in patients with CD might in part be due to the persistence of abdominal obesity rather than to the presence of hypercortisolism. Therefore, it would be interesting to assess the long-term effects of medical therapy on coagulation and fibrinolysis.

Currently, there are no guidelines on type, dosage and duration of thromboprophylaxis for patients with CD before and after (surgical) treatment [1]. It was previously reported that the incidence of VTE among patients with CS decreased significantly in those who received thromboprophylaxis [75]. In the group of patients that did not receive thromboprophylaxis, 15/75 patients (20%) developed pulmonary embolism or deep vein thrombosis during the course of their disease, as opposed to 14/232 patients (6%) that were treated with unfractionated heparin (15.000-22.500 units daily for at least 2 weeks) postoperatively [75].

The reported postoperative period in which VTE occurs in patients with CS varies among different studies. However, all the cases of postoperative VTE are discovered within 3 months following surgery [20, 75, 92-94], as has also been reported for other types of high risk surgery [95]. We have been able to find 20 cases of postoperative VTE in which the exact interval between surgery and VTE has been described [20, 93, 94, 96]. Of these twenty patients, 16 developed VTE within 4 weeks following surgery, 8 of which already presented with VTE in the first two weeks postoperatively. There is also an increased incidence of VTE in active CD before surgery and therefore, it should be considered to administer thromboprophylaxis to patients with CD from the time of diagnosis, while awaiting surgery, until at least 4 weeks postoperatively. Similarly, thromboprophylaxis is recommended to be continued until maximally 4 weeks after discharge in patients that have undergone major surgery for cancer [95].

So far, no prospective placebo-controlled trials have been reported that evaluated the effects of thromboprophylaxis in patients with CD. We would recommend a randomized controlled trial in which patients with CD are randomized between low-molecular weight heparin (LMWH) in a high prophylactic dose (i.e. nadroparin 5700 U or enoxaparin 40 mg once daily), starting 24h after surgery, for the duration of immobilization or duration of hospital stay, as is the current standard of care in many centers, and prolonged prophylaxis with LMWH for a period of 4 weeks postoperatively. The percentage of patients with VTE and bleeding complications should be compared between the two groups. Importantly, since the prevalence of CD is very low, this study should be carried out in a multi-center setting. Until a prospective study has yielded an optimal regimen for thromboprophylaxis, we would suggest giving LMWH in a high prophylactic dose (nadroparin 5700 U, dalteparin 5000 U or enoxaparin 40 mg sc once daily). Because some patients may have an increased risk of thrombosis until 3 months after surgery, prophylaxis should be tailored for the individual patients not only considering the additional risk factors of thrombosis (age, obesity etc.), but also the risk of bleeding. Thus, the benefit of prophylaxis should be carefully weighed against the risk of bleeding. Importantly, if LMWH is started preoperatively, it should be temporarily stopped at the time of surgery because of peroperative bleeding risk.

The effects of medical combination therapy with pasireotide, cabergoline and ketoconazole on quality of life and cortisol diurnal rhythm are described in Chapter 7 of this thesis. First, our data confirmed previous observations that patients with CS have a severely impaired quality of life, the cause of which is multifactorial and includes deterioration of physical, emotional, mental and social functioning [73, 74, 97-99]. Based on 5 different quality of life questionnaires, our patients' quality of life had not improved after 80 days of successful combination therapy but, despite the occurrence of several adverse effects, had not worsened either. Pasireotide, for instance, often causes gastrointestinal discomfort and hyperglycemia [7, 8]. In addition, 9 patients that had normal serum concentrations of IGF-1 before the start of the study period had become IGF-1 deficient after 80 days of treatment. These factors might at least partially have hindered any improvement in quality of life. Steroid withdrawal induced by successful drug therapy may have been another possible explanation for the fact that

we did not observe any amelioration, since patients with adrenal insufficiency report impaired QoL [100]. This hypothesis is supported by our findings that quality of life tended to improve after the initial steroid withdrawal phase in patients with sustained biochemical remission. Moreover, these data are in accordance with previously reported results that the quality of life of patients with CS ultimately improves after successful surgery [73, 101-105], but also after prolonged treatment with pasireotide [7]. Importantly, in our study as well as in other studies [73, 98, 99], it was shown that despite the fact that quality of life can improve to a certain degree after successful treatment, the impairment of quality of life in these patients is partially irreversible. Therefore, it is very important to rapidly and aggressively treat patients with CD, in order to reverse the detrimental effects of glucocorticoid excess as soon as possible.
Remarkably, 6/12 patients that had a disturbed circadian rhythm of cortisol secretion at baseline recovered their normal diurnal pattern within 80 days of treatment. This suggests that normalization of cortisol production by medical therapy allows for recovery of hypothalamic control of normal corticotroph cell function in patients with CD. Conversely, the fact that the circadian cortisol rhythm did not recover in the other 6 patients suggests that recovery of the cortisol diurnal rhythm requires prolonged remission in a subset of patients. Of note, it is not known whether any of the drugs that were used, in particular the centrally acting agents pasireotide and cabergoline, might influence the cortisol day-night profile. Our study was the first to evaluate the effects of medical therapy on cortisol diurnal rhythm, but in a cross-sectional study, a normal circadian rhythm was observed in patients that had been surgically cured from CS for a mean of 8.2 years [106]. Finally, whereas the physiological circadian rhythm of cortisol levels is usually disturbed, our data again show that it can be preserved in a subset of patients with CD [106-108]. This suggests that a normal pattern of diurnal cortisol variation does not necessarily rule out the diagnosis of CD.

Whereas hypertension is an important hallmark of CD, the pathogenesis of glucocorticoid-related hypertension is not yet fully understood [109]. In Chapter 8, it is described that untreated, hypertensive patients with CD have low/suppressed plasma concentrations of renin and aldosterone, respectively, which are accompanied by elevated levels of angiotensinogen. This observation suggests that hypertension in CD is not renin-mediated, but does not rule out increased mineralocorticoid activity due to elevated cortisol concentrations that exceed the capacity of 11β-hydroxysteroiddehydrogenase type II. Thus, cortisol-mediated MR stimulation may have suppressed both renin and aldosterone production. However, several studies reported the inability of the MR antagonist spironolactone to prevent GC-induced hypertension [110, 111], in part pleading against mineralocorticoid hypertension in these patients. A very remarkable finding was that renin concentrations were low even in patients who were using Ang II type 1 receptor antagonists, ACE-inhibitors or MR-antagonists, the use of which has been shown to result in an increase of renin [112]. Whereas renin concentrations are generally suppressed by the use of β-adrenoceptor blockers [113], no differences were observed between patients that used these drugs and those who did not. This denies that renin concentrations might have been suppressed because of drug-induced effects. The discrepancy between previous studies that reported normal/elevated renin levels in patients with CS [114-117] and our results might be explained by differences in conditions (e.g. supine vs. upright position). Importantly, using renin activity assays in the presence of elevated angiotensinogen levels may result in overestimation of renin levels, which may be an alternative explanation for elevated renin in CS.
Concentrations of NT-proBNP were measured after renin levels had found to be low, to determine whether CD is accompanied by volume overload. Since NT-proBNP was in the low-normal range, we conclude that there is no activation of BNP in patients with CD.
The elevated angiotensinogen levels might represent another explanation for the low renin concentrations. Decreased renin levels could compensate for the elevated angiotensinogen levels to prevent a further rise in blood pressure [118]. The increased angiotensinogen concentrations might be caused by excessive glucocorticoid production, which apparently is more important than the angiotensinogen-lowering effects of RAAS-blockade [119]. It has previously been demonstrated that treatment with dexamethasone results in a rapid increase of hepatic angiotensinogen mRNA expression and, consequently, angiotensinogen levels in rats [120].
Parallel with UFC excretion levels, a significant decrease in blood pressure occurred in our patients during medical combination therapy [8]. Unexpectedly, angiotensinogen remained elevated, while renin and aldosterone

remained low/suppressed in most patients, despite biochemical remission and normalization of blood pressure. Hypertension has been reported to persist in an important percentage of patients after surgical cure [121]. This suggests that hypertension might partially be irreversible in patients with CD.
The persistently elevated angiotensinogen levels may be caused by a persisting stimulatory GC effect on angiotensinogen production [120]. However, we would have expected angiotensinogen production to decrease following normalization of UFC. Apart from the elevated angiotensinogen concentrations, the lack of increase in renin and aldosterone plasma concentrations might be caused by direct inhibitory effects of pasireotide and/or cabergoline. Endogenous somatostatin and octreotide decrease the volume of the adrenal zona glomerulosa and serum concentrations of aldosterone in rats, whereas the zona fasciculata and zona reticularis remain unaffected in the same studies [54, 122, 123]. The D_2R antagonist metoclopramide increased aldosterone levels and the Ang II-stimulated increase in aldosterone concentration in both rats and humans on a high sodium diet. These effects were counteracted by dopamine and were not observed in subjects on a normal sodium diet, suggesting that aldosterone secretion is under tonic dopaminergic control [124].
Whereas the lack of change in plasma concentrations of angiotensinogen, renin and aldosterone after 80 days could be hypothesized to be due to either sustained effects of cortisol or to be drug-mediated, we would have expected these plasma concentrations to move towards the normal range after prolonged biochemical remission. However, renin and aldosterone remained suppressed even after 4 years of biochemical remission, which could be due to the fact that the blood pressure of these patients was less strictly regulated at the follow-up visit compared to the initial study period. In contrast to renin and aldosterone, angiotensinogen appeared to have decreased at follow-up, although not statistically significant. Together, these data confirm that complications of CD are partially irreversible despite biochemical cure [121].
Although the decrease in blood pressure following biochemical remission might well be explained by the normalization of cortisol concentrations, it was also investigated whether pasireotide and cabergoline could also have had a direct blood pressure-lowering effect. Human arterial endothelial cells express sst_2, sst_4 and especially sst_1 [125]. Wistar rats have been reported to express all ssts, both at mRNA and protein level in their aortic wall [126]. In the study that is described in Chapter 8, pasireotide and cabergoline did not affect the Ang II-mediated vasoconstriction in iliac arteries of the spontaneous hypertensive rat in the absence or presence of hydrocortisone. However, pasireotide, octreotide and, to a lesser extent cabergoline induced an inhibition of Ang II-stimulated constriction in the presence of dexamethasone. Therefore, it cannot be ruled out that pasireotide and, in patients that were treated with both drugs, cabergoline might have directly contributed to the decrease in blood pressure.

Taken together, it seems that the complications of CD that were investigated do not normalize parallel to UFC excretion. Whereas cortisol levels normalized in 15/17 patients within 80 days of medical combination therapy, their hypercoagulable state persisted and their quality of life remained impaired. Moreover, while blood pressure normalized with decreased UFC excretion levels, plasma concentrations of renin, angiotensinogen and aldosterone remained suppressed or elevated.
As has been extensively discussed above, the lack of change in these parameters might be explained by the relatively short period of biochemical remission. In six patients, UFC excretion only normalized when ketoconazole was added to pasireotide and cabergoline at day 60. Thus, these patients had not been normocortisolemic for more than 3 weeks at the end of the study period, which apparently is too short to reverse the detrimental effects of chronic glucocorticoid excess. Indeed, other studies showed that quality of life and plasma concentrations of coagulation proteins improved in patients with CD after sustained biochemical remission. However, in contrast to our data, these studies evaluated the effects of successful surgery on these parameters. This is an important difference because, as was discussed earlier, the drugs that were used in our study might have interfered with some of the outcome measures. In conclusion, medical combination therapy with pasireotide, cabergoline and ketoconazole is very efficacious with respect to normalization of UFC excretion levels in patients with CD. Short-term remission, however, is insufficient to reverse the severe complications that have developed during years of hypercortisolism. Finally, it is difficult to distinguish the (lack of) effects of cortisol normalization from possible adverse effects of drug therapy.

5. *Future perspectives*

The results that are described in this thesis have led to a better understanding of the pathophysiology of corticotroph pituitary tumors and their medical treatment. They also raise new questions that remain to be answered.

The single most important issue to be resolved in order to develop an optimal drug for the treatment of CD is the etiology of the disease. Despite many efforts, it remains unclear why ACTH-producing pituitary tumors develop. Unraveling the pathogenesis of CD will probably lead to the identification of molecular targets for better drug therapy.

With respect to the pathogenesis, it was shown in Chapter 2 that CD might also be caused by adenomatous hyperplasia or hyperplasia of ACTH-secreting cells of the intermediate pituitary lobe. As mentioned above, recent work showed that PAX7 is a selector gene that drives POMC-positive pituitary cells towards melanotroph differentiation [30]. Therefore, to corroborate the results that are presented in this thesis, future studies could aim to determine the expression of both PC2 and PAX7 in pituitary tumors from patients with CD, with a concomitant determination of sst and D_2R expression. Such studies could contribute to a further understanding of the development of ACTH-secreting pituitary tumors and possibly to better individualized treatment approaches.

It has been extensively discussed in this chapter that monotherapy with pasireotide or cabergoline has led to control of hypercortisolism in approximately 20-40% of the patients [7, 9-11]. Leaving adverse effects out of consideration, these percentages indicate that these compounds, at least when given alone, are not ideal candidates for drug therapy in CD, although alternative pituitary-targeting treatment approaches are not yet available. A much higher success rate has been obtained when combining both drugs, either or not together with ketoconazole [8]. Remarkably, successful treatment with this combination of drugs did not result in recovery of the hemostatic profile and RAAS of these patients after 11 weeks. Future studies should learn which determinants prevent these complications of CD to resolve with biochemical remission. Furthermore, it would be interesting to find out whether complications of CD ultimately improve or even disappear with sustained normocortisolism induced by medical (combination) therapy.

The effects of combined treatment with pasireotide and cabergoline have only been studied in a small number of patients in our trial. The efficacy of this approach needs further investigation in future clinical trials, not only regarding biochemical cure of CD but also taking complications of the disease into account. Moreover, chimeric compounds may become available that target both the sst_5 and D_2R. It would be interesting to see whether such compounds can effectively control hypercortisolism in patients with CD and if so, whether they are more efficacious compared to pasireotide and cabergoline combined, due to a possible synergistic effect.

Importantly, even though pasireotide was used in a much lower dosage in this study, antidiabetic agents were necessary in a considerable number of patients. Obviously, adding second or third drugs to treat adverse effects of the first one is not a desirable situation. Still, the efficacy of a long-acting form of pasireotide is being investigated in an ongoing clinical trial. Taken together, combination therapy with these agents is a reasonably good option for patients with unsuccessfully operated CD or patients that are ineligible to surgery. However, intriguing observations have been made recently by other groups, having identified the epidermal growth factor receptor (EGFR) as a promising novel target for medical treatment of CD [14] and retinoic acid as a substance that potentially possesses ACTH-lowering ability [16]. In fact, retinoic acid has already been used clinically in a limited number of patients with CD with promising results [17]. These data warrant further investigation to evaluate the clinical efficacy of retinoic acid and EGFR-directed medical therapy in CD.

Another novel approach with drug therapy for CD was described in Chapter 9. Octreotide is generally ineffective with respect to control of hypercortisolism in CD [33], which is probably due to down-regulating effects of glucocorticoids on the sst_2 expression [31, 32]. Although we showed that this down-regulation likely is a dynamic process that can be abrogated by cortisol- or ACTH-lowering therapy, successive treatment with ketoconazole and octreotide did not prove to be successful in a limited number of patients that were described in the previous chapter. A possible explanation for the observation that patients escaped under octreotide monotherapy might be that the period of normocortisolism that was induced by ketoconazole monotherapy and maintained under

ketoconazole/octreotide combination therapy was too short to induce re-expression of functional sst_2 on the adenoma cells. Alternatively, UFC excretion levels may not have been decreased far enough, still allowing for a certain degree of sst_2 down-regulation. In this respect, it should be investigated why an increased sst_2 mRNA expression after normalization of cortisol levels did not result in increased protein expression levels. Are there indeed translational issues with the sst_2 gene that remain to be discovered?
As stated above, an alternative approach would be to evaluate the effects of octreotide after having blocked the effects of cortisol at the level of the pituitary adenoma using mifepristone.

With respect to the adrenal cortex, future studies should clarify the functional role of the sst_5 and D_2R in the zona reticularis. This could be done *in vitro* by incubating primary cultures of human adrenal glands with compounds that target these receptors. Alternatively, serum concentrations of androgens could be measured in patients that are treated with pasireotide or cabergoline, e.g. because of acromegaly (pasireotide, cabergoline), Parkinson's disease (cabergoline) or prolactinomas (cabergoline). Patients that are treated with the D_2R antagonist metoclopramide represent another potentially interesting group to evaluate the role of the D_2R in the zona reticularis. It has previously described that metoclopramide, but not native dopamine and bromocriptine influenced basal aldosterone levels in healthy volunteers. This may suggest that not only agonists, but also antagonists are useful to study functional receptor activity in the adrenal cortex.

To conclude, this thesis aimed to provide further insight in corticotroph pituitary tumor biology and medical treatment options for CD. Although progress has been made in the understanding of somatostatin receptor physiology in these tumors, some issues that were described above remain unsolved and warrant further study. Furthermore, the effects of medical combination therapy with pasireotide, cabergoline and ketoconazole on complications of Cushing's disease were studied. Despite the rapid occurrence of biochemical remission with this regimen, the complications of the disease generally remained present. Whether these parameters ultimately improve or even normalize after prolonged successful drug therapy is yet to be investigated.

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CHAPTER 1

SUMMARY/ SAMENVATTING

SUMMARY

With an incidence of 1-2 cases per million per year, Cushing's disease is a rare endocrine disorder. It is caused by an adrenocorticotropin-producing pituitary tumor that chronically stimulates the adrenal cortex to produce excess amounts of cortisol, leading to a plethora of signs and symptoms. These include muscle weakness, central obesity, abdominal striae, hypertension, impaired glucose tolerance, venous thromboembolism, osteoporosis and psychiatric disorders. When left untreated, patients with Cushing's disease have an impaired life expectancy, which is predominantly caused by an increased risk for cardiovascular disease. Transsphenoidal surgery is the primary choice of treatment and leads to a long-term remission rate of approximately 60-70%. This indicates that in the 30-40% of patients with residual/recurrent disease, alternative treatment options are required. Pituitary irradiation is often the second line treatment modality, but has major drawbacks. First, it can take years before the adrenocorticotropin (ACTH) hypersecretion starts to decrease and moreover, radiotherapy often renders patients dependent on life-long hormone substitution therapy because of detrimental effects on the production of (other) pituitary hormones. To develop useful compounds for drug therapy of Cushing's disease, it is crucial to gain insight in the pathogenesis of the disease and to identify molecular targets for possible new drugs. In recent years, it has become clear that the somatostatin receptor subtype 5 (sst_5) and dopamine receptor subtype 2 (D_2R) are often highly expressed by corticotroph pituitary tumor cells. More importantly, both preclinical and clinical studies have revealed beneficial effects of drugs that target these receptors. One of the most important clinical trials in this respect was performed in our center. Seventeen patients with Cushing's disease were included in this study and treated with the somatostatin analog pasireotide, which has a high binding affinity for the sst_5. In case of persistent hypercortisolism after 4 weeks of treatment, cabergoline (a D_2R agonist) was added to pasireotide. After an additional 4 weeks of treatment, ketoconazole (an imidazole antifungal agent that directly suppresses adrenocortical cortisol production) was added to these drugs in patients in whom cortisol excretion was still elevated. Ultimately, 15/17 patients had become normocortisolemic after 11 weeks of combination therapy.
In this thesis, it was aimed to gain further insight in the etiology of Cushing's disease and the biology of the expression of the aforementioned receptors in the pituitary tumor as well as in the adrenal gland. Chapters 2-5 focus on these subjects. Additionally, the effects of medical combination therapy on several complications of the disease were investigated. The results of these studies are described in Chapters 6-8.

Pro-opiomelanocortin is the precursor molecule of ACTH and is cleaved by so-called prohormone convertases (PC). PC1 is expressed in both the anterior and intermediate pituitary lobes (the latter of which is believed to regress soon after birth and to be absent in adults) and cleaves pro-opiomelanocortin into ACTH and β-lipotrophin. PC2, which is predominantly expressed in the intermediate lobe, further processes these products. It is generally accepted that Cushing's disease originates from ACTH-producing adenomas in the anterior pituitary lobe. However, it has been described that after successful surgery in some patients, pituitary lesions were histologically classified as hyperplasia or adenomatous hyperplasia of ACTH-producing cells in the region between the anterior and posterior pituitary lobes. In Chapter 2, it is described that indeed, the subset of tumors that are identified as (adenomatous) hyperplasia contains less macroadenomas and expresses higher levels of PC2 and neurofilament protein as compared to typical anterior lobe-adenomas. These results suggest that Cushing's disease might also originate from the intermediate pituitary lobe.

Corticotroph pituitary tumors predominantly express the sst_5 and D_2R. Clinical studies with cabergoline and pasireotide have demonstrated that when used as monotherapy, these drugs can induce sustained normocortisolism in approximately 20-40% of the patients. In contrast to the sst_5 and D_2R, the sst_2 is expressed to a much lesser extent by corticotroph tumor cells. This most likely results from down-regulating effects of high circulating concentrations of glucocorticoids in patients with Cushing's disease. This could explain previous observations that octreotide, which has a high binding affinity for the sst_2, is generally ineffective. It was therefore hypothesized that ACTH- and/or cortisol-lowering therapy would result in increased sst_2 expression

levels. This in turn could have consequences for the clinical efficacy of somatostatin analogs that preferentially bind this receptor. In the study that is described in Chapter 3, evidence was found that ACTH-producing pituitary adenomas from patients with normalized preoperative cortisol levels induced by drug therapy have higher sst_2 mRNA expression levels than adenomas from patients who, despite medical pre-treatment, had elevated preoperative urinary free cortisol excretion. This shows that the glucocorticoid-mediated sst_2-downregulation may be a dynamic process which can be reversed after cortisol-lowering therapy. Remarkably, this difference in sst_2 expression could not be confirmed at the protein level. In accordance, octreotide less potently inhibited the *in vitro* ACTH secretion by cultured human corticotroph adenoma cells compared to pasireotide, which has a high binding affinity for both the sst_2 and sst_5. It remains unknown, therefore, whether increased sst_2 protein expression levels on these adenoma cells would result in higher octreotide efficacy.

The functional role of the sst_5 and D_2R in corticotroph tumor cells has been well established. As already mentioned, both preclinical and clinical studies have demonstrated ACTH- and cortisol-lowering effects of pasireotide and cabergoline, which are agonists of these receptors. However, the expression levels of somatostatin and dopamine receptors in the adrenal gland as well as the direct effects of their agonists on steroidogenesis had not been extensively studied thus far. Chapter 4 describes the study that was performed to evaluate the protein expression levels of these receptors in the adrenal gland. In agreement with the very low sst_2, sst_5 and D_2R expression levels in the zona fasciculata of both normal and ACTH-dependent hyperplastic human adrenal glands, no cortisol-lowering effects of their agonists were found in primary cultures. We therefore conclude that the beneficial effects of pasireotide and cabergoline in patients with Cushing's disease should be attributed to their ACTH-lowering effects rather than to a direct adrenal-mediated effect. A remarkable finding was the extraordinary high protein expression levels of both the sst_5 and D_2R in the zona reticularis. The functional role of these receptors in the zona reticularis remains to be elucidated in future studies.

Chapter 5 shows results of a comparative study on the effects of ketoconazole and fluconazole, two antifungal agents, on steroidogenesis. Ketoconazole is one of the most frequently used cortisol-lowering drugs in patients with Cushing's syndrome. Major disadvantages of ketoconazole are its hepatotoxicity and gastrointestinal side effects that sometimes limit its long-term use. Fluconazole, on the other hand, is generally well tolerated. In this study it is shown that pharmacological concentrations of fluconazole decrease cortisol secretion by two human adrenocortical carcinoma cell lines and cultured primary human adrenal cells. Similar to ketoconazole, fluconazole appears to exert its effects by blocking the activity of both the 17-hydroxylase and 11β-hydroxylase enzymes. Although the EC_{50} of fluconazole was higher compared to that of ketoconazole, this compound might become an alternative to decrease cortisol levels in patients with hypercortisolism. Conversely, patients that are treated with fluconazole because of mycosis should be closely monitored since they might be at risk for developing adrenal insufficiency.

Patients with Cushing's disease have an increased risk of developing venous thromboembolism. A limited number of studies previously evaluated plasma concentrations of proteins that are involved in hemostasis as well as the effects of successful surgery on these concentrations in patients with Cushing's syndrome. The effects of drug therapy on this complication of Cushing's disease are, however, not known yet. The results that are shown in Chapter 6 indicate that the hypercoagulable state in patients with Cushing's disease is caused by an increased production of procoagulant proteins (such as Factor VIII, fibrinogen and Von Willebrand Factor) and an impaired fibrinolytic potential (characterized by increased levels of inhibitors of the fibrinolytic system). These alterations ultimately lead to both a shortened activated partial thromboplastin time and an increased plasma clot lysis time. Despite successful medical combination therapy with pasireotide, cabergoline and ketoconazole, which induced normalization of cortisol excretion levels in 15/17 patients after 11 weeks of treatment, no improvement was observed in the hemostatic profile. It was therefore concluded that these

patients still had an increased risk for developing venous thrombosis. This in turn might have consequences for the duration of thromboprophylaxis that should be administered.

In most patients with Cushing's disease, the normal cortisol day-night profile is disturbed. Moreover, this disease causes a severe impairment in health-related quality of life. Both these characteristics were confirmed in the study that is presented in Chapter 7. Twelve out of the seventeen patients with Cushing's disease had a disturbed cortisol diurnal rhythm and quality of life was dramatically worse in these patients compared to controls according to virtually all subscales of the four questionnaires that were used. Remarkably, 6/12 patients with a disturbed baseline circadian cortisol rhythm regained a normal day-night profile after 11 weeks of medical combination therapy with pasireotide, cabergoline and ketoconazole. This suggests that successful drug therapy can induce regained hypothalamic control of normal corticotroph cell function in patients with CD. Despite the fact that 15/17 patients were biochemically cured at the end of the study, no ameliorations were found in quality of life. On the other hand, quality of life-parameters did not deteriorate either, despite the occurrence of several side effects. These patients' quality of life ultimately improved after sustained biochemical remission in three patients that had been on pasireotide/cabergoline combination therapy for up to 1 year and in the whole group of patients after an average period of more than 2 years, irrespective of the ultimate treatment modality.

Hypertension is another important hallmark of Cushing's disease. The pathogenesis of glucocorticoid-mediated hypertension has not been fully elucidated yet and little is known about the role of the renin-angiotensin-aldosterone system in blood pressure-regulation of patients with Cushing's disease. In Chapter 8, it is described that among our 17 patients with Cushing's disease, important changes in the renin-angiotensin-aldosterone system were found. Plasma concentrations of angiotensinogen were elevated, while renin- and aldosterone levels were suppressed in these patients, even in those who used blockers of the renin-angiotensin-aldosterone system. These data show that Cushing's disease is associated with a low-renin hypertension, but do not rule out the possibility that elevated cortisol concentrations chronically stimulate the mineralocorticoid receptor, thereby mimicking aldosterone action. Successful medical combination therapy with pasireotide, cabergoline and ketoconazole resulted in significantly decreased systolic and diastolic blood pressure, but plasma concentrations of these components of the renin-angiotensin-aldosterone system did not change. In an *in vitro* study, we found that pasireotide and, to a lesser extent, cabergoline inhibited the angiotensin II-mediated vasoconstriction of the iliac artery of the spontaneous hypertensive rat.

Chapter 9 contains preliminary data of a multicenter clinical trial that was performed to investigate the efficacy of successive treatment with ketoconazole and octreotide in patients with Cushing's disease. The rationale behind this study is that cortisol-lowering therapy may abrogate the glucocorticoid-induced sst_2 down-regulation on corticotroph tumor cells. This in turn could potentially increase the ACTH-lowering potential (i.e. clinical efficacy) of both sst_2-preferring somatostatin analogs and pasireotide. Preclinical data that support this hypothesis are outlined in Chapter 3. In Chapter 9, the effects of this treatment regimen in three patients with Cushing's disease are described.

Finally, Chapter 10 is the last chapter of this thesis. In the general discussion, the results of the aforementioned studies are further addressed. Possible limitations of these studies, as well as challenges for future investigation are discussed.

SAMENVATTING

Met een incidentie van 1-2 gevallen per miljoen per jaar is de ziekte van Cushing een zeldzame endocriene aandoening. Het wordt veroorzaakt door een adrenocorticotroop hormoon producerende hypofysetumor die de bijnierschors chronisch stimuleert tot het produceren van overmatige hoeveelheden cortisol. Dit leidt tot een verscheidenheid aan klachten en symptomen, waaronder spierzwakte, centrale obesitas, abdominale striae, hypertensie, glucose-intolerantie, veneuze trombose, osteoporose en psychiatrische aandoeningen. Onbehandeld hebben patiënten met de ziekte van Cushing een verslechterde levensverwachting, wat met name veroorzaakt wordt door een verhoogd risico op hart- en vaatziekten. Transsfenoidale chirurgie is de eerste keus behandeling en leidt op lange termijn tot remissie in ongeveer 60-70% van de patiënten. Dit betekent dat alternatieve behandelingen nodig zijn in de 30-40% van de patiënten met persisterende/recidiverende ziekte. Hypofysebestraling is vaak de tweedelijnsbehandeling maar heeft belangrijke nadelen. Op de eerste plaats kan het jaren duren voordat de adrenocorticotroop hormoon (ACTH) hypersecretie begint af te nemen en bovendien maakt radiotherapie patiënten vaak levenslang afhankelijk van hormoon substitutie therapie vanwege ongunstige effecten op de productie van (andere) hypofysehormonen. Om bruikbare middelen te ontwikkelen voor geneesmiddeltherapie voor de ziekte van Cushing is het cruciaal om inzicht te verkrijgen in de pathogenese van de ziekte en om moleculaire doelwitten voor mogelijke nieuwe geneesmiddelen te identificeren. In de laatste jaren is duidelijk geworden dat de subtype 5 somatostatine receptor (sst_5) en de dopamine receptor subtype 2 (D_2R) vaak sterk tot expressie worden gebracht door corticotrope tumorcellen in de hypofyse. Belangrijker nog is dat zowel preklinische als klinische studies hebben aangetoond dat geneesmiddelen die aangrijpen op deze receptoren gunstige effecten hebben. Een van de belangrijkste studies in dit opzicht werd verricht in ons centrum. Zeventien patiënten met de ziekte van Cushing werden in deze studie geïncludeerd en behandeld met het somatostatine analogon pasireotide, dat een hoge bindingsaffiniteit heeft voor de sst_5. In geval van persisterend hypercortisolisme na 4 weken behandeling werd cabergoline, een D_2R agonist, aan pasireotide toegevoegd. Na nog eens 4 weken behandeling werd ketoconazol, een imidazol antimycoticum dat direct de cortisolsynthese in de bijnierschors remt, toegevoegd aan deze middelen in de patiënten die nog steeds een verhoogde cortisolexcretie hadden. Uiteindelijk werden 15/17 patiënten normocortisolemisch na 11 weken combinatiebehandeling. In dit proefschrift werd getracht verder inzicht te verkrijgen in de etiologie van de ziekte van Cushing en de biologie van de expressie van voornoemde receptoren in de hypofysetumor en de bijnier. Hoofdstukken 2-5 behandelen deze onderwerpen. Ook werden de effecten van medicamenteuze combinatietherapie op verscheidene complicaties van de ziekte van Cushing onderzocht. De resultaten van deze studies worden beschreven in de hoofdstukken 6-8.

Pro-opiomelanocortine is het voorlopermolecuul van ACTH en wordt gesplitst door zogeheten prohormoon convertases (PC). PC1 wordt zowel in de hypofyse voorkwab als in de hypofyse middenkwab tot expressie gebracht (men gelooft dat de middenkwab snel na de geboorte in regressie gaat en daarom afwezig is in volwassenen) en splitst pro-opiomelanocortine in ACTH en β-lipotropine. PC2, dat voornamelijk in de intermediaire lob tot expressie wordt gebracht, verwerkt deze producten verder. Algemeen aanvaard wordt dat de ziekte van Cushing ontstaat uit ACTH-producerende adenomen in de hypofyse-voorkwab. Het is echter beschreven dat hypofyse laesies na een operatie histologisch werden geclassificeerd als hyperplasie of adenomateuze hyperplasie van ACTH-producerende cellen in de regio tussen de hypofyse voor- en achterkwab. In Hoofdstuk 2 wordt beschreven dat de subgroep tumoren die wordt geïdentificeerd als (adenomateuze) hyperplasie inderdaad minder macroadenomen bevat en hogere PC2 en neurofilament eiwitexpressie heeft vergeleken met typische voorkwabadenomen. Deze resultaten suggereren dat de ziekte van Cushing wellicht ook in de middenkwab van de hypofyse kan ontstaan.

Corticotrope hypofysetumoren brengen voornamelijk de sst_5 en D_2R tot expressie. Klinische studies met cabergoline en pasireotide hebben laten zien dat deze middelen als monotherapie blijvend normocortisolisme

kunnen induceren in ongeveer 20-40% van de patiënten. In tegenstelling tot de sst_5 en D_2R wordt de sst_2 in veel mindere mate tot expressie gebracht door corticotrope tumorcellen. Dit is waarschijnlijk het resultaat van down-regulerende effecten van hoge circulerende glucocorticoid concentraties in patiënten met de ziekte van Cushing. Dit zou de eerdere observatie kunnen verklaren dat octreotide, dat een hoge bindingsaffiniteit heeft voor de sst_2, over het algemeen niet effectief is. De hypothese werd daarom gesteld dat ACTH- en/of cortisolverlagende therapie resulteert in verhoogde sst_2 expressieniveaus. Dit zou weer consequenties kunnen hebben voor de klinische effectiviteit van somatostatine analoga die een voorkeur hebben voor deze receptor. In de studie die in Hoofdstuk 3 wordt beschreven werd bewijs gevonden dat ACTH-producerende hypofyseadenomen van patiënten met genormaliseerde preoperatieve cortisolwaarden door geneesmiddeltherapie hogere sst_2 mRNA expressieniveaus hebben dan adenomen van patiënten die, ondanks medicamenteuze voorbehandeling, verhoogde preoperatieve cortisolwaarden hadden. Dit laat zien dat de glucocorticoid-gemedieerde sst_2 down-regulatie wellicht een dynamisch proces is dat omkeerbaar is na cortisolverlagende therapie. Opvallend genoeg werd het verschil in sst_2 expressie niet bevestigd op eiwitniveau. Hiermee in overeenstemming remde octreotide de in vitro ACTH secretie door gekweekte humane corticotrope adenoomcellen in mindere mate dan pasireotide, dat een hoge bindingsaffiniteit heeft voor zowel de sst_2 als de sst_5. Om deze reden blijft onbekend of verhoogde sst_2 eiwitexpressie op deze adenoomcellen zou resulteren in een hogere effectiviteit van octreotide.

De functionele rol van de sst_5 en D_2R in corticotrope tumorcellen is uitgebreid vastgesteld. Zoals eerder genoemd hebben zowel preklinische als klinische studies aangetoond dat pasireotide en cabergoline, agonisten van deze receptoren, ACTH- en cortisolverlagende effecten hebben. De expressie van somatostatine- en dopamine receptoren in de bijnier en de directe effecten van hun agonisten op de steroidogenese waren tot nog toe echter niet uitgebreid onderzocht. Hoofdstuk 4 beschrijft de studie die werd verricht om de eiwitexpressie niveaus van deze receptoren in de bijnier vast te stellen. In overeenstemming met de erg lage sst_2, sst_5 en D_2R expressie in de zona fasciculata van zowel normale als ACTH-afhankelijke hyperplastische humane bijnieren werden geen cortisolverlagende effecten van hun agonisten gevonden in primaire kweken. Daarom concluderen we dat de gunstige effecten van pasireotide en cabergoline in patiënten met ziekte van Cushing toegeschreven zouden moeten worden aan hun ACTH-verlagende effect en niet aan een direct bijnier-gemedieerd effect. Een opvallende bevinding was de buitengewoon hoge eiwitexpressie van zowel de sst_5 als de D_2R in de zona reticularis. De functionele rol van deze receptoren in de zona reticularis zou daarom moeten worden opgehelderd in toekomstige studies.

Hoofdstuk 5 laat resultaten zien van een vergelijkende studie naar de effecten van ketoconazol en fluconazol, twee antimycotica, op de steroidogenese. Ketoconazol is een van de meest gebruikte cortisolverlagende geneesmiddelen in patiënten met de ziekte van Cushing. Belangrijke nadelen van ketoconazol zijn hepatotoxiciteit en gastro-intestinale bijwerkingen die soms langdurig gebruik beperken. Fluconazol daarentegen wordt over het algemeen goed getolereerd. In deze studie wordt aangetoond dat farmacologische concentraties fluconazol de cortisolsecretie door twee humane bijniercarcinoom cellijnen en gekweekte primaire humane bijniercellen vermindert. Net als ketoconazol lijkt fluconazol haar effecten uit te oefenen door de activiteit van zowel het 17-hydroxylase als het 11β-hydroxylase te blokkeren. Hoewel de EC_{50} van fluconazol hoger was dan die van ketoconazol zou deze stof een alternatief kunnen worden voor ketoconazol om de cortisolspiegels in patiënten met hypercortisolisme te verlagen. Omgekeerd zouden patiënten die met fluconazol worden behandeld vanwege een mycose nauwlettend in de gaten gehouden moeten worden omdat ze een bijnierinsufficiëntie zouden kunnen ontwikkelen.

Patiënten met de ziekte van Cushing hebben een verhoogd risico op het ontwikkelen van veneuze trombose. Een beperkt aantal studies heeft eerder plasma concentraties onderzocht van eiwitten die betrokken zijn bij de hemostase, evenals de effecten van succesvolle chirurgie op deze concentraties in patiënten met het syndroom van Cushing. De effecten van geneesmiddeltherapie op deze complicatie van de ziekte van Cushing waren echter nog onbekend. De resultaten in Hoofdstuk 6 laten zien dat de verhoogde stollingsneiging in patiënten

met de ziekte van Cushing worden veroorzaakt door verhoogde productie van stollingseiwitten (zoals Factor VIII, fibrinogeen en Von Willebrand Factor) en een verslechterde fibrinolyse (gekenmerkt door verhoogde spiegels van remmers van de fibrinolyse). Deze veranderingen leiden uiteindelijk tot een verkorte geactiveerde partiële tromboplastinetijd en een verlengde plasma 'clot lysis time'. Ondanks succesvolle medicamenteuze combinatiebehandeling met pasireotide, cabergoline en ketoconazol, resulterend in normalisatie van de vrije cortisol excretie in de urine in 15/17 patiënten na 11 weken, werd geen verbetering gevonden in de hemostase. Daarom werd geconcludeerd dat deze patiënten nog steeds een verhoogd risico hadden op het ontwikkelen van veneuze trombose. Dit kan weer gevolgen hebben voor de duur waarmee tromboprofylaxe zou moeten worden toegediend.

Het normale cortisol dag-nachtritme is in de meeste patiënten met de ziekte van Cushing verstoord. Bovendien veroorzaakt deze ziekte een ernstig verslechterde gezondheid-gerelateerde kwaliteit van leven. Beide werden bevestigd in de studie die in Hoofdstuk 7 wordt gepresenteerd. Twaalf van de zeventien patiënten met de ziekte van Cushing hadden een verstoord cortisol dagritme en de kwaliteit van leven was dramatisch verslechterd in deze patiënten vergeleken met controles volgens praktisch alle subschalen van de vier vragenlijsten die zijn gebruikt. Opvallend genoeg herstelde het cortisol dagritme na 11 weken medicamenteuze combinatiebehandeling met pasireotide, cabergoline en ketoconazol in 6/12 patiënten met een verstoord baseline cortisol dagritme. Dit suggereert dat succesvolle geneesmiddeltherapie de hypothalame controle over de normale corticotrope celfunctie kan herstellen in patiënten met de ziekte van Cushing. Ondanks het feit dat 15/17 patiënten biochemisch gecureerd waren aan het eind van de studie werden geen verbeteringen gevonden in de kwaliteit van leven. Aan de andere kant verslechterde de kwaliteit van leven parameters ook niet, ondanks het optreden van verscheidene bijwerkingen. De kwaliteit van leven van deze patiënten verbeterde uiteindelijk na langdurige biochemische remissie in drie patiënten die gedurende een jaar waren behandeld met pasireotide/cabergoline combinatietherapie en in het hele cohort patiënten na gemiddeld meer dan twee jaar, ongeacht de uiteindelijke behandelingsmodaliteit.

Hypertensie is een ander belangrijk kenmerk van de ziekte van Cushing. De pathogenese van glucocorticoid-gemedieerde hypertensie is nog niet volledig opgehelderd en er is weinig bekend over de rol van het renine-angiotensine-aldosteron systeem in de bloeddrukregulatie in patiënten met de ziekte van Cushing. In Hoofdstuk 8 wordt beschreven dat belangrijke veranderingen in het renine-angiotensine-aldosteron systeem werden gevonden in onze 17 patiënten met de ziekte van Cushing. Plasma concentraties van angiotensinogeen waren verhoogd terwijl renine- en aldosteronspiegels onderdrukt waren in deze patiënten, zelfs in patiënten die blokkers gebruikten van het renine-angiotensine-aldosteron systeem. Deze data laten zien dat de ziekte van Cushing geassocieerd is met een laag-renine hypertensie, maar sluiten niet uit dat verhoogde cortisol concentraties de mineralocorticoidreceptor chronisch stimuleren en daardoor de aldosteronwerking simuleren. Succesvolle medicamenteuze combinatietherapie met pasireotide, cabergoline en ketoconazol resulteerde in significante dalingen in systolische en diastolische bloeddruk, maar plasma concentraties van deze componenten van het renine-angiotensine-aldosteron systeem veranderden niet. In een in vitro studie vonden we dat pasireotide en in mindere mate ook cabergoline de angiotensine II-gemedieerde vasoconstrictie van de A. iliaca van de spontane hypertensieve rat remmen.

Hoofdstuk 9 bevat preliminaire data van een multicenter klinische studie die werd verricht om de effectiviteit van opeenvolgende behandeling met ketoconazol en octreotide te onderzoeken in patiënten met de ziekte van Cushing. De rationale achter deze studie is dat cortisolverlagende therapie de glucocorticoid-gemedieerde sst_2 down-regulatie op corticotrope tumorcellen zou kunnen tegengaan. Dit zou mogelijk de ACTH-verlagende werking (dus klinische effectiviteit) kunnen verhogen van zowel pasireotide als somatostatine analoga die voornamelijk de sst_2 binden. Preklinische data die deze hypothese onderbouwen worden weergegeven in hoofdstuk 3. In hoofdstuk 9 worden de effecten van dit behandelschema beschreven in drie patiënten met de ziekte van Cushing.

Hoofdstuk 10 is tenslotte het laatste hoofdstuk van dit proefschrift. In de algemene discussie worden de resultaten van voornoemde studies verder behandeld. Mogelijke beperkingen van deze studies, evenals uitdagingen voor toekomstig onderzoek worden bediscussieerd.

LIST OF PUBLICATIONS

1. **R. van der Pas**, C. de Bruin, F. W. G. Leebeek, M. P. M. de Maat, D. C. Rijken, A. M. Pereira, J. A. Romijn, R. T. Netea-Maier, A. R. Hermus, P. M. J. Zelissen, F. H. de Jong, A. J. van der Lely, W. W. de Herder, S. W. J. Lamberts, L. J. Hofland, R. A. Feelders. The Hypercoagulable State in Cushing's Disease Is Associated with Increased Levels of Procoagulant Factors and Impaired Fibrinolysis, But Is Not Reversible after Short-Term Biochemical Remission Induced by Medical Therapy. J Clin Endocrinol Metab 2012; 97(4): 1303-10.

2. **R. van der Pas**, W.W. de Herder, L.J. Hofland, R.A. Feelders. Recent developments in the Medical Treatment of Cushing's syndrome. Endocrine Related Cancer 2012; 19(6): R205-23.

3. **R. van der Pas**, L.J. Hofland, J. Hofland, A.E. Taylor, W. Arlt, J. Steenbergen, P.M. van Koetsveld, W.W. de Herder, F.H. de Jong, R.A. Feelders. Fluconazole Inhibits Human Adrenocortical Steroidogenesis In Vitro. Journal of Endocrinology 2012; 215(3):403-12.

4. F. Gatto, R.A. Feelders, **R. van der Pas**, J.M. Kros, A.M. Waaijers, D.M. Sprij-Mooij, S.J. Neggers, A.J. van der Lelij, F. Minuto, S.W.J. Lamberts, W.W. de Herder, D. Ferone, L.J. Hofland. Immunoreactivity score using an anti-sst2A receptor monoclonal antibody strongly predicts the biochemical response to adjuvant treatment with somatostatin analogs in acromegaly. J Clin Endocrinol Metab 2013; 98(1): E66-71.

5. **R. van der Pas**, F.W.G. Leebeek, L.J. Hofland and R.A. Feelders. Hypercoagulability in Cushing's syndrome: prevalence, pathogenesis and treatment. Clinical Endocrinology 2013; 78(4): 481-88.

6. **R. van der Pas**, C. de Bruin, A.M. Pereira, J.A. Romijn, R.T. Netea-Maier, A.R. Hermus, P.M. Zelissen, F.H. de Jong, A.J. van der Lely, W.W. de Herder, S.M. Webb, S.W.J. Lamberts, L.J. Hofland, R.A. Feelders. Cortisol Diurnal Rhythm and Quality of Life after Successful Medical Treatment of Cushing's Disease. Pituitary 2012; epub ahead of print.

7. **R. van der Pas**, W.W. de Herder, L.J. Hofland, R.A. Feelders. Recent developments in the Medical Treatment of Cushing's disease. Drugs 2013; 73(9); 907-18.

8. **R. van der Pas**, R.A. Feelders, C. de Bruin, A.M. Pereira, D.M. Sprij-Mooij, A.M. Waaijers, S. Schulz, S.W.J. Lamberts, L.J. Hofland. Normalization of cortisol levels in Cushing's disease after medical pretreatment before surgery: consequences for somatostatin receptor subtype expression and in vitro response to somatostatin analogs. Submitted.

9. **R. van der Pas**, J.H.M. van Esch, C. de Bruin, A.H.J. Danser, D.M. Sprij-Mooij, I.M. van den Berg-Garrelds, A.H. van den Meiracker, L.J. Hofland, R.A. Feelders. Cushing's disease and hypertension: role of the renin-angiotensin-aldosterone system and effects of medical therapy. Submitted.

10. **R. van der Pas**, S.W.J. Lamberts, F. Gatto, A.M. Pereira, J.M. Kros, S.G. van Duinen, R.A. Feelders, L.J. Hofland. Different entitities of corticotroph adenomas on the basis of prohormone convertase expression and the relationship with dopamine D2 receptor and somatostatin receptor subtype 2 and 5 expression. Submitted.

11. F. Gatto, R.A. Feelders, **R. van der Pas**, J.M. Kros, F. Dogan, P.M. van Koetsveld, A.J. van der Lelij, S.J. Neggers, F. Minuto, W.W. de Herder, S.W.J. Lamberts, D. Ferone, L.J. Hofland. Beta arrestin-1 and 2 and G protein coupled receptor kinase 2 expression in pituitary adenomas: role in the regulation of response to somatostatin analogue treatment in patients with acromegaly. Submitted.

12. **R. van der Pas**, R.A. Feelders, C. van Eijck, P.M. van Koetsveld, S. Schulz, F. van Nederveen, L.J. Hofland. Pasireotide, octreotide and cabergoline do not decrease basal- and ACTH stimulated cortisol secretion by primary cultures of human adrenal glands. In preparation.

ABOUT THE AUTHOR

Rob van der Pas was born November 19th, 1985 in 's-Hertogenbosch (The Netherlands). After obtaining his high-school degree at 'Het Stedelijk Gymnasium van 's-Hertogenbosch' in 2003, he studied medicine at the Erasmus University in Rotterdam. In 2009, he finished his graduation project ("Comparison of in vitro effects of fluconazole and ketoconazole on ACTH-producing pituitary adenoma cells and on cortisol producing adrenocortical carcinoma cells") under supervision of Prof. dr. L.J. Hofland and Dr. R.A. Feelders. After obtaining his medical degree cum laude in 2009, the author started working as a PhD student in the laboratory of neuroendocrinology of the Erasmus Medical Center. He investigated basal and clinical aspects of the medical treatment of Cushing's disease and these studies were again performed under supervision of Prof. dr. L.J. Hofland and Dr. R.A. Feelders. He has now started his residency in Internal Medicine in the St. Elisabeth Hospital in Tilburg under supervision of Dr. P.L. Rensma.

PHD PORTFOLIO

Courses	Year	Hours
Molmed Basic Introduction Course on SPSS	2009	14
Molmed course Research Management	2010	16
35th Erasmus endocrinology course	2009	20
36th Erasmus endocrinology course	2010	20
Molmed course basal and translational oncology	2010	50
'Basiscursus regelgeving en organisatie voor klinisch onderzoekers'	2012	25
Oral presentations		
Dutch Endocrine Meeting, Noordwijkerhout	2010	48
Dutch Adrenal Network	2010	15
Dutch Endocrine Meeting, Noordwijkerhout	2011	48
Dutch Endocrine Meeting, Noordwijkerhout	2012	48
Science Days Internal Medicine, Antwerp	2012	46
Poster presentations		
Science Days Internal Medicine, Antwerp	2010	46
European Congress of Endocrinology, Prague	2010	70
Science Days Internal Medicine, Antwerp	2011	46
European Congress of Endocrinology, Rotterdam	2011	70
93rd Endocrine Society Annual Meeting, Boston, USA	2011	46
93rd Endocrine Society Annual Meeting, Boston, USA	2011	46
Targeting the Pituitary: Expert Knowledge Forum, Berlin	2011	38
Targeting the Pituitary: Expert Knowledge Forum, Berlin	2011	38
Annual Molecular Medicine Day	2012	38
European Congress of Endocrinology, Florence	2012	70
94th Endocrine Society Annual Meeting, Houston, USA	2012	46
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