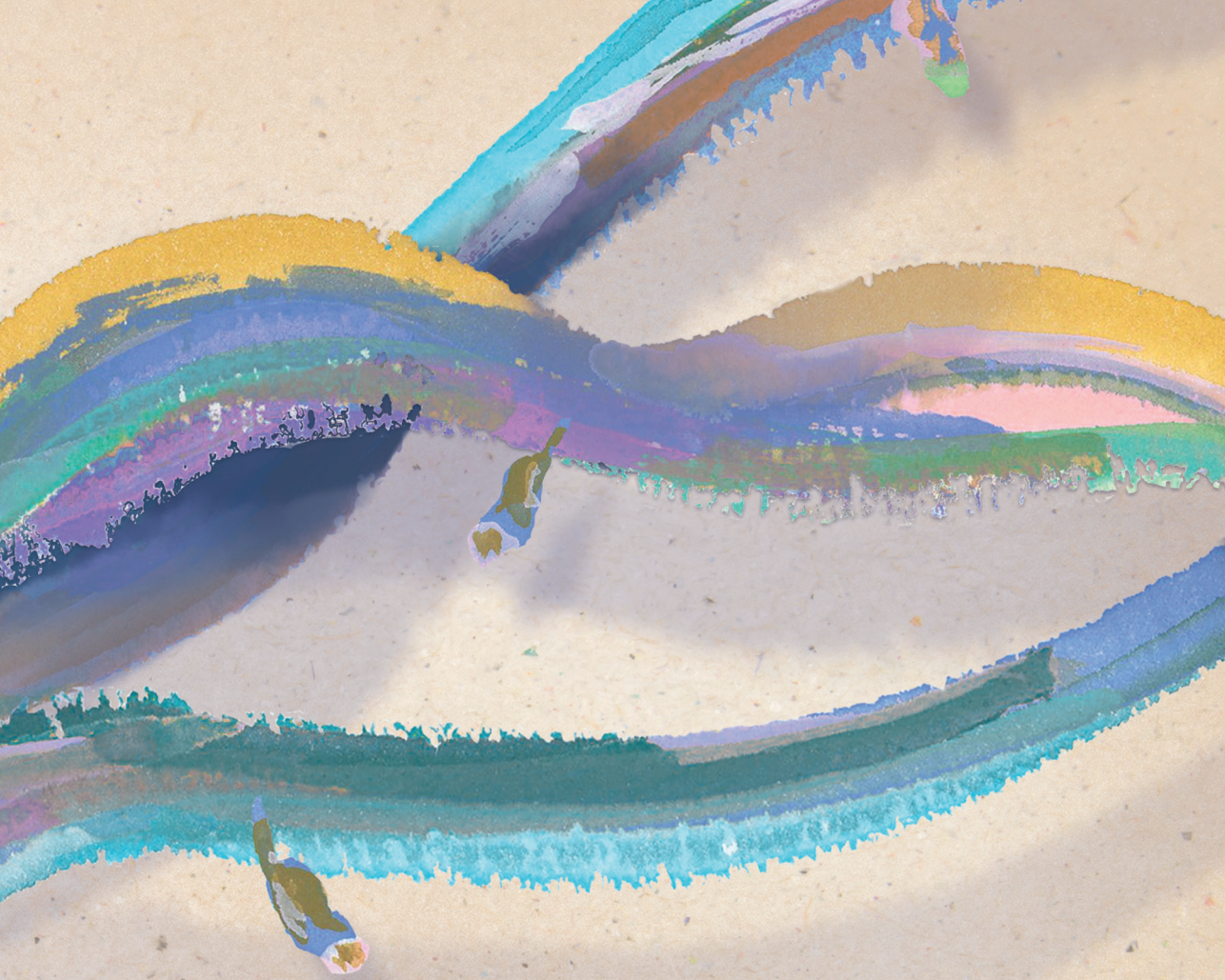

Novel Insights into Diagnosis and Treatment of Adrenocortical Tumors

Sara G. Creemers

NOVEL INSIGHTS INTO DIAGNOSIS AND TREATMENT OF ADRENOCORTICAL TUMORS

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NOVEL INSIGHTS INTO DIAGNOSIS AND TREATMENT OF ADRENOCORTICAL TUMORS

Nieuwe inzichten in de diagnose
en behandeling van bijnierschorstumoren

Proefschrift

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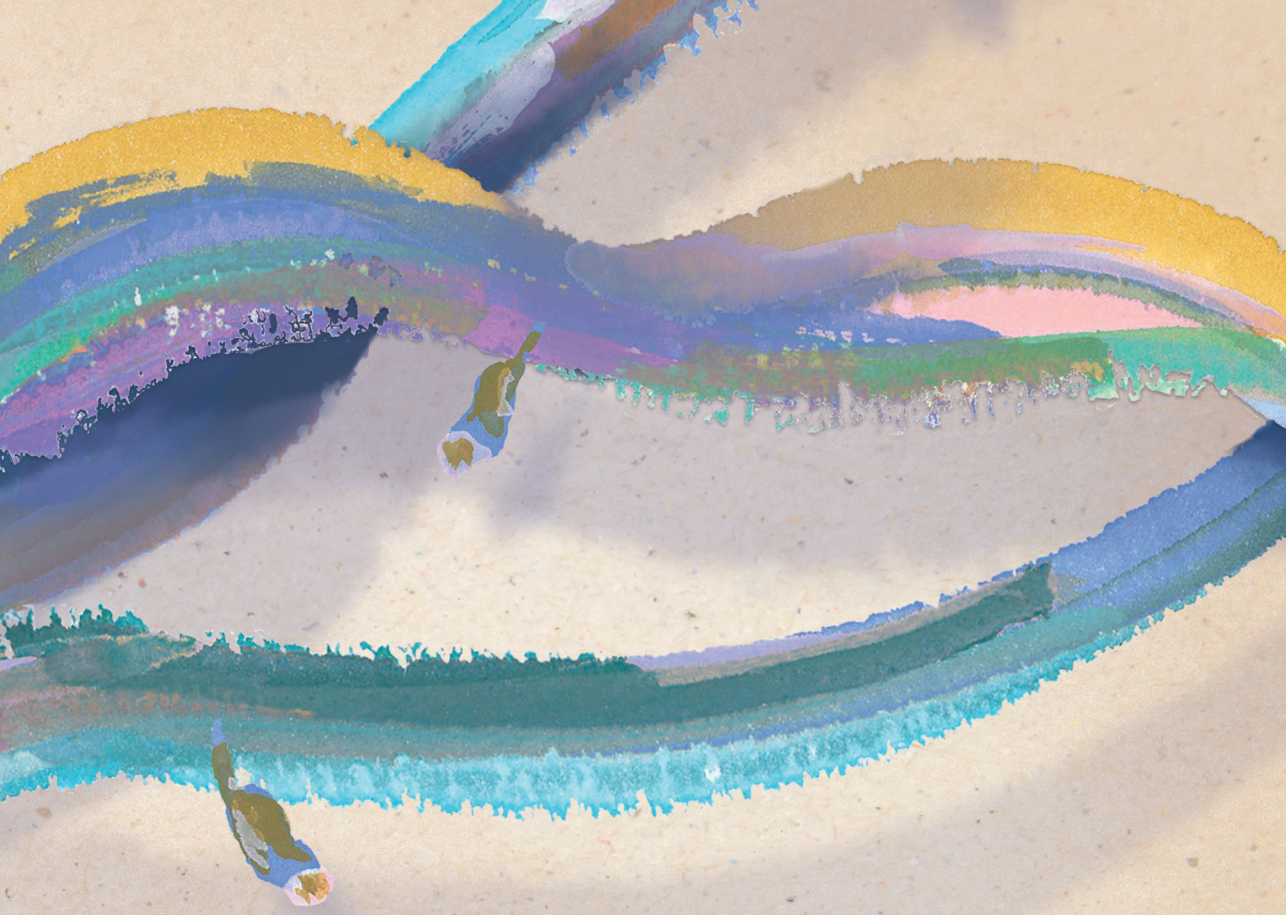

CHAPTER 1

General Introduction

PARTLY BASED ON:

Cushing's syndrome: an update on current pharmacotherapy and future directions
Creemers SG, Hofland LJ, Lamberts SW, Feelders RA
Expert Opin Pharmacother, 2015, 16(12):1829-44

Future directions in the diagnosis and medical treatment of adrenocortical carcinoma
Creemers SG, Hofland LJ, Korpershoek E, Franssen GJ, van Kemenade FJ, de Herder WW, Feelders RA
Endocr Relat Cancer, 2016, 23(1):R43-69

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THE ADRENAL GLAND

The adrenals are two symmetric endocrine glands, localized above the kidneys in the retroperitoneum. The adrenal has two distinct structures, i.e. the medulla and the cortex (Fig. 1). In the medulla, particularly epinephrine and norepinephrine are produced. The adrenal cortex, the focus of this thesis, exists of three layers: the outermost or zona glomerulosa produces aldosterone, the middle or zona fasciculata is the largest producing cortisol, while the innermost or zona reticularis produces the adrenal androgens. The adrenal steroidogenesis is facilitated by several cytochrome P450 and hydroxysteroid dehydrogenase enzymes, converting the common precursor cholesterol (Fig. 2).

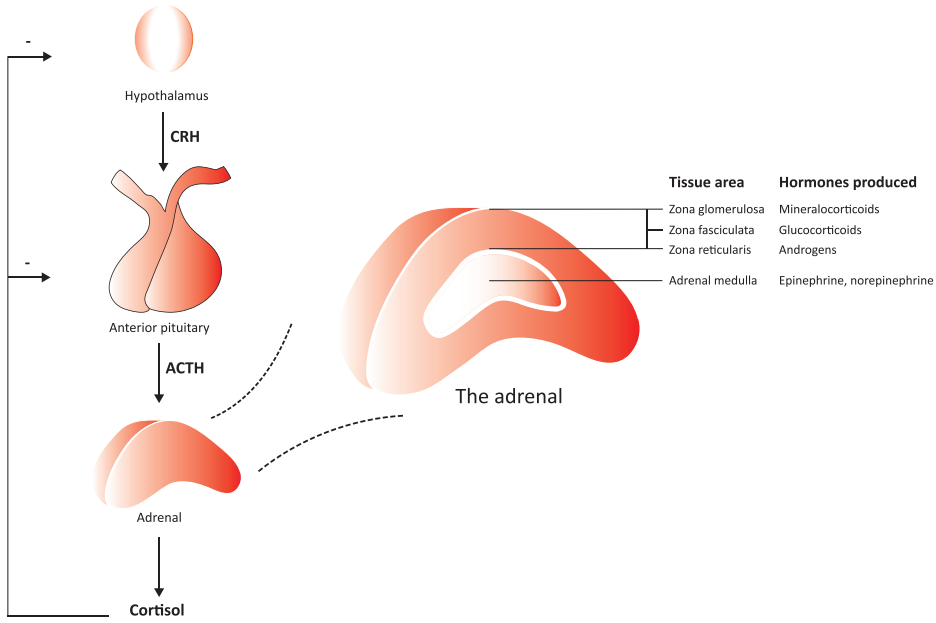

Figure 1. Overview of the hypothalamic-pituitary-adrenal (HPA) axis and the adrenal gland with the hormones that are produced in the specific areas. ACTH, adrenocorticotropic hormone; CRH, corticotropin releasing factor.

The production of hormones by the adrenal cortex is regulated by several physiological systems. For example, cortisol production is in the healthy situation regulated by the hypothalamic-pituitary-adrenal (HPA) axis (Fig. 1). Stimulation of the hypothalamus by the central nervous system stimulates release of corticotropin-releasing hormone (CRH), which in turn stimulates adrenocorticotropic hormone (ACTH) production by the anterior pituitary (1). ACTH is carried via the bloodstream to its effector organ, the adrenal cortex, where it stimulates adrenocortical steroidogenesis by binding to the melanocortin type

2 receptor (MC2R). When ACTH binds to the MC2R in the adrenal, several steroidogenic enzymes in the zona fasciculata of the adrenal cortex that are required for cortisol synthesis are induced. Cortisol has important effects on glucose and lipid metabolism, the immune system, and plays a role in maintaining blood pressure. It also decreases the production of both CRH and ACTH, thereby regulating its own production through a negative feedback loop (Fig. 1) (1). ACTH also induces production of adrenal androgens, but to a lesser extent compared to cortisol. Aldosterone is primarily controlled by the renin-angiotensin-aldosterone system (RAAS) and potassium levels, and secondary by ACTH.

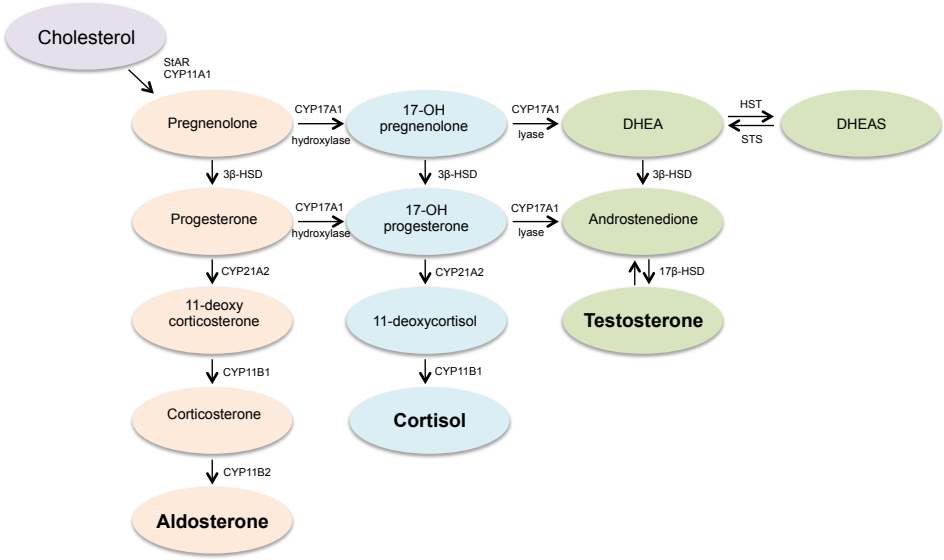

Figure 2. Simplified scheme of adrenocortical steroidogenesis. The first step in steroidogenesis requires cholesterol to enter the mitochondria, facilitated by Steroid Acute Regulatory protein (StAR) and cholesterol side-chain cleavage enzyme (CYP11A1). This is the rate limiting step of steroidogenesis. CYP, cytochrome P450 enzyme; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulphate; HSD, hydroxysteroid dehydrogenase; HST, hydroxysteroid sulfotransferase; OH, hydroxy; STS, sulfotransferase.

In the adrenal cortex, neoplasms like adenomas and carcinomas may develop, or hyperplasias can occur. These adrenal masses are prevalent and often incidentally discovered during diagnostic imaging procedures performed for other indications. Autopsy studies show a prevalence of 1.0 - 8.7% (2, 3), whereas radiological studies report a frequency of clinically inapparent adrenal masses of less than 1% for patients under 30 years of age, a percentage which increases up to 10% in those 70 years of age or older (4-6). Adrenal masses can be accompanied by two distinct clinical problems, i.e. hormonal

overproduction or malignant tumor growth. In some patients, a combination of these clinical problems may occur. An overproduction of cortisol by the adrenal gland can result in Cushing's syndrome (CS), a metabolic disorder often involving obesity and high blood pressure. Incidence rates vary from 0.7 to 2.4 per million population per year (7). The first part of this thesis focuses on novel developments in the treatment of hormonal overproduction by the adrenal cortex. The clinical problem of malignant growth of an adrenocortical tumor, e.g. adrenocortical carcinoma (ACC), is also rare, with incidence rates varying from 0.7 to 2.0 cases per million population each year (8-10). The second part of this thesis focuses on novel insights into diagnosis and treatment of ACC, with or without overproduction of steroids.

PART I CUSHING'S SYNDROME

Endogenous CS is characterized by chronic exposure to excess levels of glucocorticoids. Most characteristic features of CS include central obesity, full and plethoric facial appearance, increased supraclavicular fat deposit, proximal muscle weakness and easy bruisability (7, 11). Curative treatment decreases mortality and morbidity, although substantial morbidity can persist, including metabolic complications such as diabetes, dyslipidemia and obesity; cardiovascular and thromboembolic complications due to multiple risk factors; osteoporosis; and psychological and cognitive disturbances (12-17). In CS patients with (persistent) hypercortisolism, cardiovascular and cerebrovascular events are the main causes of excess mortality (7, 15, 18, 19). Very high cortisol levels can, due to saturation of the renal enzyme corticosteroid 11β-hydroxysteroid dehydrogenase 2 (HSD11B2), activate the mineralocorticoid receptor, leading to hypertension and severe hypokalemia (20). Despite cure or long-term control, quality of life is often impaired in patients with CS (21).

Causes of Cushing's syndrome

CS is most frequently ACTH-dependent (~80% of cases), caused by an ACTH-producing pituitary adenoma (Cushing's disease (CD), ~70%) and more rarely by ectopic ACTH production by non-pituitary tumors (~10%) (7). Primary adrenal ACTH-independent CS (~20% of cases) is usually caused by an adrenocortical adenoma (ACA) and less frequently by a functional adrenocortical carcinoma (ACC) (7, 11). Rare causes of adrenal CS involve bilateral micronodular or macronodular adrenal hyperplasia (BMAH) or primary pigmented nodular adrenocortical disease (PPNAD). Some causes of CS can also result in subtle hypercortisolism without evident clinical signs (subclinical CS), which is based on literature estimated to be present in 5-20% of patients with incidentalomas (22). However, this prevalence could be overestimated.

Diagnosis of Cushing's syndrome

Testing for diagnosis of chronic hypercortisolism is required in case specific symptoms, like hypertension or osteoporosis, occur in patients younger than expected. Also in patients with unexplained, severe, and resistant features, irrespective of age, assessment of hypercortisolism should be done. First, use of exogenous glucocorticoids should be excluded. The most used screening tests for endogenous hypercortisolism include: 24-h urinary free cortisol excretion (UFC), low-dose dexamethasone suppression test, and midnight plasma cortisol or late-night salivary cortisol levels (11). Once CS is diagnosed, the cause should be identified in order to determine the therapeutic strategy.

Treatment of CS

Tumor-directed surgery is the first-line treatment approach for patients with any form of CS, aiming for definite cure (1). For adrenal CS, patients usually undergo uni- or bilateral laparoscopic adrenalectomy. In case of CD, transsphenoidal removal of the pituitary tumor is the primary treatment, which is associated with recurrence risks of up to 25% (23, 24). These rates are highly dependent on surgical experience and size of the tumor. Repeat pituitary surgery is often accompanied by hypopituitarism, and is associated with even lower success rates (25). In case surgical resection of the primary tumor is not successful or not feasible, second-line treatments are indicated, which include radiotherapy, bilateral adrenalectomy, and pharmacotherapy. Radiotherapy has the disadvantage that it might only be effective after several years, requiring alternative therapies in the intervening time. Besides, there is a high risk of hypopituitarism (26). In case rapid eucortisolism is required or in case hypercortisolism cannot be controlled otherwise, bilateral adrenalectomy is a definite treatment option. Medical therapy in CS can be indicated: 1) to improve the clinical and metabolic condition of patients awaiting surgery, although evidence is lacking that this improves surgical outcome; 2) in patients with acute complications of severe hypercortisolism; 3) as bridging therapy in patients treated with radiotherapy; 4) in patients not feasible for surgery (metastasized disease, low a priory chance on surgical cure, high operation risk), and 5) in patients with persistent or recurrent CS after surgery (7).

Pharmacotherapeutic modalities for the treatment of Cushing's syndrome

There are several targets for pharmacotherapy of CS identified so far (Fig. 3), which will be described in the following sections. A combination of therapies might be necessary to achieve eucortisolism in patients with moderate-to-severe hypercortisolism.

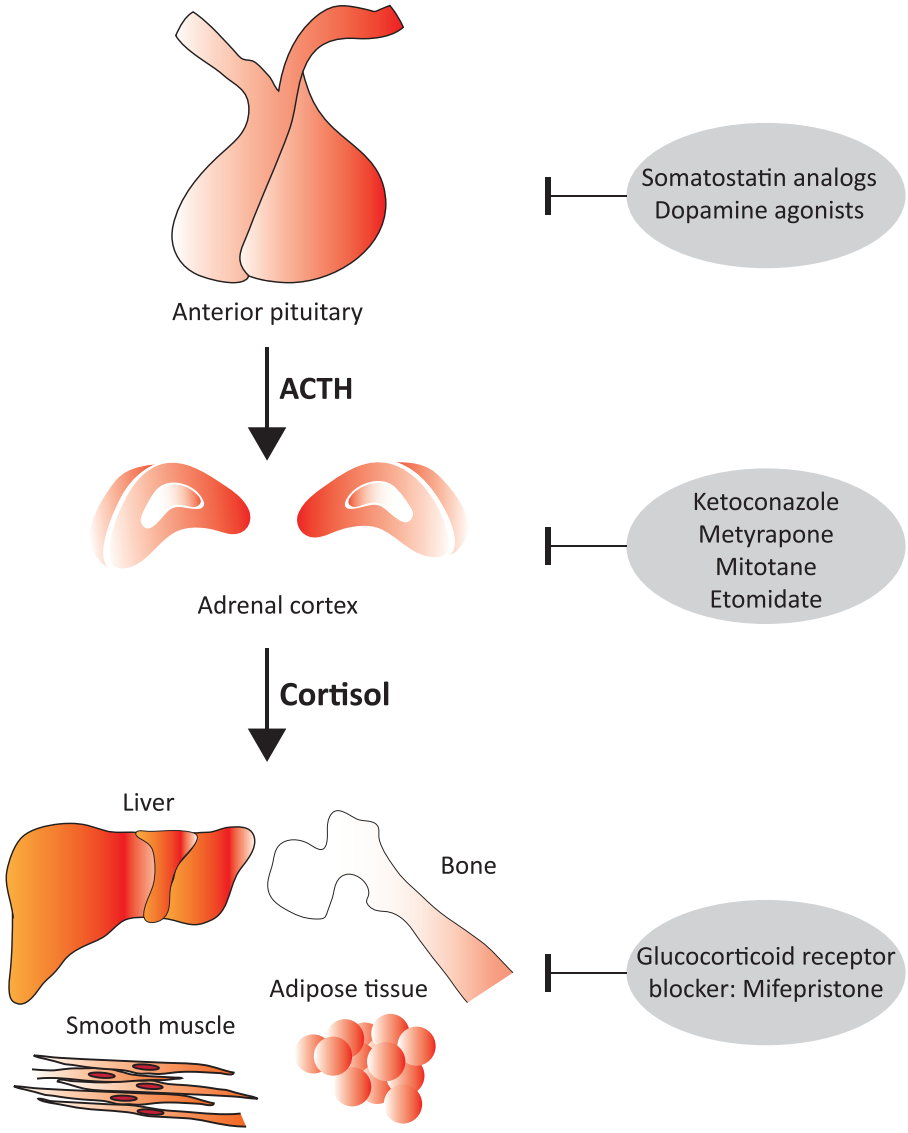

Figure 3. Targets and drugs for the medical treatment of Cushing's syndrome. ACTH, adrenocorticotropic hormone.

Pituitary-targeting drugs

Dopamine and somatostatin receptors have been identified as targets for medical treatment of CD, aiming to inhibit ACTH secretion (27). Pasireotide binds with high affinity to somatostatin receptor subtypes (SST) 1, 2, 3, and 5, but in corticotroph pituitary adenomas mainly acts on SST_5, the predominantly expressed receptor (28, 29). SST_2 expression is generally low and thought to be mediated by high glucocorticoid levels in CD. Consequently, the SST_2-targeting somatostatin analogs octreotide and lanreotide are relatively ineffective (30, 31). Pasireotide can induce hyperglycemia via inhibition of insulin secretion, directly via binding to the SST_5 on pancreatic islet-cells, and indirectly via suppression of incretin hormone production by K and L cells (32). Pasireotide might also cause gastrointestinal side effects (33). A recent phase III study, including 150 patients with CD, showed normalization of 24 hour UFC in 40% of patients receiving once a month 10 to 30 mg of long-acting intramuscular pasireotide after 7 months (34).

Dopamine receptor subtype 2 (D_2 receptor) has been found in 80% of ACTH-secreting pituitary adenomas (35). Cabergoline is a dopamine agonist with particular high affinity to the D_2 receptor. The most common side effects include nausea, dizziness, headache and gastrointestinal complaints (36). Controversy exists as to whether chronic use of cabergoline causes valvular heart disease. Studies with long-term follow-up are required to assess potential cardiac involvement at chronic high dosages. In the last years, six series were published with a total of 141 patients evaluating monotherapy with cabergoline in patients with CD (36-41). On average, studies reported normalization of UFC in 38% of patients with follow-up periods ranging from 6 weeks until 37 months. In a study combining pasireotide, ketoconazole and cabergoline, 17 patients with CD were treated in a stepwise treatment regimen (42). Pasireotide monotherapy was the initial treatment, which was extended based on UFC levels with cabergoline and ketoconazole after 4 and 8 weeks, respectively. Using this approach, 88% (15/17) of patients reached normal UFC levels.

Recent studies have reported the value of the chemotherapeutic agent temozolomide in patients with aggressive corticotroph pituitary adenomas or carcinomas that are refractory to surgery or irradiation (43-47), alone or in combination with pasireotide (48).

Adrenocortical steroidogenesis inhibitors

Ketoconazole is an imidazole derivative, originally developed as antifungal agent. It is known to block several steps in adrenal steroidogenesis, including CYP11A1 (cholesterol side-chain cleavage enzyme), CYP17A1 (17-hydroxylase and 17,20-lyase), CYP11B1 (11β-hydroxylase), and CYP11B2 (aldosterone synthase) (49-51). Important side effects of

ketoconazole include hepatotoxicity, rash, gastrointestinal symptoms, and gynaecomastia and hypogonadism in men, which together can lead to discontinuation of therapy (Table 1, (52-55)). Liver function should be carefully monitored during treatment. Increases in liver aminotransferases, which normally occur within 4 weeks of treatment initiation or dose change, should however only lead to dose reduction or cessation in case they rise to more than three times the upper limit of normal (56). Ketoconazole may also have extra-adrenal effects, like effects on corticotroph tumor cells in patients with CD (55, 57, 58). This might explain the impairment of ACTH release found in a subset of patients during prolonged treatment with ketoconazole (59, 60). Ketoconazole is a CYP3A4 inhibitor, emphasizing cautiousness as it comes to combination treatment. In 2013, the European Medicines Agency made ketoconazole only available for treating CS under controlled conditions. Response rates to ketoconazole vary between studies, with an overall response rate of 53% in 5 retrospective studies together (Table 1). It is likely that the effectiveness of ketoconazole represents an underestimation, since not all patients in these studies received the maximum dosage. This can also be the result of side effects, which is the reason for withdrawal in approximately 20% of patients (61).

In contrast to ketoconazole, metyrapone specifically blocks the distal steps in the synthesis of cortisol via inhibition of CYP11B1 and CYP11B2 (62, 63). Recent *in vitro* data suggest that metyrapone has more potent inhibitory effects on CYP11B2 (63). Due to this distal block, mineralocorticoid precursors and adrenal androgens can increase, enhanced by increased ACTH levels due to decreased cortisol-mediated negative feedback (24, 62, 64). Accumulation of adrenal androgens may lead to worsening of acne or hirsutism in female patients. Accumulation of 11-deoxycorticosterone, a steroid precursor with weak mineralocorticoid activity, may cause hypertension, edema and hypokalemia (Table 1) (24, 62, 64). Hypokalemia has however not been found to be an important issue in the two largest retrospective studies investigating metyrapone, probably due to careful monitoring and managing of serum potassium levels (62, 65). The most frequent side effects are gastrointestinal upset and hypoadrenalism, which conditions may have a significant overlap (61). Because gynaecomastia is not a side effect of metyrapone, this drug instead of ketoconazole might be preferably used in male patients (66). Metyrapone has a rapid onset of action and a short half-life of about 2 hours (62). Since the precursor 11-deoxycortisol cross-reacts in the standard immunoassay for cortisol, monitoring might be difficult and requires a more reliable method like LC-MS/MS (67). In several countries, metyrapone is not commercially available. Taken all studies together, metyrapone monotherapy has resulted in normalization of cortisol levels in 43% of 287 patients with CS (Table 1) (12). Additional long-term studies of outcome after treatment with metyrapone are required.

Table 1. Studies investigating the use of ketoconazole and metyrapone in Cushing's syndrome

	Overall response rate	Response rate (n/N)	Response rate (%)
Ketoconazole	53% (161/304)	30/34	88% (53)
		7/15*	47% (68)
		17/38	45% (54)
		9/17	53% (69)
		98/200	49% (70)
Metyrapone	43% (123/287)	66/87	76% (62)
		13/13*	100% (71)
		6/23	26% (69)
		38/164	43-76% (65)**

In these studies, ketoconazole or metyrapone were the only medical therapy. Only studies with at least 10 patients are reported. Response rate was identified by normalization of urinary free cortisol, serum cortisol, or early morning cortisol.

Mitotane has adrenolytic properties and is therefore mainly used to treat ACC (detailed description in PART II of the introduction, section 'Mitotane treatment') (72, 73), but has also shown to have cortisol-suppressing effects in patients with CS. Mitotane is a strong CYP3A4 inducer, which can reduce bio-availability of cortisol, as well as of drugs (74). Besides induction of CYP3A4 and the adrenolytic properties, mitotane at lower concentrations reduces bioavailability of cortisol by the action on steroidogenic enzymes, like suppression of CYP11A1, and possibly other steps as CYP11B1 and CYP11B2 (75, 76). The drug is thereby thought to induce the cortisol binding protein (CBG), resulting in a decreased serum free cortisol fraction (77). Adrenal crisis needs to be avoided by exogenous corticosteroids (78). A retrospective analysis in 76 patients with CD resulted in remission in 72% of patients with a medium follow-up time of 6.7 months (79).

Etomidate is an imidazole derivative that inhibits CYP11B2 and to a lesser degree CYP11A1 (51). Originally it was used as an anesthetic agent, but was soon reported to cause adrenal insufficiency (80). Etomidate is given parentally at a dose between 0.03 and 0.3 mg/kg/h, has a rapid onset of action (81), and is used in critically ill patients with acute and/or life-threatening CS when oral treatments are ineffective or impossible (80, 82, 83).

Follow-up	Multi-center	Side effects	Daily dose and administration
> 4 months, maximum 6 years Median 19 weeks Mean 23 months Median 108 months 1 year	x	Gastrointestinal upset, hepatotoxicity with liver function derangement *Men*: hypogonadism, gynaecomastia	400 – 1600 mg per day Administer with acidified beverages and avoid proton pump inhibitors
1 – 16 weeks 6 – 238 months Median 4 months Mean 8 months	x	Gastrointestinal upset, hypoadrenalism, hypertension, edema, hypokalemia *Women*: acne, hirsutism	CD or ACA: 750 – 1000 mg EAS or ACC: 1500 mg – 4 g Administer with milk or light snack

Overall response rate represents a weighted mean. All studies were retrospective. *Only patients with ectopic ACTH syndrome. **Dependent on the biochemical targets for treatment.

Glucocorticoid receptor blocker

Mifepristone is a progesterone and glucocorticoid receptor (GR; type II) antagonist, which binds to the GR with an affinity 18 times higher than that of cortisol (84). It has a rapid onset of action. Following binding to the receptor at tissue level, the GR changes into an inappropriate conformation. Mifepristone treatment may lead to increased ACTH secretion and subsequently increased cortisol levels via antagonism of GR at the pituitary and hypothalamic level. Due to mineralocorticoid effects of increased cortisol levels, serious adverse events, i.e. worsening of hypokalemia and hypertension, can occur (84, 85). Other common side effects include fatigue, headache, nausea, abdominal complaints, arthralgia, vomiting, and edema (84). Unfortunately, no biochemical parameter is available to adjust the mifepristone dose, so clinical features are required to monitor treatment response. Overdosing and consequently clinical adrenal insufficiency may occur, requiring interruption of treatment and high doses of dexamethasone administration. Chronic treatment can thereby be accompanied by endometrium hyperplasia in female patients (86). Finally, long-term mifepristone treatment may potentially induce corticotroph tumor growth, particularly in patients with macroadenomas (87). Fifty patients with CS were included in the multicenter SEISMIC study, of whom 43 with CD (84). Treatment improved clinical status in 87% of patients, including a decreased AUC_{gluose}, weight loss, decrease in diastolic blood pressure and improvement of diabetes mellitus.

PART II ADRENOCORTICAL CARCINOMA

Patients with ACC present in 40-60% of the cases with clinical symptoms due to hormone excess, like hypercortisolism in ~55% of the hormone-secreting ACC (78, 88). Patients may also present with symptoms due to local or distant tumor growth, i.e. flank pain, abdominal discomfort, back pain, or abdominal fullness (78, 88). About 10-25% of the ACC cases are diagnosed incidentally, and this percentage is still thought to be increasing due to the wide use of imaging studies in medicine. At the time of presentation, most ACC are very large, measuring on average 10-13 cm, but can still be localized (78, 89, 90). On the basis of the European Network for the Study of Adrenal Tumors (ENSAT) classification, advanced ACC is defined by locoregional spread (stage III) or distant metastases (stage IV), and represents 18-26% and 21-46% of ACC patients at diagnosis, respectively (Table 2) (91-93). Most ACC occur sporadically, but they can also be part of various genetic syndromes such as Li Fraumeni syndrome (94), Beckwith Wiedemann syndrome (95), Multiple Endocrine Neoplasia type 1 (96), or Lynch syndrome (97).

Table 2. The ENSAT staging system for adrenocortical carcinoma

ENSAT stage	T	N	M
I	1	0	0
II	2	0	0
III	1, 2	1	0
	3, 4	0, 1	0
IV	1 - 4	0, 1	1

Tumors are classified as follows: T1, tumor ≤ 5 cm; T2, tumor > 5 cm; T3, tumor infiltration into surrounding (fat) tissue; T4, tumor invasion into adjacent organs or venous tumor thrombus in vena cava or renal vein; N0, no spread into nearby lymph nodes; N1, positive lymph node(s); M0, no distant metastasis; M1, presence of distant metastasis. ENSAT, European Network for the Study of Adrenal Tumors.

Diagnosis of adrenocortical carcinoma

For decades, there has been debate regarding the optimal diagnostic strategy for patients with ACC. Early and correct classification is relevant to establish the appropriate therapeutic strategy. In the last years, as a result of extensive research and international collaborations, existing diagnostic tools have been improved, and new approaches have been proposed.

Biochemical evaluation

A thorough hormonal evaluation is recommended in all patients with (suspected) ACC, even in the patients with apparently nonfunctional tumors (78). Biochemical evaluation, which is in part guided by hormone-related clinical symptoms of patients, is performed by

measurement of steroid hormones potentially produced by the tumor. For several reasons, it is important to perform biochemical evaluation prior to surgery (98): 1) it can further add to judge the risk of malignancy, since this risk increases in case of androgen or estrogen production; 2) in case of glucocorticoid excess cortisol lowering- or antagonizing therapy can be indicated; 3) patients with cortisol-producing adrenal tumors need hydrocortisone replacement post-surgery; 4) hormonal parameters can be used as tumor markers; 5) pre-surgical testing for pheochromocytoma-related hormones can avoid complications during surgery (99).

Imaging

Initial evaluation of adrenal masses is usually performed with assessment of the radiological characteristics on (contrast-enhanced) computed tomography (CT) scan, magnetic resonance imaging (MRI), and/or positron emission tomography with ^{18}F-2-deoxy-D-glucose (mostly combined with CT: FDG-PET/CT) (98, 100). The most important predictor for malignancy is the size of a tumor, although size alone is not sufficient for an accurate discrimination between ACCs and ACAs (90). Specificity for malignancy increases from 52 to 80% for tumors larger than 4 to 6 cm, respectively (90). It is considered that only in case of a homogenous adrenal lesion with low CT density of ≤ 10 Hounsfield Units (HU), it is reliable to rule-out an ACC on non-contrast CT (100). FDG-PET/CT can be considered as additional imaging technique. Although these findings together will not always indicate a clear diagnosis, characteristics on CT scan are currently used to guide the decision on adrenalectomy. The general consensus is that surgery is recommended in tumors larger than 4-6 cm (101, 102), although exceptions exist if all other characteristics point towards a benign lesion. In tumors with several suspicious imaging features, further evaluation is warranted. It is generally not recommended to use fine needle aspiration biopsy (FNAB) in ACC, because of the risk on hemorrhage, tumor spill, and the limited diagnostic value (100, 103).

Pathology

Generally, the distinction between adrenocortical and non-adrenocortical tumors can be made on the basis of hematoxylin and eosin-stained slides. ACC is thought to have an independent origin and progression of an ACA into an ACC may represent an exceptionally random event (104). In case of doubt of the origin, it is recommended to perform immunohistochemistry with steroidogenic factor-1 (SF1), the most sensitive and specific marker (100, 105). For determination of malignancy, the Weiss score (WS) is the most widely used classification system, which includes pathological assessment of adrenocortical tumors (100, 106, 107). It consists of nine morphological parameters with, since 1989, a threshold for malignancy of at least three criteria present in the tumor (Table 3) (108). In addition to conventional ACC, distinct subtypes, such as oncocytic, myxoid,

and sarcomatoid variants have been described. The WS suffers a high interobserver variability, and is difficult to apply in ACC variants and pediatric adrenocortical tumors (109, 110). Thereby, the WS is challenged and lacks reliability in tumors with a WS of 2 or 3, since cases have been described with a WS of 2 that metastasized during follow-up (111-113). Adrenocortical tumors with a WS of 2 or 3 can in some cases thus be considered as borderline malignant. It is recommended that adrenocortical tumors that cannot be readily classified, and all suspected ACC, are reviewed by an expert adrenal pathologist (100). Different more simplified algorithms have been proposed with only the most reliable parameters included, like the revised WS by Aubert et al. (114). The Helsinki score consists of the sum of 3 x mitotic rate + 5 x presence of necrosis + maximum proliferation index (Ki67) (115). This scoring system was able to diagnose metastatic ACC with 100% sensitivity and 99.4% specificity, whereas the revised WS of Aubert et al. had a sensitivity of 100% and specificity of 96.9%. To prevent overdiagnosis in oncocytic variants with the classic Weiss score, an alternative diagnostic system was proposed, the Lin-Weiss-Bisceglia (LWB) system (116), and also validated to correctly predict malignancy in this ACC variant (117). Because of the remaining difficulties with the Weiss score and the LWB system, and because still a definite diagnosis can only be made in case of locoregional invasive tumor growth or the presence of metastasis, pathologists have put effort in developing new techniques to refine the diagnostic assessment of adrenocortical tumors.

Table 3. Characteristics of the Weiss score

Histological criteria	Weight of Criteria	
	0	1
Nuclear grade	1 and 2	3 and 4
Atypical mitotic figures	No	Yes
Mitotic rate	< 5 per 50 HPF	≥ 6 per 50 HPF
Clear cells	> 25%	≤ 25%
Diffuse architecture	≤ 33% surface	> 33% surface
Confluent necrosis	No	Yes
Venous invasion	No	Yes
Sinusoid invasion	No	Yes
Invasion of tumor capsule	No	Yes

Presence of three or more criteria is related to malignancy of the adrenal cortex. HPF, high power fields.

Ki67, a marker for proliferation, has raised attention for its use in the differential diagnosis of adrenocortical tumors (118, 119), with a mean sensitivity of 78% and specificity of 96% (120). The general agreement is that ACCs have a Ki67 labeling index of ≥ 5%. It is recommend that the Ki67 is introduced in the routine pathology for adrenocortical tumors (100).

Volante et al. demonstrated that disruption of reticular networks, defined as the loss of continuity of reticular fibers or basal membrane network as highlighted by histochemical staining, was present in all ACCs included in their study (n = 92, (121)). By adding at least one of the following three parameters – necrosis, high mitotic rate or vascular invasion – this reticulin algorithm identified malignancy with a sensitivity and specificity of 100% (121). A study aiming to validate the presence of reticulin fibre disruptive changes in 178 adrenocortical tumors showed that a specific training increased the interobserver reproducibility to 86% (122). Specifically for cortical tumor variants like oncocytic and myxoid subtypes, this algorithm might be applicable (123-125).

Urine steroid metabolomics

Urine steroid metabolomics might offer an alternative diagnostic tool for malignancy of adrenal tumors and is based on aberrant amounts of adrenal steroids secreted by ACCs. In a series of 102 patients with ACAs and 45 with ACCs, urinary steroid profiling differentiated ACCs from ACAs with a sensitivity and specificity of 90% (126). These findings, however, have to be validated.

Molecular markers

Several larger molecular studies have greatly expanded our knowledge in the field of ACC pathogenesis, and have demonstrated molecular heterogeneity in ACC regarding epigenetics, miRNA expression, gene expression (transcriptome), recurrent mutations, and chromosome alterations. Differences between ACC and ACA have also been identified, which will be shortly discussed below (Fig. 4). *IGF2* is the most widely known overexpressed gene in ACC, but does not fully discriminate ACC from ACA (127, 128). Insights and interest in the imprinted *IGF2* gene comes from an association of ACC with the Beckwith-Wiedemann syndrome, in which the 11p15 region (IGF2, H19, and CDKN1C genes) shows altered expression (95, 129). ACC are thereby found to harbor global hypomethylation, whereas CpG islands in promoter regions are hypermethylated compared to normal adrenals and ACAs (130-132). Several studies have focused on the relevance of microRNAs (miRNAs), short noncoding sequences regulating gene expression post-transcriptionally (133). MiR-483-5p and miR-483-3p are the most consequently overexpressed miRNAs in ACCs compared to ACAs, whereas miR-195 is often found to be underexpressed (134-137).

Studies of adrenocortical tumors using comparative genomic hybridization (CGH) have shown a complex pattern of chromosomal alterations in ACCs, while ACA present few regions of chromosomal gains and losses (138-143). More recently, frequent recurrent copy number variations were identified at 5p15 and deletions at 22q12.1 (144). Regions contain *TERT*, encoding telomerase reverse transcriptase, and the *ZNRF3* gene, which is recently reported to act as a tumor suppressor gene (145), respectively. The studies together show

the diversity and heterogeneity of chromosomal gains and losses in ACC. TP53 is one of the most frequent mutations identified in ACC (15-35% of cases), discovered on the basis of the association with the Li-Fraumeni syndrome (146). The second frequently mutated driver gene in ACC is *CTNNB1* (β-*catenin*), with a prevalence of 20-30% of samples (144, 147-151). Recently, *ZNRF3* was identified as a new tumor suppressor gene driving ACC pathogenesis (10-21% of cases), with inactivation, most frequently by a homozygous deletion (144, 151). Several other genes are frequently mutated in ACCs, but because of the lack of a discriminative value and the relative rarity of genetic abnormalities in ACCs, mutation studies are not primarily used to diagnose ACCs, but to identify potential novel targets for therapy and prognosis stratification.

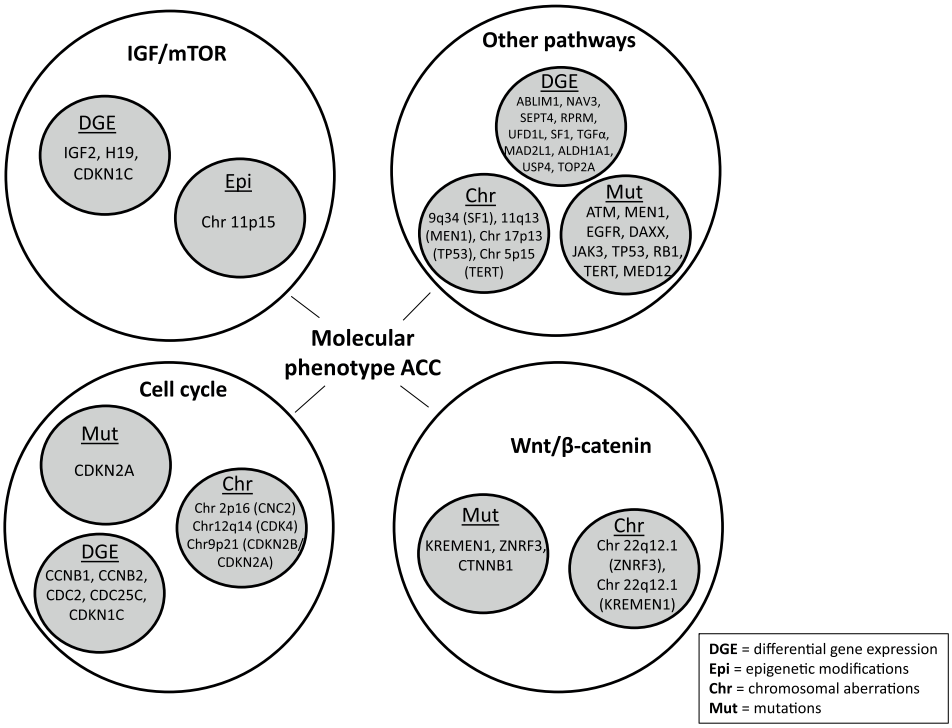

Figure 4. Most frequently altered pathways in adrenocortical carcinomas (ACC) compared to adrenocortical adenomas, with molecular aberrations involving the IGF/mTOR-, cell cycle-, and the Wnt/β-catenin pathway. Alterations are organized per molecular aberration. Chr, chromosomal aberrations; DGE, differential gene expression, consisting of both up- and downregulated genes in ACC; Epi, epigenetic modifications; Mut, mutations.

Liquid biopsies

In other types of cancer, as well as in ACC, researchers have been exploring the use of liquid biopsies. A liquid biopsy is based on minimally invasive blood tests and provides a potentially powerful and reliable clinical tool for individual molecular profiling of patients in real time (152). Circulating tumor cells have been found in the blood of ACC patients, but further research is necessary to validate these findings and investigate the potential to monitor disease progression and drug response (153). To date, three studies expanded on using serum miRNAs, of which miR-483 harbors the highest potential for use as a noninvasive biomarker in ACC (137, 154, 155). Another potential liquid biopsy, circulating cell-free tumor DNA (ctDNA) analysis, is now emerging and particularly appealing due to the ease in collection of the plasma, without the need for prior enrichment and isolation of a rare population of cells (156). Circulating tumor DNA release largely depends on tumor type and disease stage and generally presents only a variable and small fraction of the total circulating cell-free DNA (157, 158). CtDNA has not yet been investigated in ACC.

Treatment of adrenocortical carcinoma

Treatment of ACC is dependent on the ENSAT tumor stage at diagnosis (Fig. 5). During all steps, clinical trials have to be considered, as these are the best way to improve our knowledge and patient care.

Surgery

For localized ACC, successful tumor-directed surgery is the only potentially curative treatment. However, even after complete resection, recurrence rates are high (30-50%), and are even higher in patients with incomplete resection (93, 161-165). To reduce the amount of recurrences with the goal of a R0 resection (microscopically free margins), it is recommended to perform adrenalectomy only in specialized centers (100). Open adrenalectomy with lymph node dissection is regarded as standard treatment for ACC (166). For patients with ENSAT stage I-II ACC with a diameter < 6 cm, laparoscopic resection is reasonable if oncological standards are respected (100). In patients with stage IV disease, debulking surgery can be beneficial, in case of severe hormone excess which cannot be controlled otherwise, a limited number of organs with tumor metastases (≤2) involved, and a resectable tumor mass (167). When this is not the case, medical therapy should be started as soon as possible (Fig. 5).

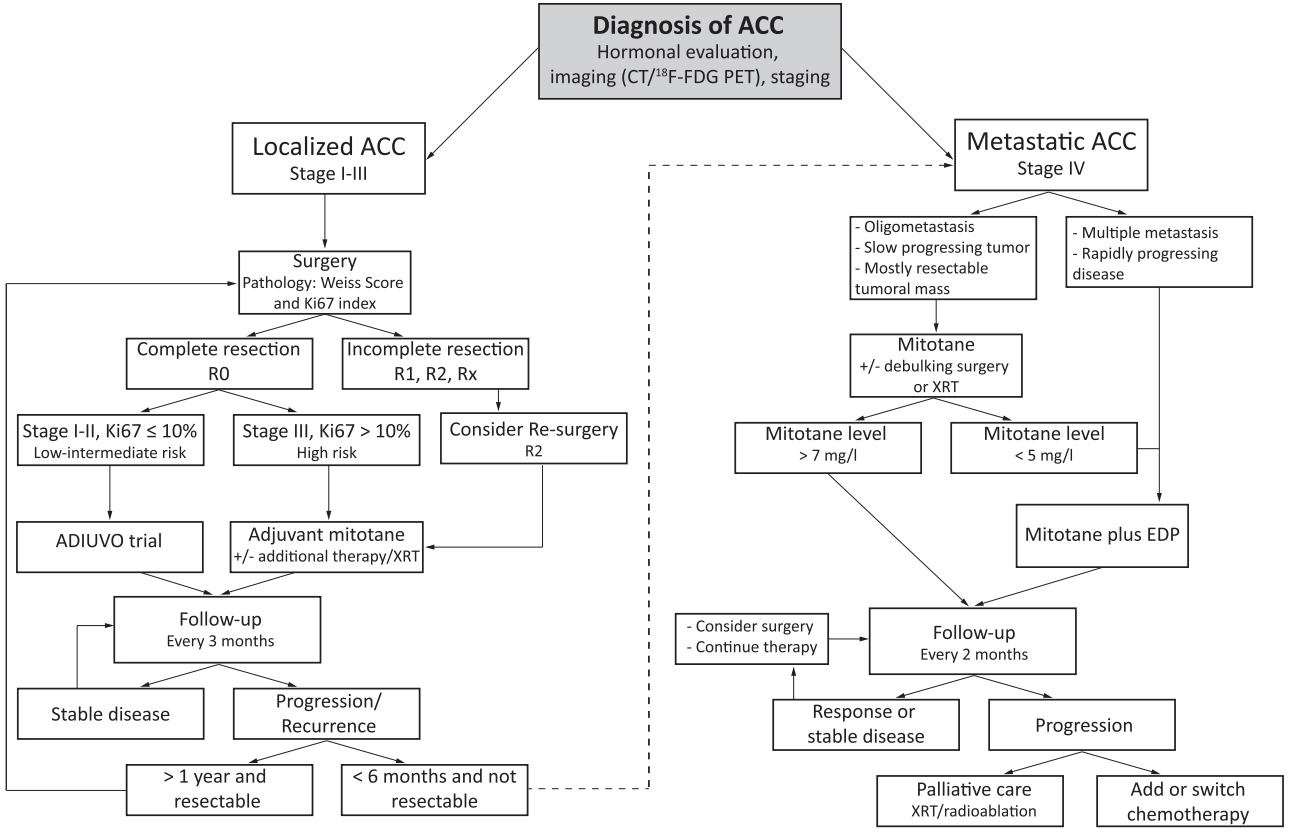

Figure 5. Algorithm for the management of adrenocortical carcinoma (ACC). CT, computed tomography; EDP, etoposide, doxorubicine and cisplatin; ^{18}F-FDG PET, 18F-fluorodeoxyglucose positron emission tomography; Ki67, proliferative index; R1, incomplete microscopic resection; R2, incomplete macroscopic resection; Rx, unknown margin status; XRT, radiotherapy (73, 159, 160).

Mitotane treatment

Mitotane [1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane (o,p'-DDD)] was first described to have therapeutic effects on the adrenal cortex in 1949 (168). To date, it is the only approved drug for treatment of ACC (169). Mitotane is thought to act primarily by disruption of mitochondria and thereby activating an apoptotic process (170). Recently, endoplasmic reticulum stress was identified as a key molecular pathway activated by mitotane, in which Sterol-O-Acyl-Transferase 1 (SOAT1) was identified as a key molecular target (171). Mitotane is difficult to manage clinically and mitotane use is often accompanied by severe adverse effects, limiting long-term tolerance (162). Side effects mainly consist of gastrointestinal (nausea and diarrhea), neurological (confusion and sleepiness), metabolic and endocrine effects. As mentioned in PART I of this chapter, mitotane induces CYP3A4 activity, which indicates potentially relevant drug interactions (172). This issue needs to be considered when designing clinical trials in patients with ACC. Mitotane can lower cortisol levels by several mechanisms, as also described in PART I of this chapter, making glucocorticoid replacement therapy required.

The high recurrence rates after surgery provide the rationale for adjuvant treatment of ACC. However, the efficacy of mitotane as adjuvant treatment modality for patients with ACC has been only investigated retrospectively and studies report discordant results (164, 173-175). The main challenge of the retrospective design is that patients may have received mitotane based on unfavorable clinical or tumor characteristics, which makes the comparison between groups challenging. Currently, adjuvant mitotane treatment is recommended in patients with high recurrence risk postsurgically (i.e. stage III, Ki67 > 10%, R1 or Rx resection; Fig. 5), if tolerated for at least two years (100). The ADIUVO study, a phase III trial, is now being conducted to address the need for adjuvant mitotane treatment in patients who undergo R0 resection and have low-to-intermediate recurrence risk (stage I-III, Ki67 ≤ 10%).

For patients with unresectable or metastastic ACC, all therapies should be considered palliative. The first line treatment option is mitotane (Fig. 5). About one-third of the patients with advanced ACC treated with mitotane either obtain complete response, partial response or stable disease (Table 4) (120). A subgroup of patients also has very slow disease progression while on mitotane therapy. In case the blood mitotane concentration is still below 5 mg/L after 3 weeks of treatment, or in case of progression of advanced disease, a combination of mitotane with chemotherapy can be considered (Fig. 5).

These response rates indicate that we face the important challenge of identifying a subpopulation of patients who do benefit from mitotane treatment. To date, reaching the target mitotane plasma concentration (14-20 mg/L) is the most important predictive

factor and careful monitoring is thus of great importance. Several studies have shown that patients with advanced ACC who reached this target concentration had fewer recurrences and showed a prolonged recurrence-free survival (176-178). Several other factors have been proposed that may be helpful in the prediction of treatment response. Low ribonucleotide reductase large subunit 1 (*RRM1*) gene expression was associated with a shorter disease-free survival and overall survival in patients treated with mitotane (179). As a possible mechanism, Germano et al. showed that the *RRM1* gene interferes with mitotane metabolism in ACC cells (180). Besides, high protein expression of CYP2W1, independent of ENSAT stage, has been associated with a longer overall survival and time to progression in patients treated with mitotane. This difference in survival was not significant in patients who underwent follow-up only (181). SOAT1 expression, which has previously been identified as a key molecular target of mitotane, also correlated with response to mitotane *in vivo* (171). Finally, it is hypothesized that patients with cortisol-producing ACC specifically benefit from mitotane treatment, although data have been inconsistent (89, 174, 182). The most important explanation is the decrease in cortisol production with concomitant improvement of comorbidities associated with CS.

Radiotherapy

For decades, ACC was considered a radiotherapy resistant cancer and studies reported poor and contradictory results of postoperative radiotherapy (160). More recently, several studies have shown a role of radiotherapy in improved control of local disease postoperatively (56 – 100% of patients had local control), although no effects on disease-free and overall survival were found (189-193). Prospective studies are required to establish the value of adjuvant radiotherapy for local disease control or for palliation.

Chemotherapeutic drugs

Although several cytotoxic drugs have been studied in advanced ACC, only a few large trials have been performed. The first randomized trial showed that for patients with advanced ACC, a combination of mitotane with etoposide, doxorubicine, and cisplatin (M-EDP) had a longer median progression-free survival as compared to patients receiving streptozotocin and mitotane (5.0 vs 2.1 months) (194). No effect on overall survival was observed. Although the median overall survival is only 14.8 months, this regimen (M-EDP) is the preferred choice in case of multiple metastases, rapidly progressing disease, or in case of progression of advanced disease after mitotane monotherapy. This regimen is often associated with dose-limiting adverse side effects. EDP is usually administered for a maximum of 6-8 cycles, whereas mitotane is maintained until progression. Other possible chemotherapeutic options if patients fail M-EDP are gemcitabine with or without capecitabine or mitotane (195-197), and streptozotocin with mitotane (194, 198). Overall, response rates are very low. Since cytotoxic therapies are not effective in many patients,

research is focusing on factors associated with sensitivity and identifying patients who are likely to respond. One of the possible explanations of low efficacy of chemotherapy in ACC includes the multidrug resistance gene, *MDR1*, which is highly expressed in the adrenal gland (199-203). It is supposed to be one of the mechanisms of adrenal cells to handle their high steroid environment. P-glycoprotein, the protein encoded by the *MDR1* gene, is an enzyme that pumps a variety of structurally unrelated compounds, like chemotherapy, out of the cell. There is still limited knowledge about the exact role of P-gp inhibition in enhancing efficacy of chemotherapy in ACC patients. Mitotane has been studied as a P-gp inhibitor, but data on its role appeared to be equivocal (202, 204, 205). High protein expression of the excision repair cross complementing group 1 (ERCC1) is thought to be a predictor of response to platinum-based chemotherapy, since it was associated with a worse overall survival in ACC patients treated with these compounds (206). In poorly differentiated endocrine carcinomas, the chemotherapeutic drug temozolomide (TMZ) has shown efficacy in 17 of the 25 patients (207). TMZ is used as cytostatic drug incorporated in the standard care for patients with malignant gliomas (208). Epigenetic marks regulating O6-methylguanine-DNA methyltransferase (MGMT) expression are now used as a predictive marker for response to TMZ in glioblastoma patients (209). In neuroendocrine tumors, the combination of capecitabine and TMZ (CAPTEM) is under investigation, which combination potentially leads to synergistic effects (210). In ACC, capecitabine has been studied in combination with gemcitabine, resulting in moderate activity (195), but TMZ has not been investigated in ACC yet.

Pathway-driven therapies

New insights in molecular and genetic alterations underlying ACC pathogenesis have led to the identification of several potential therapeutic targets, although results have been largely disappointing. Targeting the IGF-mTOR pathway, the vascular endothelial growth factor receptor, and the epidermal growth factor receptor, have become the main focuses for development of targeted therapy in ACC. Only a small subset of patients appears to benefit from targeted therapies in ACC (211-215). There is evidence that monotherapy with tyrosine kinase inhibitors (TKIs) causes compensatory activation of other signaling pathways (216), and because of the lack of efficacy of monotherapy in clinical trials, the general view is that combination therapy is potentially more effective in patients with ACC. Sunitinib, a multi-TKI, resulted in stable disease in 5/35 patients (217). Discouraging results were obtained from clinical trials with several other multi-TKI (218-221). Thus, an important challenge is to search for predictive factors.

Table 4. Efficacy of mitotane as therapy for advanced/metastatic adrenocortical carcinomas

Study	Multi-center	With mitotane (n)	Without mitotane (n)	Follow-up	RR With mitotane
(183)		21	25	5 y	NR
(184)		7	43	2.4 y	7/7
(185)		8	6	Minimal 12 mo	NR
(186)		11	36	NR	NR
(187)		7	11	NR	2/7
(173)	x	47	130	Median 43-67.6 mo	23/47
(182)		86	80	NR	NR
(164)		22	196	Mean 88 mo	12/22
(174)	x	251	273	Median 50 mo	NR
(175)	x	84	235	Median 43.7 mo	NR
(175)	x	142	76	Median 69.8 mo	NR
(188)	x	88	119	Median 44 mo	44/88
Total		767	1,230		51% (88/171)

Total rates represent weighted means. All studies are retrospective. DFS, disease-free survival; mo, months; NR, not reported; OS, overall survival; RR, recurrence rate;

Prognostic markers

Clinical features

Several studies report a decreased overall survival for patients with cortisol-secreting ACC (89, 222, 223). The exact mechanism remains to be elucidated, although it is known that overt CS represents an important cause of postsurgical and postchemotherapy morbidity, and cortisol can have immunosuppressive effects favoring tumor progression (165). An older age has also been associated with an adverse prognosis (88, 89), but data are inconsistent (173, 224).

Pathological parameters

Among the pathological parameters, the resection status, and the Ki67 index are the most important and validated prognostic factors for patients with ENSAT stage I-III ACC, together with the ENSAT stage (93, 100, 225). In a large study (n = 319, validation cohort n = 250) evaluating the prognostic value of histopathological, clinical and immunohistochemical markers, Ki67 alone most powerfully predicted recurrence-free and overall survival (175). In a large ENSAT study including 444 patients with stage III or IV ACC, several factors appeared to be important for prognostication, namely a modified ENSAT classification (III, IVa, IVb, IVc), tumor grade (Weiss > 6 and/or Ki67 ≥ 20%), resection status, age, and

RR Without mitotane	DFS	OS	Comments
NR	=	=	
35/43	=	NR	Comparison between no adjuvant treatment (*n* = 44) and adjuvant treatment (mitotane *n* = 7, radiotherapy *n* = 3)
NR	↓	NR	
NR	=	=	
8/11	=	=	
110/130	↑	=	Italian and German control group
NR	=	NR	
160/190	↑	=	
NR	↑	=	
NR	=	↑	Data of German cohort. Effect on OS only significant in multivariable analysis.
NR	=	=	Validation cohort
53/119	=	=	No effect in multivariable analysis
74% (366/493)			

y, years; =, no statistically significant difference between mitotane or no mitotane administration; ↓, decreased survival time, ↑ increased survival time under adjuvant mitotane treatment.

tumor- or hormone-related symptoms (161). The Weiss score has also been associated with prognosis, however findings are inconsistent between different studies. Mitotic activity has been reported as the most significant determinant of survival (110, 226).

Molecular Characteristics

Several molecular markers have been proposed for prognostic classification of ACC. Using gene expression profiles (transcriptome), two subgroups of ACCs have been identified: cluster C1A and cluster C1B, with a remarkably worse outcome in cluster C1A (127, 227-229) (151). Cluster C1B could be further divided into three subgroups, with inactivating *TP53* mutations (C1A-p53), activated β-catenin (C1A-β-catenin), and one group with an unidentified molecular alteration (C1A-x) (230). Barreau et al. made a correlation of DNA methylation with survival outcome in patients with ACC (131). In this study, a CpG island methylation phenotype (CIMP) was defined as having a higher methylation compared to ACAs. The CIMP group could further be divided into CIMP-high and CIMP-low, of which the high group was associated with a poor prognosis (131). Remarkably, the C1A-p53 and C1A-x subgroups with poor prognosis showed a CIMP profile, whereas the C1A-β-catenin and the good-prognosis (C1B) group showed a non-CIMP profile.

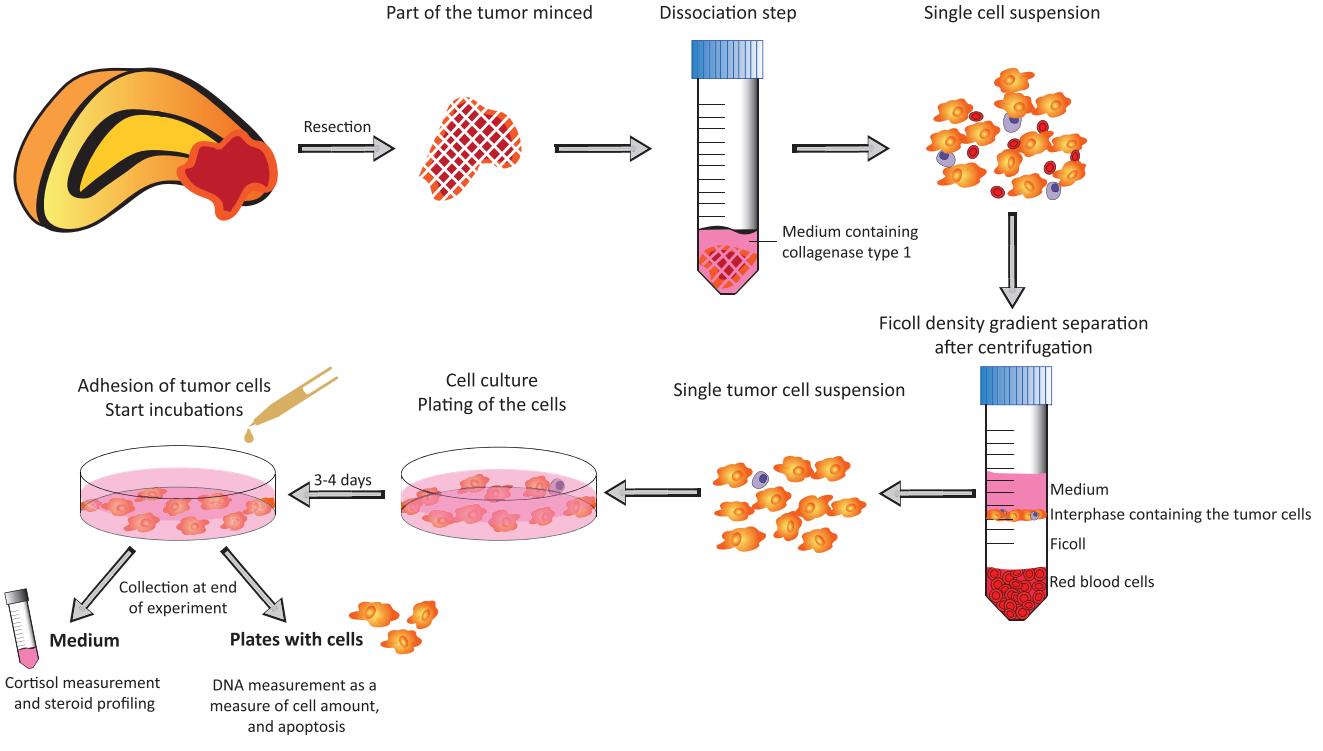

Figure 6. The different steps for the processing of adrenocortical tissues to obtain primary cultures. Immediately after surgery, a part of the specimen is minced into small pieces of 2-3 mm^3, and dissociated using medium supplemented with collagenase type 1 at 37°C for up to two hours. If necessary, the obtained suspension is further dissociated into single cells through a sterile needle (not shown). Ficoll density gradient separation is used to separate contaminating red blood cells from tumor cells. Contamination of lymphocytes plays only a minor role in adrenocortical tissues, and these cells do not attach during culturing. The tumor cells are plated, and allowed to attach for 3-4 days. The medium is refreshed before the incubation starts. At the end of the experiments, medium or plates are analyzed, dependent on the research aim.

More specifically, expression levels of several genes have been correlated with clinical outcome in ACC, like the steroidogenic factor 1 (SF1), matrix metalloproteinase type 2, glucose transporter GLUT1, pituitary tumor transforming gene 1 (PTTG), the transforming growth factor β signaling mediator SMAD and the transcription factor GATA-6 (231-235).

AIMS AND OUTLINE OF THIS THESIS

PART I

Several agents are now under investigation in order to improve efficacy and to reduce side effects of medical treatment for CS. Levoketoconazole (COR-003), the single 2S,4R enantiomer of ketoconazole, is now developed as a new investigational drug for the treatment of hypercortisolism in CS (236). Levoketoconazole blocks CYP11B1 and CYP11B2, CYP17A1, and CYP11A1 (237). It is suggested to be a more potent inhibitor of cortisol synthesis, to induce less liver toxicity, and to have a reduced hepatic metabolism compared to racemic ketoconazole (236). A clinical trial with levoketoconazole has recently been finalized in patients with CS, of which initial results show normalized UFC in 38% (n/N = 40/94) of CS patients treated with levoketoconazole after a 6 months maintenance phase (238).

Osilodrostat (LCI699) is another novel steroidogenesis inhibitor, which was originally developed for its inhibitory effects on aldosterone production, and blood pressure lowering abilities (239-241). However, in these studies, a blunted cortisol response to synthetic ACTH was observed, which raised its attention for treatment of CS. The efficacy of osilodrostat has been assessed in an extended phase II study in patients with CD (242). Of the 19 patients, 78.9% had normalized UFC levels after 22 weeks. Treatment was generally well tolerated (242). A phase III trial was recently completed. However, effects of osilodrostat on other enzymes and steroid precursors of the adrenal steroidogenesis are yet unknown.

The aim of the first part of this thesis is to explore the *in vitro* effects of the two novel steroidogenesis inhibitors, levoketoconazole and osilodrostat, focusing on both adrenal steroidogenesis and potential pituitary-directed effects. Effects will be assessed in HAC15 cells and in primary cultures of adrenocortical tumors or adrenal hyperplasias (Fig. 6). In **Chapter 2**, the effects of levoketoconazole are compared to those of racemic ketoconazole, which may provide insights to answer the question whether levoketoconazole could be an alternative to racemic ketoconazole for the treatment of CS. In order to investigate the potential of osilodrostat as a novel treatment for CS, this compound is compared with metyrapone as well as with ketoconazole. The results of this *in vitro* comparative study are presented in **Chapter 3**.

PART II

The general aim of the second part of this thesis is to provide novel insights into diagnostic and therapeutic strategies in ACC. As it comes to preclinical studies in ACC, one of the challenges is the limited availability of ACC cell lines, and due to its rarity the limited availability of primary cultures (Fig. 6). In this thesis, we aim to combine cell line studies, primary ACC cultures obtained during several years of research, analyses of ACC, as well as other adrenocortical specimens, blood samples, and clinical patient characteristics, in an attempt to make the translation from preclinical research concepts to the potential use in clinic.

As stated earlier, the pathological diagnosis of ACC remains challenging and there is a need for a more unequivocal classification of adrenocortical tumors. Early and correct adjunction of adrenocortical tumors is important in order to establish an early appropriate therapeutic strategy. The IGF2 gene is the most frequently overexpressed gene in malignant adrenocortical tumors. In **Chapter 4**, we aim to investigate whether DNA methylation patterns of several IGF2 regulatory regions can discriminate adrenocortical carcinoma from adenoma by calculating an IGF2 methylation score. In **Chapter 5**, these findings are validated through collaboration with the ENSAT consortium in a multicenter European cohort study. In this study, the IGF2 methylation score is also correlated with follow-up clinical characteristics and outcome in patients with ACC.

In ACC, the hypothesis that the molecular heterogeneity drives the heterogeneous and variable clinical features, treatment response and disease course is currently accepted. To endeavor novel non-invasive approaches for monitoring and/or classifying patients with ACC, we aim to identify cell-free circulating DNA derived from the tumor in patients with ACC in **Chapter 6**. In other types of cancer, many efforts are made into research focusing on liquid biopsies, by assessing specific molecular markers in the bloodstream of patients. These studies revealed that ctDNA might be associated with disease course.

Surgery is the only curative treatment for patients with ACC, but systemic therapies are needed as adjuvant treatment or in the advanced setting. Mitotane is only effective in a subset of patients with ACC and is associated with severe toxicity. There is an urgent need for markers to identify patients who will respond to mitotane in order to prevent overtreatment, unnecessary adverse effects, and to safe costs. In **Chapter 7**, we aim to explore the efficacy of mitotane *in vitro* in a unique large panel of human primary ACC cultures, and to investigate the relationship with clinical characteristics and potential predictive markers for response to mitotane. To further explore the efficacy of currently used treatment modalities, and to investigate ways to improve efficacy, the purpose of **Chapter 8** is to explore the role of P-glycoprotein in ACC. The effect of P-glycoprotein

inhibition on sensitivity is investigated for all compounds of the M-EDP regimen, the first-line chemotherapeutic regimen in ACC. In **Chapter 9**, we aim to investigate the therapeutic potential of a compound not previously investigated in ACC, temozolomide. We furthermore assess the potential role of the *MGMT* gene in sensitivity to TMZ in ACC.

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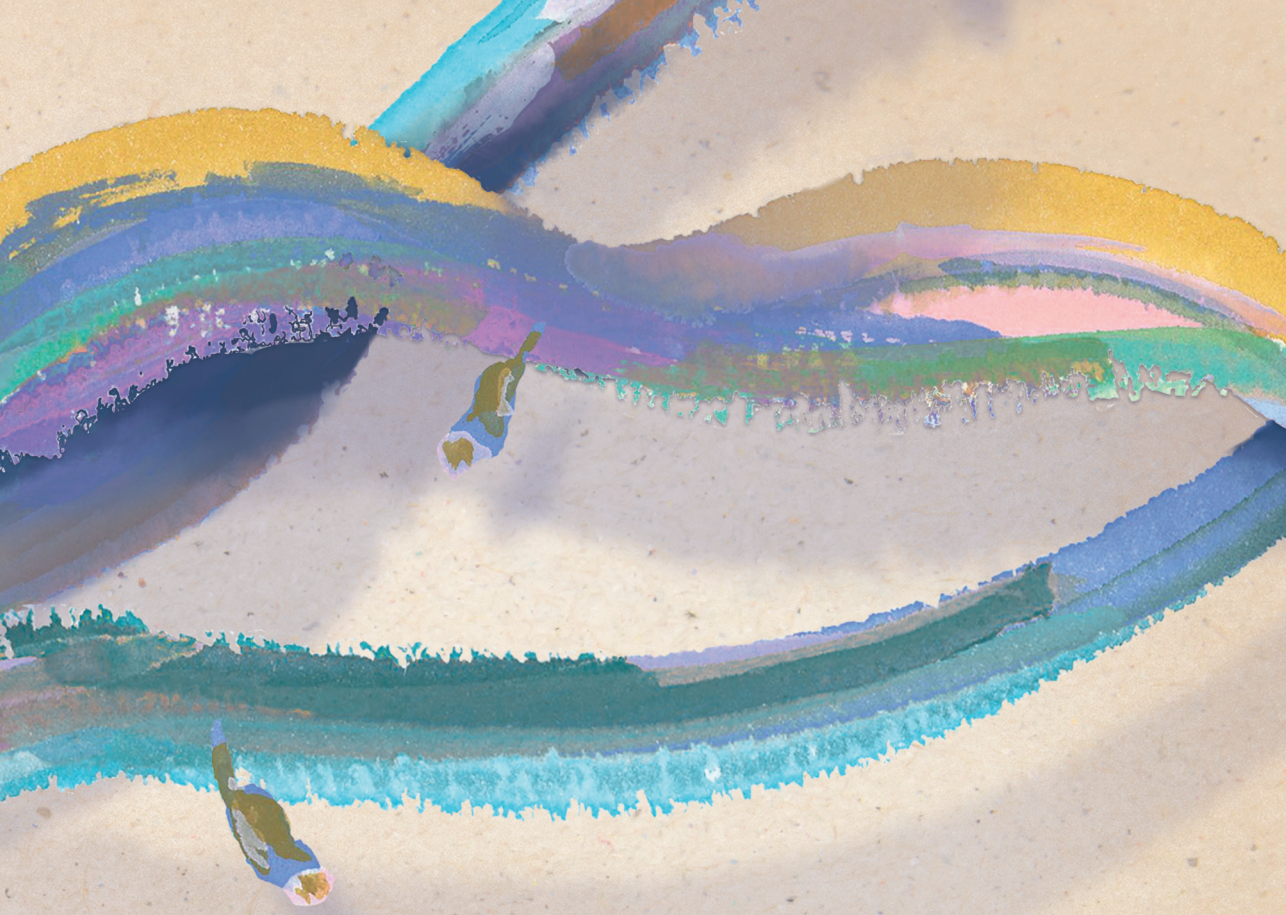
PART I



CHAPTER 2

Levoketoconazole, the single 2S, 4R enantiomer of ketoconazole, as a potential novel steroid synthesis inhibitor for medical treatment of Cushing's syndrome

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ABSTRACT

Introduction: Racemic ketoconazole (RK) is a steroidogenesis inhibitor used for the treatment of Cushing's syndrome. Levoketoconazole (COR-003), the single 2S,4R enantiomer, is potentially more potent and safe compared to RK. We compared the *in vitro* effects of levoketoconazole and RK on adrenal steroidogenesis, and on ACTH secretion by corticotroph pituitary adenoma cells.

Materials and methods: HAC15 cells and 15 primary human adrenocortical cultures, with or without ACTH, and murine (AtT20) and human corticotroph adenoma cultures, were incubated with levoketoconazole or RK (0.01 - 10 μM). Cortisol and ACTH were measured in the supernatant using a chemiluminescence immunoassay system (Immulite 2000XPi). Steroid profiling was carried out by liquid chromatography-mass spectrometry (LC-MS/MS).

Results: In HAC15 cells, levoketoconazole inhibited cortisol production at lower concentrations (IC_{50}: 0.300 μM) compared to RK (0.611 μM; $P < 0.0001$). IC_{50} values of levoketoconazole for basal cortisol production in primary adrenocortical cultures varied over a 24-fold range (0.00578 μM to 0.140 μM), with a trend towards higher sensitivity to levoketoconazole compared to RK. At equimolar concentrations, levoketoconazole had significantly stronger inhibitory effects on steroid concentrations determined by LC-MS/MS in HAC15 cells and adrenocortical cultures compared to RK. In AtT20 cells, levoketoconazole inhibited cell growth and ACTH secretion, and 5 μM of levoketoconazole inhibited cell number in one of two primary human corticotroph pituitary adenoma cultures (-44%, $P < 0.001$).

Conclusion: Levoketoconazole is a potent inhibitor of cortisol production in human adrenocortical cells, with variable degrees of suppression between tissue specimens. Levoketoconazole inhibits adrenal steroid production more potently compared to RK, and might also inhibit ACTH secretion and growth of pituitary adenoma cells. Together with the previously reported potential advantages, this indicates that levoketoconazole can be a promising novel treatment for Cushing's syndrome.

INTRODUCTION

Endogenous Cushing's syndrome (CS) is characterized by chronic glucocorticoid excess and is associated with significant comorbidities potentially leading to increased mortality (1). CS can be caused by adrenocorticotropic hormone (ACTH) overproduction by a pituitary adenoma or by non-pituitary tumors, or by autonomous cortisol production by an adrenal tumor or hyperplasia (1). The primary treatment modality of CS is surgical resection of the underlying cause (1). Medical therapy can be applied as pretreatment before surgery, in case of surgical failure, in the acute setting with complications of (severe) hypercortisolism or in patients with inoperable neuroendocrine- or adrenocortical tumors (2). Medical therapy can be divided into pituitary-targeting drugs, adrenal steroidogenesis inhibitors, and glucocorticoid receptor antagonists (2). The most important adrenal blocking drugs include ketoconazole, metyrapone, mitotane, and etomidate.

Ketoconazole, originally developed as an antifungal agent, is one of the most widely used cortisol lowering drugs for the treatment of CS. It is a 50/50 racemic mixture of two enantiomers (2S,4R and 2R,4S) (2, 3). The most severe side effect is hepatotoxicity (3, 4). Levoketoconazole (COR-003) is the purified 2S,4R enantiomer of ketoconazole. Based on early *in vitro* analyses, levoketoconazole is thought to inhibit CYP11B1, CYP17A1, and CYP21A2 enzymes more potently compared to the 2R,4S enantiomere (5). Increased potency was also shown in a preclinical study in rats, where levoketoconazole more potently inhibited serum corticosterone, the main glucocorticoid in rats, compared to 2R,4S ketoconazole (5). This may allow for a lower dose of levoketoconazole compared to racemic ketoconazole to achieve the same efficacy, and thus an increased therapeutic index. *In vitro* studies in rats suggest that levoketoconazole may have a favorable safety profile compared to racemic ketoconazole, based on less potent inhibition of CYP7A, compared to the 2R,4S enantiomer (6). Decreased CYP7A activity may lead to decreased bile acid production and functional cholestasis, which may cause hepatotoxicity. A clinical study in patients with type 2 diabetes mellitus showed decreased low-density lipoprotein cholesterol levels after 14 days of treatment with levoketoconazole 200-600 mg (7), suggesting that levoketoconazole may have beneficial metabolic effects. In a comparative study in 24 healthy subjects, levoketoconazole (400 mg daily) inhibited serum cortisol slightly more potently compared to racemic ketoconazole (5). Besides, levoketoconazole plasma levels appeared to be 3-fold higher compared to those of the 2R,4S enantiomer (5), suggesting a lower hepatic metabolism of levoketoconazole. Headache and nausea were the most commonly reported adverse events (5, 7). Initial results of a prospective, open-label, phase III maintenance-of-benefit study investigating levoketoconazol resulted in normalized UFC in 38% (n/N = 40/94) of CS patients after a 6 months maintenance phase (8) . Currently, an international multicenter single-arm, open-label, dose titration study is

being conducted to assess the efficacy and safety of ascending doses of levoketoconazole in patients with elevated urinary free cortisol (UFC) concentrations due to Cushing's syndrome (NCT01838551) (9).

Taken together, levoketoconazole might inhibit cortisol synthesis more potently, might have a reduced hepatic metabolism, and may have less hepatotoxic effects compared to racemic ketoconazole. The aim of this study is to compare the direct effects of levoketoconazole on basal and ACTH-stimulated adrenocortical steroid production to those of racemic ketoconazole. *In vitro* studies were performed in HAC15 cells and in primary adrenocortical cultures by assessing the concentrations of steroids in the supernatant after treatment with both compounds. Finally, we assessed the pituitary-directed effects of both levoketoconazole and racemic ketoconazole on cell amount and ACTH secretion in pituitary corticotroph cells.

MATERIALS AND METHODS

Cell culture and compounds

Human adrenocortical carcinoma HAC15 (kind gift by dr. W. Rainey) and mouse corticotroph AtT20 cells (ATCC number: CRL-1795) were used. Dulbecco's Modified Eagle Medium F12 containing 5% fetal calf serum was used for HAC15 cells, whereas AtT20 cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum. Both media were supplemented with L-glutamine (2 mmol/L) and penicillin (10^5 U/L). Medium and supplements were obtained from Fisher Scientific (Landsmeer, the Netherlands), except penicillin, which was obtained from Bristol-Meyers Squibb (Woerden, the Netherlands). HAC15 and AtT20 cells were cultured in 75cm^2 flasks at 37°C in a humidified incubator (Greiner Bio-One, Alphen a/d Rijn, the Netherlands) at 5% CO_2. Short tandem repeat (str) profiling using a Powerplex Kit (Promega, Leiden, the Netherlands) of HAC15 cells provided results consistent with the ATCC database, confirming the identity of the cell line. Once a week, cells were harvested with trypsin (0.05%)-EDTA (0.53 mM) and resuspended in culture medium. Levoketoconazole (COR-003) and racemic ketoconazole (both from Cortendo AB, Savedalen, Sweden) were dissolved in absolute ethanol (EtOH), according to manufacturer's instructions, and stored at -20°C at a stock concentration of 10^{-2} M. At the start of each experiment, both drugs were freshly diluted in EtOH to the correct concentrations. Synacten (synthetic ACTH, Novartis Pharma, Arnhem, the Netherlands) stock solution was stored at 4°C and diluted in culture medium on the day of use. The final concentration of ACTH was chosen based on a dose-response curve in HAC15 cells and on previously reported studies (10). For HAC15, 200,000 and 100,000 cells were plated in 0.5 ml medium in 24-wells plates for

experiments of 1 and 3 days, respectively. One day after seeding the HAC15 cells, medium was refreshed and cells were treated 1 or 3 days in quadruplicate with levoketoconazole or racemic ketoconazole (0.05 – 5 μM), with or without 10 nM ACTH (10). To study effects of both compounds on pituitary AtT20 cells, concentrations of 0.1 to 10 μM of both drugs were used and incubations were performed for 1, 3, and 7 days. For 7 days experiments, medium and compounds were refreshed after 3 days. Controls were vehicle treated. If compounds had an effect on cell number, steroid levels were corrected for total amount of DNA per well as a measure of cell number. DNA concentrations were determined using the bisbenzimide fluorescent dye (Hoechst 33258, Sigma-Aldrich, Zwijndrecht, the Netherlands), as previously described (11). Media were collected at the end of the experiments and stored at -20°C until analysis. Regarding AtT20 and primary pituitary adenoma culture experiments, media were supplemented with the protease inhibitor Trasylol (final concentration 5 IU per ml, Sigma-Aldrich, Zwijndrecht, the Netherlands) before storage in order to prevent degradation of ACTH. All cell culture experiments were carried out at least twice in quadruplicate.

Processing of human tissues

To obtain primary cultures, adrenal specimens (adrenocortical adenomas, adrenal hyperplasias and adrenocortical carcinomas) were collected after adrenalectomy at the Erasmus University Medical Center, Rotterdam, the Netherlands, between April 2016 and May 2018. The study was conducted under guidelines that have been approved by the Medical Ethics Committee of the Erasmus Medical Center. Furthermore, informed consent was obtained from all patients. Immediately after surgery, specimens were minced into small pieces (2-3 mm^3), washed in culture medium, centrifuged for 5 minutes at 600 g and stored overnight in culture medium at 4°C. The next day, the specimens were centrifuged again, after which the supernatant was removed. Dissociation of the fragments was performed using collagenase type 1 (10-25 ml; 2mg/ml: Sigma-Aldrich, Zwijndrecht, the Netherlands), followed by incubation at 37°C for up to two hours. We used Ficoll (GE healthcare, Eindhoven, the Netherlands) density gradient separation once or twice as required in order to separate contaminating red blood cells from the adrenal cells. Cell viability was determined by trypan blue exclusion and visually counted using Türk solution. Dissociated cells were plated at a density of 10^5 cells per well. ACTH-secreting corticotroph pituitary adenoma tissue was available after transsphenoidal surgery from two patients with Cushing's disease. Single-cell suspensions of the pituitary adenoma tissues were prepared as previously described (12).

Culture conditions for primary cultures were similar as described in the section 'Cell culture and compounds', but with small adjustments: ACTH was used at a concentration of 85 pM (250 pg/mL), treatment was started 3-4 days after plating of the cells and cells

were incubated for 3 days. For pituitary primary cultures, levoketoconazole and racemic ketoconazole were only tested at a concentration of 5 μM. Owing to a limited number of cells obtained from the specimens, not all experiments could be performed in every primary culture.

Measurement of steroid hormone concentrations

For construction of the dose-response curves, cortisol and ACTH were measured in the culture media using an Immulite 2000 XPi immunoassay analyzer (Siemens Medical Solutions USA, Inc). Samples for LC/MS-MS steroid measurements were those closest to 50% inhibition or maximal inhibition of cortisol as determined by the immunoassay. In these selected culture conditions, androstenedione, cortiosterone, cortisol, 11-deoxycortisol (11-DOC), dehydroepiandrosterone (DHEA), DHEA sulphate (DHEAS), progesterone, 17-hydroxyprogesterone (17-OHP), and testosterone were simultaneously measured using a Waters® Acquity™ UPLC HSS T3 1.8 μm column and a Waters XEVO-TQ-S system (Waters, Milford, MA, USA) equipped with an ESI source operating in the electrospray positive mode except for DHEAS (negative ESI). Intra- and inter-assay coefficients of variation for the steroid assays were <7 and <8% for androstenedione, <8 and <4% for corticosterone, <6 and <6% for cortisol, <10 and <6% for 11-deoxycortisol, <7 and <8% for DHEA, <8 and <13% for DHEAS, <6 and <7% for progesterone, <6 and <6% for 17-OHP, and <6 and <9% for testosterone. Multiple reaction monitoring was applied for the detection of the analytes using both quantifiers and qualifiers.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 6.0 (Graphpad Software, San Diego, CA). Non-linear regression curve fitting program was used to calculate the half maximal inhibitory concentrations (IC_{50}). IC_{50} values were only calculated when the curve reached a clear bottom and the top of the curve did not extend 100%. Effects of both compounds on the steroid profile were measured as absolute change compared to control and compared using the Student's t-test. When assessing differences between effects of both compounds, the percentage change was evaluated and compared in order to correct for differences in the vehicle treated control cells. Values of $P < 0.05$ were considered statistically significant and data are presented as mean ± SEM.

RESULTS

Effects of racemic ketoconazole and levoketoconazole on cortisol production *in vitro*

HAC15 cells

After 3 days of treatment, levoketoconazole more potently suppressed cortisol production in HAC15 cells compared to racemic ketoconazole, with an approximate two fold lower IC_{50} value (Fig. 1D; 3 days IC_{50} 0.300 µM, 95% CI 0.221 - 0.407 vs. 0.611 µM, 95% CI 0.425 – 0.878, $P < 0.0001$). IC_{50} values of both compounds did not significantly change when HAC15 cells were treated for 1 day (Fig. 1A), or were stimulated with ACTH (Fig. 1, B and E). ACTH stimulation resulted in a mean increase in cortisol of 34% and 61% after 1 and 3 days of incubation, respectively (Fig. 1C, F; both $P < 0.0001$). In the conditions as mentioned above, no effects on cell amount were observed.

Primary adrenocortical cultures

Characteristics of patients of whom a primary culture was obtained are listed in Table 1, with corresponding numbers that will be used to refer to throughout the Results section. Effects of levoketoconazole and racemic ketoconazole were assessed in 14 primary cultures of human adrenocortical tissue: six cortisol-producing adrenocortical adenomas (ACA), 3 ACTH-dependent adrenal hyperplasias, 3 ACTH-independent adrenal hyperplasias and 3 cortisol-producing adrenocortical carcinoma (ACC). DNA measurement was performed in 28 of 37 primary adrenal culture plates, and showed no effects of the drugs on cell number in these cultures at any of the concentrations tested.

IC_{50} values and dose-response curves for cortisol production of both compounds in the different primary adrenocortical cultures are listed in Table 2 and shown in Fig. 2 and Supplementary Fig. 1. IC_{50} values for levoketoconazole in the basal condition in ACA primary cultures varied between 0.0631 and 0.140 µM, whereas in ACTH-dependent adrenal hyperplasia the three IC_{50} values varied between 0.0220 and 0.179 µM. In the basal condition, the mean IC_{50} of levoketoconazole was 0.110 µM (95% CI 0.0867 – 0.139) in ACA ($n = 4$), 0.0562 µM (95% CI 0.0336 – 0.0940; $P = 0.0014$ vs ACA) in ACTH-dependent adrenal hyperplasia ($n = 3$), and 0.0383 µM (95% CI 0.0253 – 0.0578; $P < 0.0001$ vs ACA) in ACC ($n = 3$). In eight of the eleven conditions in which a direct comparison between levoketoconazole and racemic ketoconazole could be made in the same patient, higher IC_{50} values were observed of racemic ketoconazole compared to levoketoconazole (mean percentage increase in IC_{50} versus racemic ketoconazole 116%, range 29 – 303%). The difference, however, only reached statistical significance in three cultures corresponding to two patients (ACA no. 2 and ACTH-dependent adrenal hyperplasia no. 3). In the three remaining cultures, IC_{50} values were highly comparable between both compounds (mean difference in IC_{50} 4%, range 1 – 8%). Levoketoconazole also inhibited cortisol production in

ACC cultures (Supplementary Fig. 1). Cortisol production significantly increased in nine of the eleven primary cultures with ACTH stimulation, varying from 34% to 2239% (Table 2). In one of the six primary cultures in which basal and ACTH stimulated levoketoconazole IC_{50} values could be compared, a lower IC_{50} value was observed under ACTH stimulation (P = 0.0095, ACC no. 3).

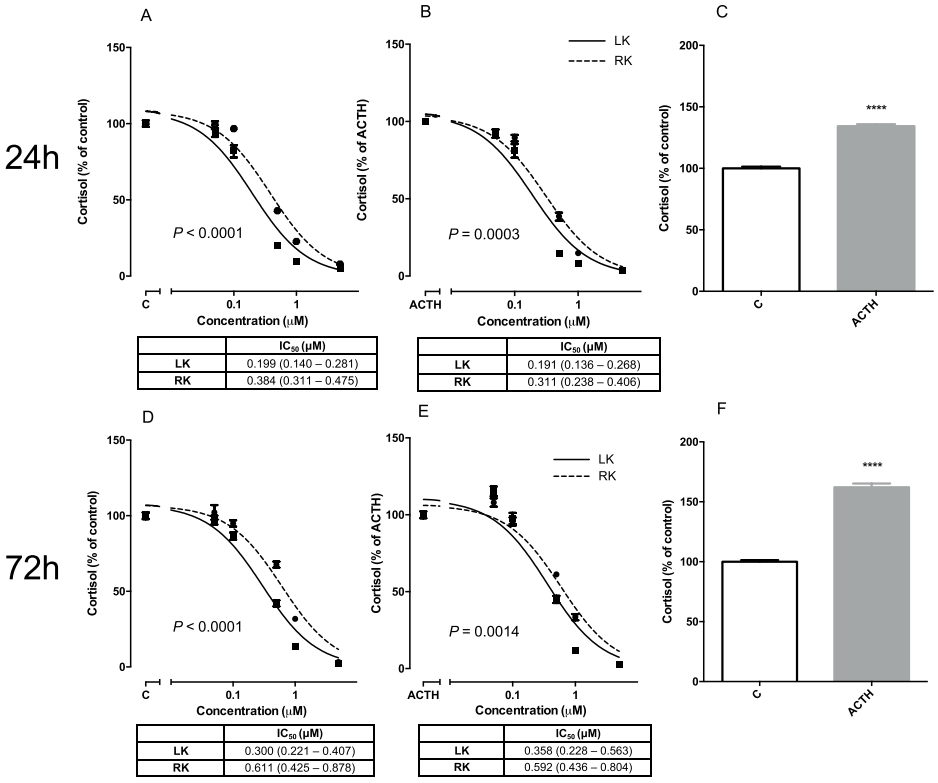

Figure 1. Dose-dependent effects of levoketoconazole (LK, solid lines, ■) and racemic ketoconazole (RK, dotted lines, ●) on cortisol production by HAC15 cells in the basal condition (A, D) and when stimulated with 10 nM ACTH (B, E) after 24 hours (24h; A, B) and 72 hours (72h; D, E) of incubation. Effects of ACTH after 24 hours (C) or 72 hours (F) of treatment. IC_{50} values are depicted in micromolar with 95% confidence interval. P-value compares IC_{50} value of levoketoconazole and racemic ketoconazole. Values are depicted as mean ± SEM and as percentage of vehicle treated control or ACTH stimulated HAC15 cells. **** P < 0.0001 versus control. ACTH, adrenocorticotropic hormone; C, control.

Table 1. Clinical and tumor characteristics of patients of whom a primary culture was obtained

Patient no.	Sex	Side	Age at surgery (yrs)	Size of lesion (cm)	Weiss score	Steroid production
Cortisol-producing adrenal adenomas						
No. 1	F	Left	57	2.5	0	Cortisol
No. 2	F	Left	67	4	0	Cortisol
No. 3	F	Left (Bilateral)	63	4.2	0	Cortisol
No. 4	F	Right	66	3.9	0	Cortisol
No. 5	M	Right	55	6.8	2	Cortisol
No. 6	F	Left	38	3.3	0	Cortisol
ACTH-dependent adrenal hyperplasias						
No. 1	F	Bilateral	79	-	-	Cortisol
No. 2	F	Left (Bilateral)	69	-	-	Cortisol
No. 3	F	Bilateral	32	-	-	Cortisol
ACTH-independent hyperplasias						
No. 1	F	Left (Bilateral)	50	-	-	Cortisol
No. 2	F	Left	73	-	-	Cortisol
No. 3	M	Left	66	-	-	Cortisol
Adrenocortical carcinoma						
No. 1	F	Right	61	5	9	Cortisol
No. 2	M	Left	64	18.5	7	Cortisol
No. 3	F	Right	67	15	8	Cortisol and androgens

ACTH-dependent adrenal hyperplasias are based on ectopic ACTH syndrome (no. 1 and 2) or ACTH-secreting corticotroph pituitary adenoma (no. 3). ACTH, adrenocorticotropic hormone; cm, centimeter; F, female patient; M, male patient; yrs, years.

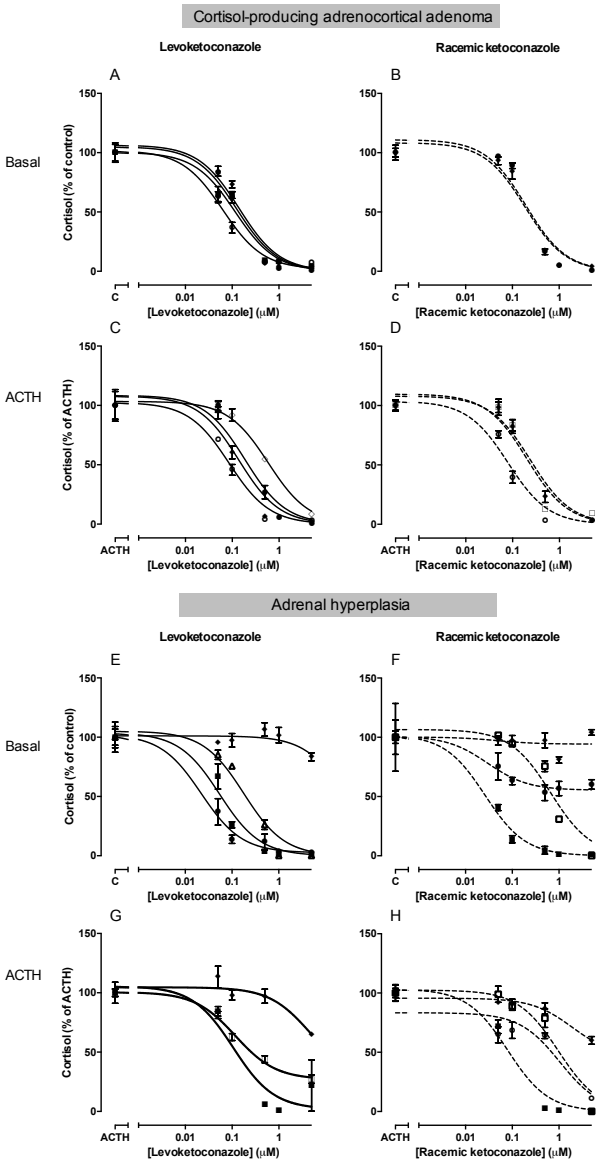

Figure 2. Dose-dependent effects of levoketoconazole (left panel, solid lines) and racemic ketoconazole (right panel, dotted lines) on cortisol production in primary human adrenocortical cultures. Upper panel represent cortisol-producing adrenal adenoma cultures and lower panel primary adrenal hyperplasia cultures, both ACTH-dependent and -independent. No IC_{50} values were calculated from the dose-response curves that did not reach a bottom (E, F), or had a top of the curve above 100% (G). Symbols are presented in Table 2. Basal cultures represent vehicle treatment without (A, B, E, F). Panels C, D, G, H show results after ACTH stimulation (85 pM). Values are depicted as mean ± SEM and as percentage of vehicle treated control. ACTH, adrenocorticotropic hormone. C, control.

Effects of racemic ketoconazole and levoketoconazole in the steroid hormone profile on adrenal cells

HAC15 cells

To determine the effects of levoketoconazole and racemic ketoconazole on steroid precursors and adrenal androgens, multi-steroid analysis was carried out using LC/MS-MS (Supplementary Table 1). In both the basal and ACTH-stimulated condition of HAC15, effects of treatment with levoketoconazole on the steroid profile were comparable. Except for DHEA, DHEAS, and testosterone, all steroids statistically significantly accumulated under ACTH stimulation, varying from an increase of 7% of progesterone, to 359% increase of corticosterone. In the ACTH-stimulated condition, production of all steroids was significantly inhibited, including cortisol (-22 nmol/L, -65%) and 11-DOC (Fig. 3; -1524 nmol/L, -47%). In both conditions, levoketoconazole inhibited almost all steroids to a slightly greater extent compared to racemic ketoconazole (Fig. 3 HAC15; all $P < 0.01$), except DHEA, which was more strongly inhibited by racemic ketoconazole (Fig. 3 HAC15; $P < 0.05$). No differences were seen between compounds for DHEAS. To evaluate the overall effects of the compounds on the steroid profile, absolute changes were added together. The total sum of decrease of steroids was stronger under levoketoconazole compared to racemic ketoconazole treatment (basal: -3,007 vs -1,920 nmol/L; ACTH: -2,813 vs -1,590 nmol/L).

ACTH-dependent adrenal hyperplasia

The effects of two concentrations (0.05 and 5 μM) of both compounds on the steroid profile were studied in ACTH-dependent adrenal hyperplasia no. 1, resulting in a dose-dependent effect on the components of the steroid profile (Supplementary Table 1). DHEA and DHEAS were below the limit of quantitation. In this primary culture, ACTH stimulation resulted in an increase of corticosterone, 17-OHP, 11-DOC, cortisol, androstenedione, and testosterone (mean increase +96%; Supplementary Table 1). Progesterone slightly decreased (-11%), whereas testosterone did not change. In the ACTH-stimulated condition at a concentration of 0.05 μM (Fig. 3 EAS), levoketoconazole significantly inhibited cortisol (-235 nmol/L, -37%, $P = 0.0019$), androstenedione (-9.6 nmol/L, -50%; $P < 0.0001$), and testosterone (-0.3 nmol/L, -45%, $P = 0.0049$ vs control). In contrast to the basal condition, corticosterone and 17-OHP accumulated after treatment with levoketoconazole (+98 nmol/L, +29%, $P = 0.0124$; +8.3 nmol/L, +41%, $P = 0.0257$; respectively), whereas 11-DOC did not change (Fig 3. EAS). When focusing on the difference between levoketoconazole and racemic ketoconazole, accumulation of progesterone (+167% vs +96%; $P < 0.01$), and decrease of cortisol (-37% vs -11%; $P < 0.05$) were stronger after exposure to levoketoconazole (Fig. 3 EAS). Accumulation of 11-DOC was in contrast higher under racemic ketoconazole ($P < 0.01$ vs levoketoconazole). In the basal condition at 0.05 μM, no significant changes between both compounds were observed (Supplementary Table 1). The total change of

Table 2. Efficacy of levoketoconazole, and racemic ketoconazole on inhibition of cortisol production in human primary adrenocortical cultures

Diagnosis			Basal condition	
			Levoketoconazole	Racemic ketoconazole
Cortisol-producing adrenocortical adenomas (ACA)	No. 1	■	0.116 (0.0762 – 0.177)	NT
	No. 2	●	0.125 (0.0809 – 0.194)	0.266 (0.158 – 0.450) #
	No. 3	◆	0.140 (0.0782 – 0.251)	0.138 (0.0700 – 0.274)
	No. 4	○	0.0631 (0.0443 – 0.0899)	NT
	No. 5	□	NT	NT
	No. 6	◇	NT	NT
ACTH-dependent hyperplasias	No. 1	■	0.0262 (0.00859 – 0.122)	0.0544 (0.0311 – 0.0951)
	No. 2	●	0.0220 (0.00986 – 0.0492)	0.0296 (0.00515 – 0.170)
	No. 3	◇	0.179 (0.112 – 0.284)	0.661 (0.364 – 0.120) ####
ACTH-independent hyperplasias	No. 1	◆	Ambiguous	Ambiguous
	No. 2	○	NT	NT
	No. 3	□	NT	NT
ACC	No. 1	■	0.00578 (0.00270 – 0.0124)	NT
	No. 2	○	0.0571 (0.0309 – 0.105)	0.0763 (0.0430 – 0.135)
	No. 3	◆	0.0731 (0.0477 – 0.112)	0.0676 (0.0534 – 0.0857)

IC_{50} values are presented in micromolar (μM) after 3 days of treatment. ACTH (85 pM) stimulated cortisol represents the mean percentage increase of cortisol production compared to vehicle-treated control, with the applicable P-value. Column 3 represents the symbols used in Fig. 2 and Supplementary Fig. 1.

steroids in the basal condition was comparable between levoketoconazole and racemic ketoconazole (-343 vs -219 nmol/L, respectively), whereas under ACTH stimulation, the total sum of change of the steroids was a decrease of 139 nmol/L under levoketoconazole, whereas there was an increase of 125 nmol/L by racemic ketoconazole.

At a hundred times higher concentration of 5 μM of the drugs, all steroids except progesterone were strongly inhibited by both levoketoconazole and racemic ketoconazole (all decrease > 66%; Supplementary Table 1) both in the basal as well as ACTH-stimulated condition. In both conditions, the total sum of change of the steroids was comparable between levoketoconazole and racemic ketoconazole.

Cortisol-producing adrenocortical adenoma

In ACA primary culture no. 2, two concentrations (0.1 and 0.5 μM) of levoketoconazole and racemic ketoconazole were tested in the basal condition, which also resulted in

	ACTH-simulated condition	
ACTH stimulated cortisol (%)	Levoketoconazole	Racemic ketoconazole
NT	NT	NT
+145% ****	0.188 (0.0937 – 0.378)	NT
+66% ****	0.187 (0.102 – 0.342)	0.241 (0.127 – 0.460)
+230 ****	0.0934 (0.0685 – 0.127)	0.0895 (0.0597 – 0.134)
+615 ****	NT	0.204 (0.116 – 0.360)
+132% **	0.586 (0.272 – 1.267)	NT
+228% ****	0.0799 (0.0484 – 0.132)	0.106 (0.0262 – 0.428)
NT	NT	NT
+6%	0.240 (0.176 – 0.326)	0.967 (0.326 – 2.87) ####
+34% ***	Ambiguous	Ambiguous
-14% *	Ambiguous	1.394 (0.2770 – 7.012)
+2239% ****	0.117 (0.0763 – 0.180)	NT
NT	NT	NT
NT	NT	NT
+67% *	0.0321 (0.0158 – 0.0651)	NT

Ambiguous means that the IC_{50} value could not be calculated, because dose-response curves were not suitable. ACC, adrenocortical carcinoma; ACTH, adrenocorticotropic hormone. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, and **** $P < 0.0001$ vs vehicle treated control. # $P < 0.05$ or #### $P < 0.0001$ versus IC_{50} value of levoketoconazole in the same patient. NT, not tested.

a dose-dependent effect on the steroid profile (Supplementary Table 1). At 0.1 μM, levoketoconazole inhibited cortisol (-264 nmol/L, -40%, $P = 0.0006$), androstenedione (-37 nmol/L, -79%, $P < 0.0001$), and testosterone (-0.4 nmol/L, -66%, $P < 0.0001$), whereas the other steroids increased (Supplementary Table 1; Fig. 3 ACA). Levoketoconazole inhibited cortisol and androstenedione more potently compared to racemic ketoconazole (-40% vs -14%, $P < 0.01$; -79% vs -66%, $P < 0.05$; respectively), while corticosterone accumulated more strongly under racemic ketoconazole (+54% vs +213%, $P < 0.0001$; Fig. 3 ACA). The total change of steroids at 0.1 μM was a decrease of 229 nmol/L under levoketoconazole, whereas there was an increase of 56 nmol/L by racemic ketoconazole. At a five times higher concentration of 0.5 μM, the same tendency was observed, although with a more pronounced absolute change of all steroids in both up- and downwards directions by both compounds (Supplementary Table 1). No difference was observed in the total sum of change of the steroids between levoketoconazole and racemic ketoconazole.

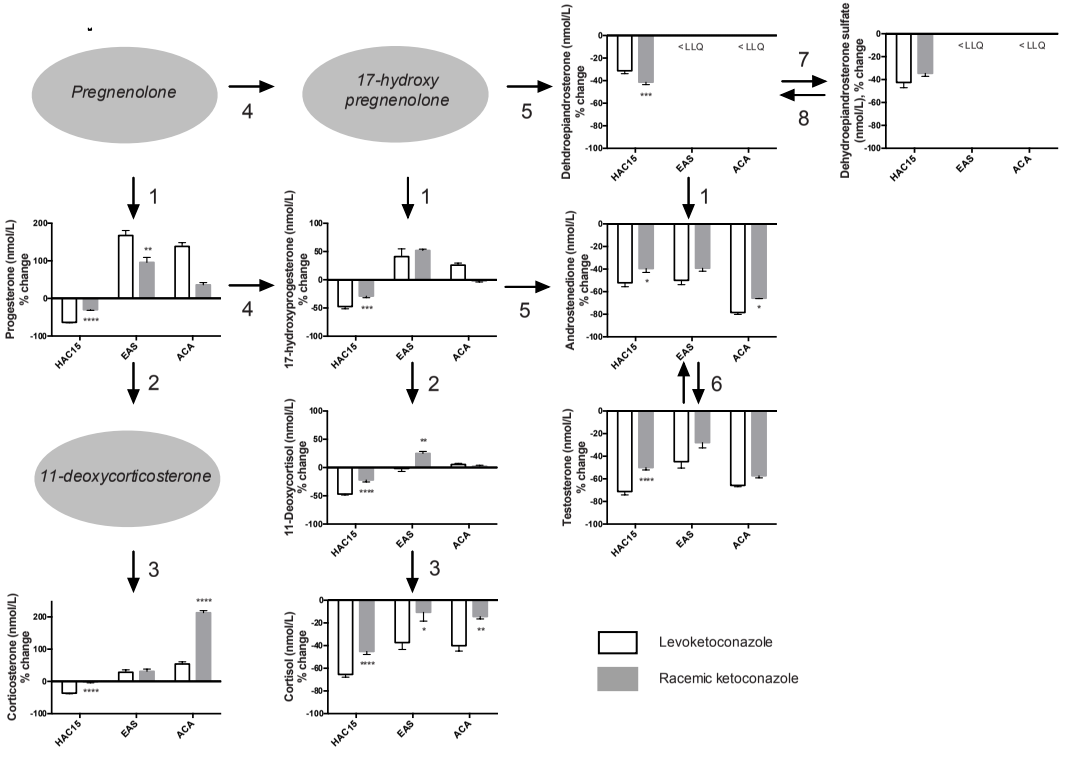

Figure 3. Effects of levoketoconazole (white bars) and racemic ketoconazole (grey bars) on the steroid hormone profile in three different adrenocortical cultures. The displayed conditions were chosen based on the most pronounced differences between levoketoconazole and racemic ketoconazole and were different for HAC15 (ACTH stimulation, concentration 0.5 μM), ectopic ACTH syndrome associated (ACTH-dependent) adrenal hyperplasia no. 1 (EAS; ACTH stimulation, concentration 0.05 μM) and cortisol-producing adenoma no. 2 (ACA; basal condition, concentration 0.1 μM). Symbols are presented in Table 2. Arrows represent steroidogenic enzymes: (1) 3β-hydroxysteroid dehydrogenase, (2) CYP21A2, (3) CYP11B1, (4) CYP17A1 hydroxylase, (5) CYP17A1 lyase, (6) 17β-hydroxysteroid dehydrogenase III, (7) sulfotransferase, and (8) steroid sulfatase. Values are depicted as percentage change ± SEM compared to ACTH stimulation (HAC15 and EAS) or vehicle treated control (ACA). Note the difference in scale of the y-axes. ACTH, adrenocorticotropic hormone; LLQ, lower limit of quantitation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ vs the effect of levoketoconazole.

Effects of levoketoconazole and racemic ketoconazole on corticotroph pituitary cells

Levoketoconazole and racemic ketoconazole inhibited cell number after 3 and 7 days of treatment in corticotroph pituitary murine AtT20 cells (Fig. 4, A and B), whereas no effect was seen after 24h of treatment (data not shown). IC_{50} values for inhibition of cell number after 7 days were 1.05 µM (95% CI 0.576 - 1.91) and 5.81 µM (95% CI 0.948 - 35.5) of levoketoconazole and racemic ketoconazole, respectively (P = 0.0892). Only levoketoconazole showed inhibition of ACTH secretion, corrected for cell amount, after 3 days of treatment (P = 0.0436 vs racemic ketoconazole), where both levoketoconazole and racemic ketoconazole inhibited ACTH secretion after 7 days of treatment (Fig. 4, E and F). Maximal inhibition of ACTH secretion after 7 days of treatment with 10 µM was 38% and 34% for levoketoconazole and racemic ketoconazole, respectively (Fig. 4F).

In two primary ACTH-secreting corticotroph pituitary adenoma cultures, the effects of levoketoconazole and racemic ketoconazole were examined on both cell amount and ACTH secretion after 7 days of treatment. In primary culture no. 2, levoketoconazole significantly inhibited cell number after 7 days of treatment (P < 0.001 vs control; Fig. 4D). In both cultures, there was a significant difference between levoketoconazole and racemic ketoconazole, favoring a stronger effect by levoketoconazole (Fig. 4, C and D). No effects were observed on ACTH secretion corrected for cell number after 7 days of treatment in both primary cultures (Fig. 4, G and H).

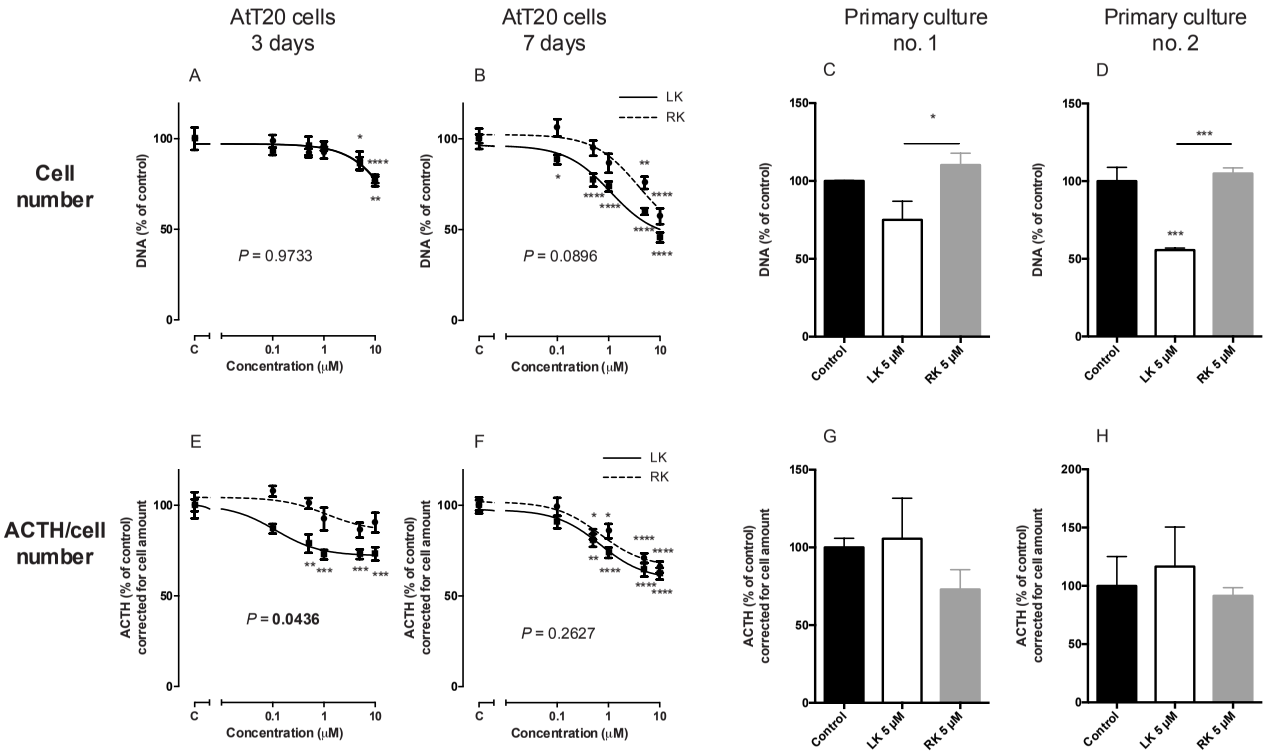

Figure 4. Effects of levoketoconazole (LK, solid lines, ■) and racemic ketoconazole (RK, dotted lines, ●) on cell amount (upper row, A-D) and ACTH secretion corrected for cell amount (bottom row, E-H) in mouse pituitary AtT20 cells and in two primary human corticotroph pituitary adenoma cultures. Primary cultures were incubated with treatment of LK or RK for 7 days. Values are depicted as mean ± SEM and as percentage of vehicle treated control. P-values compare dose response curves of levoketoconazole and racemic ketoconazole in AtT20 cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ vs control or as stated by the lines.

DISCUSSION

Ketoconazole is frequently used for medical treatment of CS, but is often accompanied by serious adverse effects, mainly gastrointestinal. Levoketoconazole, the 2S,4R enantiomer of ketoconazole, might have a favorable toxicity profile, a higher potency, and a lower hepatic metabolism (5, 6). To the best of our knowledge, this is the first study evaluating the direct effects of levoketoconazole on primary human adrenocortical cell cultures. We show that levoketoconazole is a potent inhibitor of cortisol secretion and might be slightly more potent compared to racemic ketoconazole *in vitro*.

The basis for interest in this purified form of racemic ketoconazole includes a study of Rotstein et al., showing large differences in selectivity for inhibition of the cytochromes P450 involved in steroid synthesis by different stereoisomers of ketoconazole (6). In HAC15 cells, we found a twofold lower IC_{50} value for inhibition of cortisol production of levoketoconazole compared to racemic ketoconazole. In primary human adrenocortical cultures, levoketoconazole appears to be a potent inhibitor of cortisol secretion. Sensitivity to levoketoconazole seems to be slightly higher compared to racemic ketoconazole in primary cultures as well, although the difference only reached statistical significance in two patients. We also demonstrate that potency of levoketoconazole is highly variable between patients and tissue specimens with a 24-fold difference in IC_{50} value, indicating that there might also be heterogeneity in response to levoketoconazole in clinical studies, due to differences in sensitivity at the cellular level. Sensitivity to racemic ketoconazole varied with a 10-fold difference in IC_{50}. A direct comparison is difficult, because this was partly based on other primary cultures. To date, no research has been performed yet focusing on determinants of sensitivity to stereoisomers of ketoconazole on cellular level. Direct effects might be stronger in ACC and hyperplasia compared to ACA, implying tissue entity specific effects. However, these differences have to be interpreted with caution, considering the low number of cultures. Previously, a single-nucleotide polymorphism in the CYP17A1 gene has been shown to be associated with the response to ketoconazole and metyrapone in CS patients (13). Considering the small sample size and individual dose titration schemes, these results have to be confirmed in larger populations. Additional underlying hypothetical explanations of variable sensitivity include differences in basal enzyme levels between specimens and tissues, other genetic abnormalities, differences in breakdown of levoketoconazole in the cell, or cell-dependent differences in uptake. Further research could focus on elucidating this issue in an attempt to make the first step towards selecting patients in which ketoconazole enantiomeres might be effective.

In a pharmacokinetic study investigating administration of 200 mg ketoconazole in healthy volunteers, plasma concentrations up to 11 μM could be reached (14). Plasma levels of levoketoconazole can be expected to be even higher, since it has been suggested that liver extraction of this enantiomere is lower (5). Effects as observed in the present study were found at even lower concentrations.

In order to obtain insights into the mechanism of action of levoketoconazole on adrenal steroidogenesis, multi-steroid analysis by LC-MS/MS was used. For reliable measurements, it seems essential to select primary adrenocortical cultures with no molecular alterations. For example, in 35-65% of the cortisol-producing ACA, recurrent activating mutations in PRKACA, encoding the catalytic subunit α of protein kinase A (PKA), have recently been identified (15). This suggests that ACTH-dependent adrenal hyperplasias include the most solid candidate specimens. From measurement of the steroid profile, it appears that differences between levoketoconazole and racemic ketoconazole are most pronounced at concentrations approximating the IC_{50} value for cortisol inhibition. Maximum inhibitory effects seem to be highly comparable. We show that effects of levoketoconazole on the steroid profile are variable between patients; in some cases the production of all steroids are inhibited, whereas in other cultures there is accumulation of progesterone, corticosterone, 17-OHP, and 11-DOC. These differences might be related to the relative amounts of the various steroidogenic enzymes in the tissue samples. The changes in the steroid profiles suggest that levoketoconazole inhibits several steroidogenic enzymes and that effects of levoketoconazole and racemic ketoconazole seem overall comparable. Differences in percentage change are subtle, favoring a more potent effect of levoketoconazole compared to racemic ketoconazole. In HAC15 cells, adrenal androgens are inhibited more strongly by levoketoconazole, whereas this was not confirmed in most of the primary human adrenocortical cultures. In male patients, inhibition of adrenal or testicular androgen production by ketoconazole can result in hypogonadism and gynecomastia (16, 17). Exact percentages are however unknown. Long-term treatment with ketoconazole only slightly affects testosterone levels, potentially explaining the few androgen-related reported side effects (17). The absence of strong accumulation equal to the total sum of inhibition of steroids, suggests an inhibition of the proximal steps of the steroid biosynthetic pathway, like cholesterol side chain cleavage enzyme or steroidogenic acute regulatory protein (StAR). We hypothesize that the extent of this proximal inhibition might be slightly higher for levoketoconazole compared to racemic ketoconazole, as demonstrated by a greater negative balance for levoketoconazole in the majority of adrenal cultures. We do have to acknowledge that we did not measure all steroids of the profile, which can influence the balance.

In the ACTH-dependent adrenal hyperplasia, corticosterone accumulated at 0.05 μM and decreased at 5 μM levoketoconazole and racemic ketoconazole under ACTH stimulation. This implies that specificity of levoketoconazole and racemic ketoconazole on inhibition of steroidogenesis enzymes is dependent on concentration. In a study in which human adrenal tissue slices were incubated with ketoconazole, it has been shown that CYP17A1 lyase is inhibited at the lowest concentration (IC_{50} 2 μM), followed by CYP17A1 hydroxylase (IC_{50} 18 μM), CYP11B2 (18-hydroxylase, IC_{50} 28 μM), and CYP11B1 (IC_{50} 35 μM) (18). The relatively potent inhibition of CYP17A1 might explain the difference in effect between corticosterone and cortisol, and furthermore the accumulation of only corticosterone at lower concentrations.

We also demonstrated that both levoketoconazole and racemic ketoconazole affect corticotroph ACTH-secreting cells. The inhibitory effect of ketoconazole on ACTH secretion by pituitary adenomas has been described before, showing decreased ACTH secretion in two primary human corticotroph pituitary adenoma cultures (19). This might be mediated by cAMP, as was indicated to be the case in rat pituitary cells (20). The inhibitory effect of ketoconazole on corticotroph ACTH secretion could be one of the explanations of the unexpected absence of increased ACTH in patients with a corticotroph pituitary adenoma treated with ketoconazole for a longer period (16). In this study, levoketoconazole and racemic ketoconazole inhibited cell growth and ACTH production corrected for cell amount in a dose- and time-dependent manner in AtT20 cells. Furthermore, in one of the two human corticotroph pituitary adenoma cultures, levoketoconazole inhibited cell growth, whereas this effect was not observed after treatment with racemic ketoconazole. No effect was observed on ACTH secretion in these two corticotroph pituitary adenoma cultures, which might be due to the applied correction for cell amount.

In conclusion, we show that levoketoconazole is a potent inhibitor of cortisol secretion in primary human adrenocortical cells, which might inhibit steroidogenesis more potently compared to racemic ketoconazole. In addition, levoketoconazole may have pituitary-directed effects. Together with the previously reported potential advantages of increased efficacy *in vivo*, a favorable safety profile and increased therapeutic index, this makes levoketoconazole a very promising novel treatment option for Cushing's syndrome.

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SUPPLEMENTARY MATERIAL

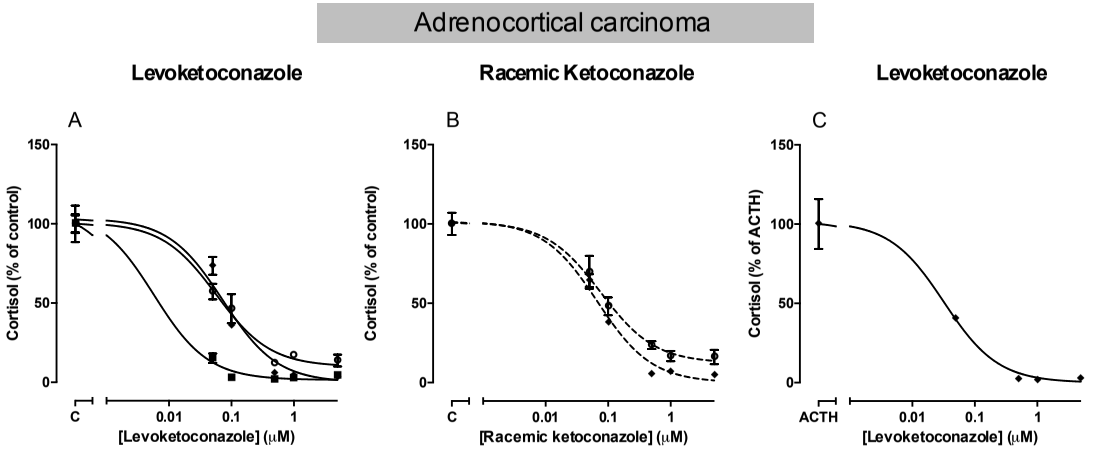
Supplementary Table 1. Overview of effects of levoketoconazole, and racemic ketoconazole on the steroid profile in human adrenocortical cultures

			Prog	Corticosterone	17-OHP
HAC15	Basal	LK 0.5 µM	**-0.9 ± 0.1** (-55%)	**-0.3** ± 0.1.(-39%)	**-27 ± 1.7,**(-57%)
		RK 0.5 µM	**-0.3 ± 0.1** (-19%****)	+0.2 ± 0.1 (+19%****)	**-17 ± 2.1** (-37%****)
	Effect ACTH	10 nM	**+119%**	**+359%**	**+35%**
	ACTH	LK 0.5 µM	**-2.2 ± 0.1** (-64%)	**-1.1 ± 0.1** (-36%)	**-25 ± 2.7** (-47%)
		RK 0.5 µM	**-1.0 ± 0.1** (-30%****)	-0.0 ± 0.3 (-1.5%****)	**-14 ± 1.7** (-29%**)
ACTH-dependent adrenal hyperplasia no. 1	Basal	LK 0.05 µM	**+5.1 ±0.9** (+123%)	-67 ± 69 (-33%)	-0.5 ± 1.1 (-4.6%)
		RK 0.05 µM	**+4.5 ±0.7** (+108%)	-69 ± 71 (-32%)	+0.7 ± 0.9 (+7.5%)
		LK 5 µM	**+13 ± 0.5** (+363%)	**-194 ± 48** (-99%)	**-14 ± 0.3** (-93%)
		RK 5 µM	**+20 ± 1.4** (+537%***)	**-167 ± 6.3** (-98%)	**-15 ± 0.8** (-92%)
	Effect ACTH	85 pM	**-11%**	**+145%**	**+94%**
	ACTH	LK 0.05 µM	**+6.0 ± 0.5** (+167%)	**+98 ± 28** (+29%)	**+8.3 ± 2.8** (+41%)
		RK 0.05 µM	**+3.4 ± 0.5** (+96%**)	**+106 ± 32** (+31%)	**+8.1 ± 1.1** (+52%)
		LK 5 µM	**+38 ± 3.8** (+1201%)	**-374 ± 9.5** (-100%)	**-32 ± 1.6** (-90%)
		RK 5 µM	**+40 ± 3.3** (+1292%)	**-398 ± 6.7** (-100%)	**-28 ± 1.1** (-90%)
Cortisol-producing ACA no. 2	Basal	LK 0.1 µM	**+2.4 ± 0.2** (+138%)	**+50 ± 6.5** (+54%)	**+5.1 ± 0.9** (+26%)
		RK 0.1 µM	**+0.5 ± 0.1** (+36%)	**+176 ± 5.6** (+213%****)	-0.5 ± 0.6 (-2.4%)
		LK 0.5 µM	**+40 ± 1.8 (+2350%)**	**+218 ± 3.8** (+239%)	**+21 ± 3.9** (+107%)
		RK 0.5 µM	**+22 ± 1.1** (+1567%)	**+144 ± 24** (+174%*)	**+11 ± 2.0** (+58%*)

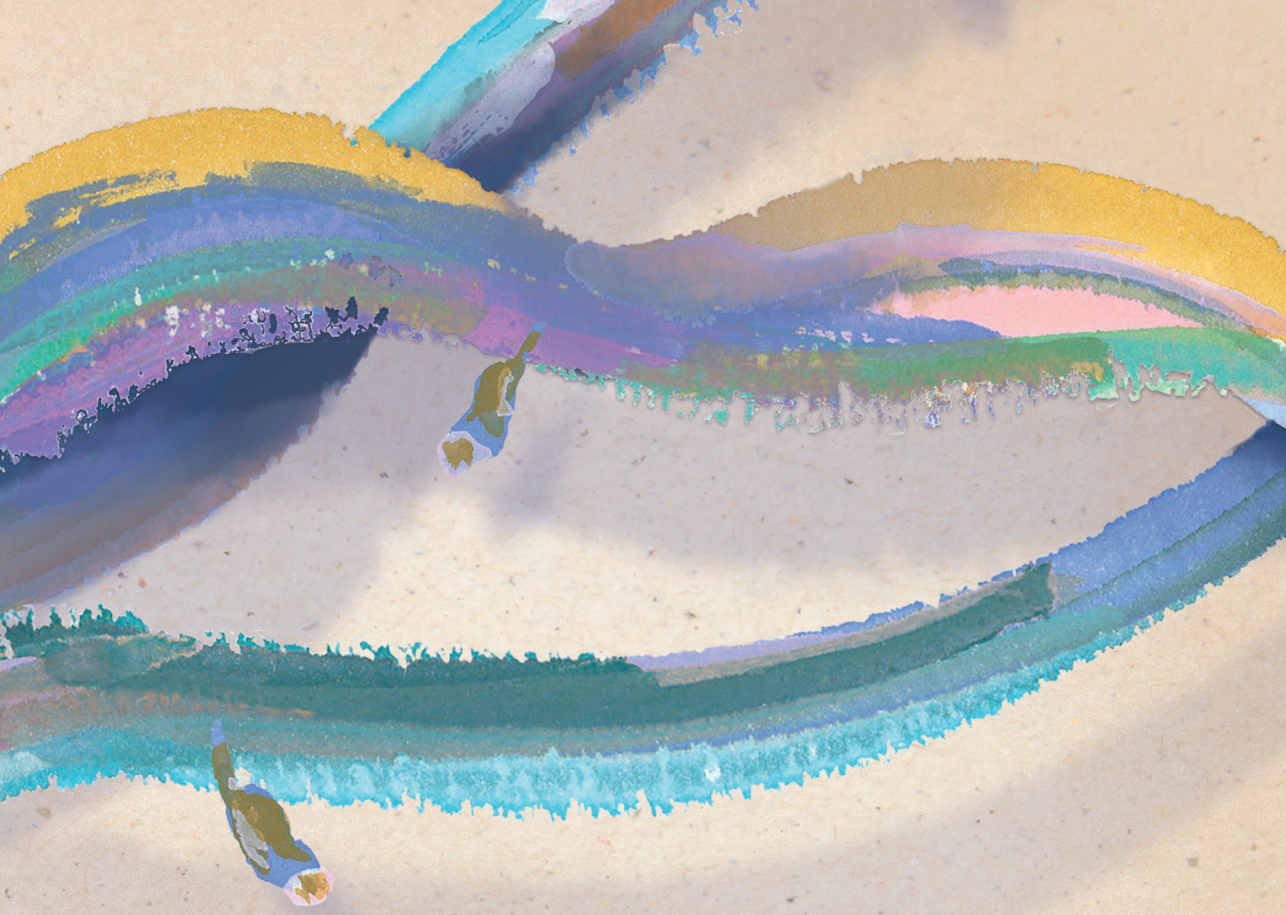
Effects of levoketoconazole (LK) and racemic ketoconazole (RK) on levels of progesterone (prog), corticosterone, 17-hydroxyprogesterone (17-OHP), 11-deoxycortisol (11-DOC), cortisol, dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), androstenedione, and testosterone after 3 days of treatment. Numbers of the primary cultures correspond to the numbers in Table 1 and 2. Data are presented as absolute change ± standard error of the difference (in nmol/L) compared to vehicle treated control (basal) or compared to ACTH stimulation with vehicle (ACTH).

11-DOC	Cortisol	DHEA	DHEAS	Androstenedione	Testosteron
-1129 ± 119 (-48%)	**-16 ± 11** (-69%)	**-31 ± 25** (-44%)	-659 ± 68 (-56%)	-1105 ± 117 (-56%)	**-39 ± 5.1** (-64%)
-521 ± 112 (-23%****)	**-11 ± 0.8** (-47%****)	**-40 ± 2.6** (-56%**)	**-496 ± 38** (-44%*)	**-809 ± 80** (-47%**)	**-26 ± 2.5** (-58% ***)
+42%	**+77%**	**-35%**	**-38%**	+7%	+7%
-1524 ± 71 (-47%)	**-22 ± 1.3** (-65%)	**-14 ± 1.8** (-31%)	**-250 ± 34** (-43%)	**-944 ± 79** (-52%)	**-31 ± 2.1** (-71%)
-665 ± 115 (-22%****)	**-15 ± 1.4** (-45%****)	**-17 ± 2.7** (-41%**)	**-197 ± 22** (-35%)	**-661 ± 67** (-40%*)	**-20 ± 1.4** (-50%****)
-96 ± 24 (-37%)	-178 ± 79 (-64%)	< LLQ	< LLQ	**-6.5 ± 1.2** (-64%)	**-0.3 ± 0.1** (-48%)
-62 ± 19 (-27%)	**-87 ± 22** (-57%)	< LLQ	< LLQ	**-5.9 ± 1.2** (-55%)	**-0.2 ± 0.1** (-32%)
-281 ± 17 (-99%)	**-230 ± 71** (-99%)	< LLQ	< LLQ	**-8.0 ± 0.8** (-95%)	**-0.4 ± 0.1** (-90%)
-271 ± 18 (-98%)	**-165 ± 16** (-99%)	< LLQ	< LLQ	**-8.2 ± 1.0** (-94%)	**-0.3 ± 0.1** (-66%)
+41%	**+209%**	< LLQ	< LLQ	**+64%**	**+22%**
-6.0 ± 23 (+1.6%)	**-235 ± 45** (-37%)	< LLQ	< LLQ	**-9.6 ± 1.0** (-50%)	**-0.3 ± 0.1** (-45%)
+77 ± 23 (+25%**)	-62 ± 66 (-11%*)	< LLQ	< LLQ	**-7.5 ± 1.5** (-39%)	**-0.2 ± 0.1** (-28%)
-435 ± 14 (-99%)	**-640 ± 12** (-100%)	< LLQ	< LLQ	**-15 ± 0.6** (-98%)	**-0.6 ± 0.1** (-85%)
-403 ± 11 (-99%)	**-644 ± 64** (-100%)	< LLQ	< LLQ	**-15 ± 1.1** (-98%)	**-0.4 ± 0.0** (-85%)
+15 ± 5.8 (+5.4%)	**-264 ± 40** (-40%)	< LLQ	< LLQ	**-37 ± 1.7** (-79%)	**-0.4 ± 0.0** (-66%)
+6.4 ± 9.3 (+2.4%)	-93 ± 40 (-14%**)	< LLQ	< LLQ	**-33 ± 2.2** (-66%*)	**-0.3 ± 0.0** (-57%)
-98 ± 5.8 (-36%)	**-633 ± 25** (-96%)	< LLQ	< LLQ	**-45 ± 1.5** (-97%)	**-0.5 ± 0.0** (-83%)
-38 ± 17 (-14%***)	**-563 ± 40** (-87%)	< LLQ	< LLQ	**-47 ± 2.2** (-95%)	**-0.4 ± 0.02** (-79%)

Significant absolute changes compared to control are depicted in bold. The percentage change compared to control is displayed between brackets. * P<0.05, ** P<0.01, and *** P<0.001 compares the percentage change of RK and LK for the same steroid in the same condition in the same culture. ACA, adrenocortical adenoma; ACTH, adrenocorticotropic hormone; LLQ, lower limit of quantitation.



Supplementary Figure 1. Effects of levoketoconazole (solid lines) and racemic ketoconazole (dotted lines) on cortisol production in primary human adrenocortical carcinoma cultures. The symbols correspond to the symbols as presented in Table 2 and thus correspond to the same patient. Controls represent vehicle treatment without (A, B) or with (C) ACTH stimulation (85 pM). Values are depicted as mean ± SEM and as percentage of vehicle treated control. ACTH, adrenocorticotropic hormone; C, control.



CHAPTER 3

Osilodrostat is a potential novel steroidogenesis inhibitor for the treatment of Cushing's syndrome: an in vitro study

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ABSTRACT

Context: Metyrapone and ketoconazole, frequently used steroidogenesis inhibitors for treatment of Cushing's syndrome, can be associated with side effects and limited efficacy. Osilodrostat is a CYP11B1 and CYP11B2 inhibitor, with unknown effects on other steroidogenic enzymes.

Objective: To compare the effects of osilodrostat, metyrapone, and ketoconazole on adrenal steroidogenesis, and pituitary adenoma cells *in vitro*.

Methods: HAC15 cells, seventeen primary human adrenocortical cell cultures, and pituitary adenoma cells were incubated with osilodrostat, metyrapone, or ketoconazole (0.01-10 μM). Cortisol and ACTH were measured using chemiluminescence immunoassays, and steroid profiles by liquid chromatography-mass spectrometry.

Results: In HAC15 cells, osilodrostat inhibited cortisol production more potently (IC_{50}: 0.035 μM) than metyrapone (0.068 μM; $P < 0.0001$), and ketoconazole (0.621 μM; $P < 0.0001$). IC_{50} values of osilodrostat and metyrapone for basal cortisol production varied with a 25-fold and 18-fold difference, respectively, with comparable potency. Aldosterone production was inhibited more potently by osilodrostat versus metyrapone and ketoconazole. Osilodrostat and metyrapone treatment resulted in strong inhibition of corticosterone and cortisol, 11-deoxycortisol accumulation, and modest effects on adrenal androgens. No pituitary-directed effects of osilodrostat were observed.

Conclusions: Under our study conditions, osilodrostat is a potent cortisol production inhibitor in human adrenocortical cells, comparable with metyrapone. All steroidogenesis inhibitors showed large variability in sensitivity between primary adrenocortical cultures. Osilodrostat might inhibit CYP11B1 and CYP11B2, in some conditions to a lesser extent CYP17A1 activity, and a proximal step in the steroidogenesis. Osilodrostat is a promising novel treatment option for Cushing's syndrome, and *in vivo* differences with metyrapone are potentially driven by pharmacokinetic differences.

INTRODUCTION

Cushing's syndrome (CS) is characterized by chronic exposure to excess glucocorticoids, resulting in significant multisystem morbidity, and when untreated, increased mortality (1). Adrenocorticotropic hormone (ACTH) dependent CS can be caused by a corticotroph pituitatry adenoma (Cushing's disease, CD) or, more rarely, by ectopic ACTH secretion by a neuroendocrine tumor (ectopic ACTH syndrome, EAS). ACTH-independent CS is in most cases caused by an unilateral cortisol-producing adrenocortical adenoma (ACA) and less frequently by an adrenocortical carcinoma (ACC) or bilateral adrenal hyperplasia (1, 2). The first line treatment modality in all types of CS is surgery (3). There are however several indications, like after surgical failure, metastatic or occult disease, or when surgery is contra-indicated, in which other treatment modalities like medical therapy are indicated (3). Traditionally, medical treatment options for CS can be divided into three categories: I) pituitary-targeting drugs, e.g. pasireotide and cabergoline; II) glucocorticoid receptor blockers, e.g. mifepristone; and III) adrenocortical steroidogenesis inhibitors that directly suppress cortisol production via inhibition of steroidogenic enzymes (3).

Two of the most frequently used steroidogenesis inhibitors are metyrapone and ketoconazole. Although it is known that metyrapone selectively inhibits the last step in the cortisol biosynthesis via inhibition of CYP11B1 (11β-hydroxylase), it also inhibits CYP11B2 (aldosterone synthase) (4, 5). More recently, *in vitro* data obtained in a rodent model even suggested that metyrapone has greater potency to inhibit CYP11B2, a feature not previously recognized (6). Ketoconazole, originally developed as an antifungal agent, is known to inhibit several steps in adrenal steroid synthesis (7-9). However, although several medical therapies are currently available, not all patients respond and many patients experience side effects (3). Metyrapone can cause hypertension, edema, hypokalemia, acne and hirsutism, due to an increase of mineralocorticoid precursors and adrenal androgens (5). The most important adverse events of ketoconazole include hepatotoxicity and gastrointestinal symptoms (10-13).

Osilodrostat (LCI699) is a new adrenal blocking drug which, based on preclinical *in vitro* studies (14), was thought to specifically inhibit CYP11B2 and at higher concentrations also CYP11B1 (15). It was originally developed for its inhibitory effects on aldosterone production, and blood pressure lowering abilities (15-17). In these clinical studies however, a blunted cortisol response to synthetic ACTH and increased levels of 11-deoxycorticosterone were observed. In 14 patients with primary hyperaldosteronism, basal cortisol levels remained unchanged under treatment, but morning ACTH levels were approximately 2 fold higher than baseline values (17). Doses that were used varied between 1 to 2 mg osilodrostat per day. Recently, an extended phase II study using a dose escalation schedule of osilodrostat

starting at 4 mg per day showed normalized urinary free cortisol (UFC) or ≥50% decrease of UFC from baseline in 79% of the 19 patients with CD after 22 weeks of treatment (18). Plasma levels of cortisol and aldosterone decreased, whereas levels of their precursors, 11-deoxycortisol and 11-deoxycorticosterone, increased (18). Most common adverse events were nausea, diarrhea, asthenia, and adrenal insufficiency (n = 6 for each). Hirsutism and/or acne due to increased testosterone levels were reported in 4 out of 14 female patients (18). Initial results of a phase III study investigating the effects of osilodrostat showed that 53% (n = 72) of patients had normalized UFC without up-titration after 12 weeks. Of these patients, 86% of patients randomized to continue osilodrostat treatment for 8 weeks showed normalized UFC compared to 29% of patients in the placebo group (19).

The aim of the present study is to further explore the effects of osilodrostat on basal and ACTH-stimulated cortisol production and adrenocortical steroidogenesis in human adrenocortical cells, compared to those of metyrapone and ketoconazole. In addition, we examined possible pituitary-directed effects of osilodrostat on cell amount and ACTH secretion in corticotroph pituitary adenoma cells.

MATERIALS AND METHODS

Cell culture and compounds

Cortisol and aldosterone producing human adrenocortical carcinoma HAC15 (as a kind gift by dr. W. Rainey) and AtT20 mouse corticotroph tumor cells (ATCC number CRL-1795) were used. Short tandem repeat profiling of HAC15 showed a genetic profile identical to H295R, which is consistent with a previous report that HAC15 is a clone of H295R (20). Cells were routinely cultured in 75 cm^2 flasks at 37 °C in a humidified incubator at 5% CO_2, harvested with trypsin (0.05%)-EDTA (0.53 mM), and resuspended in culture medium, as previously described (21). HAC15 cells were cultured in Dulbecco's Modified Eagle Medium F12 (D-MEM/F12) containing 5% fetal calf serum, whereas AtT20 cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum. Both media were supplemented with L-glutamine (2 mmol/L) and penicillin (10^5 U/L; Bristol-Meyers Squibb, Utrecht, the Netherlands). Media and supplements, except penicillin, were obtained from Fisher Scientific, Landsmeer, the Netherlands.

Stock solution of osilodrostat, metyrapone (both Novartis Pharma, Arnhem, the Netherlands), and ketoconazole (Sigma-Aldrich, Zwijndrecht, the Netherlands) were dissolved in 0.01N hydrochloric acid, distilled water, and absolute ethanol, respectively, according to manufacturer's instructions, and stored at -20°C at a stock concentration of 10^{-2} M. At the start of each experiment, osilodrostat, metyrapone, and ketoconazole were

diluted to the correct concentration in the same solution as it was dissolved in. Synacten (synthetic ACTH, Novartis Pharma) stock concentration was stored at 4°C and diluted in culture medium at the day of use. Angiotensin II (Sigma-Aldrich) stock concentration was stored at -20°C and diluted in distilled water at the day of use. The used concentrations of ACTH and Angiotensin II were based on a dose-response curve performed in HAC15 cells on cortisol and aldosterone production, respectively, and according to previously reported studies (22, 23). One day after seeding the cells, incubations were started. Control cells were vehicle treated. For HAC15, cells were plated at a density of 100,000 cells per well in 0.5 ml medium. Osilodrostat, metyrapone, or ketoconazole (0.01 - 5 μM) were added for three days, with or without 10 nM ACTH or 100 nM Angiotensin II, for evaluation of the effects on the steroid profile, and aldosterone production, respectively. To assess the effect of osilodrostat on mouse corticotroph pituitary cells, AtT20 cells were incubated with osilodrostat for 1, 3, and 7 days (0.01 - 10 μM). For 7 days experiments, medium and compounds were refreshed after 3 days.

Cells and media were collected at the end of experiments and stored at -20°C until analysis. At the end of both the AtT20 and primary corticotroph pituitary adenoma culture experiments, media were collected and supplemented with the protease inhibitor Trasylol (final concentration 5 IU per ml, Sigma-Aldrich) to prevent degradation of ACTH. All cell culture experiments were carried out in quadruplicate at least twice. DNA measurement was performed using the bisbenzimide fluorescent dye (Hoechst 33258, Sigma-Aldrich, Zwijndrecht, the Netherlands), as previously described (24). In case compounds had an effect on cell number, steroid levels were corrected for total DNA per well as a measure of cell number.

Processing of adrenocortical and pituitary tissue

Adrenocortical tissues (ACA, hyperplasia, ACC) and ACTH-secreting corticotroph pituitary adenomas were collected during surgery at the department of Surgery at Erasmus MC from January 2015 until February 2018. The study was approved by the Medical Ethics Committee of Erasmus MC and informed consent was obtained from all patients. Directly after surgery, the adrenal specimens were minced into small fragments of about 2-5 mm^3 and washed twice with culture medium (Fisher Scientific, Landsmeer, the Netherlands). The fragments were dissociated for 2 hours at 37°C, using collagenase type 1 (2 mg/ml; Sigma-Aldrich, Zwijndrecht, the Netherlands). The suspension was filtered through a sterile gauze (single layer) to obtain single cell suspensions, and subsequently Ficoll density gradient separation was used to remove any remaining cell debris. Cell viability was determined by trypan blue exclusion, visually counted using Türk solution, and plated at a density of 10^5 cells per well. Corticotroph pituitary adenoma tissues from patients with CD (n = 2) were available after transsphenoidal surgery. Single-cell suspensions of

the pituitary adenoma tissues were prepared as previously described (25). Primary human adrenal and pituitary adenoma culture experiments were similar to experiments in HAC15 and AtT20 cells, with small adjustments: ACTH was used at a concentration of 85 pM, Angiotensin II was used at a concentration of 10 nM, treatment was started 3-4 days after plating the cells and preceded by medium refreshment, and in primary corticotroph pituitary adenoma cultures osilodrostat was only tested at a concentration of 1 μM. Owing to a limited number of cells obtained from some specimens, not all experiments could be carried out in every primary culture.

Measurement of steroid hormone concentrations

For the dose-response curves, both cortisol and ACTH were measured in supernatants using an Immulite 2000 XPi immunoassay analyzer (Siemens Medical Solutions USA, Inc). In addition, in selected conditions, androstenedione, cortiosterone, cortisol, 11-deoxycortisol (11-DOC), dehydroepiandrosterone (DHEA), DHEA sulphate (DHEAS), progesterone, 17-hydroxyprogesterone (17-OHP), and testosterone were measured simultaneously using a Waters® Acquity™ UPLC HSS T3 1.8 μm column and a Waters XEVO-TQ-S system (Waters, Milford, MA, USA) equipped with an ESI source operating in the electrospray positive mode except for DHEAS (negative ESI). Intra- and inter-assay coefficients of variation for the steroid assays were <7 and <8% for androstenedione, <8 and <4% for corticosterone, <6 and <6% for cortisol, <10 and <6% for 11-deoxycortisol, <7 and <8% for DHEA, <8 and <13% for DHEAS, <6 and <7% for progesterone, <6 and <6% for 17-OHP, and <6 and <9% for testosterone. Aldosterone was measured by mass spectrometry, equipped with an ESI source operating in the electrospray negative mode, using a protein precipitation method with a mixture of methanol, zinc sulphate and phosphoric acid. The lower limit of quantification was 46 pmol/L. Intra- and inter-assay coefficients of variation were 6.6 and 10.8% respectively.

Multiple reaction monitoring was applied for the detection of the analytes using both quantifiers and qualifiers. Samples for LC/MS-MS steroid measurements were those closest to 50% inhibition or maximal inhibition of cortisol as determined by the immunoassay.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 6.0. Non-linear regression curve fitting program was used to calculate the half maximal inhibitory concentration (IC_{50}, in μM) of the steroidogenesis inhibitors for cortisol and aldosterone production. Effects of the three compounds on the components of the steroid profile compared to control were compared using Student's *t*-test, and in case of multiple concentrations using ANOVA with Tukey's multiple comparisons test. Student's t-test was used to compare

the effects of similar concentrations of osilodrostat with metyrapone and ketoconazole on the steroid profile. Significance was accepted at the 0.05 level of probability. Data are presented as mean ± SEM.

RESULTS

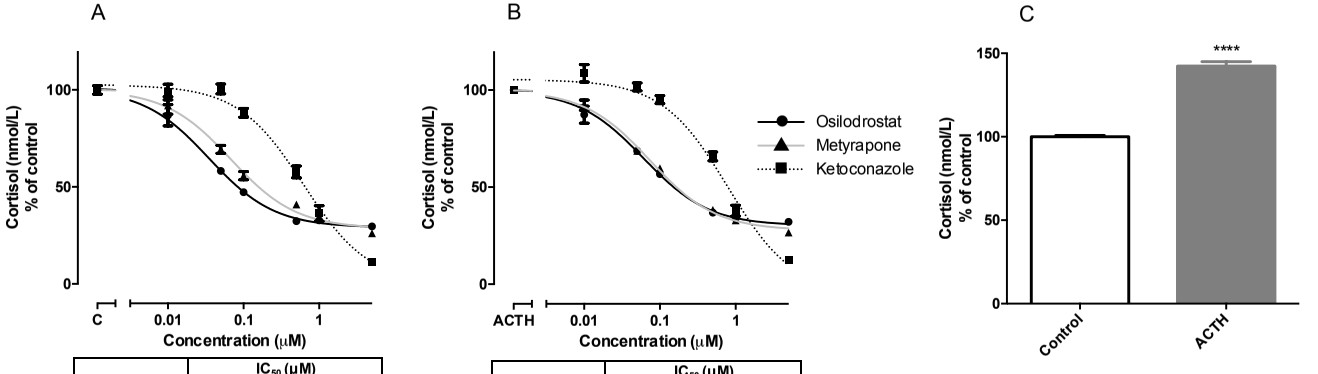
Effects of osilodrostat, metyrapone, and ketoconazole on basal and ACTH-stimulated cortisol production *in vitro*

HAC15 cell line

After 3 days, osilodrostat inhibited cortisol production at significantly lower concentrations (IC_{50} 0.0347 µM 95% CI 0.0294 – 0.0410) than metyrapone (0.0678 µM 95% CI 0.0543 – 0.0848, $P < 0.0001$), and ketoconazole (0.621 µM 95% CI 0.488 - 0.833, $P < 0.0001$) (Fig. 1A). Mean cortisol stimulation by ACTH was 42% (± 4%) in all experiments (Fig. 1C). For osilodrostat, the IC_{50} value increased 1.7 fold when HAC15 cells were stimulated with ACTH ($P < 0.0001$ vs basal condition), whereas potency under ACTH stimulation did not significantly change for metyrapone and ketoconazole. Comparing the three compounds under ACTH stimulation, osilodrostat inhibited cortisol production as potently as metyrapone (IC_{50} 0.0605 µM 95% CI 0.0514 – 0.0714 vs 0.0739 µM 95% CI 0.0645 – 0.0847, $P = 0.0669$), and more potently compared to ketoconazole (IC_{50} 0.709 µM 95% CI 0.523 – 0.962, $P < 0.0001$). Addition of the inhibitors of steroidogenesis did not affect cell amounts.

Primary adrenocortical cultures

Effects of osilodrostat, metyrapone, and ketoconazole were also assessed in seventeen primary cultures of human adrenocortical tissue: eight cortisol-producing ACA, three ACTH-dependent adrenal hyperplasia, two ACTH-independent adrenal hyperplasias, two cortisol-producing ACCs, and two Conn's syndrome associated adrenal hyperplasias. Patient and tissue characteristics are outlined in Table 1. IC_{50} values of osilodrostat, metyrapone, and ketoconazole for cortisol production in primary adrenocortical cultures are listed in Table 2 and dose-response curves are displayed in Fig. 2 and supplementary Fig. 1. DNA measurement was performed in 37 of the 58 adrenal culture plates in which dose-responses of the compounds on either cortisol or aldosterone were assessed, and showed no effects of any of the drugs on cell number in these cultures. The 85 pM ACTH induced cortisol increase varied from 48% to 737% in primary adrenocortical cultures (Table 2).



	IC_{50} (μM)
Osilodrostat	0.0347 (0.0294 – 0.0410)
Metyrapone	0.0678 (0.0543 – 0.0848)
Ketoconazole	0.621 (0.488 – 0.833)

	IC_{50} (μM)
Osilodrostat	0.0605 (0.0514 – 0.0714)
Metyrapone	0.0739 (0.0645 – 0.0847)
Ketoconazole	0.709 (0.523 – 0.962)

Figure 1. Dose-dependent effects of osilodrostat (black solid lines, ●), metyrapone (grey solid lines, ▲), and ketoconazole (black dotted lines, ■), on basal (A) and 10 nM ACTH-stimulated (B) cortisol production in HAC15 cells after 72h of incubation. (C) Effects of 10 nM ACTH after 72h of incubation in HAC15 cells. Controls represent vehicle treated (A) or vehicle with 10 nM ACTH (B) treated HAC15 cells. Values are depicted as mean ± SEM and as percentage of control. **** $P < 0.0001$ vs control. ACTH, adrenocorticotropic hormone; C, control.

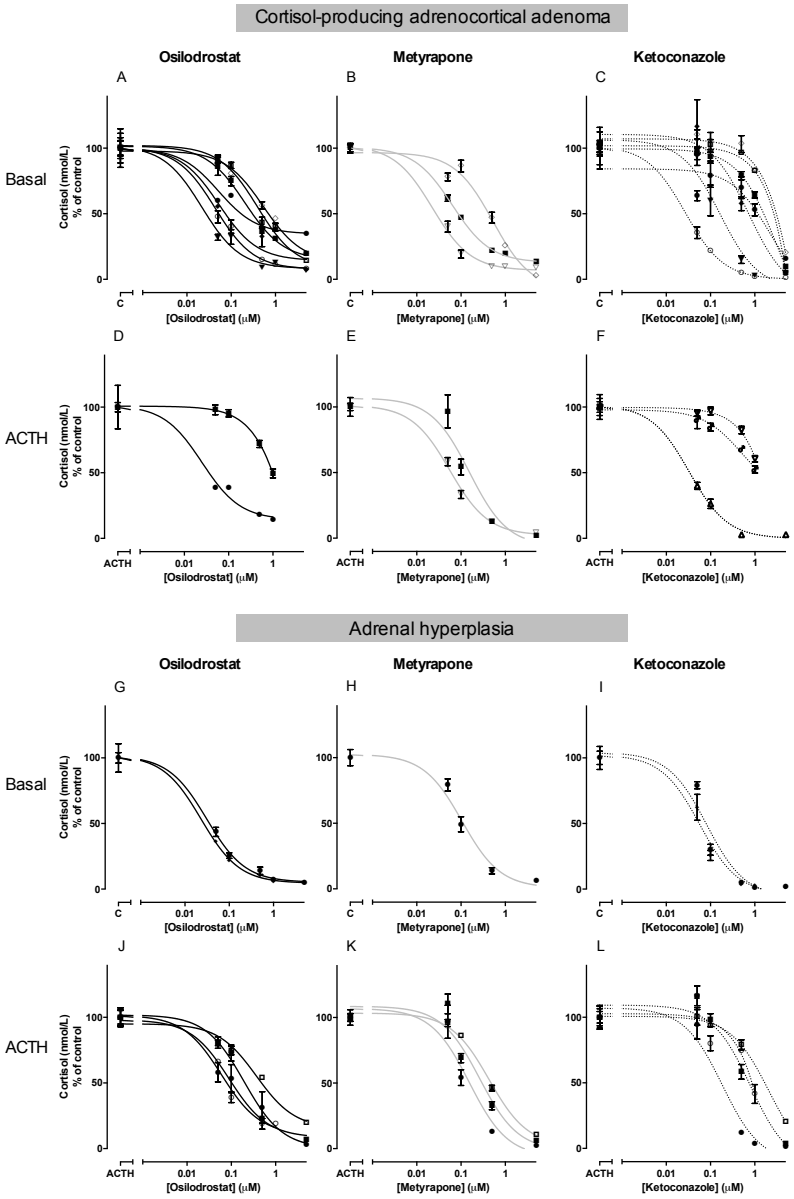


Figure 2. Effects of osilodrostat (left panel), metyrapone (middle panel), and ketoconazole (right panel) on basal and ACTH-stimulated cortisol production in primary human adrenocortical cultures. Upper panel represents cortisol-producing adrenocortical adenoma cultures and lower panel primary adrenal hyperplasia cultures, both ACTH-dependent and -independent. Symbols are presented in Table 2. Controls represent vehicle treatment without (A-C, G-I) or with (D-F, J-L) ACTH stimulation (85 pM). Values are depicted as mean ± SEM and as percentage of control. ACTH, adrenocorticotropic hormone; C, control.

Table 1. Clinical and pathological characteristics of patients of whom a primary culture was obtained

Patient no.	Sex	Side	Age at surgery (yrs)	Size of lesion (cm)	Weiss score	Steroid production
Cortisol-producing adrenocortical adenoma						
No. 1	F	Left	51	2.9	0	Cortisol
No. 2	M	Left	45	6	2	Cortisol
No. 3	M	Right	52	4.8	1	Cortisol
No. 4	F	Left (Bilateral)	65	6	0	Cortisol
No. 5	F	Left (Bilateral)	57	4.5	0	Cortisol
No. 6	F	Right	64	2.5	0	Cortisol
No. 7	F	Right (Bilateral)	62	4	0	Cortisol
No. 8	F	Right	66	3.9	0	Cortisol
ACTH-dependent adrenal hyperplasia						
No. 1	F	Bilateral	79	-	-	Cortisol
No. 2	F	Right (Bilateral)	75	-	-	Cortisol
No. 3	F	Left (Bilateral)	69	-	-	Cortisol
ACTH-independent hyperplasia						
No. 1	M	Right (Bilateral)	70	-	-	Cortisol
No. 2	F	Left (Bilateral)	50	-	-	Cortisol
Cortisol-producing adrenocortical carcinoma						
No. 1	F	Right	61	5	5	Cortisol
No. 2	M	Left	64	18.5	6	Cortisol
Conn's Syndrome						
No. 1	F	Left	57	-	-	Aldosterone
No. 2*	F	Left	44	1.5	-	Aldosterone

* Recurrent Conn syndrome, adrenal hyperplasia with dominant 1.5 cm nodule. Tissue diagnosis was based on the pathology report. ACTH-dependent adrenal hyperplasias are all based on ectopic ACTH syndrome. (Bilateral) indicates that the lesion was bilateral, but only one side was used to obtain the primary culture. ACTH, adrenocorticotropic hormone; cm, centimeter; F, female; M, male; yrs, years.

In unstimulated primary ACA cultures, IC_{50} values of osilodrostat for cortisol production varied with a 25-fold difference (Table 2, Fig. 2A; 0.0217 95% CI 0.0102 – 0.0461 to 0.534 95% CI 0.360 – 0.793), whereas there was an 18-fold difference for metyrapone, and 84-fold difference for ketoconazole. The mean IC_{50} of osilodrostat in ACA was higher (n = 7, 0.104 μM 95% CI 0.0716 – 0.151) compared to the mean IC_{50} in adrenal hyperplasia (n = 2, 0.0269 μM 95% CI 0.0210 – 0.0346, P < 0.0001 vs ACA), and not statistically significantly different from the IC_{50} of ACC (n = 2, 0.0644 μM 95% CI 0.0419 – 0.0988, P = 0.1889 vs ACA), although groups were small. The mean IC_{50} of osilodrostat was lower in adrenal hyperplasia compared to ACC (P = 0.0007). In eight conditions (basal or ACTH-stimulated), a direct comparison between osilodrostat and metyrapone could be made (Table 2). Metyrapone inhibited cortisol production more potently in three conditions compared to osilodrostat (P < 0.05), whereas osilodrostat inhibited cortisol more potently in ACTH-dependent adrenal hyperplasia no. 1 (P < 0.0001). Osilodrostat inhibited cortisol more potently compared to ketoconazole in 8 of the 11 cultures in which efficacy of both compounds were studied in the basal condition (P < 0.05 to P < 0.0001). Under ACTH stimulation, a lower IC_{50} was found for osilodrostat in two of the six primary cultures compared to ketoconazole (P < 0.01 and P < 0.001).

Efficacy of osilodrostat changed in 2 of the 3 cultures in which efficacy was compared in the basal and ACTH simulated conditions, with in one culture a higher and in the other culture a lower potency in the ACTH-stimulated condition (P < 0.01, P < 0.05, respectively).

Effects of osilodrostat, metyrapone, and ketoconazole on aldosterone production in human adrenocortical cells

In Angiotensin II stimulated HAC15 cells (Fig. 3D; mean increase of aldosterone 282%, P < 0.0001), osilodrostat inhibited aldosterone levels at more than 10 times lower concentrations compared to metyrapone (Fig. 3A; IC_{50} 0.0354 μM 95% CI 0.0269 – 0.0465 vs 0.413 μM 95% CI 0.306 – 0.557; P < 0.0001). Osilodrostat also inhibited aldosterone production much more potently compared to metyrapone in an aldosterone-producing adrenal hyperplasia causing Conn's Syndrome (Fig. 3B; IC_{50} 0.00281 μM 95% CI 0.000910 – 0.00866 vs 0.822 μM 95% CI 0.471 – 1.433; P < 0.0001). In a second aldosterone-producing adrenal hyperplasia, no differences were observed in suppressive effects of osilodrostat and metyrapone on basal aldosterone concentrations in two concentrations tested (0.1 and 5 μM, data not shown). In ACTH-dependent adrenal hyperplasia no. 1 in the basal condition, osilodrostat inhibited aldosterone significantly more potent compared to metyrapone (Fig. 3C; IC_{50} 0.00469 μM 95% CI 5.516E-5 – 0.398 vs 0.364 μM 95% CI 0.05515 – 2.397; P < 0.0001), and ketoconazole (0.315 μM 95% CI 0.0516 – 1.916; P < 0.0001 vs osilodrostat). In this primary culture, osilodrostat inhibited aldosterone production at significantly lower concentrations compared to those needed for cortisol inhibition (IC_{50} aldosterone 0.00469 μM 95% CI 5.516E-5 – 0.398 vs cortisol 0.0311 μM 95% CI 0.0242 – 0.0399; P = 0.0164).

Table 2. Efficacy of osilodrostat, metyrapone, and ketoconazole for cortisol production by human primary adrenocortical cultures.

			Basal condition		
			Osilodrostat	Metyrapone	Ketoconazole
Cortisol-producing adrenocortical adenomas (ACA)	No. 1	●	0.0519 (0.0296 – 0.0910)	NT	2.198 (0.375 – 1.290)
	No. 2	■	0.206 (0.127 – 0.333)	0.0632 (0.0541 – 0.0739) ****	1.392 (0.912 – 2.127) ****
	No. 3	◆	0.053 (0.019 – 0.15)	NT	0.570 (0.13 – 2.55) *
	No. 4	○	0.044 (0.032 – 0.059)	NT	0.027 (0.016 – 0.045)
	No. 5	□	0.395 (0.270 – 0.576)	NT	1.826 (0.551 – 6.05) ****
	No. 6	◇	0.534 (0.360 – 0.793)	0.412 (0.258 – 0.667)	2.270 (0.776 – 6.64) ****
	No. 7	▼	0.0217 (0.0102 – 0.0461)	NT	0.138 (0.0705 – 0.269) ****
	No. 8	▽	NT	0.0226 (0.0164 – 0.0310)	NT
ACTH-dependent adrenal hyperplasia	No. 1	●	0.0311 (0.0242 – 0.0399)	0.105 (0.0658 – 0.168) ****	0.0702 (0.0423 – 0.117) **
	No. 2	■	NT	NT	NT
	No. 3	◆	0.0232 (0.0146 – 0.0369)	NT	0.0509 (0.0300 – 0.0863) *
ACTH-independent adrenal hyperplasia	No. 1	○	NT	NT	NT
	No. 2	□	NT	NT	NT
ACC	No. 1	●	0.100 (0.0446 – 0.226)	NT	0.0728 (0.0376 – 0.141)
	No. 2	□	0.0431 (0.0328 – 0.0568)	0.0266 (0.0179 – 0.0396) *	0.107 (0.0609 – 0.188) **

IC_{50} values are presented in micromolar (μM). ACTH (85 pM) stimulated cortisol represents the mean percentage increase of cortisol production compared to control, with **** $P < 0.0001$ versus control. Column 3 includes the symbols that are used in Fig. 2 and Supplementary Fig. 1. * $P < 0.05$,

	ACTH stimulated condition		
ACTH stimulated cortisol (% change)	Osilodrostat	Metyrapone	Ketoconazole
+737% ****	0.0251 (0.00939 – 0.0700)	NT	0.499 (0.0875 – 2.851)
+538% ****	0.988 (0.641 – 1.524)	0.440 (0.323 – 0.600) ***	1.315 (0.753 – 2.296)
NT	NT	NT	NT
NT	NT	NT	NT
NT	NT	NT	NT
NT	NT	NT	NT
NT	NT	NT	NT
+207% ****	NT	0.0557 (0.0391 – 0.0793)	0.0328 (0.0213 – 0.0504)
+343% ****	0.0887 (0.0371 – 0.212)	0.138 (0.0644 – 0.296)	0.138 (0.0578 – 0.329)
+127% ****	0.194 (0.118 – 0.321)	0.263 (0.123 – 0.533)	0.799 (0.289 – 1.206) ***
NT	NT	NT	NT
+92% ****	0.0602 (0.0377 – 0.0960)	NT	0.826 (0.112 – 6.074) **
+48% ****	0.369 (0.224 – 0.610)	0.430 (0.318 – 0.581)	1.476 (0.568 – 3.837) **
NT	NT	NT	NT
NT	NT	NT	NT

** $P < 0.01$, and *** $P < 0.001$, and **** $P < 0.0001$ compared to the IC_{50} of osilodrostat. ACC, adrenocortical carcinoma; ACTH, adrenocorticotropic hormone; NT, not tested.

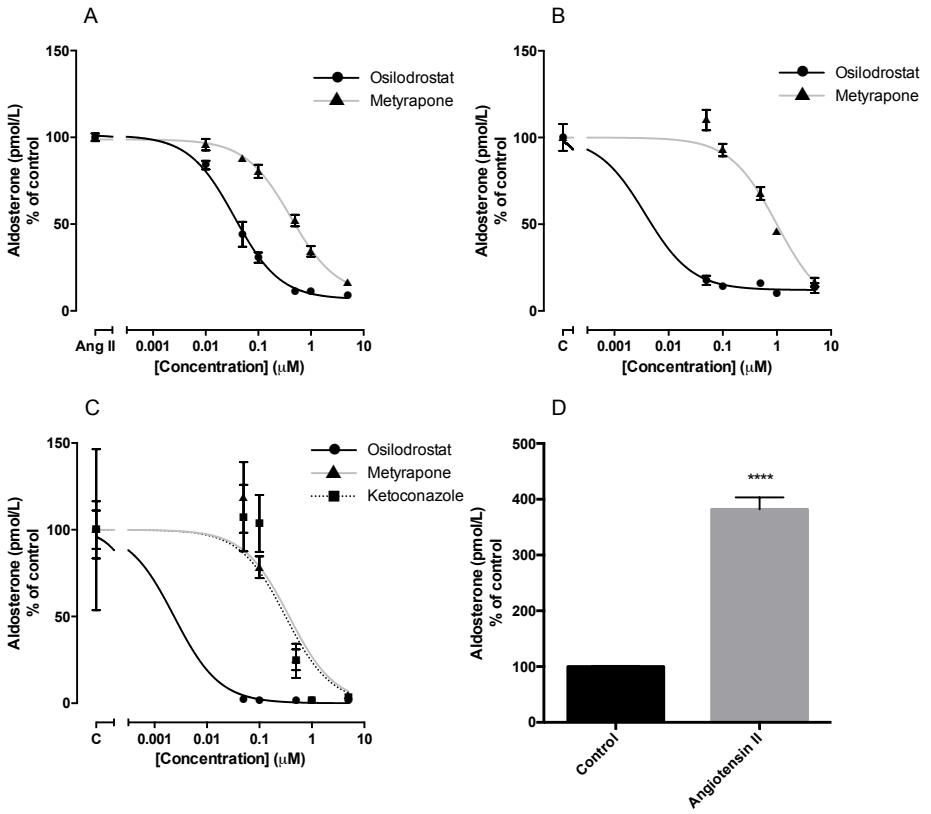


Figure 3. Effects of osilodrostat (black solid lines, ●), metyrapone (grey solid lines, ▲), and ketoconazole (black dotted lines, ■) on 100 nM Angiotensin II stimulated aldosterone production in HAC15 cells (A), and basal aldosterone production in aldosterone-producing adrenocortical hyperplasia no. 1 (B), and in ACTH-dependent adrenal hyperplasia no. 1 (C) after 72h of incubation. (D) Effect of 100 nM Angiotensin II in HAC15 cells after 72h of incubation. Values are depicted as mean ± SEM and as percentage of control. **** $P < 0.0001$ vs control. Ang II, Angiotensin II; C, control.

Effects of osilodrostat, metyrapone, and ketoconazole on the steroid hormone profile in adrenocortical cells

HAC15 cell line

To examine other effects of osilodrostat on steroidogenesis next to inhibition of cortisol and aldosterone production, multi-steroid analysis was carried out using LC/MS-MS in culture media in several conditions (Fig. 4, Supplementary Table 1 and 2). HAC15 cells were studied in the basal and ACTH-stimulated condition. ACTH increased the production of all steroids (range 11 - 216%), except for DHEA and DHEAS, of which the concentrations decreased. Under ACTH stimulation, osilodrostat only induced a strong decrease in

cortisol and corticosterone concentrations (-51 nmol/L, -87%, $P < 0.0001$; -4.7 nmol/L, -84%, $P = 0.0005$; respectively), accompanied by accumulation of 11-DOC (+316.4 nmol/L, +12%, $P = 0.0005$). When focusing on differences between osilodrostat and metyrapone, metyrapone had a slightly stronger percentual inhibitory effect on 17-OHP ($P < 0.0001$), 11-DOC ($P < 0.0001$), cortisol ($P < 0.05$), androstenedione ($P < 0.0001$), and testosterone ($P < 0.0001$; all vs osilodrostat; Fig. 4). Both in the basal and the ACTH stimulated condition, ketoconazole strongly blocked production of all steroids (all > 79% decrease), except progesterone, which accumulated.

To evaluate the overall effects of the compounds on the steroid hormone profile and say something about inhibition of the proximal steroidogenic enzymes in the adrenal cortex, the absolute changes of the steroids were added together. In total in the basal condition, all measured steroids together were inhibited with 1,414 nmol/L by osilodrostat, whereas this was 976 nmol/L for metyrapone. Under ACTH stimulation this was reversed, with a total increase of concentration of steroids of 171 nmol/L under osilodrostat, and a decrease of 900 nmol/L by metyrapone. In both conditions, the total decrease of steroids was strongest under treatment with ketoconazole, with a decrease of 3727 and 4376 nmol/L in the basal and ACTH-stimulated condition, respectively.

ACTH-dependent adrenal hyperplasia

The steroid hormone profiles were analyzed in two primary cultures of ACTH-dependent adrenal hyperplasia (Supplementary Table 1, Fig. 4). In the first primary culture, osilodrostat significantly suppressed the levels of corticosterone (-89 nmol/L, -98%, $P = 0.0003$), cortisol (-200 nmol/L, -99%, $P = 0.0003$), and androstenedione (-15 nmol/L, -47%, $P = 0.026$), accompanied by accumulation of progesterone (+1.9 nmol/L, +58%, $P = 0.0009$). Metyrapone predominantly showed the same percentual effects on the steroid profile as osilodrostat, except for a stronger accumulative effect on progesterone by osilodrostat (+58% vs +9%; $P < 0.01$), and a less strong inhibition of 17-OHP (-2% vs -38%; $P < 0.0001$; Fig. 4). In both conditions, ketoconazole strongly inhibited production of all steroids except progesterone, which increased. Besides progesterone, ACTH stimulated the concentration of all steroids. In the ACTH-stimulated condition, no differences in percentual change were observed between osilodrostat and metyrapone. In contrast to the basal condition, ketoconazole caused a decrease in corticosterone under ACTH stimulation (-445 nmol/L, -99%, $P < 0.0001$). The total inhibition of measured steroids in the basal condition was similar for osilodrostat and metyrapone, and under ACTH stimulation a decrease of 758 nmol/L was found for osilodrostat, and of 438 nmol/L for metyrapone. Ketoconazole inhibited the total amount of steroids more potently compared to osilodrostat and metyrapone in the basal and ACTH-stimulated condition, with inhibition of 598 and 1748 nmol/L, respectively.

In ACTH-dependent adrenal hyperplasia culture no. 2, three concentrations of the compounds (0.1, 0.5, and 5 μM) were tested only in the ACTH stimulated condition (Supplementary Table 1, Fig. 4). Progesterone, DHEA, and DHEAS were below the limit of quantitation. ACTH increased the levels of all steroids. Osilodrostat induced a dose-dependent decrease in both cortisol and corticosterone (at 5 μM: -427 nmol/L, -96%, P < 0.0001; -55 nmol/L, -93%, P < 0.0001; respectively), accompanied by a dose-dependent accumulation of 11-DOC (at 5 μM: +218 nmol/L, +728%, P < 0.0001). Androstenedione and testosterone showed an increase under osilodrostat treatment (both P < 0.0001). The percentual increases in 17-OHP (+36% vs +5%; P < 0.0001), and 11-DOC (+1086% vs +728%; P < 0.0001) were stronger by metyrapone compared to osilodrostat. At 5 μM, there was a slightly greater negative balance of the total steroids of 257 nmol/L by osilodrostat compared to 182 nmol/L by metyrapone. In this primary culture, ketoconazole strongly inhibited all steroids, except 17-OHP. At 5 μM, the total decrease in steroids was 687 nmol/L for ketoconazole.

Cortisol-producing adrenocortical adenoma

The effect of the compounds was additionally studied in two primary cortisol-producing ACA cultures (Supplementary Table 2). When specifically focusing on the difference between osilodrostat and metyrapone (only in ACA primary culture no. 2), 17-OHP accumulated more strongly by metyrapone (+6% vs + 22%, P < 0.05), whereas accumulation of 11-DOC was only present after treatment with osilodrostat (+100% vs -6%; P < 0.001). Levels of progesterone and 17-OHP did not change under treatment with osilodrostat, whereas there was an increase under metyrapone (P < 0.001, P < 0.01 vs osilodrostat, respectively). Total reduction in concentration of measured steroids was strongest for ketoconazole (basal: -573 nmol/L; ACTH: -1474 nmol/L), followed by metyrapone (basal: -283 nmol/L; ACTH: -946 nmol/L) and osilodrostat (basal: -116 nmol/L; ACTH: -48 nmol/L).

Effects of osilodrostat on cell growth and ACTH secretion by pituitary tumor cells

No effects of osilodrostat were observed on cell amount and ACTH production by mouse pituitary AtT20 cells after 1, 3, and 7 days of treatment with osilodrostat (0.1 to 10 μM, Supplementary Fig. 2). In addition, no inhibitory effects were observed of osilodrostat (1 μM) on both cell growth and ACTH production in two primary human corticotroph pituitary adenoma cultures after 3 and 7 days of treatment (Supplementary Fig. 2).

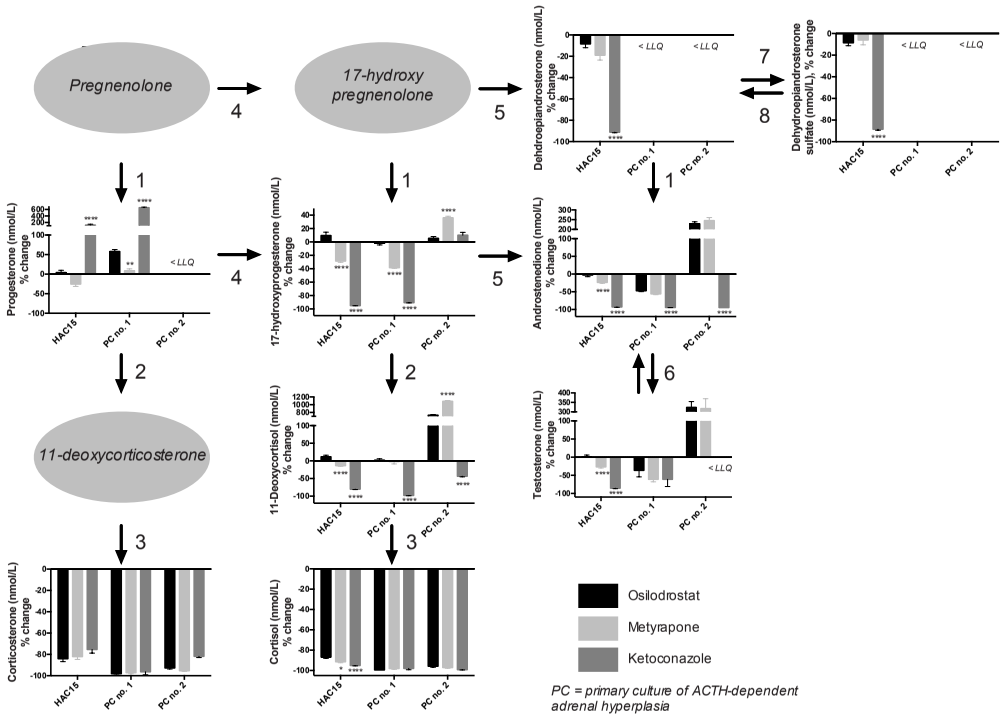


Figure 4. Effects of 5 μM osilodrostat (black bars), metyrapone (light grey bars), and ketoconazole (dark grey bars) on the steroid hormone profile in three different adrenocortical cultures. Primary cultures (PC) represent ACTH-dependent adrenal hyperplasia. The displayed conditions were chosen based on the most pronounced differences between the three compounds and were under ACTH stimulation (HAC15 and ACTH-dependent adrenal hyperplasia no. 2), and the basal condition (ACTH-dependent adrenal hyperplasia no. 1). Numbers of the primary cultures correspond to the numbers in Table 1 and 2. Arrows represent steroidogenic enzymes: (1) 3β-hydroxysteroid dehydrogenase, (2) CYP21A2, (3) CYP11B1, (4) CYP17A1 hydroxylase, (5) CYP17A1 lyase, (6) 17β-hydroxysteroid dehydrogenase III, (7) sulfotransferase, and (8) steroid sulfatase. Values are depicted as percentage change ± SEM compared to ACTH stimulation (HAC15 and ACTH-dependent adrenal hyperplasia no. 2) or vehicle treated control (ACTH-dependent adrenal hyperplasia no. 1). Note the differences in scales of the y-axes. ACTH, adrenocorticotropic hormone; LLQ, lower limit of quantitation; PC, primary culture. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ vs the effect of osilodrostat.

DISCUSSION

Medical therapy for CS is indicated in case of surgical failure or when surgery is contraindicated. In this respect, it is very important to aim at complete normalization of cortisol production in order to reduce morbidity and mortality. Pharmacotherapy for CS can, however, be associated with limited efficacy and severe side effects restricting prolonged use. Therefore, new therapeutic options are urgently needed. In this study, we show the effects of osilodrostat, a novel steroidogenesis inhibitor, on human adrenocortical steroidogenesis *in vitro*, and compared the potency of osilodrostat on steroid inhibition with those of metyrapone and ketoconazole. In addition, we evaluated potential pituitary-targeting effects of the drug *in vitro*.

In HAC15 cells, osilodrostat inhibits cortisol production approximately 2 times more potently compared to metyrapone, and 18 times more potently compared to ketoconazole. The effects of osilodrostat and metyrapone on cortisol production in primary cultures were largely similar, whereas osilodrostat did in general inhibit cortisol production at significantly lower concentrations compared to ketoconazole.

Sensitivity to steroidogenesis inhibitors appeared to be highly variable between adrenal patient tissues, with IC_{50} values varying by a factor 25, factor 18, and factor 84, for osilodrostat, metyrapone, and ketoconazole, respectively. A direct comparison between the compounds is difficult, because these IC_{50} values are partly based on different primary cultures. The primary cultures that responded to a lesser extent to osilodrostat, also seemed to respond less strongly to metyrapone and ketoconazole. A higher mean IC_{50} of osilodrostat was found for ACA (0.104 μM), followed by ACC (0.0644 μM), and the lowest mean IC_{50} was found in ACTH-dependent adrenal hyperplasia (0.0269 μM). These differences between tissue entities have to be interpreted with caution, considering the relative low number of primary cultures. Our study suggests that clinically observed differences in effects of steroidogenesis inhibitors between patients may be caused by differences on tissue level as well, rather than (only) pharmacokinetic differences. Compounds could thereby be metabolized or inactivated by the adrenal cortex, causing lower concentrations of steroidogenesis inhibitors in certain primary cultures. In CS patients, a single-nucleotide polymorphism in the CYP17A1 gene has been shown to be associated with the response to ketoconazole and metyrapone (26). Considering the small sample size and individual dose titration schemes, these results have to be interpreted with caution and confirmed in larger populations. Sensitivity might also depend on basal enzyme expression levels, differences in genotype, or cell-specific uptake and/or outward transport. Further research could focus on elucidating this issue in an attempt to make the first step towards selecting patients in which specific steroidogenesis inhibitors might be effective.

Osilodrostat is known to inhibit CYP11B2 more potently compared to CYP11B1 (15). In ACTH-dependent adrenal hyperplasia no. 1, we indeed found an almost 7-fold higher IC_{50} value for the effect on cortisol production compared to the IC_{50} value for suppressing aldosterone production. To the best of our knowledge, this is the first study to report the effects of osilodrostat on human adrenocortical steroidogenesis *in vitro*. Since ACTH-dependent cortisol-producing adrenal hyperplasias are suggested to contain no molecular alterations, these specimens are considered most useful to investigate the effects of the compounds on the steroid profile. This contrasts with e.g. cortisol-producing ACA, where recurrent activating mutations in PRKACA, encoding the catalytic subunit α of protein kinase A (PKA), have been identified in 35-65% of cases (27). Measurement of the steroid profile showed a clear inhibition of CYP11B1 by osilodrostat, as demonstrated by a strong decrease of cortisol levels and no effect on or even accumulation of its precursor 11-DOC. An increase of 11-DOC was also found in the serum of patients treated with osilodrostat (18). In HAC15 and ACTH-dependent adrenal hyperplasia no. 1, there might also be a block of CYP17A1 lyase as demonstrated by a stronger absolute inhibition of androstenedione compared to 17-OHP. This was not observed for metyrapone. Since we did not observe a strong accumulation equal to the total inhibition of steroids, there might also be inhibition of (one of the) proximal steps of the steroid biosynthetic pathway, like cholesterol side-chain cleavage enzyme and/or the StAR protein. We hypothesize that the extent of this proximal inhibition might be variable between patients, considering for example the different balance of the absolute change in steroids between the two ACTH-dependent adrenal hyperplasias at 5 μM osilodrostat treatment (-758 vs -257 nmol/L). In the two ACA cultures in the basal condition, osilodrostat might result in less strong proximal inhibition as demonstrated by a less pronounced sum of balance in change of steroids (+51 and -116 nmol/L). The upstream inhibition was variable between osilodrostat and metyrapone, with proximal inhibition that was alternately higher for osilodrostat or metyrapone. Ketoconazole might inhibit the proximal steps in the adrenal steroidogenesis more strongly compared to both osilodrostat and metyrapone, given the increased negative balance in the total amount of steroids in the different primary cultures. We do have to acknowledge that we did not measure all steroids of the profile. In order to study to which exact extent the separate enzymes are inhibited, cell lines transfected with the respective enzymes treated with osilodrostat could be used. In general, the effects of osilodrostat and metyrapone on the steroid profile were highly comparable, with subtle differences that were not comparable in every culture.

In this *in vitro* study, we observed in some cultures a slight decrease in levels of adrenal androgens under osilodrostat treatment, but not as strong as that of mineralocorticoids and glucocorticoids. *In vivo* however, an increase in testosterone might be expected due to (compensatory) ACTH stimulation. This assumes that osilodrostat treatment,

like metyrapone, might in female patients be limited by testosterone related side effects. Correspondingly, increased levels of testosterone were observed in 4 of the 14 female patients included in the clinical trial investigating osilodrostat (18). In HAC15 cells and cortisol-producing ACA culture no. 1, androstenedione and testosterone were inhibited more strongly by metyrapone, whereas metyrapone caused slightly stronger accumulation of adrenal androgens in ACTH-dependent adrenal hyperplasia no. 1 and 2, and ACA no. 2. This indicates that patients may respond slightly differently to osilodrostat and metyrapone with respect to the levels of adrenal androgens, although the clinical relevance of this difference is yet unknown.

As expected, ketoconazole had a distinctive effect on the steroid profile compared to osilodrostat and metyrapone, as a result of inhibition of multiple steroidogenic enzymes (7-9). In some conditions, ketoconazole caused accumulation of corticosterone. In ACTH-dependent adrenal hyperplasia no. 2, the direction of change of corticosterone was dose-dependent. In a previous study, in which human adrenal tissue slices were incubated with ketoconazole, it was shown that the different steroidogenic enzymes are inhibited with distinct IC_{50} values of ketoconazole (9).

For the steroidogenesis inhibitor ketoconazole, direct effects on corticotroph pituitary adenoma cells have been shown (28). Therefore, we aimed to examine potential effects of osilodrostat on pituitary adenoma cells as well. However, no effects of osilodrostat were observed on both cell growth and ACTH secretion of pituitary adenomas in this *in vitro* study. Corresponding to this, in the clinical trial using osilodrostat, diameter changes of less then 2.0 mm were observed at week 22 in the 6 patients in whom the pituitary tumor size could be followed, which is a change that is considered not clinically meaningful (18, 29).

Taking the effects on the production of cortisol and the steroid profile together, it is concluded that direct effects of osilodrostat and metyrapone on adrenocortical cells are highly comparable, with no clear higher potency for cortisol inhibition of one of both compounds. However, despite the similarities of *in vitro* potency of osilodrostat and metyrapone, clinically it appears that there is a large difference in the administered dosage between the compounds, for a response (normalization of UFC or ≥ 50% decrease of baseline) in CD patients. The median metyrapone dosage for normalization of UFC in CD patients appears to be 1375 mg (range 500 – 3500 mg) per day (4), whereas dosages of 4 to 100 mg osilodrostat per day were administered in the phase II clinical trial to normalize UFC (30). Furthermore, in the extended phase II study, osilodrostat dosages were escalated to a maximum of 60 mg per day (18). Initial results of the phase III study investigating osilodrostat demonstrated the use of a mean dose of approximately 8 to 15 mg per day in CD patients (19). Based on our *in vitro* study, *in vivo* differences seem to be driven

particularly by pharmacokinetic differences between both compounds. Indeed, it has been suggested that osilodrostat has an approximately 2-fold longer half-life compared to metyrapone (~4-5 vs ~2 hours) (30-32). This may result in more stable plasma levels of osilodrostat. Regarding pharmacokinetics, the plasma concentration of osilodrostat reached a maximum of 204 ng/mL (0.9 μM), measured at day 70, 12 hours after the final administration, in a patient who was uptitrated to the maximum dosage of 100 mg per day for 14 days (30). Little is known about the plasma concentration of metyrapone and its variance, although 4 hours after administration, a plasma concentration of 500 ng/mL (2.2 μM) may be reached (750 mg single dose, www.accessdata.fda.gov/scripts/cder/daf). The conditions in which the concentrations were measured are not comparable, making a solid comparison between the plasma levels of both compounds impossible. Given the lower required dose, there is a rationale of reduced side effects and a favorable safety profile for osilodrostat compared to metyrapone. However until now, this has not been shown in clinical trials and no head-to-head comparison has been made.

In the proof-of-concept study, patients needed highly variable dosages of osilodrostat to achieve normalized UFC (range 4 to 100 mg/day; factor 25 difference), which dose corresponded to the plasma levels (0.34 to 204 ng/mL) (30). Metyrapone varied only from 500 to 3500 mg per day (factor 7 difference) to achieve normalization of UFC in CD patients (4). Furthermore, hypocortisolism related adverse events were reported in 32 to 51% (n/N = 6/19 and n/N = 70/137) of the patients treated with osilodrostat (18, 19), compared to 7% of CD patients in a retrospective study on metyrapone treatment (4). The highly variable necessary dosages to achieve eucortisolism and the occurrence of adrenal insufficiency in 25% of patients both stress the need for careful uptitration of osilodrostat in patients with CD to prevent hypoadrenalism.

In conclusion, we show that osilodrostat is a potent inhibitor of *in vitro* cortisol and aldosterone secretion in human adrenocortical cells. We demonstrate highly variable sensitivity to steroidogenesis inhibitors with respect to cortisol production between adrenal tissues of patients, which together with differences in pharmacokinetics potentially explain clinically observed differences between patients treated with the same compound. Under the conditions of our study, effects of osilodrostat and metyrapone on the steroid profile are highly comparable, where osilodrostat seems to block CYP11B1 and CYP11B2, in some conditions to a lesser extent CYP17A1 lyase activity, and a proximal step in the steroidogenesis pathway. Differences between osilodrostat and metyrapone *in vivo* are potentially the result of pharmacokinetic differences rather than the pharmacodynamic effects on the adrenal cortex. These data indicate that osilodrostat is a promising novel treatment option for patients with CS. Additional information from phase III trials will provide important further data on efficacy and safety of osilodrostat.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Effects of osilodrostat, metyrapone, and ketoconazole on the steroid profile in human adrenocortical cells

			Progesterone	Corticosterone	17-OHP
HAC15	Basal	Osilodrostat 5 µM	+0.7 ± 0.4 (+70%)	**-6.4 ± 0.2** (-95%)	-4.6 ± 5.0 (-9%)
		Metyrapone 5 µM	**+0.4 ± 0.1** (+31%)	**-6.3 ± 0.3** (-95%)	**-10 ± 3.1** (-24%****)
		Ketoconazole 5 µM	**+6.9 ± 0.3** (+644%****)	**-3.4 ± 0.1** (-92%)	**-33 ± 2.0** (-93%****)
	ACTH	Osilodrostat 5 µM	+0.2 ± 03 (+4%)	**-4.7 ± 1.0** (-84%)	+3.6 ± 2.6 (+10%)
		Metyrapone 5 µM	**-0.6 ± 0.2** (-25%)	**-3.9 ± 0.7** (-82%)	**-12 ± 1.4** (-28%****)
		Ketoconazole 5 µM	**+5.6 ± 0.5** (+121%****)	**-2.7 ± 0.5** (-75%)	**-37 ± 0.9** (-95%****)
ACTH-dependent adrenal hyperplasia no. 1	Basal	Osilodrostat 5 µM	**+1.9 ± 0.2** (+58%)	**-89 ± 7.4** (-98%)	-0.4 ± 1.4 (-2%)
		Metyrapone 5 µM	+0.2 ± 0.2 **(+9%**)**	**-76 ± 5.2** (-97%)	**-8.8 ± 0.6** (-38%****)
		Ketoconazole 5 µM	**+25 ± 0.6** (+655%****)	**-144 ± 13** (-96%)	**-14 ± 0.9** (-91%****)
	ACTH	Osilodrostat 5 µM	**+2.3 ± 0.3** (+82%)	**-241 ± 5.7** (-98%)	-3.5 ± 6.1 (-7%)
		Metyrapone 5 µM	**+0.9 ± 0.2** (+44%)	**-76 ± 5.2** (-97%)	**-11 ± 3.8** (-27%)
		Ketoconazole 5 µM	**+60 ± 1.4** (+999%****)	**-445 ± 16** (-99%)	**-32 ± 3.1** (-85%****)
ACTH-dependent adrenal hyperplasia no. 2	ACTH	Osilodrostat 0.1 µM	< LLQ	**-22 ± 4.8** (-37%)	+0.05 ± 0.1 (+2%)
		Metyrapone 0.1 µM	< LLQ	**-17 ± 4.5** (-28%)	**+0.5 ± 0.1** (+21%*)
		Ketoconazole 0.1 µM	< LLQ	**+91 ± 4.1** (+124%****)	**+0.3 ± 0.1** (+15%)
		Osilodrostat 0.5 µM	< LLQ	**-44 ± 4.1** (-74%)	-0.1 ± 0.2 (-5%)
		Metyrapone 0.5 µM	< LLQ	**-40 ± 4.3** (-67%)	**+1.1 ± 0.1** (+46%****)
		Ketoconazole 0.5 µM	< LLQ	**+183 ± 4.5** (+250%****)	**+0.6 ± 0.1** (+30%****)

11-Deoxycortisol	Cortisol	DHEA	DHEAS	Androstenedione	Testosterone
-282 ± 144 (-10%)	**-79 ± 5.1** (-87%)	**-10 ± 1.7** (-38%)	**-284 ± 50** (-33%)	**-738 ± 118** (-38%)	**-11 ± 3.3** (-21%)
-146 ± 73 (-6%)	**-77 ± 3.7** (-88%)	**-9.5 ± 2.8** (-38%)	**-217 ± 35** (-29%)	**-500 ± 61** (-31%)	**-11 ± 1.6** (-28%)
-1539 ± 80 (-79%****)	**-65 ± 2.1** (-95%*)	**-29 ± 3.5** (-96%****)	**-778 ± 72** (-91%****)	**-1254 ± 67** (-96%****)	**-32 ± 2.3** (-89%****)
+316 ± 137 (+12%)	**-51 ± 4.3** (-87%)	-2.3 ± 2.6 (-8%)	-51 ± 38 (-9%)	-40 ± 89 (-3%)	+0.4 ± 3.1 (+1%)
-398 ±157 (-13%****)	**-50 ± 3.4** (-91%*)	**-4.4 ± 1.9** (-19%)	-40 ± 39 (-6%)	**-382 ± 112** (-23%****)	**-9.6 ± 4.0** (-27%****)
-2434 ± 176 (-80%****)	**-40 ± 0.8** (-95%****)	**-24 ± 2.4** (-91%****)	**-452 ± 21** (-88%****)	**-1364 ± 93** (-93%****)	**-28 ± 3.4** (-85%****)
+8.1 ± 32 (+2%)	**-200 ± 17** (-99%)	<LLQ	<LLQ	**-15 ± 2.2** (-47%)	-0.6 ± 0.3 (-36%)
-6.9 ± 24 (-2%)	**-173 ± 11** (-98%)	<LLQ	<LLQ	**-16 ± 0.7** (-56%)	**-0.9 ± 0.1** (-61%)
-285 ± 6.5 (-98%****)	**-171 ± 14** (-98%)	<LLQ	<LLQ	**-8.3 ± 0.5** (-94%****)	**-0.3 ± 0.1** (-61%)
+347 ± 144 (+59%)	**-845 ± 15** (-99%)	<LLQ	<LLQ	**-17 ± 2.4** (-33%)	-0.3 ± 0.2 (-18%)
+557 ± 86 (+90%)	**-894 ± 32** (-99%)	<LLQ	<LLQ	**-14 ± 2.5** (-31%)	**-0.8 ± 0.2** (-44%)
-469 ± 13 (-100%****)	**-842 ± 24** (-100%)	<LLQ	<LLQ	**-19 ± 1.0** (-97%****)	**-0.6 ± 0.1** (-84%***)
+83 ± 2.5 (+278%)	**-194 ± 23** (-44%)	<LLQ	<LLQ	**+6.5 ± 0.4** (+205%)	**+0.1 ± 0.0** (+271%)
+90 ± 2.3 (+339%)	**-158 ± 11** (-36%)	<LLQ	<LLQ	**+6.3 ± 0.4** (+229%)	**+0.2 ± 0.0** (+403%)
+0.4± 1.1 (+1725%****)	**-99 ± 40** (-16%****)	<LLQ	<LLQ	**-0.4 ± 0.1** (-22%****)	<LLQ
+154 ± 6.1 (+513%)	**-371 ± 13** (-83%)	<LLQ	<LLQ	**+8.5 ± 0.7** (+268%)	**+0.2 ± 0.0** (+348%)
+221 ± 6.1 (+837%****)	**-333 ± 7.8** (-77%)	<LLQ	<LLQ	**+11 ± 0.4** (+411%****)	**+0.2 ± 0.0** (+477%)
+1.9 ± 1.1 (+9450****)	**-371 ± 40** (-60%****)	<LLQ	<LLQ	**-1.3 ± 0.1** (-72%****)	<LLQ

Supplementary Table 1. Continued

			Progesterone	Corticosterone	17-OHP
ACTH-dependent adrenal hyperplasia no. 2 (Continued)	ACTH	Osilodrostat 5 µM	< LLQ	**-55 ± 4.2** (-93%)	+0.1 ± 0.1 (+5%)
		Metyrapone 5 µM	< LLQ	**-56 ± 4.3** (-95%)	**+0.9 ± 0.1** (+36%****)
		Ketoconazole 5 µM	< LLQ	**-60 ± 3.4** (-82%)	+0.2 ± 0.1 (+10%)

Effects of osilodrostat, metyrapone, and ketoconazole on levels of progesterone, corticosterone, 17-hydroxyprogesterone (17-OHP), 11-deoxycortisol, cortisol, dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), androstenedione, and testosterone in HAC15 cells and two ACTH-dependent adrenal hyperplasias. Numbers of the primary cultures correspond with the numbers in Table 1 and 2.

Supplementary Table 2. Effects of osilodrostat, metyrapone, and ketoconazole on the steroid profile in primary human adrenocortical cultures.

			Progesterone	Corticosterone	17-OHP
Cortisol-producing ACA no. 1	Basal	Osilodrostat 1 µM	+0.1 ± 0.1 (+10%)	**-10 ± 0.9** (-67%)	+0.7 ± 0.5 (+6%)
		Ketoconazole 1 µM	**+2.1 ± 0.3** (+263%****)	**+23 ± 6.7** (-68%****)	**+1.9 ± 0.3** (+22%*)
	ACTH	Osilodrostat 1 µM	+0.2 ± 0. (+8%)	**-126 ± 17** (-85%)	-0.6 ± 6.1 (-1%)
		Ketoconazole 1 µM	**+4.7 ± 2.3** (+238%***)	**+131 ± 51** (+65%****)	+3.0 ± 1.7 (+10%)
Cortisol-producing ACA no. 2	Basal	Osilodrostat 1 µM	+0.1 ± 0.1 (+13%)	**-36 ± 4.9** (-37%)	-0.8 ± 3.7 (-1%)
		Metyrapone 1 µM	**-0.1 ± 0.0** (-19%)	**-36 ± 3.8** (-42%)	**-16 ± 2.1** (-19%*)
		Ketoconazole 1 µM	**+0.7 ± 0.1** (+77%***)	**+125 ± 26** (+92%****)	**-27 ± 3.4** (-54%****)
	ACTH	Osilodrostat 1 µM	**+0.0 ± 0.0** (+21%)	**-483 ± 39** (-53%)	**+7.8 ± 1.2** (+38%)
		Metyrapone 1 µM	+0.0 ± 0.0 (+8%)	**-741 ± 10** (-78%**)	**+14 ± 0.9** (+75%****)
		Ketoconazole 1 µM	**+0.1 ± 0.0** (+50%*)	**+1245 ± 86.** (+137%****)	**-3.3 ± 0.7** (-21%****)

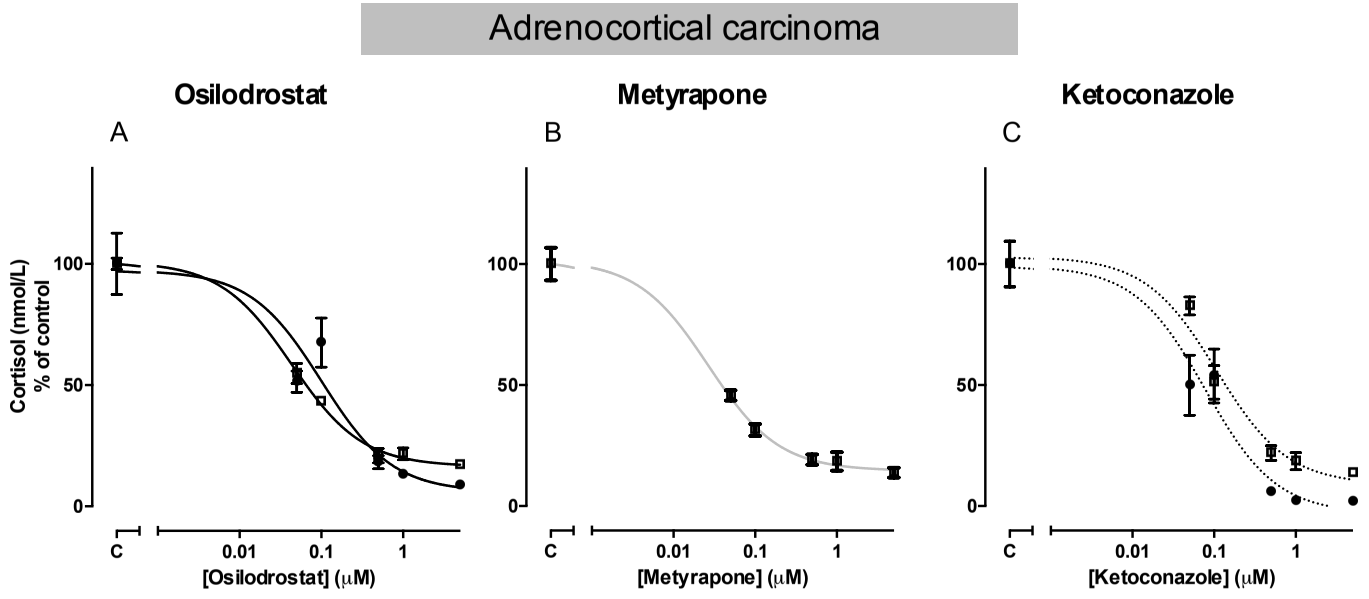
Effects of osilodrostat, metyrapone, and ketoconazole on levels of progesterone, corticosterone, 17-hydroxyprogesterone (17-OHP), 11-deoxycortisol, cortisol, dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), androstenedione, and testosterone in two primary cortisol-producing adrenal adenoma cultures. Numbers of the primary cultures correspond with the numbers in Table 1 and 2.

11-Deoxycortisol	Cortisol	DHEA	DHEAS	Androstenedione	Testosterone
+218 ± 5.7 (+728%)	**-427 ± 13** (-96%)	<LLQ	<LLQ	**+7.2 ± 0.4** (+228%)	**+0.2 ± 0.0** (+324%)
+287 ± 5.8 (+1086%****)	**-421 ± 8.0** (-97%)	<LLQ	<LLQ	**+6.7 ± 0.5** (+245%)	**+0.1 ± 0.0** (+317%)
-8.7 ± 1.1 (-44%****)	**-617± 39** (-99%)	<LLQ	<LLQ	**-1.7 ± 0.1** (-95%****)	<LLQ

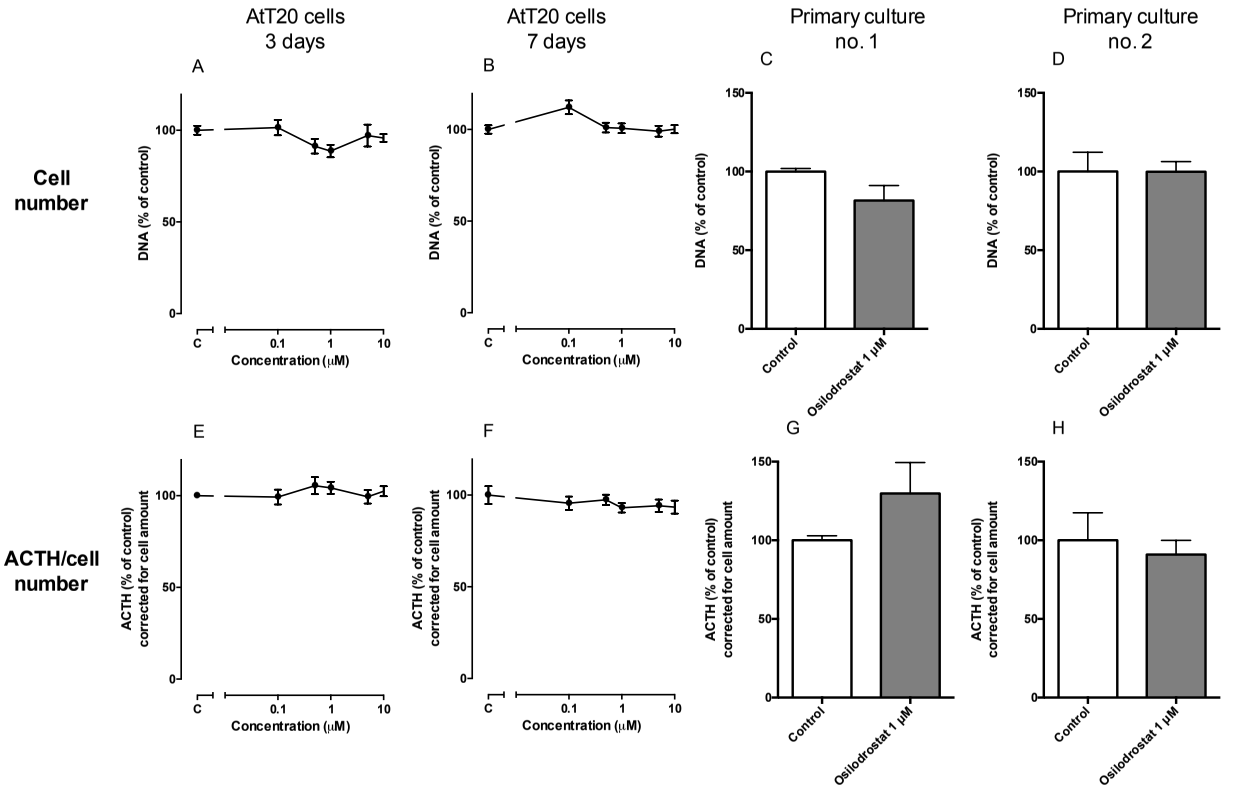
Data are presented as absolute concentration (nmol/L) change ± standard error of the difference compared to vehicle treated control (basal) or compared to ACTH stimulation (ACTH), with percentage change compared to control between brackets. Significant absolute changes compared to control are depicted in bold. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ compared to the percentage change by osilodrostat. ACTH, adrenocorticotropic hormone; LLQ, lower limit of quantitation.

11-Deoxycortisol	Cortisol	DHEA	DHEAS	Androstenedione	Testosterone
+105 ± 13 (+100%)	**-49 ± 4.0** (-80%)	**+**0.0 ± 0.1 (+0%)	<LLQ	**+4.3 ± 0.8** (+36%)	**+0.2 ± 0.0** (+41%)
-6.4 ± 7.2 (-6%****)	**-67 ± 23** (-47%)	**-1.6 ± 0.1** (-60%****)	<LLQ	**-6.0 ± 0.5** (-71%****)	**-0.2 ± 0.0** (-47%****)
+600 ± 83 (+117%)	**-734 ± 114** (-90%)	<LLQ	<LLQ	**+17 ± 5.8** (+43%)	**+**0.1 ± 0.2 (+6%)
+36 ± 16 (+13%****)	-276 ± 155 (-90%)	<LLQ	<LLQ	**-7.7 ± 3.0** (-56%***)	-0.2 ± 0.1 (-46%)
+527 ± 23 (+202%)	**-630 ± 34** (-75%)	-0.5 ± 0.4 (-9%)	<LLQ	**+**22 ± 11 (+18%)	**+2.0 ± 0.6** (+28%)
+454 ± 16 (+158%***)	**-704 ± 11** (-87%)	**-0.7 ± 0.2** (-11%)	<LLQ	**+19 ± 6.3** (+13%)	**+1.2 ± 0.5** (+13%)
-143 ± 26 (-41%****)	**-430 ± 60** (-38%****)	**-3.9 ± 0.3** (-65%****)	<LLQ	**-88 ± 2.7** (-82%****)	**-6.6 ± 0.2** (-80%****)
+2944 ± 103 (+2022%)	**-2908 ± 310** (-51%)	**+1.3 ± 0.3** (+22%)	<LLQ	**+369 ± 14** (+331%)	**+21 ± 0.4** (+382%)
+3838 ± 116 (+2993****)	**-4495 ± 90** (-77%****)	**+1.5 ± 0.3** (+26%)	<LLQ	**+415 ± 13** (+404%****)	**+22 ± 1.0** (+525%****)
-24 ± 8.0 (-24%****)	**-2660 ± 244** (-39%)	-1.0 ± 0.4 (-24%)	<LLQ	**-29 ± 2.7** (-64%****)	**-1.3 ± 0.2** (-66%****)

Data are presented as absolute concentration (nmol/L) change ± standard error of the difference compared to vehicle treated control (basal) or compared to ACTH stimulation (ACTH), with percentage change compared to control between brackets. Significant absolute changes compared to control are depicted in bold. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ compared to the percentage change by osilodrostat. ACA, adrenocortical adenoma; ACTH, adrenocorticotropic hormone; LLQ, lower limit of quantitation.

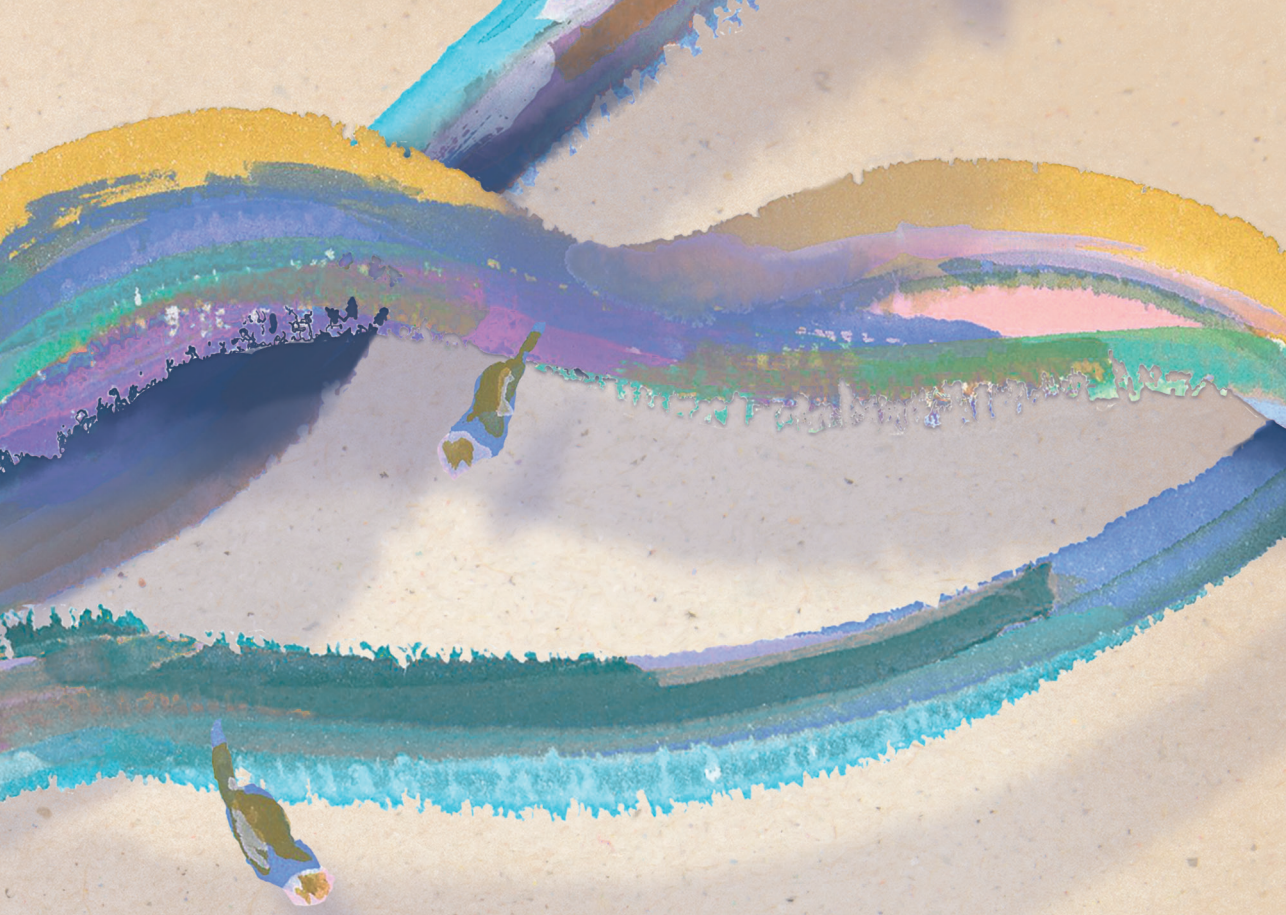


Supplementary Figure 1. Effects of osilodrostat (left, black solid lines), metyrapone (middle, grey solid line), and ketoconazole (right, black dotted lines) on basal cortisol production in primary human adrenocortical carcinoma cultures. Symbols are presented in Table 2. Values are depicted as mean ± SEM and as percentage of vehicle treated control. C, control.



Supplementary Figure 2. Effects of osilodrostat on cell amount (upper row, A-D) and ACTH secretion corrected for cell amount (bottom row, E-H) in mouse pituitary AtT20 cells (A-B, E-F) and in two primary human corticotroph pituitary adenoma cultures (C-D, G-H). Primary cultures were incubated with treatment for 7 days. Values are depicted as mean ± SEM and as percentage of vehicle treated control.

PART II



CHAPTER 4

Methylation of IGF2 regulatory regions to diagnose adrenocortical carcinomas

Creemers SG, van Koetsveld PM, van Kemenade FJ, Papathomas TG, Franssen GJH, Dogan F, Eekhoff EMW, van der Valk P, de Herder WW, Janssen JAMJL, Feelders RA, and Hofland LJ

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ABSTRACT

Adrenocortical carcinoma (ACC) is a rare malignancy with a poor prognosis. Discrimination of ACCs from adrenocortical adenomas (ACAs) is challenging on both imaging and histopathological grounds. High *IGF2* expression is associated with malignancy, but shows large variability. In this study, we investigate whether specific methylation patterns of *IGF2* regulatory regions could serve as a valuable biomarker in distinguishing ACCs from ACAs.P yrosequencing was used to analyze methylation percentages in DMR0, DMR2, ICR (consisting of CTCF3 and CTCF6) and the *H19* promoter. Expression of *IGF2* and *H19* mRNA was assessed by real-time quantitative PCR. Analyses were performed in 24 ACCs, 14 ACAs and 11 normal adrenals. Using Receiver Operating Characteristic analysis, we evaluated which regions showed the best predictive value for diagnosis of ACC and determined the diagnostic accuracy of these regions. In ACCs, the DMR0, CTCF3, CTCF6, and the *H19* promoter were positively correlated with *IGF2* mRNA expression ($P < 0.05$). Methylation in the most discriminating regions distinguished ACCs from ACAs with a sensitivity of 96%, specificity of 100% and an AUC of 0.997 ± 0.005. Our findings were validated in an independent cohort of 9 ACCs and 13 ACAs, resulting in a sensitivity of 89% and a specificity of 92%. Thus, methylation patterns of *IGF2* regulatory regions can discriminate ACCs from ACAs with high diagnostic accuracy. This proposed test may become the first objective diagnostic tool to assess malignancy in adrenal tumors and facilitate the choice of therapeutic strategies in this group of patients.

INTRODUCTION

Adrenocortical carcinoma (ACC) is a rare malignancy, with an estimated incidence of 0.7-2.0 cases per million population each year (1-4). The prognosis is poor, with overall 5-year survival rates of 16-44% (5). The likelihood of malignancy of an adrenal mass is based on the CT/MRI imaging phenotype (e.g. size, Hounsfield units, contrast washout) and is decisive for surgery (6). However, the discrimination of ACCs from adrenocortical adenomas (ACAs) still remains challenging on both imaging and postoperative histopathological grounds. The current pathological scoring system (Weiss score) is limited by a lack of reproducibility and lacks diagnostic accuracy in so-called 'borderline malignant' tumors with a Weiss score of 2 or 3 (7, 8). The assessment of malignant potential in adrenocortical tumor variants and/or pediatric adrenocortical neoplasms can be challenging and requires an experienced histopathologist; accordingly, applying the Weiss scoring system could tilt the diagnosis towards malignancy in oncocytic tumors and/or tumors in the pediatric setting (9, 10), whereas underdiagnosis could possibly be the case when confronted with myxoid variants (7, 8, 10). A biomarker that determines malignancy in adrenal tumors would be an important improvement in diagnostic certainty that can underpin therapeutic strategies such as adjuvant mitotane treatment and intensity and duration of follow-up (11).

The adrenal gland produces various growth factors, including the Insulin like growth factor-2 (*IGF2*). *IGF2* is an imprinted gene primarily expressed from the paternal allele (12) and is correlated with malignancy in adrenal tumors (13-17). However, there is a large variability in *IGF2* expression and *IGF2* expression levels are not fully discriminative for a distinction between ACC and ACA (17, 18). By the concept of DNA methylation, several factors are supposed to regulate *IGF2* expression (Fig. 1).

H19, a gene whose transcript is not translated, is reciprocally imprinted with *IGF2* (19, 20). Low expression of the *H19* gene may play a role in the development of ACCs in two different ways: *H19* per se is supposed to be involved in tumor suppression and methylation of the *H19* gene regulates mRNA expression of *H19* and *IGF2* (21). Previous research revealed a higher mean degree of methylation in the *H19* promoter in ACCs compared to ACAs (22), but again with a significant overlap between both tumor entities.

Also differentially methylated regions (DMRs) play a role in the regulation of *IGF2* expression. DMRs are regions in the genome with high concentration of CpGs controlling imprinting. In other types of cancer it has been proven that hypomethylation of the DMR0 correlates with *IGF2* loss of imprinting (LOI) (23). Previous research also suggests that hypomethylation of DMR2 is associated with ACCs and increased *IGF2* expression (24).

The imprinting control region (ICR) is another element that contributes to regulation of *IGF2*. Methylation of this region determines whether *IGF2* or *H19* is expressed from the allele (25), as a result of binding of the methylation-specific CTCF protein to this region (26). Two of the key CTCF-binding sites are the 3rd (CTCF3) and 6th (CTCF6) site (27).

In this study, DNA methylation is assessed by pyrosequencing, the most sensitive and accurate method to detect methylation at a single CpG (28). To the best of our knowledge, we analyse for the first time methylation in a number of different *IGF2* regulatory regions in benign and malignant adrenal tumors. We aimed to identify specific patterns correlating with malignancy in adrenal tumors. As such, our main research aim is to evaluate whether the methylation patterns of *IGF2* regulatory regions, individually or combined, could serve as a valuable biomarker in distinguishing ACCs from ACAs. Finally, to demonstrate that methylation is involved in regulation of *IGF2* expression in ACC, we examined the effect of demethylation in ACC cell lines.

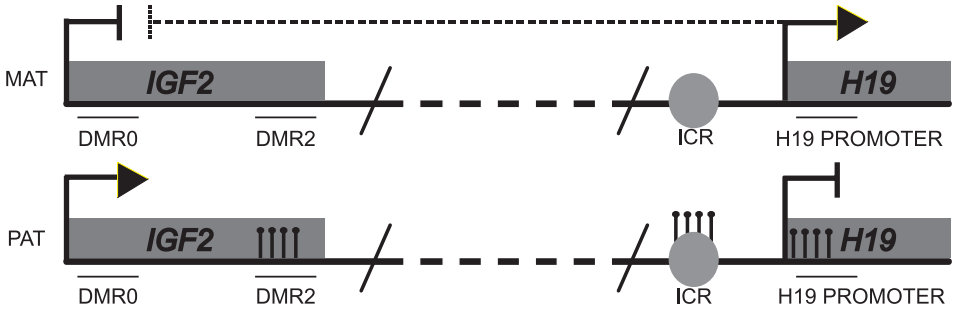

Figure 1. Overview of the imprinted region of the *IGF2* gene and *H19* gene on the maternal and paternal allele in the normal situation. Lines with dots represent known sites of DNA methylation. (arrow) expression of the gene. (sub-end arrow) repression of the gene. Methylation status of the DMR0 remains unclear. Figure is not at scale. DMR0, Differentially methylated region 0; DMR2, Differentially methylated region 2; ICR, imprinting control region, consisting of CTCF3 and CTCF6. Adapted from ref (29).

METHODS

Adrenocortical tissues

We obtained normal adrenals and adrenocortical tumors from patients during surgeries performed at the Department of Surgery, Rotterdam, Erasmus MC (EMC) between May 1995 and March 2014. We embedded the specimens in Tissue-Tek after resection and stored them at -80°C until analysis. All normal adrenals were collected during nephrectomy and confirmed by the pathologist as being normal. We randomly selected ACAs based on

availability of tumor tissue in the past 7 years. We excluded nonprimary ACCs (n = 22), ACCs with insufficient DNA or RNA yield (n = 7) or tumors defined as "borderline malignant" by the pathologist (n = 2; Supplementary Fig. 1). We collected medical history information and tumor characteristics from electronic patient records (Table 1). Follow-up data were updated from the Pathological Anatomical National Automated Archives (PALGA). An independent pathologist collected all data for pathological features, including the Weiss score, the currently used pathological scorings system for determining malignancy in adrenocortical tumors (30). A cutoff of 3 criteria or more present in the tumor was considered malignant, although only tumors with a clear pathological diagnosis were included in the analysis. We used the ENSAT classification to stage the tumors (31). To validate our findings in an independent cohort, we used a series of frozen specimens from the VU University Medical Center (VUMC), Amsterdam, the Netherlands. DNA isolation and methylation analyses were performed at the EMC. The Weiss score was independently determined both at the VUMC and at the EMC as a central review. The study was conducted under the guidelines that had been approved by the Medical Ethics Committee of the Erasmus Medical Center.

Processing of adrenocortical tissues

We used several cryostat sections of 20 µm to isolate RNA and DNA from Tissue-Tek embedded tissues and 5 µm sequential cryostat sections for Haematoxylin-eosin (HE) staining in order to confirm that we used representative tissue specimens. We considered a tissue as representative when it contained at least 80% of tumor cells in case of ACA or ACC, or when a clear normal adrenal structure was visible at microscopic evaluation.

Cell culture

We used three available human ACC cell lines: H295R and SW13 were obtained from the American Type Culture Collection and from ECACC (Salisbury, Wiltshire, UK), respectively. HAC15 cells were a kind gift from Dr. W. Rainey (Department of Physiology, Medical College of Georgia, Augusta, GA, USA). Culture conditions were described in detail previously (32). We performed incubations with and without the demethylating drug 5'-Aza-2'-deoxycytidine (AZA), purchased from Sigma-Aldrich. For treatment, we added AZA to the medium for 72 and 168 h. For 168 h of treatment, we refreshed the medium after 72 h and added AZA again. Based on the concentration of AZA that induced 50% cell growth inhibition (IC_{50}), we chose three different concentrations of AZA (0.01 µM, 0.05 µM and 1 µM) for the experiments. At the end of the incubation period, we removed medium and collected cells for DNA or total RNA isolation as described below.

Table 1. Characteristics of patients and adrenocortical tissues of the two cohorts included in this study

	EMC		VUMC	
	ACC	ACA	ACC	ACA
	n = 24	*n* = 14	*n* = 9	*n* = 13
Mean age at diagnosis (yr)	50 yr (range 9-74)	45 yr (range 26-61)	50 yr (range 22-73)	55 yr (range 17-79)
Mean follow-up (months)	42 (range 1-187)	37 (range 1-83)	20 (range 0-68)	30 (range 0-87)
Male (%)	7 (29%)	3 (21%)	2 (22%)	4 (31%)
Mean tumor size cm (SD)	13.00 (SD. 6.9)	3.05 (SD. 2.0)	16.44 (SD. 6.2)	3.33 (SD. 2.1) (*n* = 12)
Secretion				
Androgens	9 (38%)	0 (0%)	1 (13%)	1 (9%)
Glucocorticoids	13 (54%)	6 (43%)	5 (63%)	3 (27%)
Mineralocorticoids	0 (0%)	5 (36%)	0 (0%)	1 (9%)
Precursors	3 (13%)	0 (0%)	0 (0%)	0 (0%)
Estradiol	4 (17%)	0 (0%)	0 (0%)	0 (0%)
Non secreting	8 (33%)	3 (21%)	3 (38%)	6 (55%)
Weiss score (mean)	6.00 (range 3-8)	0.14 (range 0-1)	5.33 (range 3-8)	0.31 (range 0-2)
ENSAT				
I	0 (0%)	12 (86%)	0 (0%)	13 (100%)
II	13 (54%)	2 (14%)	4 (44%)	0 (0%)
III	3 (13%)	0 (0%)	4 (44%)	0 (0%)
IV	8 (33%)	0 (0%)	1 (11%)	0 (0%)
Metastasis	17 (71%)	0 (0%)	6 (67%)	0 (0%)

The two patients with tumors with an unclear diagnosis from the EMC cohort are not included in the table. In the VUMC cohort, 1 patient with ACC and 2 patients with ACA did not receive a thorough hormonal workup, so data are missing. EMC, Erasmus University Medical Center; VUMC, VU University Medical Center; SD, standard deviation; ENSAT, European Network for the Study of Adrenal Tumors.

mRNA expression analysis

We isolated total RNA from ACC cells using the High Pure RNA Purification Kit and total RNA from tissues using the High Pure RNA Tissue Kit (both from Roche) according to manufacturer's protocol. To synthesize cDNA, we added 500 ng mRNA template to 40 µl Super reverse transcriptase (RT) buffer (HT Biotechnology Ltd., Cambridge, UK) containing 40 nmol dNTP, 20U RNA'sin, 15 ng oligo-dT, 4 U Super RT (HT Biotechnology Ltd., Cambridge, UK). After 1 h incubation at 40 °C, we diluted the cDNA five times. We mixed 7.5 µl Taqman universal PCR Master Mix (Applied Biosystems) with concentrations of primers and probes described in Supplementary Table 1, with 5 µl cDNA template.

Real-time quantitative PCR was performed by TaqMan Gold nuclease assay (Perkin Elmer Corporation, Foster City, CA, USA) and the ABI-PRISM-7900 Sequence Detection System (Perkin Elmer), according to manufacturer's protocol. PCR conditions were 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Hypoxanthine-guanine phosphoribosyl transferase 1 (*HPRT1*) was used to normalize mRNA levels. We calculated PCR efficiencies (E) for the primer-probe combinations (Supplementary Table 1, Sigma-Aldrich) and calculated relative expression of genes using the comparative CT method $2^{-\Delta\Delta C}{}_{t}$.

Bisulfite conversion and pyrosequencing

We isolated DNA from ACC cells and adrenocortical tissues using the Wizard ® Genomic DNA Purification Kit (Promega, Leiden, the Netherlands). We treated genomic DNA (1 ng – 1 µg) with sodium bisulfite using the Epitect® Plus DNA Bisulfite Kit (Qiagen, Benelux) following manufacturer's protocol.

Each PCR mix of 50 µl contained 0.2 uM of forward and reverse primer (one of them being biotinylated), 0.25 mM dNTPs, 2 mM MgCl, 15µl 10x PCR buffer, 1.5 U enzyme and 10 ng bisulfite DNA, using the FastStart High Fidelity PCR System kit (Roche, Almere, the Netherlands). PCR programmes were as follows: initial denaturation at 95°C for 10 minutes, 45 cycles of 95°C for 30 seconds, primer specific hybrid temperature (Supplementary Table 2) for 30 seconds, 72°C for 30 seconds and final extension at 72°C for 10 minutes. We confirmed amplification of all samples with agarose gel (1%) electrophoresis.

We designed a pyrosequencing assay of previously reported CpGs involved in expression of *IGF2* using Pyromark Assay Design (Supplementary Table 2). After binding of the PCR product to streptavidin-coated Sepharose beads (GE Healthcare), we washed the template, made it single-stranded and neutralized the sample. When the negative control showed a signal during test phase of the primers, we developed new primers in the same region. We performed pyrosequencing using the PyroGold SQA reagent kit (Qiagen) according to manufacturer's protocol and performed analyses on the Pyromark Q24 system. To test reproducibility, we treated 5 times the same sample of high and low methylated DNA (EpigenDx, Hopkinton, USA) with bisulfite and analyzed the assays.

Statistical analysis

We performed statistical analysis by SPSS version 21 and Graphpad Prism 3.0. In all analyses, $P < 0.05$ was considered significant. Values represent mean ± SD, unless specified otherwise. One-way ANOVA, followed by Tukey's Multiple Comparison Test, was used to test for significant differences in methylation and expression after

treatment with AZA. To assess significant differences in methylation per region and *IGF2* mRNA expression in ACCs, ACAs and normal adrenals, we used the non-parametric Mann-Whitney Test. For all correlation analyses within the carcinoma group, we used Spearman's correlation.

We quantified the mean methylation in the different regions and *IGF2* expression in the tumor by creating a score for all tumors, defined as the absolute standard deviation score (|SDS|) per region compared to methylation or expression in normal adrenals. We used Receiver Operating Characteristic (ROC) analysis to determine regions with an individually significant predictive value for the diagnosis. We included the three most discriminating CpGs in these regions in final analysis. To assess the explained variation of *IGF2* expression by these factors, we used multiple linear regression. We determined diagnostic accuracy of the |SDS| by performing ROC analysis with mean |SDS| per region for predicting the diagnosis by the pathologist. We performed the same analysis when discriminating only metastatic ACCs versus all other tumors. We determined the performance of the predictive test by carrying out 10-fold cross-validation. The data were randomly partitioned into 10 subsamples, where after we performed ROC analysis ten times, in which each subsample was used once as the validation set. An independent cohort was used to validate test performance and the proposed cutoff values. Interobserver variability of the Weiss score was quantified by using an intraclass correlation, while the interrater variability of the binary parameter of pathological diagnosis was quantified by the kappa coefficient.

RESULTS

Methylation status of the *IGF2* regulatory regions in adrenocortical tissue

For analysis, we included 24 ACCs, 14 ACAs and 11 normal adrenals. Methylation patterns are shown in Supplementary Fig. 2. A median methylation of 81% was found in DMR0 in ACCs, 77% in ACAs and 82% in normal adrenals (Fig. 2A). In DMR2, a median methylation of 24% was found in ACCs, 33% in ACAs and 33% in normal adrenals (Fig. 2B). In both regions there were no statistically significant differences in median methylation percentages between ACCs and ACAs.

CTCF3 showed a median methylation of 78% in ACCs, 48% in ACAs and 51% in normal adrenals (Fig. 2C). Methylation in ACCs was significantly higher compared to methylation in ACAs (P = 0.018). CTCF6 demonstrated a median methylation of 57% in ACCs, 40% in ACAs and 47% in normal tissue (Fig. 2D), where methylation in ACCs was significantly higher compared to ACAs (P = 0.034). As expected, the *H19* promoter showed a higher

methylation in ACCs as well, namely 69% compared to 45% in ACAs and 45% in normal adrenals (Fig. 2E). The methylation difference between ACCs and ACAs was also statistically significant ($P < 0.001$). For none of the regions there were significant differences in methylation between ACAs and normal adrenals.

Correlations between DNA methylation in different *IGF2* regulatory regions

The methylation status of CTCF3 and CTCF6, both located in the ICR, were strongly positively correlated in ACCs ($\rho = 0.614$, $P < 0.001$). Methylation in the *H19* promoter was correlated with methylation in CTCF3, CTCF6 and the DMR0 ($\rho = 0.781$, $P < 0.001$; $\rho = 0.741$, $P < 0.001$; $\rho = 0.413$, $P = 0.010$, respectively). Methylation in DMR0 was also correlated with methylation in CTCF3 and CTCF6 ($\rho = 0.595$, $P < 0.001$; $\rho = 0.361$, $P = 0.018$, respectively), although strongly driven by two low methylated carcinomas. All correlation coefficients and *P* values are listed in Supplementary Table 3.

Expression of *IGF2* mRNA in adrenocortical tissue and correlations with methylation status of different *IGF2* regulatory regions

IGF2 expression was significantly higher in ACCs compared to ACAs ($P = 0.001$, Fig. 3A). No statistically significant difference in *IGF2* expression was found between ACAs and normal adrenals.

In ACCs, the mean DMR0, CTCF3, CTCF6 and *H19* promoter methylation were positively correlated with *IGF2* mRNA expression ($\rho = 0.485$, $P = 0.016$, Fig. 3B; $\rho = 0.625$, $P = 0.001$, Fig. 3C; $\rho = 0.417$, $P = 0.042$, Fig. 3D; $\rho = 0.521$, $P = 0.009$, Fig. 3E, respectively).

Quantification of differences in methylation between carcinomas and adenomas

We calculated SDS per region for the different neoplasms (Supplementary Table 4). *IGF2* expression, DMR2, CTCF3 and the *H19* promoter individually showed a significant predictive value for the diagnosis of ACC (Fig. 4A). When using the mean |SDS| in the 2nd until 4th CpG in DMR2, the 5th until 7th CpG in CTCF3, and the *H19* promoter in a ROC curve for predicting the diagnosis, an area under the curve of 0.997 was found (Fig. 4B). Test characteristics included sensitivity of 96% and specificity of 100% for a cutoff of |SDS| 2.617, with positive predictive value of 100% and negative predictive value of 93%. These three factors together explained 45.1% (R square) of the variation in *IGF2* expression in ACCs with df 3, F-statistic 5.470 and $P = 0.007$.

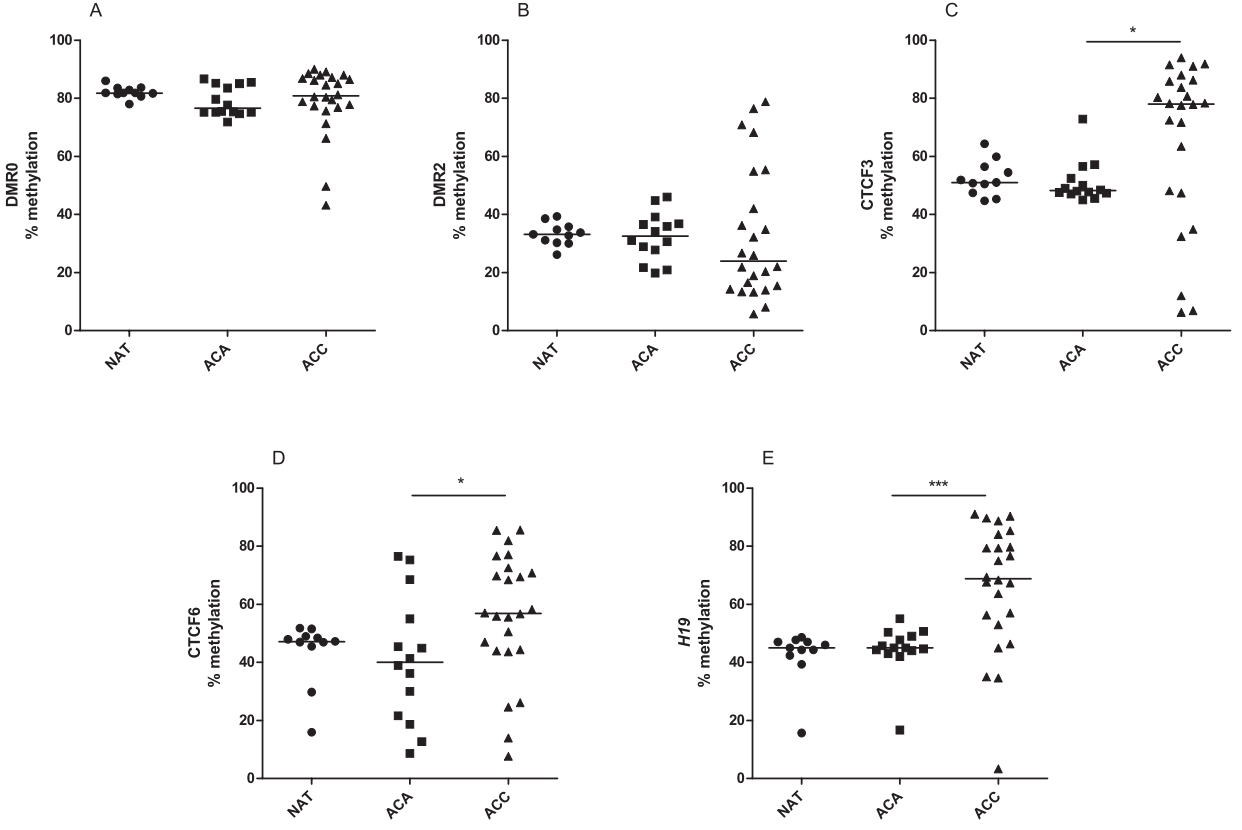

Figure 2. Methylation in the specific regions per group. Every dot represents the mean methylation for every region in a tumor of an individual patient. Lines represent medians. Mean methylations in (A) DMR0, (B) DMR2, (C) CTCF3, (D) CTCF6 and (E) H19-promoter. * $P < 0.05$, *** $P < 0.001$. ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; NAT, normal adrenal tissue.

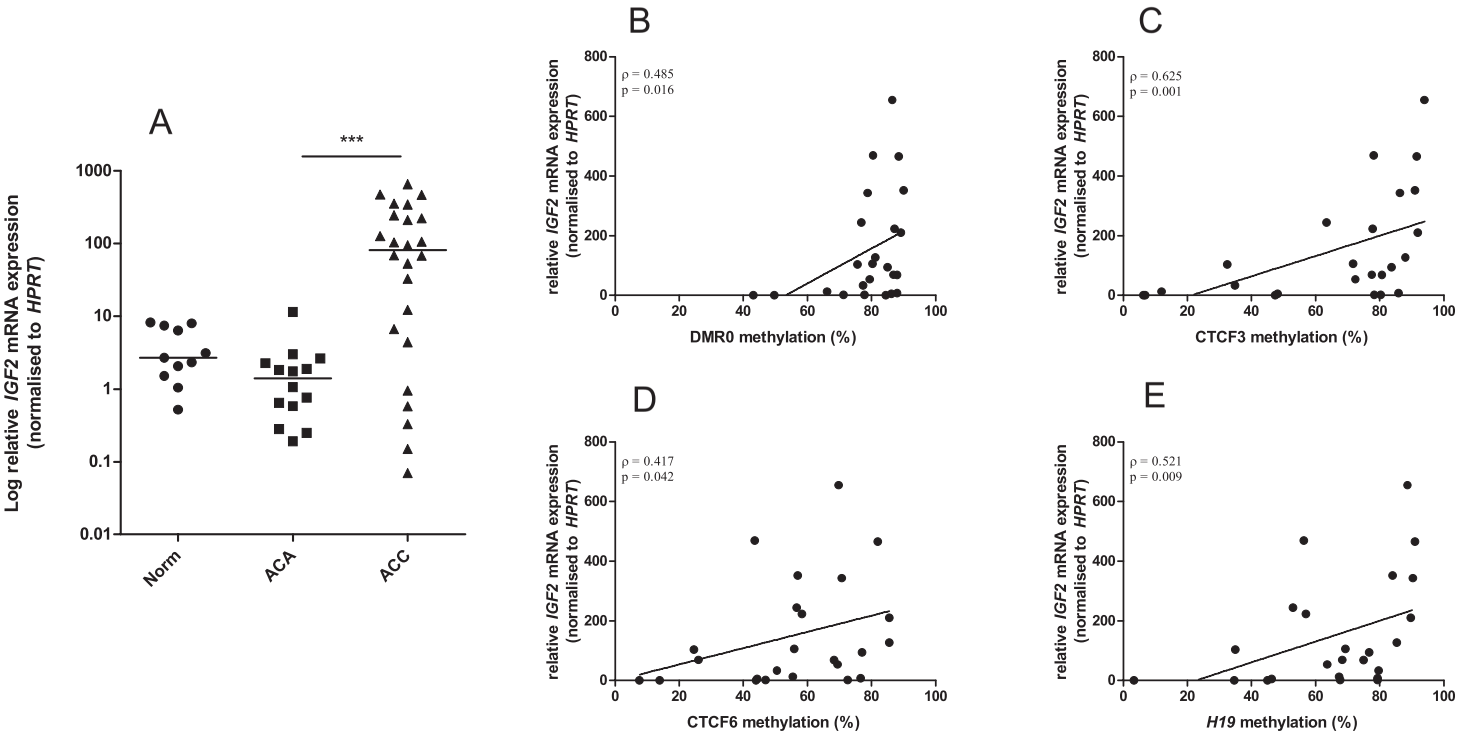

Figure 3. *IGF2* mRNA expression in adrenal tissues and correlation of *IGF2* mRNA expression in ACCs with methylation in *IGF2* regulatory regions. Expression data are presented as scatter dot plot. Expressions are normalized to *HPRT1*. ρ represents Spearman correlation coefficient. (A) Relative *IGF2* mRNA expression in different groups on a log transformed scale. Lines represent medians. *** $P < 0.001$. (B) Correlation of *IGF2* mRNA expression in ACCs with DMR0 promoter methylation, (C) CTCF3 methylation, (D) CTCF6 methylation and (E) *H19* methylation. ACA, adrenocortical adenomas; ACC, adrenocortical carcinomas; NAT, normal adrenal tissue.

Three tumors were difficult to classify based on (only) the Weiss score. Two tumors with a diameter of 9.5 cm and 6.5 cm had both a Weiss score of 1. However, the pathologist considered the first tumor as borderline malignant, because it showed minor criteria in the Lin-Weiss-Bisceglia (LWB) system, a modified Weiss system for diagnosis of oncocytic neoplasms (7). The second case was also difficult to classify, because some criteria, i.e. capsular invasion and nuclear polymorphism, were only present in parts of the tumor. Necrosis and a high mitotic rate were not present. When these two tumors with uncertain pathological diagnosis, which were therefore not included in the ACA or ACC group, were tested with the methylation scorings system, they scored |SDS| of 4.29 and 4.20 respectively. This, in turn, would lead in our test to the classification of an ACC. During follow-up (27 and 8 months respectively), no recurrence or metastases have been detected yet.

A third patient underwent surgery because of a cortisol-producing adrenal tumor with the imaging phenotype of an adenoma. Pathological examination showed a Weiss score of 3 with a preferred diagnosis of ACC. However, not all negative prognostic factors (e.g. necrosis, capsular invasion, atypical mitosis) were present in this tumor. Although the diagnosis ACC was not certain the patient was treated with mitotane as adjuvant therapy. After 20 months, lymph node- and subsequently bone metastases developed, confirming the diagnosis of ACC. The methylation score of this tumor showed a mean |SDS| of 4.68, which indicates malignancy based on our proposed test.

The mean |SDS| of the three regions did not significantly differ between ACCs with and without metastasis at the end of follow-up. When we segregated cases with adverse clinical outcome, i.e. metastatic disease, from all other cases, an AUC of 0.900 ± 0.049 ($p<0.0001$) was found.

A 10-fold cross validation resulted in a good prediction of the diagnosis in 95% of the cases, with a mean cutoff of |SDS| 2.555 ± 0.22. Mean sensitivity was 96 ± 2.0 %, specificity 100 ± 0.0% and AUC 0.997 ± 0.002. Mean coefficient of variation (CV) of regions included in final analysis in high methylated samples was 0.039 and low methylated samples 0.380. CVs for all regions are described in Supplementary Table 5.

Validation of the proposed diagnostic test in an independent cohort

After exclusion of four samples due to insufficient DNA yield, 9 ACCs and 13 ACAs were included in the validation cohort, based on the diagnosis determined by the pathologist at the VUMC. There was high agreement between the two pathologists (VUMC and EMC) regarding the Weiss score (r = 0.914), with one discordant final pathological diagnosis (κ = 0.904). The mean |SDS| was assessed in the 3 regions proposed on the basis of the EMC cohort (Fig. 5, A and B). ROC analysis, using the diagnosis as determined by the pathologist from the VUMC, showed an AUC of 0.957 ± 0.039 with a sensitivity of 89%, specificity of 92%, and a cutoff value of |SDS| 2.331. When we applied the cutoff value obtained from the EMC cohort (2.617) to the validation cohort, a sensitivity of 75% and a specificity of 92% were found. This sensitivity increased to 88% when the pathological diagnosis obtained at the EMC was used. The mean |SDS| of the tumor with discordant pathological diagnosis by the 2 pathologists was 2.49, and follow-up was only 2 months. Combination of the two cohorts (ACA *n* = 27, ACC *n* = 33) showed an AUC of 0.983 ± 0.013 with a sensitivity of 94% and a specificity of 96% using a cutoff of mean |SDS| 2.442 (Fig. 5, C and D).

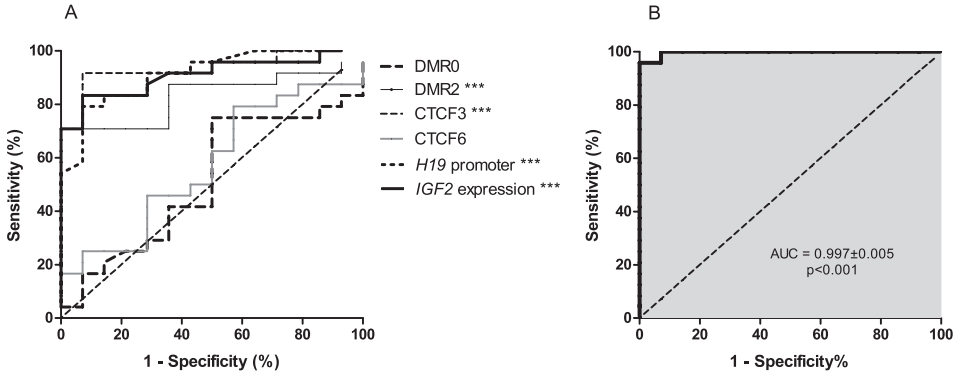

Figure 4. Receiver Operating Characteristic (ROC) curve of mean absolute standard deviation score (|SDS|) compared with methylation in normal adrenals displayed as sensitivity (y-axis) and 1-specificity (x-axis) for the diagnosis of adrenocortical carcinoma (ACC) or adrenocortical adenoma (ACA) according to the pathologist. The straight diagonal lines represent reference lines. (A) ROC curve of mean |SDS| compared to methylation in normal adrenals for methylation in all regions and *IGF2* mRNA expression separately. DMR2 (P = 0.001), CTCF3 (P < 0.001), the *H19* promoter (P < 0.001) and *IGF2* expression (P < 0.001) showed individually significant predictive value for diagnosis of ACC or ACA. (B) ROC curve of the *H19*-promoter, 2nd until 4th CpG in the DMR2 and 5th until 7th CpG in CTCF3 for the diagnosis of ACC or ACA according to the pathologist. The area under the curve (AUC) was 0.997 ± 0.005, P < 0.001. Cutoff value: Mean |SDS| 2.617, sensitivity 96%, specificity 100%.

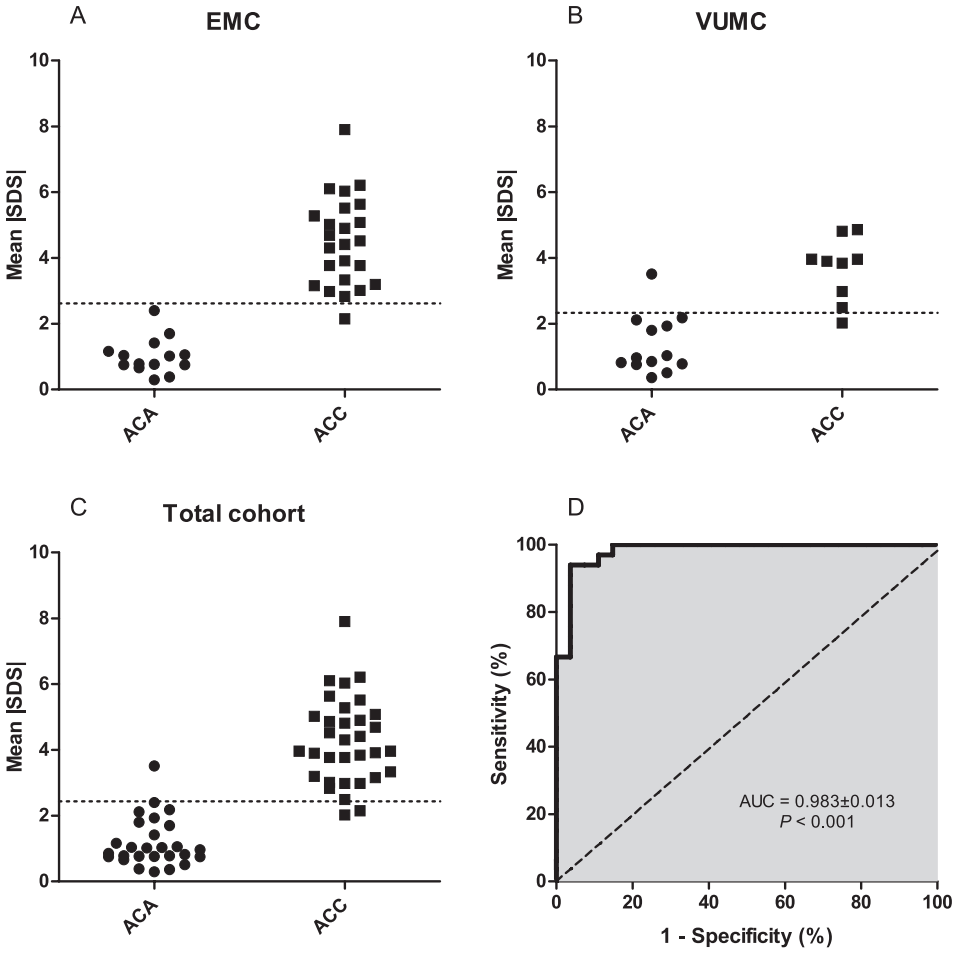

Figure 5. Mean absolute standard deviation scores (|SDS|) of the *H19*-promoter, second until fourth CpG in the DMR2, and fifth until seventh CpG in CTCF3 in the two different cohorts for adrenocortical adenomas (ACA) and carcinomas (ACC), as determined by the local pathologist on the basis of the Weiss score. Mean |SDS| in the EMC cohort (A), the VUMC cohort (B), and for the total cohort (C). Horizontal dotted lines represent the optimal cutoff value per cohort, as determined by Receiver Operating Characteristic (ROC) curve. (D) ROC of the mean |SDS| for the diagnosis of ACC or ACA according to the pathologist in the total cohort, combining the specimens from the EMC and VUMC. The area under the curve (AUC) was 0.983 ± 0.013 with a sensitivity of 94% and a specificity of 96% using a cutoff of mean |SDS| 2.442 for diagnosing malignancy. The straight diagonal line represents reference line. EMC, Erasmus University Medical Center; VUMC, VU University Medical Center.

Effect of 5'-Aza-2'-deoxycytidine (AZA) treatment on DNA methylation and expression of *IGF2* and *H19* mRNA in human ACC cell lines

In order to determine the role of DNA methylation on *IGF2* and *H19* expression, we evaluated the effect of the demethylating drug AZA in three human ACC cell lines (H295R, HAC15 and SW13). After 7 days of 1 μM AZA treatment, the mean methylation in all regulatory regions in H295R decreased with 51.9 ± 2.4% $P < 0.0001$ (mean ± se), in a both time- and concentration dependent manner (Supplementary Fig. 3, A, B, C, D, E and H, I, J, K, L). Concomitant with the decrease in methylation, we observed a strong and statistically significant decrease in *IGF2* mRNA expression after 7 days of treatment with AZA in H295R (-79.4 ± 3.8%, $P < 0.01$, mean±se), again in a both time- and concentration dependent manner (Supplementary Fig. 3, F and M). The decrease in *IGF2* mRNA expression was accompanied with strongly increased *H19* mRNA expression (Supplementary Fig. 3, G and N). We found similar results for methylation percentages and expression data in HAC15 cells (data not shown). SW13 methylation patterns were partly different compared to H295R and HAC15 and SW13 cells did not express *H19* or *IGF2* mRNA (data not shown).

DISCUSSION

In the current study, we found that a combined DNA methylation score of three different *IGF2* regulatory regions discriminates ACCs and ACAs with a sensitivity of 96% and specificity of 100%. For tumors of the adrenal cortex, the chance on malignant behavior increases with tumor size. The differentiation between ACC and ACA on histopathological grounds can be challenging, by both a poor reproducibility and by tumors classified as being of uncertain malignant behavior (33-36). Mitotane treatment is given to prevent disease recurrence in patients with malignant adrenal tumors but is associated with serious toxicity (4, 6). A reliable biomarker that indicates malignant behavior, particularly in 'borderline malignant' tumors, could be helpful for the decision on postoperative strategies, like the choice on adjuvant mitotane and the duration and intensity of follow-up.

If we would have used this test for the patient with a Weiss score of 3 who developed metastasis after 20 months, the methylation score could have been supportive for the decision of adjuvant mitotane treatment. For the two cases originally classified as tumors with 'uncertain behavior', but are identified as ACC according to our proposed test, close monitoring is currently performed. Further follow-up, which is now 27 and 8 months from diagnosis, will have to elucidate whether these tumors will recur or metastasize and whether the methylation score indeed offers benefit compared to the Weiss score in these cases. At present, no recurrences or metastases developed in both patients.

In this study, we confirmed that *IGF2* is an important marker for ACC, but as also previously described in a number of studies, does not fully discriminate ACCs from ACAs (37). Mean sensitivity and specificity for diagnosing malignancy are both around 80%, with substantial variances between studies. *H19* seems to be an important regulator, given the positive correlation of the *H19* promoter methylation with *IGF2* expression in our study, as well as in previous research (22). By pyrosequencing of different regions in a tumor of the same patient, we were able to define the most discriminating regions in this dataset and propose a diagnostic test with very high accuracy. Choosing a smaller subset of CpGs provided even better test characteristics.

We used three different adrenocortical cancer cell lines to demonstrate the mechanism of regulation of *IGF2*. By treating adrenocortical cancer cells with the demethylating agent AZA, we were able to strongly decrease the *IGF2* mRNA expression and increase the *H19* mRNA expression. Herewith we confirmed DNA methylation as a strong regulatory element in *IGF2* and *H19* expression. Although it is still unclear whether *IGF2* is mainly a contributor or an effect of malignancy (38, 39), further research should be done in order to find new insights in therapeutic possibilities of demethylating drugs, considering the reversibility of *IGF2* mRNA expression.

Pyrosequencing is a technology yielding accurate and highly reproducible knowledge about methylation percentages at a CpG (28, 40). By analyzing normal adrenals and neoplasms, we could quantify to which extent methylation in tumors deviated from normal. The methylation patterns in DMR2 and CTCF3 visually revealed 3 phenotypes in ACCs: hypomethylated, moderately methylated and hypermethylated (Fig. 2, F and I, respectively). The concept of *IGF2* regulation is based on instability of the *IGF2/H19* locus. By transforming methylation in absolute SDS, we identified both hypomethylated and hypermethylated phenotypes. Additionally, methylation of DMRs can have various effects on gene expression, both silencing and activation (41). Methylation of CpGs in promoter regions generally cause repression of the gene (42).

It is important to take into account heterogeneity of adrenocortical tumors, since this can provide different percentages of methylation in different parts of the tumor.

A notable consideration in this study is the reference diagnosis, considering the only definite diagnosis of ACC after metastasis. We have set the diagnosis of the tumors as conclusive as possible, taking into account histopathological features and presence of metastasis (71% of all ACCs). The AUC of 0.900 ± 0.049 for discriminating metastatic ACCs vs all other tumors requires the consideration that there are ACCs reported with no adverse outcome and long survival (>10 years), in literature as well as in our cohort.

The aberrant methylation patterns in ACCs can be explained either by loss of heterozygosity or by de novo (de)methylation. Probably in most ACCs, aberrant methylation patterns are caused by deletion of the maternal allele, a structural abnormality mostly seen in malignant adrenal tumors (43). However, this cannot be the case in all ACCs based on the methylation patterns. It is important to notice that the underlying cause of these methylation patterns will not influence the test characteristics.

This diagnostic test, based on analysis in only DNA, has an advantage for both reproducibility and time consumption. Besides, it makes the test more practical and useful, since RNA is more instable and fragile. We do need to take into account that our analyses are performed on frozen specimens, which is at the moment not readily available in all centers. Future research could focus on the possibility of assessing methylation percentages on DNA obtained from formalin-fixed paraffin-embedded specimens, improving the diagnostic applicability of our proposed test.

Our findings were validated in an independent cohort, which largely increases the generalizability of the proposed diagnostic test. However, larger studies, requiring multicenter collaborations, due to the rare incidence of ACCs, will be necessary to fully validate the predictive value of these specific epigenetic changes and to also validate the required cutoff values for malignancy. These results should be compared with urine steroid metabolomics, a recently introduced new potential preoperative marker for malignancy in adrenal tumors (44).

In conclusion, we show that specific methylation patterns of *IGF2* regulatory regions are a promising tool to assess malignancy in adrenal tumors and could lead to the first objective diagnostic tool for identifying ACCs.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. List of primers used for real-time quantitative PCR

Gene	PCR primers (5'-3')	Concentration	EF
HPRT1	Forward: 5'-CACTGGCAAAACAATGCAGACT-3'	0.5 pmol	1.91
	Reverse: 5'-GTCTGGCTTATATCCAACACTTCGT-3'	0.5 pmol	
	Probe: 5'-[6FAM]CAAGCTTGCGACCTTGACCATCTTTGGA[TAM]-3'	0.1 pmol	
IGF2	Forward: 5'-CCAAGTCCGAGAGGGACGT-3'	0.3 pmol	1.91
	Reverse: 5'-TTGGAAGAACTTGCCCACG-3'	0.3 pmol	
	Probe: 5'-[6FAM]ACCGTGCTTCCGGACAACTTCCC[TAM]-3'	0.1 pmol	
H19	Forward: 5'-TGCTGCACTTTACAACCACTG-3'	0.3 pmol	1.87
	Reverse: 5'-ATGGTGTCTTTGATGTTGGGC-3'	0.3 pmol	
	Probe: 5'- [6FAM]TCGGCTCTGGAAGGTGAAGCTAGAGGA[TAM]-3'	0.1 pmol	

Forward = Forward primer; Reverse = Reverse primer; concentration = amount (nmol/l) added in the total reaction volume (12.5 µl) used for each sample; EF = efficiency factor.

Supplementary Table 2. List of primers used for PCR amplification and pyrosequencing of bisulphite-converted genomic DNA

Regions	Accession no.	Nucleotide position	PCR primers (5'-3')
CTCF3 (25, 27)	AF125183	5591-5812	*Forw*: 5'-GGGGTTTTTGAGTAGTATATGGGTATT-3' *Rev*:5'- [Btn]ACCCCATCCAAAAAAAACTTAAACTA-3'
CTCF3	AF125183	5591-5812	*Forw*:5'-GGTATTTGGTTTGGGTGATT-3' *Rev*:5'- [Btn]TTCCCCTTCTATCTCACCAC-3'
CTCF6 (27)	AF125183	7881-8091	*Forw*:5'-ATGGGTATTTTTGGAGGTTTTTT-3' *Rev*:5'- [Btn]ACTCCCATAAATATCCTATTCCC-3'
DMR2 (27, 45)	AC130303	155440-155238	*Forw*:5'-AGTGGGAAAGGGGTTTAG-3' *Rev*:5'- [Btn]ATACTATTTCCCCAACTATAACCTAACCCT-3'
DMR2	AC130303	155440-155238	*Forw*:5'- [Btn]TTTTAGGAGGGTTAGGTTATAGTT-3' *Rev*:5'-ACAAAAATAATTTACTCCCCCTTC-3'
DMR2	AC130303	155440-155238	*Forw*:5'-GGGGTAGTATAGTAAGTTTTTAGGAGGGTT-3' *Rev*:5'- [Btn]AATTTACTCCCCCTTCAACCT-3'
DMR0 (46)	AC130303	155440-155238	*Forw*:5'-TTTTATTTGGGGGTTGGTAGG-3' *Rev*:5'-[Btn]CAAAACCTCCAAAACTTTATCCTT-3'
H19 (29)	AF125183	9811-10000	*Forw*:5'-GAGGGGAGATAGTGGTTTG-3' *Rev*: 5'- [Btn]ACCCCCCCAAAACCCACCT-3'

Primers were designed using Pyromark Assay Design (Qiagen, Benelux). Hybrid °C = primer specific hybrid temperature used in PCR program. p1 = sequence primer 1, p2 = sequence primer 2, p3 = sequence primer 3. [Btn] = biotynilated.

	Product length	Hybrid. °C	Sequence primers (5′-3′)	CpGs
	156	57.0°C	5′-AGTATATGGGTATTTGTGG-3′ (p1)	3
	160	57.0°C	5′-GGTTGTGATGTGTGAG-3′ (p1)	4
	223	55.0°C	5′-GGTTTTATAGTTTGGATGG-3′ (p1) 5′-GGTAGTGTAGGTTTATATATTAT-3′ (p2) 5′-TGGAAAAGTTTAGGGTTATTTAAGT-3′ (p3)	6 6 4
	127	57.0°C	5′-GAAAGGGGTTTAGGAT-3′ (p1)	8
		57.0°C	5′-CTATACTATAAAACTTCCAAACAA-3′ (p1)	6
	182	57.0°C	5′-GTTTGGAAGTTTTATAGTATAGAG-3′ (p1)	3
	220	57.0°C	5′-GTAAGGTAATATGGTGAGT-3′ (p1) 5′-GAGGGAGGTTTTATGTA-3′ (p2) 5′-TGGGGGTTGGTAGGA-3′ (p3)	1 4 1
	190	57.0°C	5′-ATGGGGTAATGTTTAGTT-3′ (p1)	3

Supplementary Table 3. Overview of correlations between mean methylation of *IGF2* regulatory regions in ACCs

		Mean DMR0	Mean DMR2	Mean CTCF3	Mean CTCF6	Mean H19
Mean DMR0	*Correlation coeff,* ρ	1.000	0.013	0.695	0.361	0.413
	Sig. (2-tailed)	.	0.938	**<0.001**	**0.026**	**0.010**
Mean DMR2	*Correlation coeff,* ρ	-	1.000	0.018	-0.036	-0.139
	Sig. (2-tailed)		.	0.912	0.828	0.404
Mean CTCF3	*Correlation coeff,* ρ	-	-	1.000	0.614	0.781
	Sig. (2-tailed)			.	**<0.001**	**<0.001**
Mean CTCF6	*Correlation coeff,* ρ	-	-	-	1.000	0.741
	Sig. (2-tailed)				.	**<0.001**
Mean H19	*Correlation coeff,* ρ	-	-	-	-	1.000
	Sig. (2-tailed)					.

Mean methylation represents average methylation of all CpGs in one patient in that specific region. ρ represents Spearman correlation coefficient. Significant correlations are given in bold.

Supplementary Table 4. Mean |SDS| per region for ACC and ACA

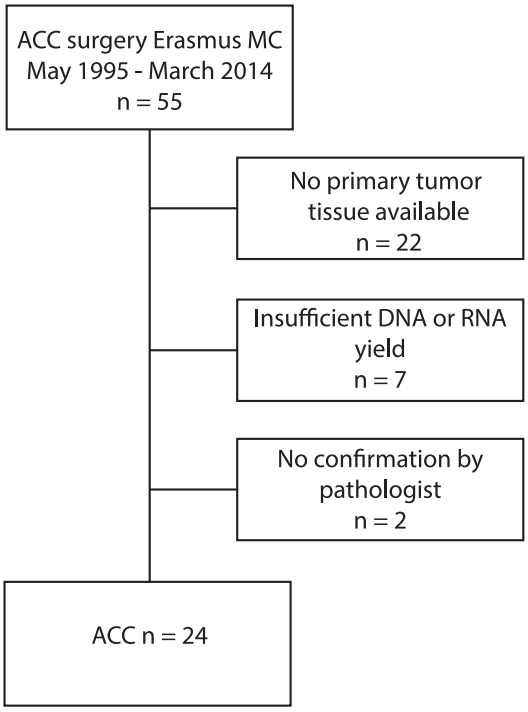
	DMR0	DMR2	CTCF3	CTCF6	*H19* promoter	*IGF2* expression
ACC (n=24)	3.89±4.64	5.32±3.30	6.35±2.64	1.89±1.21	3.40±1.81	50.29±61.17
ACA (n=14)	2.66±1.25	1.76±1.22	1.10±1.12	1.59±1.16	0.70±0.81	0.85±0.62

SDS is calculated with respect to methylation and expression in normal adrenals. ACA = adrenocortical adenoma, ACC = adrenocortical carcinoma. *IGF2* mRNA expression is transformed into |SDS| scores based on *IGF2/HPRT1* ratio.

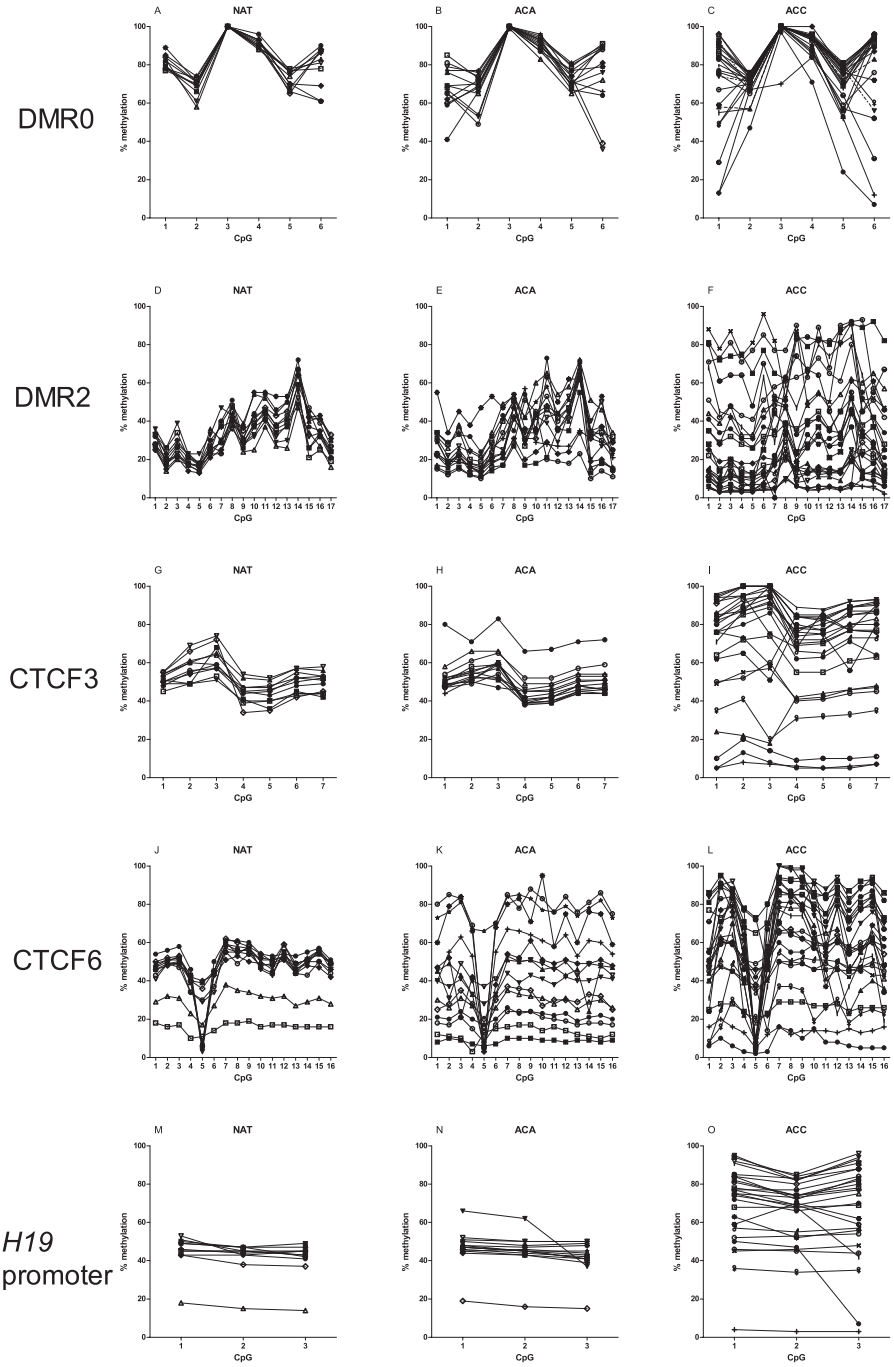
Supplementary Table 5. Overview of inter assay variability per region

	High methylated sample (n=5)			Low methylated sample (n=5)		
	Mean	SD	CV	Mean	SD	CV
DMR0	86.1	2.30	0.027	7.83	2.14	0.273
DMR2	90.2	3.13	0.035	6.26	2.71	0.433
CTCF3	90.7	1.82	0.020	8.06	2.88	0.357
CTCF6	84.6	2.11	0.025	7.90	3.15	0.399
H19	94.0	2.59	0.028	5.67	2.19	0.386

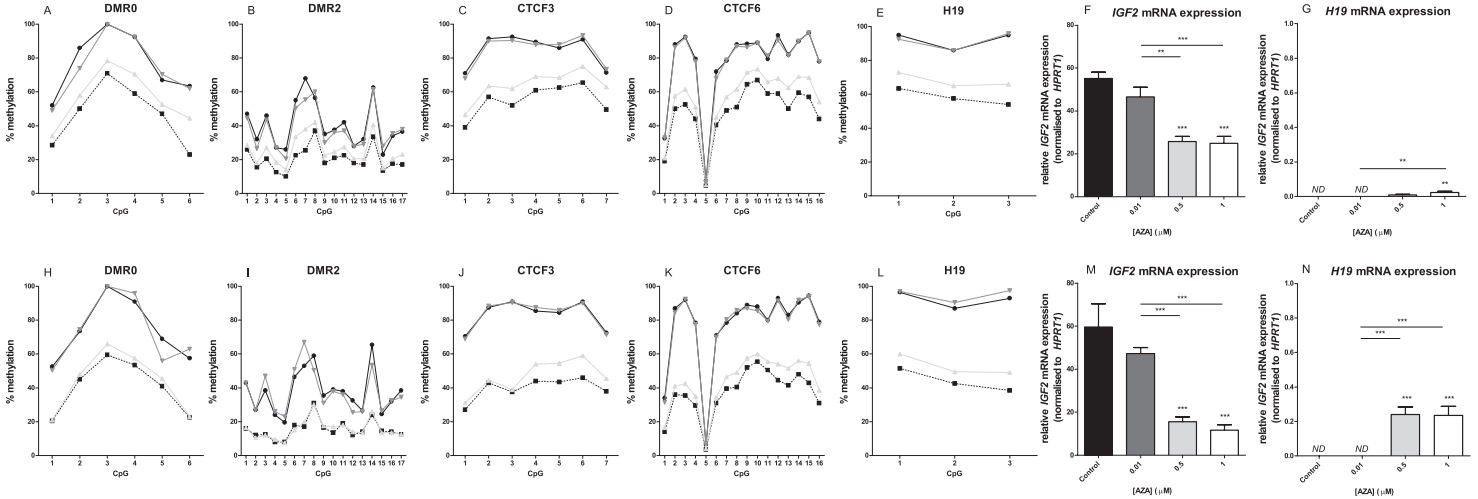
Mean represents mean of all CpGs in the 5 high or low methylated samples. SD represents mean of all SDs per CpG per region. CV = coefficient of variation.



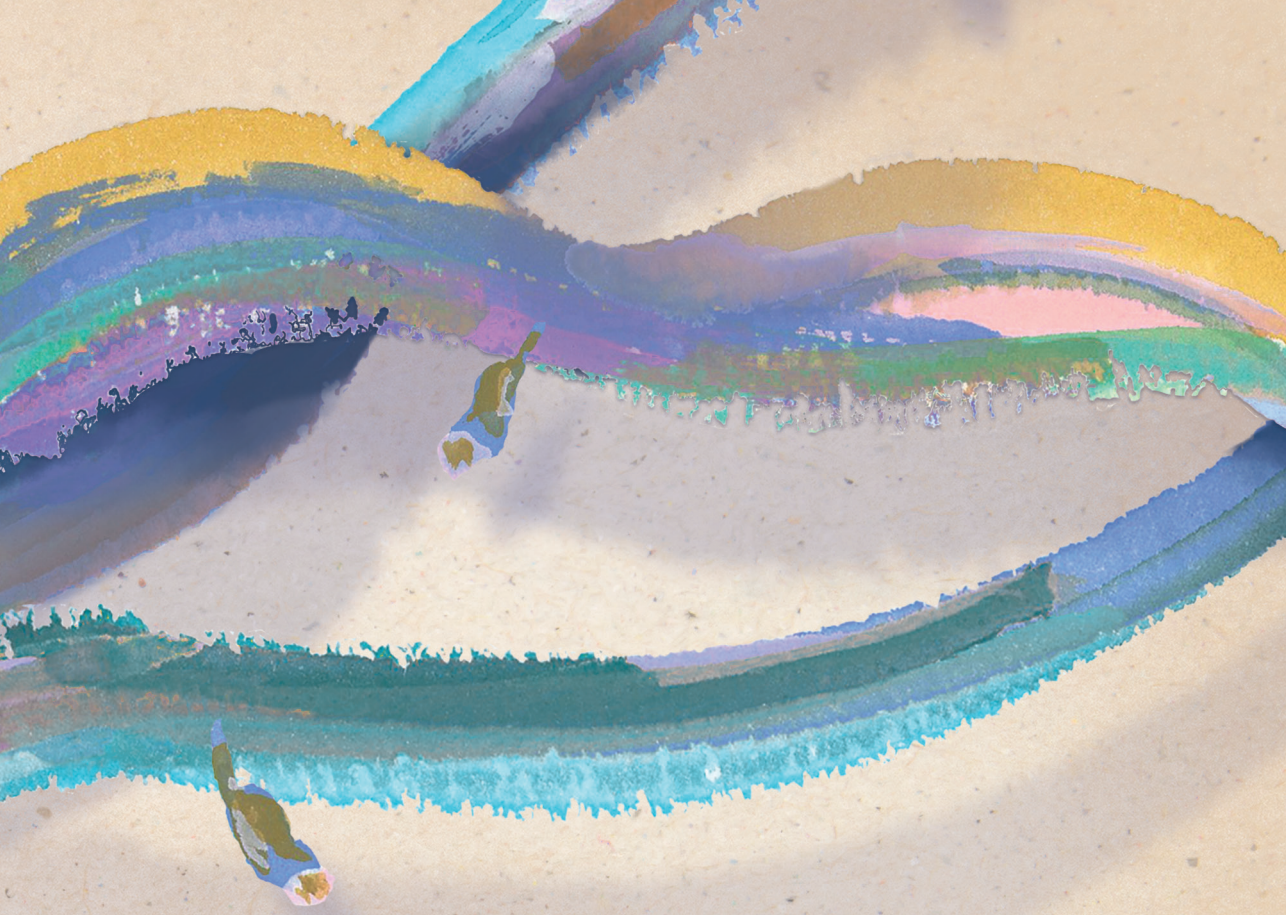
Supplementary Figure 1. Flowdiagram of enrollment of patients with ACC. Patients were excluded because of unavailability of tumor tissue, insufficient DNA or RNA yield, or because the pathologist was uncertain about the final diagnosis. 24 patients with ACC were included in final analysis.



Supplementary Figure 2. Methylation patterns in (A-C) DMR0, (D-F) DMR2, (G-I) CTCF3, (J-L) CTCF6 and (M-O) the *H19* promoter in the individual adrenocortical tissues. Every line represents a tumor of a single patient. Every dot equals an individual DNA methylation measurement. The fifth CpG in the CTCF6 is a C/T SNP (rs 10732516). ACA, adrenocortical adenomas; ACC, adrenocortical carcinomas; NAT, normal adrenal tissue.



Supplementary Figure 3. DNA methylation percentages and *IGF2* and *H19* mRNA expression in H295R ACC cells. DNA methylation in (A, H) DMR0, (B, I) DMR2, (C, J) CTCF3, (D, K) CTCF6, and (E, L) the *H19* promoter per CpG in control cells and after 3 days (upper row) and 7 days (bottom row) of treatment with different concentrations of AZA. (F, M) *IGF2* mRNA expression and (G, N) *H19* mRNA expression after 3 (F, G) days and 7 (M, N) days of treatment with different concentrations of AZA. The fifth CpG in the CTCF6 is a C/T SNP (rs 10732516). Black line = control, dark gray line = AZA treatment 0.01 μM, light gray line =AZA treatment 0.05 μM, dotted line = AZA treatment 1 μM. ** $P < 0.01$, *** $P < 0.001$ vs control or as depicted in the graphs. AZA, 5'-Aza-2'-deoxycytidine; ND, not detectable.



CHAPTER 5

The IGF2 methylation score as an objective marker for adrenocortical carcinoma: validation study of the European Network for the Study of Adrenal Tumors (ENSAT)

Creemers SG, Feelders RA, Valdes N, Ronchi CL, Volante M, van Hemel BM, Luconi M, Ettaieb MHT, Mannelli M, Chiara MD, Fassnacht M, Papotti M, Kerstens MN, Nesi G, Haak HR, van Kemenade FJ, Hofland LJ

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ABSTRACT

Adrenocortical carcinoma (ACC) is diagnosed using the histopathological Weiss Score (WS), but remains clinically elusive unless it has metastasized or grows locally invasive. Previously, we proposed the objective IGF2 methylation score as diagnostic tool for ACC. This study aims to validate this score in a multicenter European cohort analysis. Patient and tumor characteristics were obtained from adrenocortical tumor patients. DNA was isolated from frozen specimens, whereafter DMR2, CTCF3, and H19 were pyrosequenced. The predictive value of the methylation score for malignancy, defined by the WS or metastasis development, was assessed using Receiver Operating Characteristic curves, and logistic and Cox regression analyses. Seventy-six ACC patients and 118 patients with adrenocortical adenomas were included from seven centers. The methylation score and tumor size were independently associated with the pathological ACC diagnosis (OR 3.756 95% CI 2.224 – 6.343; OR 1.467 95% CI 1.202 – 1.792, respectively; Hosmer-Lemeshow test P=0.903), with an area under the curve of 0.957 (95%CI 0.930 – 0.984). The methylation score alone resulted in an AUC of 0.910 (95% CI 0.866 – 0.952). Cox regression analysis revealed that the methylation score, WS and tumor size predicted development of metastases in univariate analysis. In multivariate analysis, only the WS was predictive for development of metastasis (OR 1.682 95% CI 1.285 – 2.202; $P < 0.001$). We validated the high diagnostic accuracy of the IGF2 methylation score for diagnosing ACC in a multicenter European cohort study. Considering the known limitations of the WS, the objective IGF2 methylation score could potentially provide extra guidance on decisions on postoperative strategies in adrenocortical tumor patients.

INTRODUCTION

Adrenal tumors occur at high frequencies in the general population and are often detected incidentally. Autopsy studies show a prevalence of 1.0 - 8.7% (1, 2). Radiological studies report a frequency of clinically unapparent adrenal masses of less than 1% for patients under 30 years of age, a percentage which increases up to 10% in those 70 years of age or older (3-5). Several CT characteristics, like a large diameter (> 6 cm), lack of a well-defined margin, and increased heterogeneity, can point towards a malignant adrenal mass, but these collective findings will not always indicate a clear differential diagnosis (6). Only in rare cases, the adrenal tumor has malignant potential. Adrenocortical carcinoma (ACC) is a highly malignant tumor with five-year-survival ranging from 16 to 38% (7, 8). The Weiss Score (WS), consisting of nine histopathological criteria, is the most frequently used scoring system to differentiate between benign and malignant adrenocortical tumors (9, 10), and is also recommended in the European clinical guidelines on ACC (11). A tumor is classified as ACC at the presence of three or more Weiss criteria. The WS can be ambiguous when a score of 2 or 3 is obtained, as metastasized cases have been reported with a WS as low as 2 (12-15). In addition, the WS has been challenged due to interobserver variability and subjectivity, and may be difficult to apply in specific circumstances, even for experienced pathologists (16, 17). Consequences of malignant disease are significant, since prognosis is poor and adjuvant mitotane treatment is recommended in ACC patients after curative resection, particularly in case of patients with tumors harboring high recurrence risk (11, 18). Research is focusing on bias-free molecular markers to identify adrenocortical tumors with malignant potential. Since the diagnosis of malignancy is clinically elusive in non-metastasizing adrenocortical tumors, this can be challenging.

Recently, we showed that methylation patterns of IGF2 regulatory regions discriminate ACC from adrenocortical adenoma (ACA) with a sensitivity of 94% and a specificity of 96% (19). This IGF2 methylation score is based on the most frequent molecular alteration in ACC, i.e. increased IGF2 expression (20-24). The *IGF2* gene is an imprinted gene whose expression largely varies within ACC (24, 25). The proposed methylation score consists of the mean standard deviation score of three different IGF2 regulatory regions compared to methylation in normal adrenals (19). The original study, however, included two limited cohorts with in total 33 ACCs and 27 ACAs. The major objective of the present study is to validate the diagnostic role of the IGF2 methylation score in a multicenter cohort study via the European Network for the Study of Adrenal Tumors (ENS@T, www.ensat.org). Second aim is to correlate the IGF2 methylation score with follow-up clinical characteristics and outcome in patients with adrenocortical tumors.

METHODS

Patients and data collection

Patients with ACC or ACA from whom DNA from a snap-frozen specimen from the primary adrenocortical tumor was available, were included. Inclusion of both ACC and ACA was mandatory for each individual center. Data collected included: age at diagnosis, sex, initial tumor size, steroid secretion pattern, the WS with individual parameters, ENSAT tumor stage, follow-up duration and clinical status at the end of the follow-up period. According to availability at the participating centers, frozen specimens or 200 ng DNA isolated from frozen specimens, were collected at Erasmus University Medical Center (EMC). Ten patients had to be excluded because of insufficient DNA yield. From 5 centers, a representative section from a formalin-fixed, paraffin-embedded (FFPE) block was assessed at EMC to ascertain a blinded central histopathology evaluation. Diagnosis was based on the WS determined by the local pathologists, with a threshold of malignancy of ≥ 3 criteria present in the tumor. Two pediatric patients were excluded because of uncertain ACC diagnosis based on the WS. Of these two patients, no follow-up data were available. The inclusion of patients was approved by the local ethics committees of all participating centers and approval for use of tissues for research purposes was obtained at the coordinating center.

DNA isolation and pyrosequencing

Processing of adrenocortical tumors and DNA isolation, when necessary, was performed as previously described using the Wizard® Genomic DNA Purification Kit (Promega, Leiden, the Netherlands), according to manufacturer's protocol (19). Bisulfite conversion, PCR reactions, and pyrosequencing were also performed as previously described (19). Briefly, after binding of the PCR product to streptavidin-coated Sepharose beads (GE Healthcare), the template was washed, made single-stranded and neutralized. Pyrosequencing assays of previously reported CpGs involved in expression of IGF2 (DMR2, CTCF3 and the H19 promoter) were designed using Pyromark Assay Design. Pyrosequencing was performed using the PyroGold SQA reagent kit (Qiagen, Benelux) according to manufacturer's protocol and analyses were performed on the Pyromark Q24 system. DNA quality and quantity was assessed using the NanoDrop 2000c (ThermoFisher). PCR and corresponding sequencing primers are listed in Supplemental Table 1.

Statistical analysis

Statistical analysis were performed using SPSS24 and Graphpad Prism 6.0. The methylation percentages in the three regions were transformed into a mean standard deviation score (SDS) compared to methylation in normal adrenals, as previously described (19). Correlation between parameters was assessed using the Spearman's correlation coefficient. To assess significant differences in methylation between ACC and ACA, the non-parametric Mann-Whitney Test was used.

Logistic regression analysis was used to assess the predictive value of the IGF2 methylation score for the pathological diagnosis of ACC, adjusted for tumor size. The Hosmer-Lemeshow test was used to evaluate the goodness of fit of the model. To determine a clinically relevant cut-off value for the methylation score and to assess the discrimination of the fitted logistic regression model, Receiver Operating Characteristic (ROC) curves were constructed, followed by calculation of the area under the curve (AUC). Hazard ratios (HR) for development of metastases during follow-up, as well as death, were estimated using Cox proportional hazards regression models. Time to metastasis was defined as the time from pathological diagnosis until the time metastasis occurred. Overall survival (OS) was defined as the time from pathological diagnosis to death or last follow-up. The proportional hazards assumption was assessed with interaction of variables with time. Kaplan Meier curves were constructed and compared using the Logrank test. In an attempt to resemble the clinical situation in which the methylation score could be valuable, patients with an already proven ACC at diagnosis, i.e. with metastasized disease (ENSAT stage IV), were excluded from these analyses. For regression analyses, independent variables with a P less than 0.1 in univariate analyses were intended to be included in multivariate analysis. Agreement between the WS determined at the centers and the scoring of a representative slide at the Erasmus MC was determined using the Kappa statistic. Data are presented as mean ± SEM, unless specified otherwise. A two-sided value of $P < 0.05$ was considered statistically significant.

RESULTS

Study population

In total, 76 patients with ACC and 118 ACA patients were included from seven clinical specialist referral centers participating in ENS@T (Netherlands 3, Italy 2, Germany 1, Spain 1; Table 1). From four centers, DNA isolated from snap-frozen specimens was collected, whereas from the remaining three centers frozen specimens were shipped to the coordinating center. The location at which the DNA isolation procedure was performed did not influence the results and the predictive value of the methylation score. Clinical and tumor characteristics of the patients included in this study are listed in Table 1.

The median tumor size was 10 cm for ACC and 3.4 cm for ACA. The proportion of functional tumors (all hormones) was similar between ACC and ACA, whereas there was a clear difference between frequency of androgen, and precursor secreting tumors, which proportions were higher in ACC (both $P < 0.0001$ vs ACA). The proportion of mineralocorticoid overproduction was lower in ACC compared to ACA ($P < 0.0001$ vs ACA). Central histological evaluation of a representative HE section in cases from five

centers was performed at the Erasmus MC. The inter-observer agreement of the WS (*n* = 111) was adequate with a Cohen's kappa coefficient of 0.83 ± 0.055 (*P* < 0.001, (26)) and Pearson correlation r = 0.83 ± 0.054 (*P* < 0.001).

Of the tumors indicative of ACC on the basis of the WS, and with an available ENSAT stage (*n* = 66), 26% had metastasized disease at diagnosis (ENSAT stage IV) and 45% of tumors with follow-up data available and no metastases at diagnosis were clinically proven to be malignant by development of metastasis during follow-up. Twenty-two patients died during follow-up, all with ACC on the basis of the WS. The patients with histological suspected ACC who did not metastasize at diagnosis or during follow-up, had a median follow-up of 41.5 months (IQR 21.5 – 72.3).

Predictive value of the IGF2 methylation score for the pathological diagnosis of ACC

For all three regions, a different methylation pattern was observed for ACC compared to ACA (Fig. 1, A-C). The IGF2 methylation score was significantly higher in ACC compared to ACA (Fig. 1D; *P* < 0.0001). Within ACC, no correlation was found between the methylation score and the WS (ρ = 0.017, *P* = 0.897). For analysis of the diagnostic accuracy of the IGF2 methylation score for the pathological diagnosis of ACC and for the prediction of metastases development, confirmed ACC with metastases at diagnosis were initially excluded. The IGF2 methylation score and the tumor size appeared to be independently associated with the pathological diagnosis of ACC, with an OR of 3.756 (95% CI 2.224 – 6.343; *P* < 0.001) and 1.467 (95% CI 1.202 – 1.792; *P* < 0.001), respectively (Table 2; Hosmer and Lemeshow test *P* = 0.943).

The methylation score alone predicted the diagnosis on the basis of the WS (59 ACC, 118 ACA) with an AUC of 0.910 (Fig. 2A; 95% CI 0.867 – 0.953). When applying a cutoff value of 2.13 for the IGF2 methylation score, a sensitivity of 86% and a specificity of 84% was obtained for the pathological diagnosis of ACC. ROC curve of the fitted logistic regression model including the IGF2 methylation score and tumor size resulted in an AUC of 0.957 (Fig. 2C; 95% CI 0.930 – 0.984).

Table 1. Clinical and tumor characteristics of patients included in the present study

	All tumors *n* = 194	ACC *n* = 76	ACA *n* = 118
Age at diagnosis, mean (yrs, range)	53 yrs (16 – 83) yrs	54 (23 – 83) yrs	53 (16 – 79) yrs
Sex (Male, %)	77/194 (40%)	33/76 (43%)	44/118 (37%)
Tumor size (cm)			
Range	0.5 – 30 cm	2.1 – 30 cm	0.5 – 22 cm
Mean	6.8 cm	11.6 cm	3.8 cm
Median	5.0 cm	10.0 cm	3.4 cm
Steroid secretion			
Androgens	24/176 (14%)	21/69 (30%)	3/107 (3%)
Glucocorticoids	88/180 (49%)	40/71 (56%)	48/109 (44%)
Mineralocorticoids	32/180 (18%)	3/71 (4%)	29/109 (27%)
Precursors	13/166 (8%)	13/66 (20%)	0/98 (0%)
Estradiol	1/159 (1%)	1/61 (2%)	0/98 (0%)
Non secreting	47/175 (27%)	19/71 (27%)	28/104 (27%)
Weiss score			
Range	0 – 9	3 – 9	0 – 2
Mean	2.5	5.8	0.31
Median	1	6	0
ENSAT			
I	-	8/66 (12%)	-
II	-	25/66 (38%)	-
III	-	16/66 (24%)	-
IV	-	17/66 (26%)	-
Metastasis during follow-up (n)	17/111 (15%)	17/38 (45%)	0/73 (0%)
Follow-up months metastasis, median (IQR)	M1: 13 (4 – 24) M0: 27 (16 – 53)	M1: 13 (4 – 24) M0: 41.5 (21.5 – 72.3)	M1: - M0: 23 (14.5 – 45.5)
Death (n)	22/149 (15%)	22/71 (31%)	0/73 (0%)
Follow-up months, median (IQR)	Death: 18.5 (10.8 – 34.5) Alive: 26 (14 - 50)	Death: 18.5 (10.8 – 34.5) Alive: 35 (19.5 – 65.5)	Death: - Alive: 22 (13 – 45)

For the data on follow-up (both metastases and death), only patients with available follow-up data were included (ACA *n* = 73, ACC *n* = 38), and for the data concerning occurrence of metastases during follow-up, ENSAT tumor stage IV patients were excluded (*n* = 17). ENSAT, European Network for the Study of Adrenal Tumors; M1, metastases at diagnosis or during follow-up; M0, no metastases during follow-up.

Table 2. Predictive value of the IGF2 methylation score and tumor size for the diagnosis of adrenocortical tumors on the basis of the Weiss Score

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	P-value	OR (95% CI)	P-value
IGF2 Methylation Score	4.954 (3.130 – 7.840)	**< 0.001**	3.756 (2.224 – 6.343)	**< 0.001**
Tumor size	1.733 (1.447 – 2.076)	**< 0.001**	1.467 (1.202 – 1.792)	**< 0.001**

The Weiss score as determined by the local pathologist was used, resulting in 57 ACC and 115 ACA. Patients with proven ACC at diagnosis, i.e. metastatic disease, were excluded from analyses (n = 17). For this analysis, one outlier was excluded (ACA of 22 cm), but exclusion did not influence significance. Hosmer and Lemeshow Test P = 0.903. CI, confidence interval; OR, odds ratio.

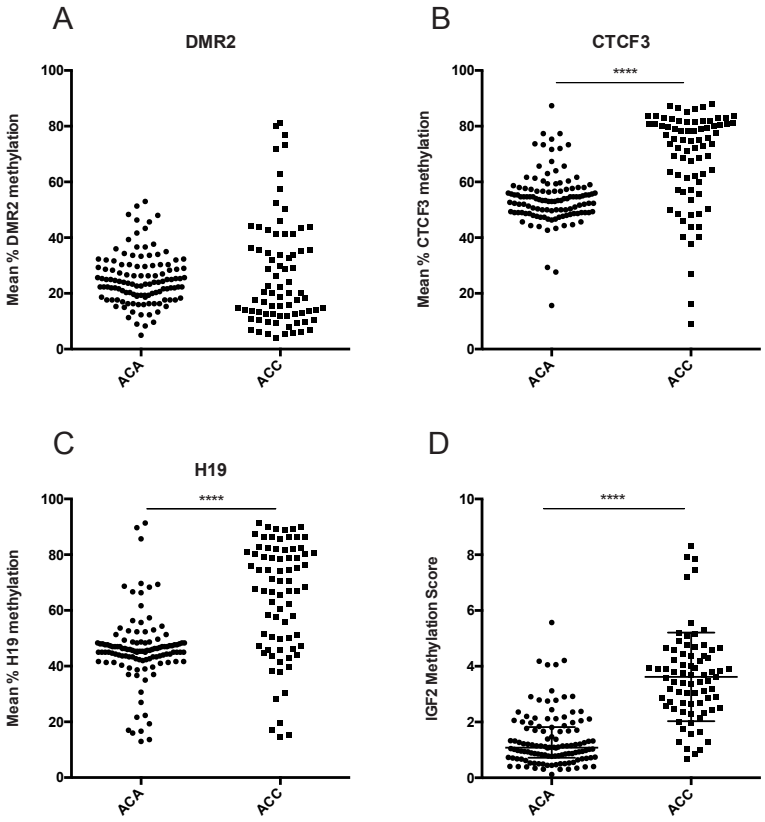

Figure 1. Mean methylation percentages in the three IGF2 regulatory regions DMR2 (A), CTCF3 (B), and the H19-promoter (C), and the IGF2 methylation score (D) for adrenocortical adenomas (ACA, n = 118) and carcinomas (ACC, n = 76). Every dot represents a patient. Lines represent medians with inter quartile range. DMR, differentially methylated region. **** P < 0.0001.

Towards clinically useful cutoff values

To provide further insights into the discriminative performance of this quantitative test, sensitivity and specificity for different cutoff values are presented in Fig. 2B. In this graph, we also demonstrate a zone that could be interpreted as a grey area, of which the implementation assures high diagnostic accuracy when the IGF2 methylation score is above or below this zone (Fig. 2B, striped area; score 1.28 - 3.15). Below the grey zone (<1.28), the negative predictive value is 97%, whereas a methylation score above the grey zone (>3.15) results in a positive predictive value of 87%. Tumors with a methylation score between 1.28 and 3.15 should then be classified as inconclusive. Overall, in our series, seventy-five of the 118 ACA (64%) could be diagnosed as ACA with a sensitivity of 97% and thus had an IGF2 methylation score below 1.28. On the other hand, thirty-three of the 59 ACC (56%) could be diagnosed as ACC with high diagnostic accuracy. The remaining tumors (n = 62, 35%) had a methylation score in the grey zone and were therefore classified as inconclusive diagnosis on the basis of the IGF2 methylation score. Of these cases, 61% were classified as ACA based on the WS (median WS 0, IQR 0 - 0), whereas 39% had a WS of 3 or more (median 6, IQR 3 - 8). Of the patients with clinically proven ACC as indicated by metastatic disease either at diagnosis or during follow-up, twenty-one (58%) of the 36 were diagnosed as ACC according to the IGF2 methylation score, two as ACA, and 13 had an IGF2 methylation score in the grey zone.

When focusing on ACC with a WS of 3 in our series (n = 12), five appeared to have a methylation score above the grey zone and would therefore be classified as ACC according to the IGF2 methylation score. The other 7 ACC with a WS of 3 would be classified as inconclusive diagnosis based on the methylation score. The median follow-up of patients with a tumor harboring a WS of 3 was 37 months (IQR 21 – 49), with 1 patient who developed metastasis after 21 months (IGF2 methylation score 1.66; grey zone). Of the six ACA with a WS of 2, four received a concluding diagnosis of an ACA on the basis of the methylations score, with a total median follow-up of 5 months (IQR 0 – 22). The two other ACA cases had an IGF2 methylation score in the grey zone.

The predictive value of the IGF2 methylation score for malignancy as defined by metastatic ACC

As secondary outcome, we aimed to assess the predictive value of the IGF2 methylation score and other variables for predicting metastases. When tumors were divided into two groups based on the methylation score, a higher IGF2 methylation score was associated with the development of metastases (Fig. 2D, P = 0.005). In univariate Cox regression analysis, the IGF2 methylation score, but also the WS and tumor size were predictive for development of metastases (Total n = 118; 16 cases, 112 censored; Table 3). In multivariate analysis however, only the WS was independently associated with metastatic disease

(Table 3; HR 1.682, 95%CI 1.285 – 2.202, $P < 0.001$). The same finding was obtained when only ACC (total $n = 53$; 16 cases, 37 censored) were included for both analyses: only the WS was independently associated with development of metastases (OR 1.443, 95%CI 1.050 – 1.984; $P = 0.024$). Regarding overall survival, univariate Cox regression analysis revealed that the same three variables were predictive for survival: the IGF2 methylation score, the WS, and the tumor size. In multivariate analysis, again only the WS was associated with increased risk of death (HR 1.466, 95%CI 1.136 – 1.891, $P < 0.001$).

Table 3. Cox regression model for the development of metastases during follow-up

	Univariate analysis		**Multivariate analysis**	
Metastases	HR (95% CI)	P-value	HR (95% CI)	P-value
IGF2 Methylation Score	1.380 (1.070 – 1.780)	**0.013**	0.861 (0.571 – 1.298)	0.476
Weiss Score	1.702 (1.308 – 2.216)	**< 0.001**	1.682 (1.285 – 2.202)	**< 0.001**
Tumor size (cm)	1.110 (1.049 – 1.174)	**< 0.001**	1.022 (0.940 – 1.111)	0.613
Patient age (years)	1.034 (0.996 – 1.074)	0.081	1.035 (0.988 – 1.083)	0.147
Survival	HR (95% CI)	P-value	HR (95% CI)	P-value
IGF2 Methylation Score	1.509 (1.235 – 1.845)	**< 0.001**	1.179 (0.875 – 1.588)	0.279
Weiss score	1.636 (1.294 – 2.069)	**< 0.001**	1.466 (1.136 – 1.891)	**0.003**
Tumor size (cm)	1.122 (1.066 – 1.181)	**< 0.001**	1.045 (0.967 – 1.130)	0.264
Patient age (years)	1.032 (0.998 – 1.067)	0.066	1.031 (0.991 – 1.073)	0.132

For the prediction of metastases, patients with ENSAT tumor stage IV disease at diagnosis were excluded ($n = 17$). Patients in which follow-up time was available were included in this analysis. In multivariate analysis, sixteen patients developed metastases during follow-up, whereas 102 patients were censored. For overall survival analysis, twenty-two patients died during follow-up and 127 patients were censored. Differences in number of patients are due to lack of knowledge about metastases in some patients and inclusion of ENSAT tumor stage IV disease in overall survival analysis. CI, confidence interval; cm, centimeters; HR, hazard ratio.

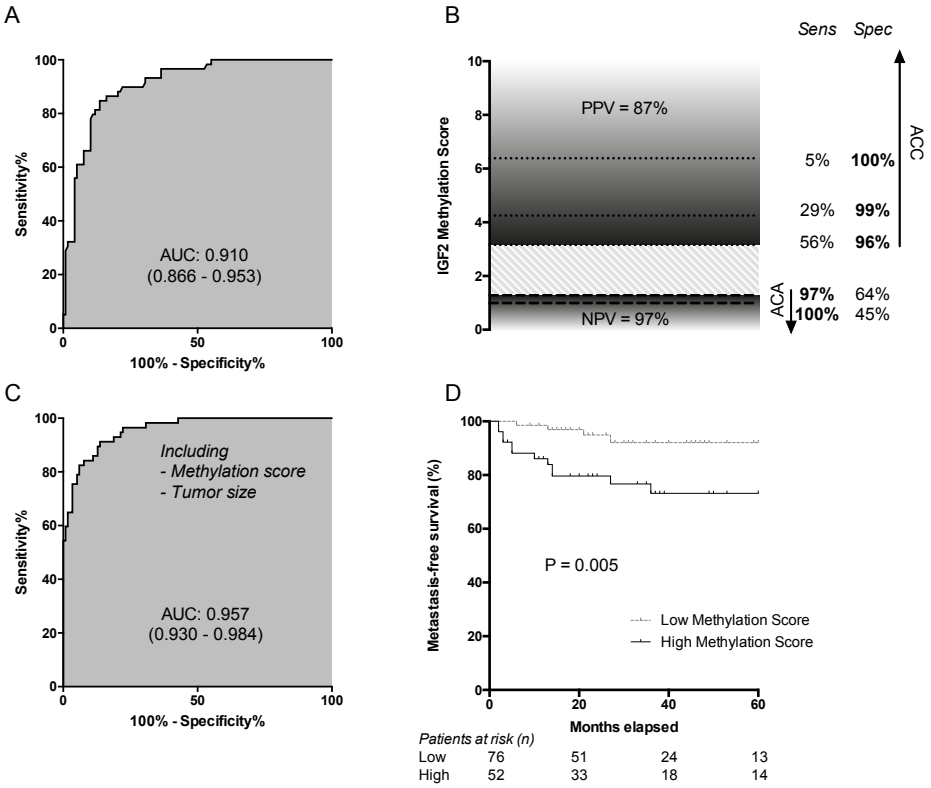

Figure 2. Discriminative value of the IGF2 methylation score for discrimination between adrenocortical adenoma (ACA, *n* = 118) and adrenocortical carcinoma (ACC, *n* = 59). ENSAT tumor stage IV patients were excluded from analyses (*n* = 17). (A) ROC curve of the IGF2 methylation score for prediction of the pathological diagnosis of ACC. (B) Sensitivity and specificity for specific cutoff values of the IGF2 methylation score for the pathological diagnosis of ACC. The striped area represents a grey zone of the methylation score with less diagnostic accuracy. PPV and NPV for the cutoff value below (1.28) or above (3.18) the grey zone. (C) ROC curve of the logistic regression model including the methylation score, and tumor size for predicting the pathological diagnosis of ACC. (D) Kaplan Meier curve for two groups based on the IGF2 methylation score for development of metastases. The two groups were divided based on an IGF2 methylation score of 2.45, which was based on the best discriminative value for the development of metastases calculated using ROC analysis. AUC, area under the curve; NPV; negative predictive value; PPV, positive predictive value; ROC, Receiver Operating Characteristic; Sens, sensitivity; Spec, specificity.

DISCUSSION

In this study, we externally validated the predictive value of methylation of IGF2 regulatory regions for the diagnosis of malignancy of adrenocortical tumors in a multicenter European cohort study and confirm that the IGF2 methylation score can serve as an objective diagnostic tool with a high sensitivity to detect adrenocortical malignancy.

Currently, the histopathological Weiss score is the most important diagnostic tool to establish adrenal malignancy. Although in this study the inter-observer agreement is quite high with a Kappa statistic of 0.83 (based on one representative slide), the WS harbors multiple challenges (9, 10), as its diagnostic applicability is low among non-expert pathologists and a group of borderline cases with a WS of 2 or 3 exist with an uncertain outcome (17). Inter-observer agreement rates in previous studies are heterogeneous. In a study by Aubert and collegues, a high inter-observer agreement was found for the total WS (r = 0.94) (9). In another study using a virtual microscopy reading, a kappa statistic of 0.70 was obtained for the diagnosis of ACC in 50 adrenocortical tumors scored by 12 pathologists (27). The inter-observer reproducibility increased after a coaching meeting to a kappa statistic of 0.75 (27). It has thereby been shown in the German ACC registry that in 13% (n/N = 21/161) of cases a diagnosis of ACC had to be revised by a reference pathologist, also containing misdiagnosis of metastases from extra-adrenal cancers and pheochromocytoma (28). In addition, after histopathological review of a large Italian series it was demonstrated that the diagnosis was changed from ACC to ACA or vice versa upon review in 3% (n/N = 9/200) of the adrenocortical tumors (29). Other disagreements were present in an additional 17 cases in this study, concerning in particular the discrimination between ACC from pheochromocytoma or metastases (29). Considering the retrospective design, this might have led to changes in the initial diagnosis by a revision or because of changes during follow-up in the current study, as well. Studying new diagnostic tests is associated with important concerns and limitations, since diagnosis of adrenal malignancy is only definite in case of locoregional invasive tumor growth or metastatic disease and thus may require long-term follow-up. The importance of studying accurate diagnostic tools for adrenal malignancy lies especially in the early decision on postoperative therapeutic strategies, i.e. adjuvant treatment with mitotane, and prognosis stratification.

In recent decades, research has focused on epigenetic changes in ACC (30, 31). Previously, these genome-wide approach studies were primarily used to identify subgroups of patients with ACC (32, 33), whereas the present study demonstrates a clinically useful cutoff value. Interest in the *IGF2* gene originates from the association of ACC with the Beckwith-Wiedemann syndrome (34), and for over 20 years IGF2 overexpression is the

most frequently detected molecular alteration in ACC. IGF2 has also been shown to be an important factor for tumor growth in the majority of ACC cases (35). The IGF2 methylation score could be regarded as a measure of instability or dysregulation of this system, explaining involvement in ACC. The IGF2 regulatory regions used in the previous study were identified on the basis of known associations with IGF2 expression or malignancy of adrenocortical tumors (19). We have now externally validated the IGF2 methylation score in a multicenter European study. Together with the application of the WS as determined by the participating centers, this largely increases the generalizability of our findings. The performance of the IGF2 methylation score is high with a sensitivity and specificity of 86% and 84%, respectively, which is slightly less accurate compared to the previous study (19). The most important advantage of the IGF2 methylation score as proposed in our study is that it is an easily applicable non-expensive objective measurement, which is not biased by inter-observer variability. Most quantitative diagnostic tests do not perfectly discriminate between groups of patients, often resulting in a significant overlap between distributions of test results for patients with and without a particular disease (36). This also applies to the WS, where a score of 2 or 3 can be considered a grey zone (12-15). Although the diagnostic accuracy of the IGF2 methylation score is already high when applying one single cutoff value, we believe that the methylation score is especially useful when the value is below or above the grey zone as presented in this study (65% of cases in this study), eventually assuring a higher diagnostic accuracy. This indicates however that the performance of the methylation score is lower in 35% of the cases with a value in the grey zone, which is a limitation of the clinical applicability. In this study, we show that also in part of the WS 3 cases, which is in clinical practice interpreted as a less solid diagnosis of malignancy compared to a higher WS, a high IGF2 methylation score could potentially help to opt for toxic mitotane treatment. As demonstrated in this study, the diagnostic accuracy of the IGF2 methylation score improves when it is combined with tumor size. Further research could focus on the combination of the IGF2 methylation with imaging characteristics, other clinical data or image analyses from histopathology in order to determine the optimal combination. These studies should also aim to further elucidate the diagnostic accuracy of the IGF2 methylation score in the clinically most relevant group of adrenocortical tumors with a WS in the grey zone (WS of 2 or 3).

We have to acknowledge that this test is and will be applied to preselected adrenocortical tumors, with a relative high pre-test probability of malignancy. Adrenocortical tumors are surgically removed in case malignancy is suspected based on imaging characteristics, or because of hormonal activity (33, 37). In this respect, assessment of the urinary steroid metabolomic profile seems a promising new tool in the decision-making on surgery in patients with adrenal masses (38). To improve practicality and increase availability of samples, further research could focus on the possibility of these analyses in DNA isolated

from formalin-fixed paraffin embedded tissues. Previous research has already shown that pyrosequencing of DNA isolated from FFPE tissues and snap-frozen specimens provides highly comparable results (39).

Besides the retrospective design, a limitation of this study is that we did not have access to executed pre-operative diagnostic tests, like various imaging techniques important for the decision on adrenalectomy. Another consideration is that patients with adenomas have shorter follow-up time compared to ACC patients, which makes it possible that development of metastases or death are underestimated in this group of patients. Development of metastases after years of follow-up have been previously reported in patients with a resected adrenocortical tumor originally classified as benign (40, 41). In our study, the occurrence of metastases and death in the total group of patients probably represent underestimations, considering the median follow-up time of 27.5 and 26 months, respectively. Thereby, regarding our secondary aim, i.e. the prediction of metastases occurring during follow-up, we acknowledge that the number of cases is very limited and the analyses should therefore be interpreted with caution. In univariate analyses, the IGF2 methylation score, the WS, and the tumor size were associated with the development of metastases and overall survival. As for survival analyses, a limitation of this study is the lack of availability of the Ki67 index, which is to date the most important prognostic factor within ACC (42). In our study, the WS was the only independent predictive factor for metastases and prognosis, although this might be affected by the limited statistical power due to a small sample size. Previously, a high mitotic count, as a measure of tumor grade, was associated with poor prognosis in ACC (43). Prospective studies are needed to further validate the diagnostic value of the IGF2 methylation score and evaluate the potential role in prediction of metastases.

In conclusion, we externally validated the high diagnostic accuracy of the previously proposed IGF2 methylation score for confirming the pathological diagnosis of ACC in a multicenter European cohort study. Considering the known limitations in clinical applicability of the WS, the objective IGF2 methylation score could provide extra guidance to multidisciplinary teams on decisions regarding postoperative strategies in patients with adrenal masses.

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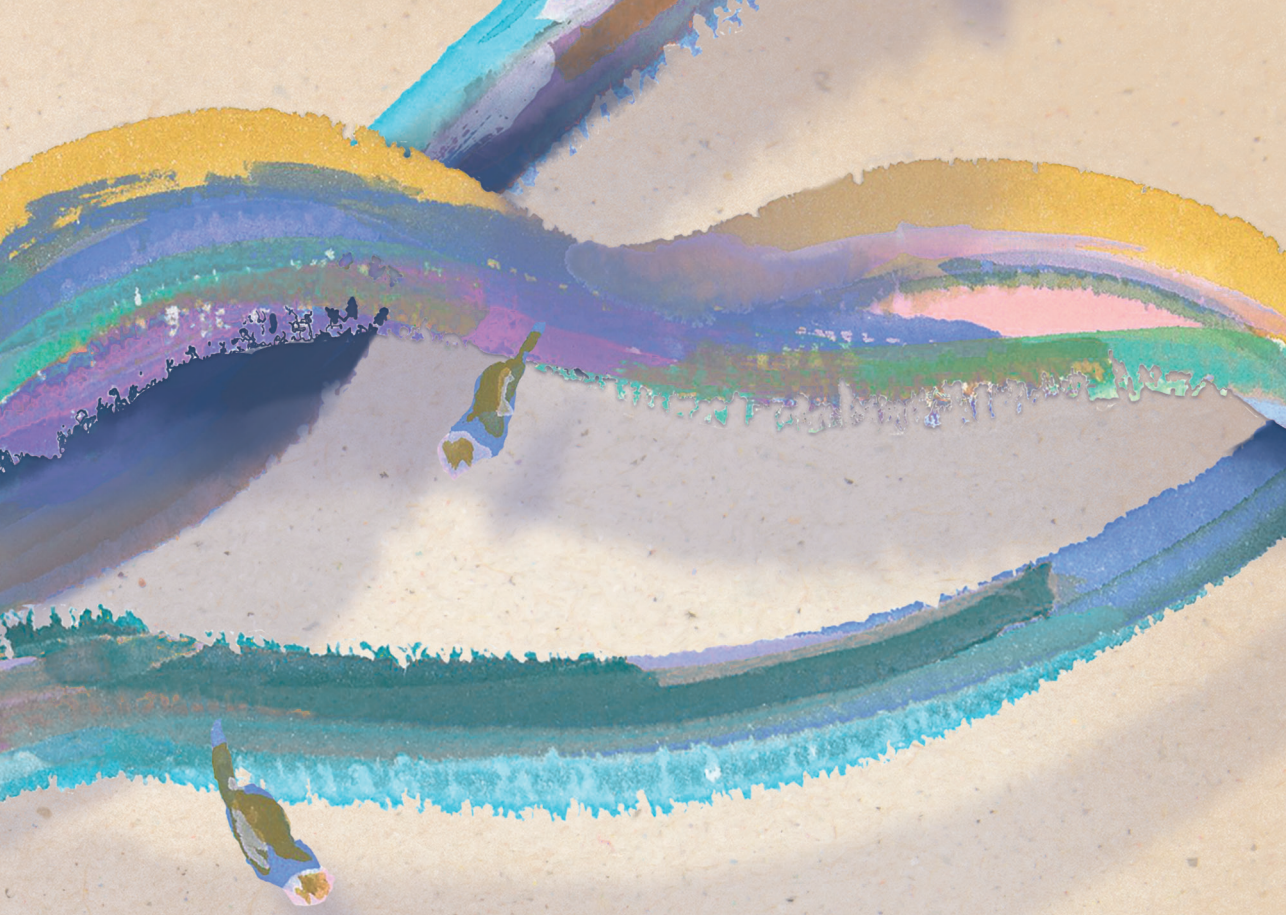
SUPPLEMENTARY MATERIAL

Supplementary Table 1. PCR and sequencing primers that were used in the present study

Region	Accession no.	Nucleotide position	PCR primers (5′-3′)
DMR2	AC130303	155440-155238	*Forw*: 5′-AGTGGGAAAGGGGTTTAG-3′ *Rev*: 5′- [Btn] ATACTATTTCCCCAACTATAACCTAACCCT-3′
CTCF3	AF125183	5591-5812	*Forw*: 5′-GGTATTTGGTTTGGGTGATT-3′ *Rev*: 5′- [Btn]TTCCCCTTCTATCTCACCAC-3′
H19	AF125183	9811-10000	*Forw*: 5′-GAGGGGAGATAGTGGTTTG-3′ *Rev*: 5′- [Btn]ACCCCCCCAAAACCCACCT-3′

Primers were designed using Pyromark Assay Design (Qiagen, Benelux). [Btn] = biotynilated. DMR, differentially methylated region; Forw, forward; Rev, reverse.

	Product length	Sequencing primers (5′-3′)	CpGs
	127	5′-GAAAGGGGTTTAGGAT-3′	2-4
	160	5′-GGTTGTGATGTGTGAG-3′	5-7
	190	5′-ATGGGGTAATGTTTAGTT-3′	1-3



CHAPTER 6

Identification of mutations in cell-free circulating tumor DNA in adrenocortical carcinoma: a case series

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ABSTRACT

Context: The disease course of adrenocortical carcinoma (ACC) patients is heterogeneous. A marker for prognosis and treatment response would facilitate choices for diagnosis and therapy. In other cancer types, circulating cell-free tumor DNA predicted tumor dynamics.

Case descriptions: The present pilot study included six patients. Next-generation sequencing (NGS) showed mutations in three ACC cases. From these patients, blood was drawn before (1 to 2 weeks) and after surgery and cell-free circulating DNA (cfDNA) was isolated. Tumor-specific mutations were found in cfDNA of one of three patients, with metastasized ACC at diagnosis. NGS of the tumor showed an *NRAS* mutation (c.182A>G:p.Q61R) in 78%, a *TP53* mutation (c.856G>A:p.E286K) in 60%, and a *TERT* gene mutation (1295250C>T) in 28% of the reads. The preoperative cfDNA showed the same mutations at a frequency of 64%, 32% and 2%, respectively. The postoperative cfDNA showed the same mutations, but at lower frequencies (52%, 16%, and 3%, respectively). The patient was postoperatively treated with mitotane and chemotherapy. No mutations were detected in the corresponding leucocyte DNA or in the cfDNA from the two other patients.

Conclusion: To the best of our knowledge, we report for the first time mutations occurring at high levels in cfDNA collected before and after surgery from one of three patients, after previous identification in the tumor. However, in the cfDNA from two patients with known mutations, we were unable to reliably detect mutations in the cfDNA. Our results indicate that mutation detection in cfDNA can vary among ACC patients, and other approaches might be required to detect the tumor response and monitor progressive disease.

INTRODUCTION

Adrenocortical carcinoma (ACC) is a rare disease with heterogeneous treatment responses and prognosis (1). Several prognostic and predictive factors have been proposed, but all have limited value (2). Many efforts in oncology have focused on noninvasive methods to monitor disease course. The discovery of circulating cell-free DNA (cfDNA) in blood and the increased concentrations found in cancer patients versus healthy individuals raised attention for its application in monitoring tumor dynamics (3). The amount of cell-free circulating DNA derived from the tumor (ctDNA) largely depends on the tumor type, disease stage, and therapeutic response (4, 5). ctDNA is derived from the primary tumor, metastases and micrometastases, and/or apoptotic circulating tumor cells (6). To identify ctDNA, tumor-specific aberrations can serve as personalized biomarkers. In ACCs, extensive efforts have led to the identification of several genes involved in ACC pathogenesis (2, 7). In the present case report, we aimed to detect ctDNA in the plasma of patients with ACC by identifying specific mutations present in both the primary tumor and the cfDNA using next-generation sequencing (NGS).

METHODS

Blood was collected in EDTA tubes 1 to 2 weeks before surgery and 5 to 6 months after surgery. For patient 3, blood was also drawn at 14 months and 2 years postoperatively. The blood samples were processed directly after collection by centrifugation for 10 minutes at 1349g at 4°C. The supernatant was carefully removed and saved in 1-mL aliquots at -80°C until analysis. DNA from 1 to 2 mL of plasma, dependent on availability, was isolated using the QIAamp Circulating Nucleic Acid Kit and the vacuum-based QIAvac 24 Plus system (Qiagen). Leucocyte preparation and DNA isolation were performed using the DNA isolation Kit for Mammalian Blood (Roche). Genomic DNA from the primary tumor was isolated from a formalin-fixed paraffin-embedded tissue sample, after selecting an area of high tumor content microscopically (>80%). DNA isolation was performed by overnight incubation at 56°C in 180 µl Tris-EDTA-buffer (pH 7.5) containing 5% Chelex, and 20 µl proteinase K (20 mg/ml), followed by 8 minutes at 100°C. The DNA yield was measured using Qubit Fluorometric Quantitation (Thermo Fisher Scientific). NGS was performed sequencing a custom-made multigene panel using the Ampliseq website (available at: www.ampliseq.com) related to major cancer pathways, such as the WNT signaling, MAPK, and PI3K/AKT pathways, and has been previously reported (8). Accordingly, the panel covered mutational hotspot areas of *APC* (exons 12-14), *AXIN1* (exon 1-6), *AXIN2* (exon 7), *CTNNB1* (exon 3), *BRAF* (exons 11/15), *KRAS* (exons 2-4), *NRAS* (exons 2-4), *HRAS* (exons 2-4), *EGFR* (exons 18-21), *PI3KCA* (exons 9/20), *AKT1* (exon 2), *AKT2* (exon 2), *AKT3* (exon

2), *PTEN* (exons 3-5/7), *ALK* (exons 23-25), *ERBB2* (exons 19-20), *PRKAR1a* (exons 4-8), *TP53* (exons 2-11), and the promoter region of *TERT* (UCSC:CRCh37/hg19 chromosome 5, nucleotides 1295228-250). NGS was performed as previously described (9). The detection threshold of a mutation was set at 1%. The patients provided informed consent, and the study was conducted under guidelines approved by the Erasmus Medical Center medical ethics committee.

NGS OF THE PRIMARY TUMOR AND LEUCOCYTES

In the present study, six patients with ACC were included. Of these six patients, mutations were found in the primary tumor of three. In these patients, mutation analyses of germline DNA isolated from leucocytes revealed no mutations. CfDNA was isolated from plasma drawn before and after surgery and analyzed for the same mutations using NGS as described in the previous section.

CASE REPORT

Patient 1

A 57-year-old male patient was referred to the Erasmus Medical Center because of an adrenal mass. The computed tomography (CT) scan showed a 15-cm left adrenal mass (Fig. 1), with lung, peritoneal, omental, and right adrenal metastases. One day after admission, blood was drawn and processed as described. Open debulking adrenalectomy was performed 10 days later, with removal of the omental metastases. The histopathology report described a tumor with a Weiss score of 6, including necrosis, nuclear atypia, mitotic count >5/50 HPF, atypical mitoses, infiltration, and diffuse architecture. Immunohistochemically, calretinin, inhibin, and melan-A were focally positive, suggestive of preexisting adrenal tissue and supporting an adrenocortical origin of the tumor. The Ki67 index was 50%. The histopathological characteristics of the tumor in the greater omentum and cytomorphology of a fine needle aspirate from a lymph node revealed features similar to those observed in the primary tumor. Six days postoperatively, mitotane was started (1500 mg/d), with the aim of increasing the dosage according to tolerability. A dosage of 6000 mg/d was reached; however, the maximum plasma level in the first three months was only 2.4 mg/L. A plasma level of ≥ 14 mg/L is considered therapeutic (10). Three weeks later, an abdominal CT scan showed disease progression. The patient developed ascites, which was drained, and also cutaneous metastases, a rare presentation of disseminated ACC (11). Mitotane was subsequently combined with etoposide, doxorubicin and cisplatin (M-EDP) according to the FIRM-act (First International Randomized Trial in Locally Advanced

and Metastatic Adrenocortical Carcinoma Treatment) protocol, as palliative treatment. The cutaneous metastases decreased in size with chemotherapy. The mitotane levels in this period varied from 3.2 to 3.9 mg/L. Stable disease, determined by radiography, was achieved after two courses of chemotherapy. However, 4 months after initiation, M-EDP was discontinued because of disease progression. Specifically, the amount of lesions in the mesentery and pelvis had increased, and subcutaneous, intramuscular and retroperitoneal lesions were still present with a mixed response. Four weeks later, blood was drawn again. At that point, signs suspicious for leptomeningeal metastases were seen on magnetic resonance imaging (MRI). The patient died 2 months later.

NGS of the primary tumor showed an *NRAS* mutation (c.182A>G:p.Q61R) in 78% of the reads and a *TP53* mutation (c.856G>A:p.E286K) in 60% of the reads. A mutation in the promoter region of the *TERT* gene (1295250C>T) was reported at a frequency of 28%. Isolation of cfDNA from the preoperative plasma yielded 123.6 ng/mL plasma. In contrast, the DNA yield from the postoperative sample was 1.99 ng/mL. NGS of the preoperative cfDNA showed the same *NRAS* mutation as found in the primary tumor in 64% of the reads, the *TP53* mutation in 32%, and the *TERT* mutation in 2% of the reads. cfDNA from the plasma collected postoperatively showed the same three mutations in 52%, 16%, and 3% of the reads, respectively (Table 1). No mutations were found in DNA isolated from leucocytes from the same blood samples.

Patient 2

A 5-cm right-sided cortisol-producing ACC was diagnosed in a 61-year-old female patient, with no signs of lymph node or distant metastases as determined by CT, fludeoxyglucose positron emission tomography/CT, and MRI. Blood was drawn 2 weeks before the first surgery. After open adrenalectomy, histopathology report described a tumor with a Weiss score of 5. The tumor showed immunoreactivity against synaptophysine, keratin and melan-A, pointing toward a primary ACC. Because of positive microscopic margins, mitotane was initiated at 6000 mg/d 1 month after surgery. At that point, a CT scan revealed no lung metastases or local recurrences. Because of toxicity, the mitotane levels did not reach 14 mg/mL in the 5 months after surgery. At 5 months postoperatively, CT and MRI scans revealed local recurrence, with a lesion in the liver suspicious for metastasis. Blood was drawn at that time.

NGS of the primary tumor showed a *CTNNB1* mutation *(*c.100G>A: p.G34R) in 34% of the reads. NGS of the preoperative (yield 6.50 ng/ml) and postoperative (yield 8.93 ng/ml) cfDNA showed no mutations (Table 1).

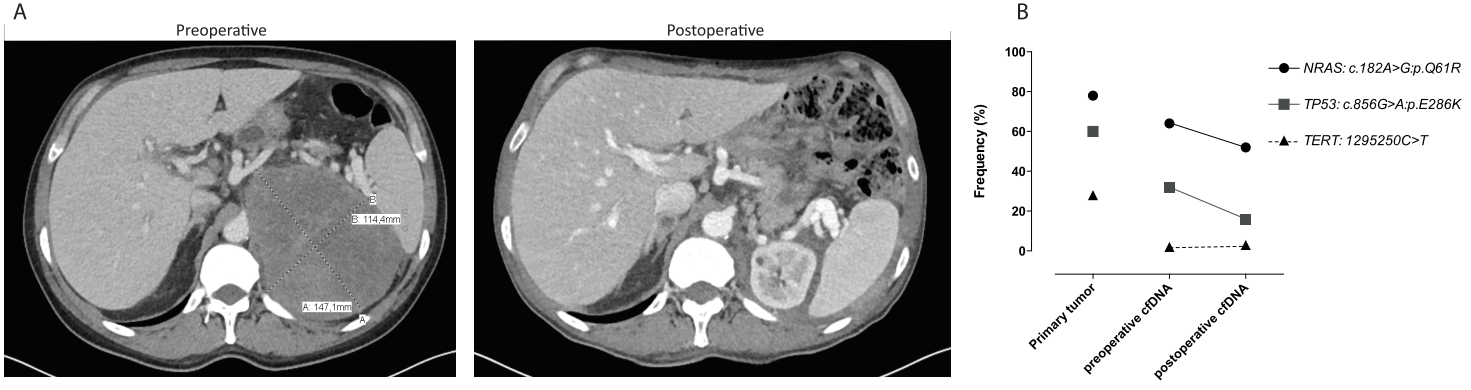

Figure 1. Overview of imaging and NGS results for patient 1. (A) Abdominal CT scans performed at Erasmus Medical Center. (left) The left adrenal mass of patient 1 at 10 days preoperatively, with a maximum diameter of 147 mm. On the same CT scan, lung, peritoneal, omental, and right adrenal metastases were detected. (right) Abdominal CT scan 5 months postoperatively and 1 month before the second blood sample was taken. The large primary adrenal tumor had been removed, but liver metastases can be seen. On the same CT scan, subcutaneous, intramuscular, retroperitoneal, and mesenteric lesions were visible. The number of metastases had increased compared with the CT scan 2 months previously, and the metastases visible on the previous CT scan showed variable responses to chemotherapy. (B) Overview of mutation frequencies of the NRAS, TP53, and TERT gene of patient 1 in the primary tumor, preoperative cfDNA, and postoperative cfDNA.

Patient 3

A 76-year-old male patient was referred to the Erasmus Medical Center because of a right adrenal mass of 14 cm. A CT scan revealed a 14-cm right adrenal mass with prominent pretracheal and aortocaval lymph nodules but no signs of metastases. Open adrenalectomy was performed. The histopathology described a tumor with a Weiss score of 5. At 3 weeks posteropatively, treatment with mitotane was started; mitotane levels of 8.4 mg/L were reached within 2 weeks. However, the patient had to withdraw from mitotane 2 months after the start of treatment because of toxicity. At 8 months postoperatively, a CT scan showed no signs of local recurrence or residual disease; however, it did show an enlarged ileocecal lymph node, for which colonoscopy was performed, and the findings ruled out a colon carcinoma. Two weeks later, blood was drawn. Subsequently, six months later, a CT scan showed increased nodules in the ileocecal and paraduodenal region on the right side, suspicious for local recurrence. Thus, blood was drawn again. Two months later, hemicolectomy and lymph node dissection were performed. The histopathology report confirmed locoregional recurrence of the primary ACC. Two months after surgery, mitotane was started but again was not tolerated by the patient. At the fourth blood sample drawn, a CT scan showed lesions in the right perirenal region suspicious for locoregional recurrence.

A mutation in the *TP53* gene (c.542G>A:p.R181H) was found in 27% of the reads in the primary tumor. At all measurement points (preoperatively and posteropatively), the *TP53* mutation was not found in the cfDNA (Table 1).

Table 1. Overview of mutations and frequencies found in the different samples of patients

Patient	Sample		Yield (ng/mL plasma)	Input DNA (ng)	Mutation 1
1	Primary tumor			10.0	*NRAS*: c.182A>G:p.Q61R
	cfDNA	Preoperative	123.6	6.18	
		6 months postoperative	1.99	0.48	
2	Primary tumor			10.0	*CTNNB1:* c.100G>A: p.G34R
	cfDNA	Preoperative	6.50	1.56	
		5 months postoperative	8.93	2.10	
3	Primary tumor			10.0	*TP53:* c.542G>A:p.R181H
	cfDNA	Preoperative	15.88	3.81	
		6 months Postoperative	44.38	10.0	
		14 months Postoperative	13.00	3.00	
		2 years Postoperative	19.75	4.74	

[a] Frequency of reads with mutations determined using NGS. [b] Total amount of reads per nucleotide position of the mutation. [c] Mean coverage per sample or mean depth. cfDNA, circulating cell-free DNA; NA, not applicable; ND, not detectable, or below the detection limit.

DISCUSSION

To the best of our knowledge, we have shown for the first time the possibility of identifying ctDNA in patients with ACC. However, from the three patients in whom we identified gene mutations in the primary tumor, we were able to identify the mutations in the cfDNA of only one patient, suggesting this minimally invasive approach will only be suitable for monitoring disease progression in a subgroup of patients with ACC.

The cfDNA from both blood samples of patient 1 appeared to include high percentages of ctDNA, as indicated by the relatively high mutation frequencies. As expected, the mutation frequencies were lower in the cfDNA than in the primary tumor DNA, because only ctDNA includes mutations and the fraction of ctDNA will be influenced by tumor heterogeneity and the cfDNA amount released by apoptotic cells from healthy or inflamed tissues unrelated to the tumor. Several reports have already shown that the fraction of circulating DNA derived from the tumor varies greatly, from 0.01% to 90% (5). The three mutations found in patient 1 were previously reported to be associated with ACC pathogenesis (12). However, the frequencies of the TERT mutation (2% and 3%) and the coverage of this position in the cfDNA from patient

%[a]	Coverage[b]	Mutation 2	%[a]	Coverage[b]	Mutation 3	%[a]	Coverage[b]	Mean Coverage[c]
78%	1337	*TP53*: c.856G>A:p.E286K	60%	694	*TERT*: 1295250C>T	28%	122	1299
64%	4580		32%	2566		2%	134	716
52%	2408		16%	1127		3%	128	125
34%	1641	NA			NA			1074
ND	2104							2350
ND	1422							1532
27%	1191	NA			NA			1074
ND	626							605
ND	2239							1961
ND	379							854
ND	3997							109

1 were low. No quantification of the background noise can be performed as yet for cfDNA with this panel; therefore, we could not consider this mutation as "real" in the cfDNA. The absence of mutations in the leucocytes served as a negative control.

The lower cfDNA yield and the lower mutation frequencies in the postoperative plasma of patient 1 could potentially be explained by the lower tumor load, because the primary tumor and most metastases were surgically removed. The patient had also undergone treatment with M-EDP before the postoperative blood sample was taken, which could have influenced ctDNA release. The mutation frequencies in the cfDNA of patients 2 and 3 were very low (< 0.05%) and should therefore be interpreted as noise. For patients with very limited ctDNA in the plasma, it might be useful and required to use more sensitive techniques to detect tumor DNA. Although it will be difficult to interpret very low (<1%) mutation frequencies in cfDNA, the clinical relevance to date is also not known. Potential other explanations for the absence of tumor-specific mutation detection in cfDNA include the absence of circulating tumor cells and intermittent or no leakage of DNA by the tumor. The differences in relative mutation frequencies detected in the tumor tissue and blood samples between and within patients are indicative of intra- or intertumor molecular heterogeneity or might result from regional differences in ctDNA stability. That ctDNA was not detected in the same patient at different disease stages

(local vs metastasized disease) might indicate that ctDNA release, stability or clearance is also, to an extent, tumor or patient related. The absence of mutations in the plasma sample from patients whose tumor was found to have a mutation has been described previously in other types of cancer (13). cfDNA is also known to be fragmented. In other types of cancer, ctDNA released by apoptosis is thought to be approximately 166 bp. In contrast, necrosis releases larger fragments of about 10,000 bp (14, 15). DNA fragmentation analysis at 100 to 12,000 bp revealed fragments in part of our cfDNA samples, which might indicate that our cfDNA samples also harbored larger DNA fragments (> 12,000 bp) or that more sensitive methods are necessary to detect DNA fragments at lower DNA concentrations (Supplemental Table 1).

Our approach considers the mutations detected by NGS in the primary tumor for identification of these tumor-specific mutations in the cfDNA. Consequently, the limitations of this approach include the required presence of ACC-associated mutations in the primary tumor and the minimal necessary DNA yield for NGS. To enable discrimination between true mutations at low frequency and background noise due to sequencing artifacts, and, thus, improve the sensitivity of NGS, molecular barcodes could be used. Hence, mutations could be identified in reads originating from different molecules, which would allow for more reliable detection of mutations. This should be investigated in more detail.

Isolation and quantification of ctDNA in ACC has several potential clinical applications. However, on the basis of this case series, it might only be applicable for a subgroup of patients with ACC, potentially those with large tumors. Research could focus on the value of ctDNA as biomarker for diagnosis, tumor dynamics, treatment response, or prognosis. Finally, it would be interesting to identify the mechanisms underlying the response to systemic therapies, because the unresponsive clones of ctDNA will potentially remain in the circulation during treatment.

In conclusion, to the best of our knowledge, this is the first study reporting mutations in the cfDNA in a patient with ACC. In addition, our results show variability in the fraction of ctDNA in the cfDNA between patients. Our results provide a basis for further research using innovative NGS approaches to enable minimally invasive techniques for monitoring progressive disease and detecting a tumor response in patients with ACC.

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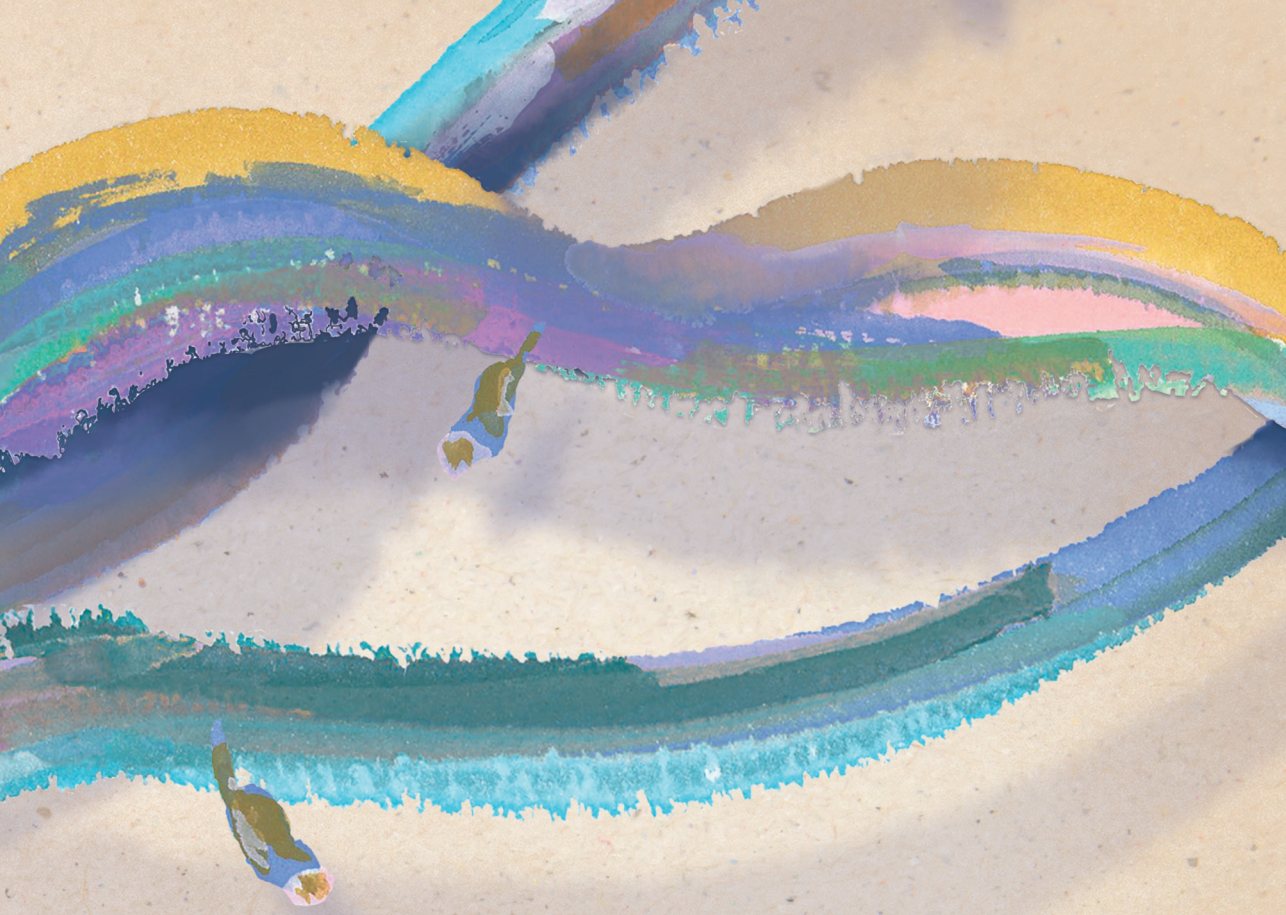
SUPPLEMENTARY MATERIAL

Supplementary Table 1. Results of DNA fragmentation analyses of the circulating cell free DNA samples

Case	Sample		Fragments detected	Fragments	DNA concentration
Case 1	Primary tumor		-	-	-
	cfDNA	Preoperative	Yes	166 bp; 303 bp and a smear	2.06 ng/µl
		6 months postoperative	No	-	0.16 ng/µl
Case 2	Primary tumor		-	-	-
	cfDNA	Preoperative	No	-	0.52 ng/µl
		5 months postoperative	No	-	0.7 ng/µl
Case 3	Primary tumor		-	-	-
	cfDNA	Preoperative	Yes	174 bp; 599 bp	1.27 ng/µl
		6 months Postoperative	Yes	Smear >2000 bp	3.55 ng/µl
		14 months Postoperative	Yes	170 bp	1.00 ng/µl
		2 year Postoperative	Yes	164 bp	1.58 ng/µl

DNA fragmentation analysis was performed using the Bioanalyzer 12000 DNA chip. Using this technique, fragments between 100 and 12,000 bp can be detected. DNA concentration was measured using Qubit Fluorometric Quantitation (Thermo Fisher Scientific). cfDNA, circulating cell-free DNA; bp, base pair.



CHAPTER 7

The efficacy of mitotane in human primary adrenocortical carcinoma cultures

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** authors contributed equally to content*

Submitted for publication

ABSTRACT

Context: Patients with adrenocortical carcinoma (ACC) often fail mitotane treatment and deal with severe toxicity, marking the relevance of predictive parameters for treatment outcome.

Objective: Determine the effects of mitotane in primary ACC cultures, and correlate sensitivity with patient and tumor characteristics.

Methods: In 32 primary ACC cultures, the effects of mitotane on cell growth and cortisol production were determined. RRM1, SOAT1, and CYP2W1 expression were assessed using RT-PCR and immunohistochemistry.

Results: The median percentage cell amount inhibition in primary ACC cultures at 50 μM mitotane was 57%. Seven patients were classified as non-responders, 14 as partial responders, and 11 as responders. The percentage cortisol-producing ACC was 14%, 43%, and 73% for non-responders, partial responders, and responders (P = 0.068). Mitotane inhibited cortisol production with a mean EC_{50} of 1.4 μM (95%CI 0.9 – 2.1), which was considerably lower compared to the EC_{50} on cell growth. *RRM1*, *SOAT1*, and *CYP2W1* mRNA expression levels were lower in ACCs compared to non-malignant adrenals, and not predictive for mitotane sensitivity *in vitro*. SOAT1 protein expression, determined using a monoclonal antibody specifically staining the adrenal cortex, was correlated with *SOAT1* mRNA expression in ACC (P = 0.0001, ρ = 0.6208).

Conclusions: Direct antitumor effects of mitotane on human primary ACC cultures are highly variable between patients, reflecting heterogeneous responses in patients. Cortisol was inhibited at lower concentrations, compared to its effect on cell amount. Cortisol secretion by ACC might be associated with enhanced mitotane sensitivity due to increased direct antitumor effects of mitotane.

INTRODUCTION

Adrenocortical carcinoma (ACC) is a rare malignancy with 5-years survival rates of 16-44% (1, 2). Although surgery is the only curative treatment modality, medical therapy can be used in metastatic disease or to prevent recurrences after radical ACC resection. Mitotane is the only accepted adrenolytic drug, but response rates and efficacy in both above mentioned settings are limited (3), and mitotane use is accompanied by severe side effects.

Markers that predict which patients benefit from mitotane treatment are of great importance in order to prevent overtreatment and side effects, as well as to safe costs. Reaching the target plasma concentration of 14 mg/L (~50 μM) is currently considered the most important predictive marker for response to mitotane, with less recurrences and a longer disease-free survival (DFS) in patients who reach this plasma concentration (1, 4, 5). Volante et al. showed a correlation of expression of ribonucleotide reductase large subunit (*RRM1)* with DFS and overall survival in ACC patients (6). In patients with low tumoral *RRM1* expression, a significantly longer DFS was found in patients who received adjuvant mitotane compared to patients who were only monitored during follow-up. This difference was not present in patients with high *RRM1* expression (6). As a possible explanatory mechanism, Germano et al. showed that RRM1 interferes with mitotane metabolism and bioavailability of the active metabolite in ACC cell line models *in vitro* (7). Recently, Sterol-O-Acyl-Transferase 1 (SOAT1) was identified as a key molecular target for mitotane, which expression was positively correlated with a longer time to progression and DFS in patients treated with mitotane (8). In another study, CYP2W1 immunoreactivity, adjusted for ENSAT stage, was positively associated with a longer overall survival and time to progression in patients treated with mitotane (9). CYP2W1 is considered an orphan human cytochrome P450 enzyme, because its physiological substrate is still unknown. Expression of this enzyme is known to be high during foetal life and in some cancers and has recently gained attention as a promising tool in targeted therapy (10).

The objective of this study was to assess for the first time the direct effects of mitotane on cell growth and cortisol production in a large series of primary human ACC cultures. Furthermore, we aimed to evaluate the relationship between mitotane sensitivity and clinical and tumor characteristics, and the previously proposed potential predictive parameters RRM1, SOAT1, and CYP2W1.

MATERIALS AND METHODS

Patients and tissue samples

ACCs, adrenocortical adenomas (ACAs), and normal adrenals (NAs) were collected during surgeries performed at the Department of Surgery at the Erasmus MC between June 1990 and August 2016. Diagnosis was made on the basis of the Van Slooten Index or the Weiss score (11, 12), dependent on the year of pathological diagnosis. Normal adrenals were collected during nephrectomy and confirmed by the pathologist as being normal. The following clinical parameters were obtained from all ACC and ACA patients: age at diagnosis, follow-up time, sex, tumor size, ENSAT stage in case of primary tumors, hormonal secretion status, systemic therapies received prior to surgery, and development of metastases. A part of the tissue specimens was embedded in Tissue-Tek directly after resection and stored at -80°C until analysis. Another part was processed to obtain primary cultures and to isolate total RNA, as described below. *In vivo* cortisol production was identified by an increased urinary free cortisol (UFC), increased midnight salivary cortisol level, a positive dexamethasone suppression test, or a combination of tests. Informed consent was obtained from all patients. The study was conducted under the guidelines that had been approved by the Medical Ethics Committee of the Erasmus Medical Center.

Primary cultures

Immediately after surgery, a part of the ACC specimen was minced into small pieces of 2-3 mm^3, washed in culture medium and centrifuged for 5 minutes at 600 g. The culture medium consisted of DMEM-F12 (Fisher Scientific, Landsmeer, the Netherlands) containing 5% fetal calf serum (FCS), penicillin ($1x10^5$ U/; Bristol-Meyers Squibb, Woerden, the Netherlands) and L-glutamine (2mmol/l; Fisher Scientific, Landsmeer, the Netherlands). The remaining tissue pellet was suspended in culture medium and was stored overnight at 4°C, where after the tissue was centrifuged again and the supernatant was removed. Tissues were dissociated in 10-25 ml medium with collagenase type-I (2mg/ml; Sigma-Aldrich, Zwijndrecht, the Netherlands) by incubation at 37°C for up to two hours. If necessary, the obtained suspension was filtered through a sterile needle. Ficoll (GE Healthcare, Eindhoven, the Netherlands) density gradient separation was used once or twice in order to separate contaminating red blood cells from the tumor cells. After centrifugation for 20 minutes at 600 g, the interphase containing the tumor cells was collected. Trypan blue exclusion was used to determine cell viability, and adrenal cells were visually counted using Türk solution. Dissociated cells were plated in quadruplicate at a density of 10^5 cells per well in a 24 wells plate in 1 ml culture medium. Medium was refreshed after 3-4 days and incubations with mitotane were initiated. Mitotane (Sigma-Aldrich, Zwijndrecht, the Netherlands) was dissolved in absolute ethanol and stored at a concentrated stock solution (10^{-2} M) at -20°C, and diluted in ethanol prior to use. After 3

days of incubation, media and mitotane were refreshed. After 7 days, media were removed and plates and media were stored at -20°C until analysis. Plated cells were routinely monitored to ascertain absence of fibroblast contamination. To determine *in vitro* cortisol production, cortisol was measured in the media of all ACC using a chemiluminescence immunoassay system (Immulite 2000XPi). Total DNA per well, reflecting cell amount, was determined using the bisbenzimide fluorescent dye (Hoechst 33258, Sigma-Aldrich, Zwijndrecht, the Netherlands), as previously described (13).

Real-time quantitative PCR

In primary cultures where there were remaining ACC cells after isolation and plating for the cell culture experiments, *CYP11B1* and *STAR* mRNA expression levels were measured. *RRM1*, *SOAT1*, and *CYP2W1* mRNA expression levels were measured in NAs, ACAs, and ACCs. RNA isolation, cDNA synthesis and RT-PCR were performed as previously described, but using other primers (Supplementary Table 1; Sigma-Aldrich, Zwijndrecht, the Netherlands) (14). The Vandesompele method was used to normalize the mRNA expression levels according to three housekeeping genes (15): hypoxanthine-guanine phosphoribosyl transferase 1 (*HPRT1*; Sigma-Aldrich, Zwijndrecht, the Netherlands), Beta-actin (*B-actin*; Thermo Fisher Scientific, Breda, the Netherlands), and glucuronidase beta (*GUSB*; Thermo Fisher Scientific, Breda, the Netherlands).

RRM1, SOAT1 and Chromogranin A immunohistochemistry

Construction of the tissue micro arrays (TMA) and immunohistochemistry of RRM1 and SOAT1 was performed as previously described in detail (16). The rabbit monocloncal RRM1 antibody (dilution 1:50; ab135383; Abcam, Cambridge, England), the mouse monoclonal SOAT1 antibody (mAb; dilution 1:1000; Sc69836; Santa Cruz Biotechnology, Heidelberg, Germany), and the rabbit polyclonal SOAT1 antibody (PoAb; dilution 1:500; Ab39327 (8); Abcam, Cambridge, England) were used. Blinded for the tissue type, the sections were independently scored by two investigators (SGC, LJH) using a semi-quantitative well-established Immunoreactivity Score (IRS), which consists of the product of the percentage positive cells (4, >80%; 3, >51-80%; 2, >10%; 1, 0) and intensity of staining (3, strong; 2, moderate; 1, mild; 0, no staining) (17). Chromogranin A immunohistochemistry was performed on slides of the normal adrenal, as previously described (18), but using the mouse Chromogranin A primary antibody (LK2H10; Ventana Medical Systems).

Statistical analysis

For statistical analysis, Graphpad Prism 6.0 (Graphpad Software, San Diego, CA) and SPSS Statistics 21 (SPSS 21.0; SPSS Inc., Chicaco, Il) were used. Response to mitotane was categorized by calculating the *in vitro* effect on cell amount at 50 μM mitotane (14 mg/L), the circulating therapeutic plasma concentration of mitotane (4), using non-linear

regression curve fitting. In two cases, the lowest concentration of mitotane caused an increase in cortisol, resulting in a top of the curve above 100%. When these curves were constrained at a top of 100%, the IC_{50} however only minimally changed. For uniformity, all curves were fitted without constraint. Patient cultures were arbitrarily classified as non-responder when the inhibitory effect on cell amount was ≤33%, as partial responders when the effect was >33% and ≤66% and responders showed a cell amount inhibition of >66% at 50 μM mitotane. Differences of categorical variables between groups were analysed using the Fisher's Exact test, considering the small sample size. Continuous variables were compared using the Kruskall-Wallis test or one-way ANOVA, dependent on the distribution. Overall survival was defined as the time from diagnosis until death or last follow-up. Survival curves were computed using the Kaplan-Meier method and differences between cortisol and non-cortisol secreting ACC were assessed by the log-rank (Mantel-Cox) test. A value of $P < 0.05$ was considered statistically significant. Values are presented as mean ± SEM, unless specified otherwise.

RESULTS

Patient characteristics and sensitivity to mitotane

Ten NAs, obtained during nephrectomy, 16 ACAs, and 45 ACCs were enrolled. Patient and tumor characteristics are listed in Supplementary Table 2. *RRM1*, *SOAT1*, and *CYP2W1* mRNA expression were assessed in 55 adrenal specimens (8 NAs, 10 ACAs, 37 ACCs), and RRM1 and SOAT1 immunohistochemistry was performed in 59 tissues (7 NAs, 14 ACAs, 38 ACCs). A primary culture was obtained from 32 ACCs, including 29 primary tumors and 3 local recurrences. 2 patients received mitotane preoperatively and all ACC patients were postoperatively treated with mitotane.

In human primary ACC cultures, mitotane suppressed cell amount and cortisol production in a dose-dependent fashion. The median percentage of cell amount inhibition by 50 μM mitotane was 57% (IQR 39 – 71). On the basis of the percentage inhibition at 50 μM mitotane (≤33%, 33-66% or >66% inhibition), seven (22 %) ACCs were classified as non-responders, 14 (44%) as partial responders, and 11 (34%) as responders (Table 1; Fig. 1, A and C-E). The mean EC_{50} value on cell growth could not be calculated for non-responders, because the dose-response curve did not reach a bottom. Fifteen of the 32 primary ACC cultures secreted cortisol *in vitro*. The mean EC_{50} of mitotane for inhibition of cortisol production, corrected for cell amount, was 15 μM for the single cortisol producing non-responder (Fig. 1F; $n = 1$, 14%), 1.7 μM (95% CI 1.2 – 2.5; $P < 0.0001$ vs non-responders) for partial responders (Fig. 1G; $n = 6$, 43%), and 0.90 μM (95% CI 0.69 – 1.2; $P < 0.0001$ vs non-responders and partial responders) for responders (Fig. 1H; $n = 8$, 73%). In 14 of the

15 primary cultures with *in vitro* cortisol production, cortisol production was inhibited at significantly lower concentrations compared to cell amount (Fig. 1, F-H; all $P < 0.01$).

In all of the ACC samples in which *CYP11B1* and *STAR* mRNA was measured ($n = 13$), *STAR* mRNA was expressed, although in one non-responder sample at a very low level (Supplementary Fig. 1). *CYP11B1* mRNA could be detected in 12 of the 13 ACC samples. This confirms adrenocortical origin of the plated cells.

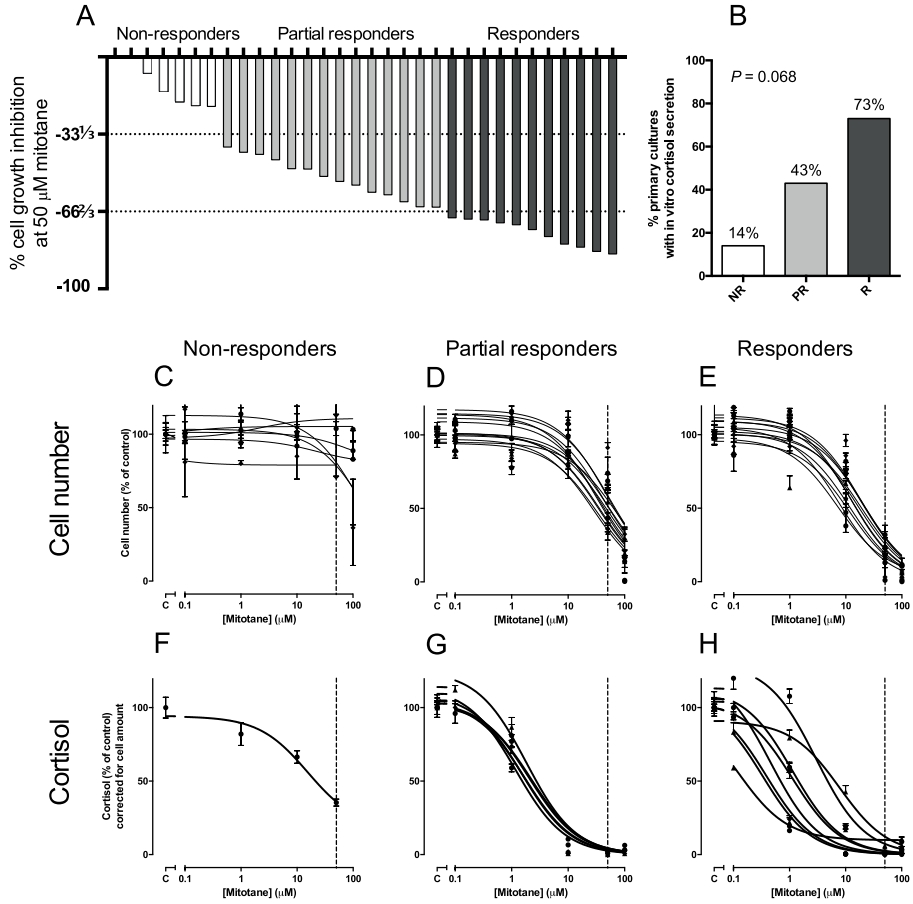

Figure 1. Effects of mitotane on cell growth and cortisol production in primary ACC cultures *in vitro*. (A) Percentage of inhibition of cell growth at 50 μM per primary culture and the identification of non-responders, partial responders, and responders. (B) The percentage of ACCs that *in vitro* produce cortisol divided per group. (C-E) Dose-reponse curves of mitotane on cell growth in non-responders (C), partial responders (D), and responders (E). (F-H) Dose-response curves of mitotane on cortisol production corrected for cell amount in non-responders (F), partial responders (G), and responders (H). Vertical dotted line represents the circulating concentration, which determined the responder classification (50 μM). Values are depicted as mean ± SEM. NR, non-responders; PR, partial responders; R, responders.

Table 1. Patient and tumor characteristics for the total group of adrenocortical carcinoma patients from which a primary culture was obtained

	Total group (ACC)	Non-responders
n (%)	32	7 (22%)
% cell amount inhibition at 50 µM (median, IQR)	57 (39 – 71)	15 (0.0 – 21)
EC_{50} (µM, 95% CI) cell amount	-	> 100 µM
EC_{50} (µM, 95% CI) cortisol	1.4 (0.90 – 2.1) *n* = 15	15 (3.2 – 75) *n* = 1
Age at diagnosis (median, IQR, yrs)	51 (43 – 57)	52 (45– 65)
Male, n (%)	17 (53%)	2 (29%)
ENSAT staging		
I	1 (4%)	0 (0%)
II	13 (46%)	5 (71%)
III	2 (7%)	0 (0%)
IV	13 (41%)	2 (29%)
Weiss score (median, IQR)	6.0 (5.0 – 7.0) *n* = 24	6.0 (4.8 – 7.0) *n* = 6
VSI (median, IQR)	22.2 (18.9 – 25.1) *n* = 30	21.0 (16.1 – 25.1) *n* = 7
Tumor diameter (median, IQR)	14.0 (8.25 – 19.0)	18.0 (14.0 – 19.0)
In vivo secretion		
Androgens	5 (16%)	1 (14%)
Cortisol	15 (47%)	1 (14%)
Mineralocorticoids	1 (3%)	0 (0%)
Precursors	2 (6%)	1 (14%)
Estradiol	2 (6%)	0 (0%)
Non secreting	13 (41%)	4 (57%)
In vitro cortisol secretion	15 (47%)	1 (14%)
Metastasis during follow-up, n (%)	20 (63%)	3 (43%)

Van Slooten Index and Weiss Score were not available for all patients, dependent on the year of diagnosis. P-value represents overall differences between the three groups. Significant p-values are shown in bold. Values represent mean ± sd.

Partial responders	Responders	P-value
14 (44%)	11 (34%)	
53 (44 – 60)	75 (70 – 82)	**<0.001**
41.6 (33.5 – 51.8)	14.2 (11.3 – 17.9)	
1.7 (1.2 – 2.5) *n* = 6	0.90 (0.69 – 1.2) *n* = 8	**< 0.0001**
43 (38 – 51)	57 (52 – 70)	**0.003**
9 (64%)	6 (55%)	0.317
0 (0%)	1 (9%)	0.375
5 (42%)	3 (33%)	
2 (17%)	0 (0%)	
5 (42%)	6 (60%)	
6.0 (5.0 – 7.0) *n* = 8	7.0 (6.0 – 7.5) *n* = 9	0.372
22.0 (18.3 – 25.3) *n* = 14	24.7 (19.6 – 28.4) *n* = 10	0.176
11.0 (6.75 – 18.25)	14.0 (8.00 – 21.0)	0.316
2 (14%)	2 (18%)	1.000
6 (43%)	8 (73%)	0.068
1 (7%)	0 (0%)	1.000
1 (7%)	0 (0%)	0.690
1 (7%)	1 (9%)	1.000
6 (43%)	3 (27%)	0.475
6 (43%)	8 (73%)	0.068
8 (57%)	9 (82%)	0.185

Non-responders, ≤33% inhibition of cell amount at 50 μM mitotane; partial responders, >33% and ≤67% inhibition of cell amount at 50 μM mitotane; responders, >67% inhibition of cell amount at 50 μM mitotane; CI, Confidence Interval; ENSAT, European Network for the Study of Adrenal Tumors, only for primary tumors; IQR, interquartile range; VSI, Van Slooten Index; yrs, years.

Correlation of sensitivity to mitotane *in vitro* with clinical parameters

Patient and tumor characteristics of the ACCs of which a primary culture was obtained are listed in Table 1. There were differences in age at diagnosis between the three groups ($P = 0.003$), however we did not find a linear correlation. In 81% of primary cultures, *in vitro* and *in vivo* cortisol production were concordant. The percentage *in vitro* cortisol-producing ACC gradually increased with a stronger response to mitotane *in vitro*, i.e. 14%, 43%, and 73% for non-responders, partial responders and responders, respectively ($P = 0.068$; Fig. 1B, Table 1). The same difference was observed for *in vivo* glucocorticoid secretion ($P = 0.068$). There were no differences in pathological characteristics, tumor size, and ENSAT stage, between the three groups. A decreased overall survival was found for cortisol versus non-cortisol secreting ACC *in vivo* (Log Rank $P = 0.043$, $n = 45$).

RRM1, *CYP2W1*, and *SOAT1* mRNA expression in adrenal tissues and correlation with sensitivity to mitotane *in vitro*

mRNA expression levels of *RRM1* and *SOAT1* were significantly lower in ACCs compared to ACAs (Fig. 2, A and B; $P < 0.05$, $P < 0.01$, respectively), whereas expression of *CYP2W1* was only decreased in ACCs compared to normal adrenals (Fig. 2C; $P < 0.05$). In ACC, *RRM1* and *SOAT1* mRNA expression levels were correlated ($P = 0.007$, $\rho = 0.436$), whereas no correlation was found between these mRNA levels and *CYP2W1* mRNA expression. In ACC, *SOAT1* mRNA expression appears slightly higher in cortisol-producing ACCs ($n = 19$) compared to non-cortisol producing ACCs ($n = 18$; $P = 0.056$). *SOAT1* and *RRM1* mRNA expression were not correlated with tumor size, ENSAT stage, or histopathological criteria in ACC. *CYP2W1* mRNA expression was negatively correlated with the VSI in ACC ($P = 0.002$, $\rho = -0.530$). No correlation was found with the WS.

mRNA expression levels of *RRM1*, *SOAT1*, and *CYP2W1* were not significantly different between non-responders, partial responders, and responders to mitotane *in vitro* (Fig. 2, D-F). A significant negative correlation was found between the percentage cell amount inhibition at 50 μM mitotane and *CYP2W1* mRNA expression ($P < 0.0281$, $\rho = 0.4306$).

RRM1 and SOAT1 immunohistochemistry in adrenal tissues and correlation with response to mitotane *in vitro*

IHC of RRM1, the most frequently described potential predictive factor for mitotane sensitivity, revealed expression both within the cytoplasm as well as in the nucleus of human adrenocortical cells (Fig. 3F). Since the relevance and exact function of expression at both localizations is unknown in ACC yet, both localizations were scored for immunoreactivity. Protein expression in the cytoplasm was significantly higher in ACCs compared to ACAs (Fig. 3A, $P < 0.01$), whereas no differences in nuclear staining were found between the different tissue entities (Fig. 3B). Cytoplasmic and nuclear RRM1 expressions were positively correlated in ACC (Fig. 3C; $P < 0.0001$, $\rho = 0.5959$). RRM1 protein expression was not correlated with mRNA expression, nor with the effect of mitotane on cell growth (Fig. 3, D and E), and cortisol production *in vitro*.

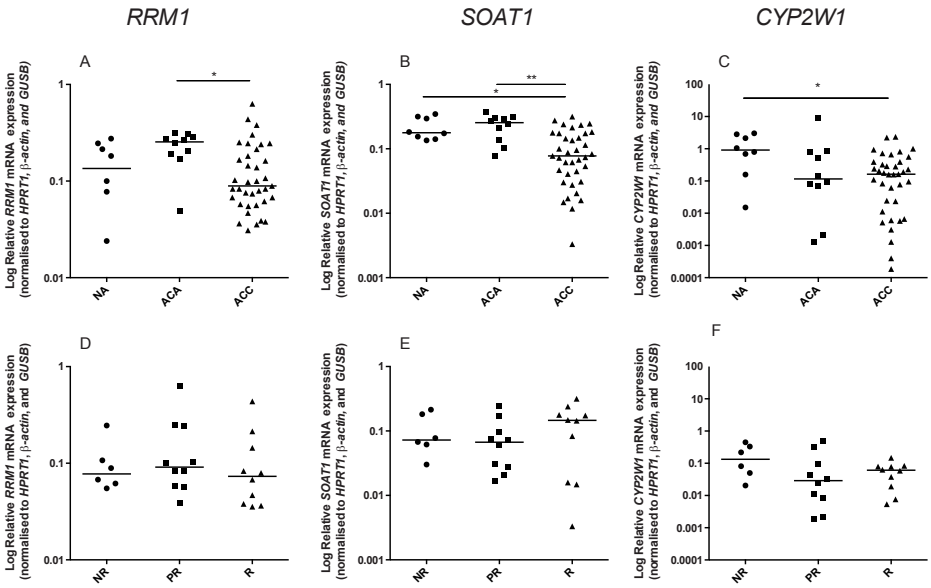

Figure 2. *RRM1* (A,D), *SOAT1* (B,E), and *CYP2W1* (C,F) mRNA expression in normal adrenals, adrenocortical adenomas and adrenocortical carcinomas (A-C), and in ACCs stratified for mitotane responsiveness *in vitro* (D-F). Lines represent medians. * $P < 0.05$, ** $P < 0.01$. ACA, adrenocortical adenomas; ACC, adrenocortical carcinomas; *B-actin*, Beta-actin; *GUSB*, glucuronidase beta; *HPRT1*, hypoxanthine-guanine phosphoribosyl transferase 1; NA, normal adrenals; NR, non-responders; PR, partial responders; R, responders; RRM1, ribonucleotide reductase M1; SOAT1, Sterol-O-Acyl-Transferase 1.

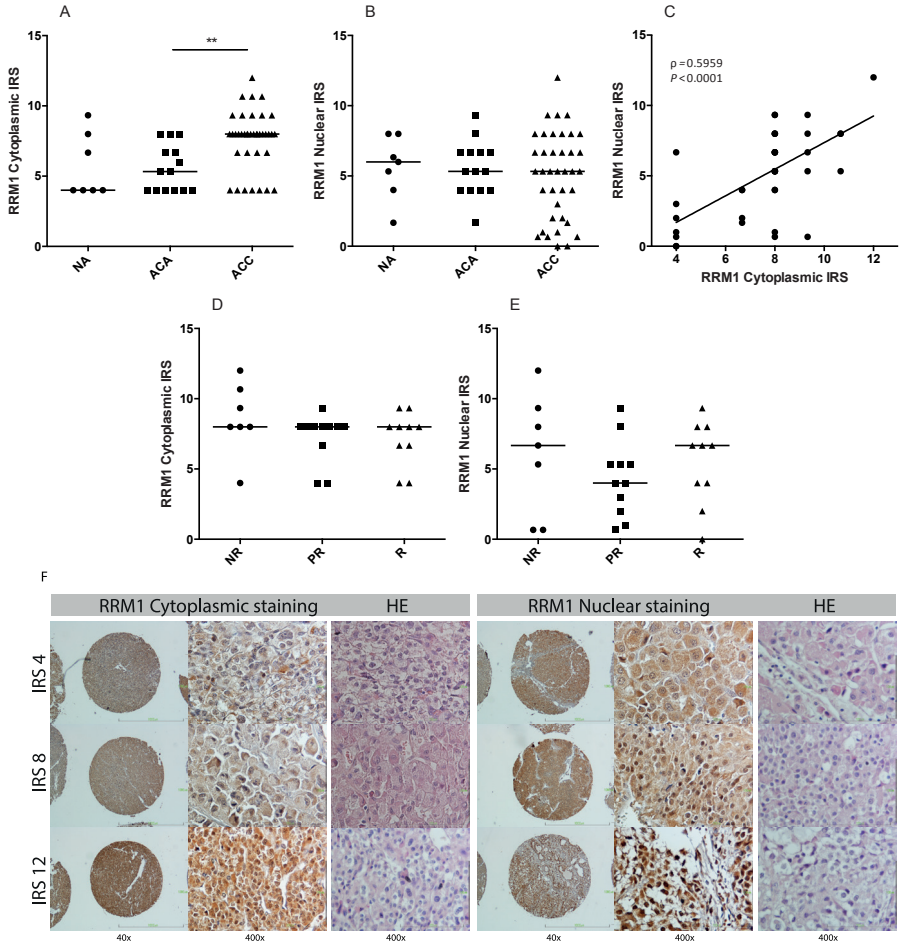

Figure 3. RRM1 protein expression in human adrenal specimens in the cytoplasm (A) and nucleus (B), and correlation of expression at both localizations in adrenocortical carcinoma (C). RRM1 protein expression in the cytoplasm (D), and nucleus (E) in adrenocortical carcinomas stratified for mitotane response in primary cultures. (F) Representative example of RRM1 immunohistochemical staining in the cytoplasm (left) and the nucleus (right) in adrenocortical carcinoma, with corresponding HE section. Sections were blinded and independently evaluated by two investigators. Microscopic magnification 40x, and 400x. ρ represents Spearman's rank correlation coefficient. Lines represent medians. ** $P < 0.01$. ACA, adrenocortical adenomas; ACC, adrenocortical carcinomas; HE, Haematoxylin eosin; NA, normal adrenals; NR, non-responders; IRS, immunoreactivity score; PR, partial responders; R, responders; RRM1, ribonucleotide reductase large subunit.

Immunoreactivity scores of SOAT1 IHC were based on expression levels relative to the normal adrenal cortex (Fig. 4, A-F). Chromogranin A expression was used to differentiate between adrenal medulla and cortex (Fig. 4, G-I), demonstrating that only the monoclonal SOAT1 Ab specifically stains the adrenal cortex (Fig. 4, A-C and G-I). Examples of SOAT1 staining, representing different IRS scores in ACC, are stated in Fig. 4, J-R. Within ACC, SOAT1 protein expression by using both antibodies was positively correlated (Fig. 5C; $P = 0.0004$, $\rho = 0.5424$). SOAT1 protein expression as assessed by either the PoAb or the mAb was not different between NAs, ACAs, and ACCs (Fig. 5, A and B). SOAT1 protein expression (mAb) was negatively correlated with ACC tumor size ($P < 0.0001$, $\rho = -0.550$, $n = 38$), and was significantly higher in ACC with *in vitro* cortisol production compared to the ACC cultures with no cortisol production ($n = 28$; $P = 0.011$). Only the SOAT1 expression as assessed by the mAb was positively correlated with *SOAT1* mRNA expression in ACC (Fig. 5D; $P = 0.0001$, $\rho = 0.6208$), where the correlation with expression as assessed by the PoAb did not reach significance ($P = 0.057$, $\rho = 0.334$). No difference was found in SOAT1 protein expression between non-responders, partial responders, and responders (Fig. 5, E and F). Although within a group of only 14 ACC, SOAT1 protein expression, as assessed with the mAb, was inversely correlated with the EC_{50} of mitotane on cortisol production in primary cultures (Fig. 5G; $P = 0.0025$, $\rho = -0.743$, $n = 14$). This indicates that higher expression of SOAT1 results in increased sensitivity to mitotane *in vitro*. This correlation appeared to be a trend using the PoAb ($P = 0.056$, $\rho = -0.521$, $n = 14$). Focusing on the correlation between higher SOAT1 protein expression (mAb) and a stronger response to mitotane as defined by the percentage cell growth inhibition at 50 μM in primary ACC cultures, there was no significant correlation (Fig. 5H; $P = 0.1201$, $\rho = 0.3064$, $n = 27$).

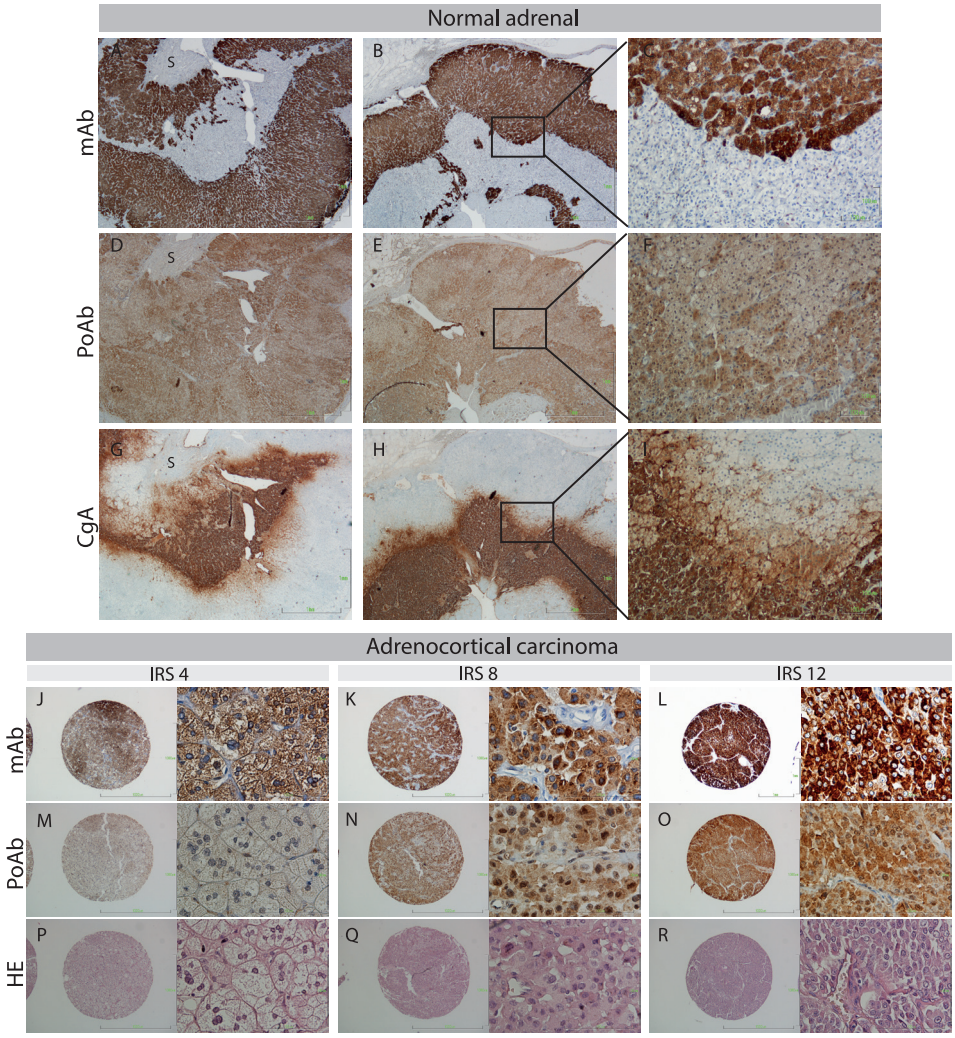

Figure 4. SOAT1 protein expression as assessed by the monoclonal and polyclonal antibody (mAb, PoAb, respectively) in human adrenal tissues. SOAT1 is primarily expressed in the cytoplasm (J-L). (A-I) SOAT1 and Chromogranin A (CgA, a marker for adrenal medulla) expression in corresponding areas in a normal adrenal. 'S' represents stromal tissue. Right panel represents a 100x microscopic magnification of the selected area. (J-R) Representative sections of SOAT1 protein expression in adrenocortical carcinomas with different IRS. Microscopic magnification 40x, and 400x in one ACC with similar IRS as determined by the mAb and the PoAb, with corresponding HE sections. CgA, chromogranin A; HE, Haematoxylin eosin. IRS, Immunoreactivity Score.

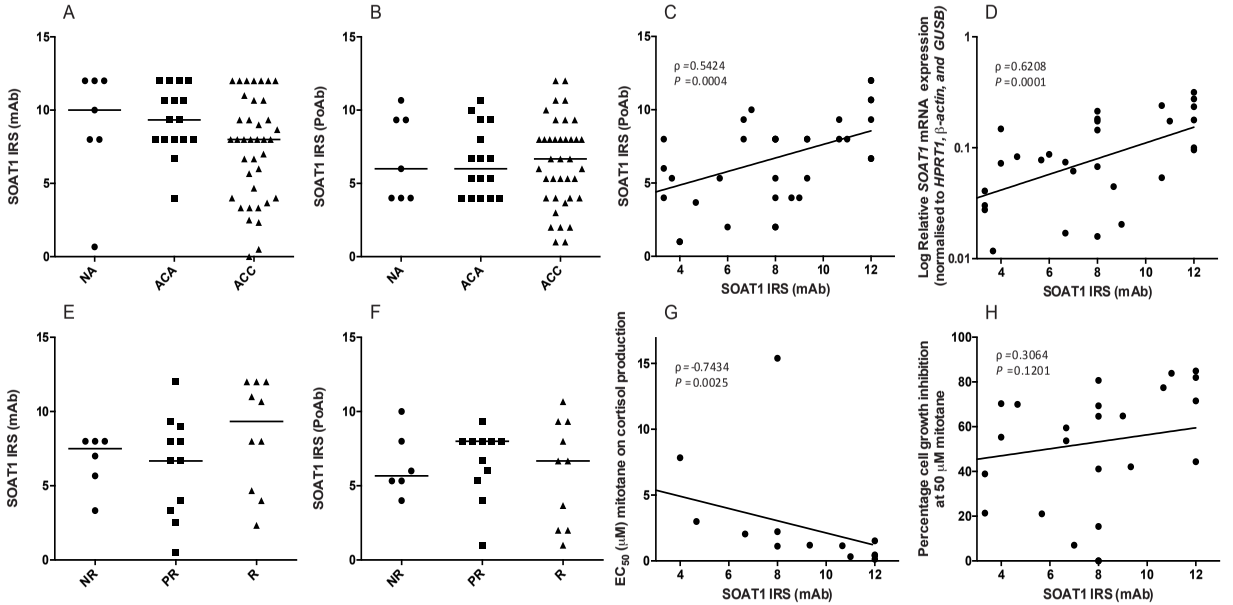

Figure 5. SOAT1 protein expression in adrenal tissues and correlation with *in vitro* mitotane sensitivity. (A, B) SOAT1 protein expression in human adrenal specimens as determined by the monoclonal antibody (mAb, A), or the polyclonal antibody (PoAb, B). (C) Correlation between SOAT1 protein expression as assessed by using both antibodies within ACC. (D) Correlation between *SOAT1* mRNA and protein expression as assessed by the mAb. (E, F) SOAT1 protein expression in non-responders, partial responders, and responders as determined by the mAb (E) and the PoAb (F). Responder classification was based on the percentage of cell growth inhibition at 50 μM mitotane *in vitro*. (G, H) Correlation between SOAT1 protein expression and the EC_{50} value of mitotane on cortisol production (G) and the percentage cell growth inhibition at 50 μM mitotane (H). Lines represent medians. ρ represents Spearman's rank correlation coefficient. ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; *B-actin*, Beta-actin; *HPRT1*, hypoxanthine-guanine phosphoribosyl transferase 1; *GUSB*, glucuronidase beta; IRS, immunoreactivity score; mAb, monoclonal antibody; NA, normal adrenals; NR, non-responders; PoAb, polyclonal antibody; PR, partial responders; R, responders; SOAT1, Sterol-O-Acyl-Transferase 1.

DISCUSSION

In this study, we demonstrate for the first time the direct effects of pharmacological concentrations of mitotane in a large series of primary human ACC cultures, including a correlation with clinical- and tumor characteristics. The general aim is to further explore potential predictive factors for response to mitotane.

The responder classification of ACC to mitotane *in vitro* was based on the effect on cell amount at a concentration of mitotane corresponding to the therapeutic circulating plasma concentration (14 mg/L, 50 μM, (1, 4, 5)). As a result, 34% of patients were classified as responder, which is consistent with clinical data, suggesting efficacy of mitotane in 25-35% of patients with advanced ACC (3). We also show that the direct antitumor effect of therapeutic concentrations of mitotane on growth of ACC cells is highly variable between ACC patients. Mitotane decreased cortisol production in all cortisol-producing ACCs, and in general at concentrations much lower than required for inhibition of cell growth. This difference suggests that a measured inhibitory effect on cortisol in patients does not necessarily resemble an antitumor effect of mitotane.

In 19% of the cases, *in vivo* and *in vitro* cortisol secretion were discordant. Discrepancy might be explained by tumor heterogeneity, considering that only a small part of the tumor is used to obtain primary cultures. Thereby, in a subset of ACC, tumor cells may minimally secrete cortisol *in vivo*, but not sufficient to be diagnosed by clinical tests. The proportion of cortisol-producing ACC *in vitro* and *in vivo* was highest in the responder group, with a gradually decreasing percentage from the partial responder to the non-responder group. Cortisol production has previously been identified as a negative prognostic factor in ACCs (19-21), which was confirmed in our study. Explanations for the decreased overall survival might be the presence of comorbidities associated with Cushing's syndrome and/or the immunosuppressive effects of cortisol favoring tumor progression. Regarding the relation between efficacy of mitotane and cortisol production by ACCs, data have been inconsistent (19, 22, 23). Two more recent studies have reported that mitotane treatment postsurgery only increased disease-free survival in patients with cortisol-secreting tumors, whereas this was not seen in the whole patient group (19, 22). One of the possible explanations of a better response to mitotane in patients with cortisol-producing ACCs might be the decrease in cortisol secretion with concomitant improvement of Cushing's syndrome co-morbidities. However, our *in vitro* findings, i.e. a trend towards more cortisol-producing ACC in the responder group, points towards a stronger direct antitumor effect of mitotane on cortisol secreting ACC cells. The exact mechanism of action of mitotane has yet to be convincingly established, although it is known that mitochondria-mediated intracellular stress induction plays a pivotal role in the basis for its action (24). The adrenal specificity is

thought to be caused by transformation of mitotane into active metabolites specifically in mitochondria of the adrenal gland (25). A reasonable hypothesis is that cortisol-producing ACC harbor increased mitochondrial function, considering the presence of a least three mitochondrial cytochrome P450s required for steroid synthesis (CYP11A1, CYP11B1 and CYP11B2). This might result in increased sensitivity to mitotane in cortisol-producing ACC. The inhibitory effects of mitotane on steroidogenesis are besides the toxicity the result of inhibition of several enzymes necessary for cortisol and aldosterone biosynthesis, like STAR, CYP11A and CYP11B enzymes (26). It has also been suggested that CYP11B1 catalyzes the initial hydroxylation step of mitotane (27), which has however not been supported by a recent report by Germano et al. using CYP11B1 silencing during mitotane action (28).

Given the rarity of ACC, it is difficult to obtain large numbers of primary cultures. Although this study presents a relative large unique series, an important consideration is that the groups are still small and statistics have to be interpreted with caution. A technical challenge as it comes to primary cultures is potential fibroblast contamination. As most important step, plates were routinely monitored to ascertain absence of fibroblast contamination. Additionally, in a subset of primary ACC cultures, *CYP11B1* and *STAR* mRNA expression were measured to confirm adrenal origin of the plated cells.

In clinical practice, as well as in the present *in vitro* study, a great variability is observed in sensitivity to mitotane between patients. Considering the severe side effects of mitotane, there is an unmet need for parameters that predict treatment response. Expression levels of several genes, like RRM1, CYP2W1, and SOAT1, have been proposed for this purpose (6, 8, 9). This is the first study that correlates these potential predictive factors with direct antitumor effects in primary cultures. We only found a significant correlation between mRNA expression of CYP2W1 and increased response to mitotane *in vitro*. This finding is in the opposite direction as has previously been described with *in vivo* response and CYP2W1 immunoreactivity (9). CYP2W1 protein expression was however not assessed in this study, because Nole and colleagues recently showed that, when using a more specific antibody compared to the antibody used by Ronchi et al. (9), CYP2W1 is only rarely expressed in ACC (29). Research is now focusing on the predictive value of CYP2W1 polymorphisms in ACC. For RRM1 and SOAT1, additionally immunohistochemistry was performed. We demonstrate RRM1 protein expression in both the nucleus as the cytoplasm of ACC. Localization of RRM1 protein expression is thought to be dependent on cell type, tissue of origin and cellular state (30). Further research could focus on the relevance of RRM1 expression at both localizations in ACC. No correlations were observed of RRM1 protein expression and *in vitro* mitotane sensitivity. SOAT1 IHC was performed with the polyclonal antibody that was used by Sbiera et al. (8), and furthermore by using

a mouse monoclonal antibody (Sc69836; Santa Cruz Biotechnology). Protein expressions as assessed by both antibodies were correlated in ACC, but only the expression as assessed using the mAb was correlated with *SOAT1* mRNA expression in ACC. Thereby, in contrast to the SOAT1 PoAb, the SOAT1 mAb showed convincing specificity for the adrenal cortex by comparing its expression to expression of chromogranin A. Chromogranin A is a peptide produced by chromaffin cells localized in the adrenal medulla (31). Together, these data suggest that the mouse monoclonal antibody used in this study might be a more reliable antibody for determining SOAT1 protein expression in ACC. The trend towards higher SOAT1 protein expression (with mAb) in patients with a stronger response to mitotane in primary cultures on cortisol production is in concordance with previously published *in vivo* data showing a prolonged progression-free survival in patients with high SOAT1 protein expression, although they used the PoAb (8). SOAT1 has an important role in cholesterol ester formation in the adrenal gland, which protects adrenal cells from potentially damaging effects of free cholesterol (32, 33). Sbiera and colleagues showed that mitotane inhibits SOAT1 expression, which leads to accumulation of toxic lipids that trigger endoplasmic reticulum stress (8). The increased mitotane response in patients with high SOAT1 expression might be explained by the fact that SOAT1 expression is a prerequisite for mitotane efficacy (8). Given the potential increased SOAT1 expression in cortisol-secreting ACC, this might be an additional explanation for increased direct antitumor effects of mitotane in cortisol-secreting ACC, although we did not show this in our *in vitro* study. Unfortunately, due to the lack of available mitotane serum levels in a subgroup of patients, and the diversity amongst the group of ACC patients regarding the indication for mitotane treatment (adjuvant or palliative), no correlations could be made with *in vivo* mitotane response. Further research is needed, therefore, to elucidate the predictive value of SOAT1 expression for mitotane response in patients with ACC.

In conclusion, direct antitumor effects of mitotane on primary ACC cultures are highly variable between patients and inhibitory effects on cortisol production seem to occur at considerably lower concentrations compared to the effects on cell amount. Cortisol secretion by ACC might be associated with enhanced mitotane sensitivity as a result of increased direct antitumor effects of mitotane. Further research should be performed to elucidate the relation between RRM1, SOAT1, and CYP2W1 expression, and mitotane sensitivity, taking into account the potential advantages of the monoclonal SOAT1 antibody.

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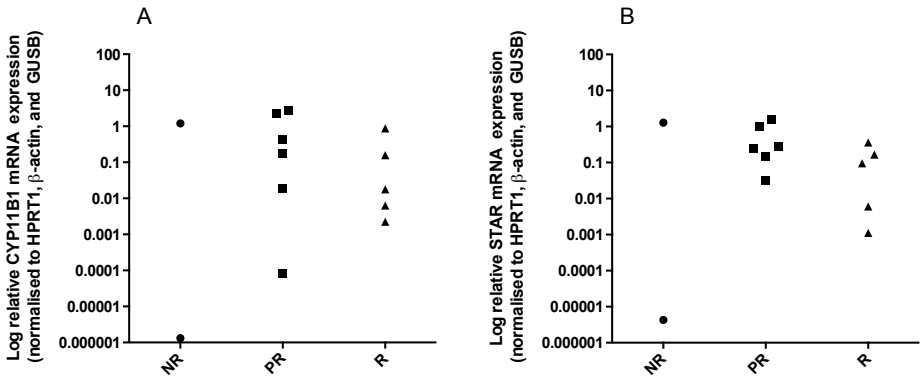
SUPPLEMENTARY MATERIAL

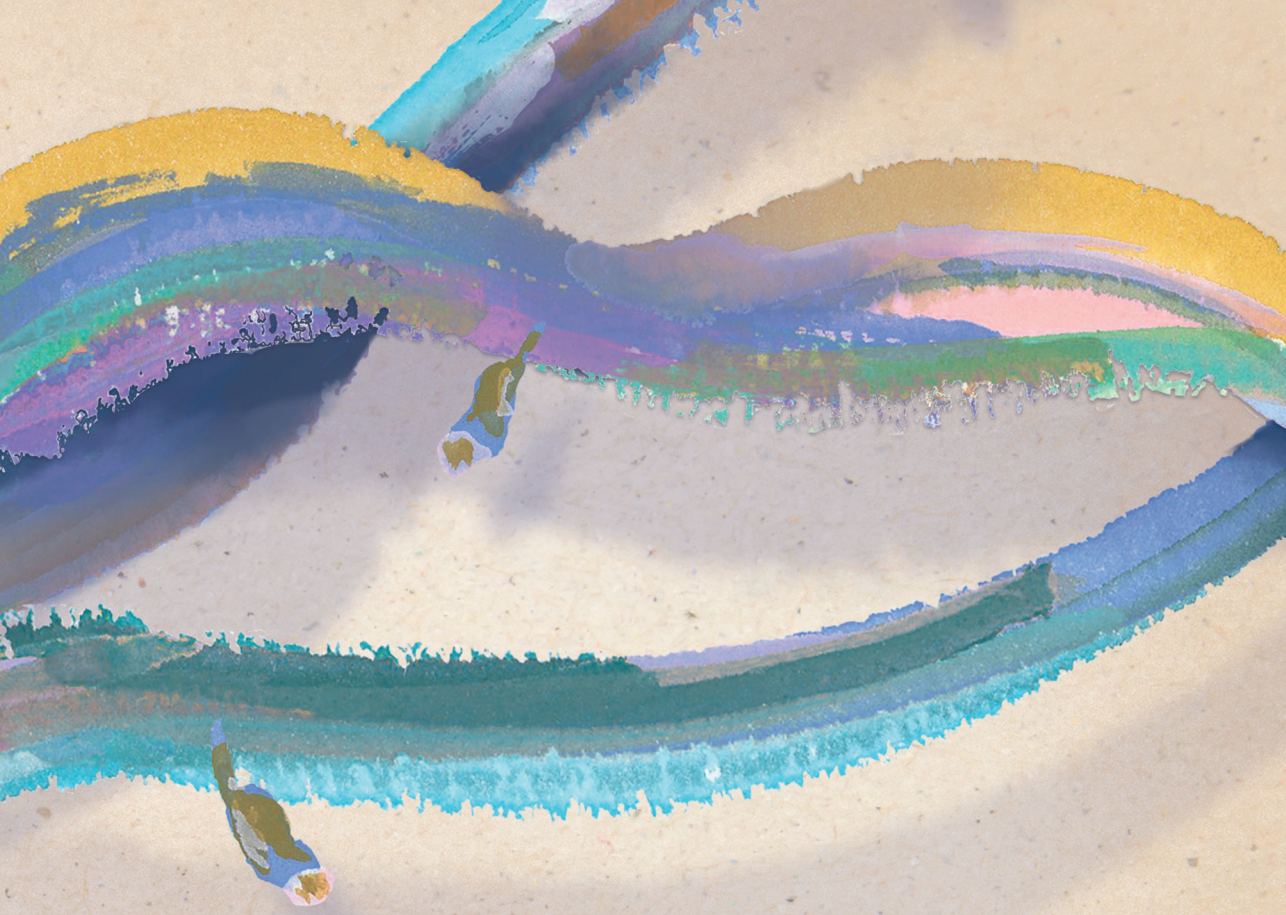
Supplementary Table 1. Primers and probes used for real time quantitative PCR

Gene	PCR primers (5'-3')	Final concentration	EF
HPRT1	Forward: 5'-CACTGGCAAAACAATGCAGACT-3' Reverse: 5'-GTCTGGCTTATATCCAACACTTCGT-3' Probe: 5'-[6FAM]CAAGCTTGCGACCTTGACCATCTTTGGA[TAM]-3'	0.5 pmol/µL 0.5 pmol/µL 0.1 pmol/µL	1.91
B-actin	Assay ID: Hs01060665_g1	Dilution 1:20	1.90
GUSB	Assay ID: Hs00939627_m1	Dilution 1:20	1.90
RRM1	Forward: 5'-ACTAAGCACCCTGACTATGCTATC-3' Reverse: 5'-CTTCCATCACATCACTGAACACTTT-3' Probe: 5'-[6FAM]CAGCCAGGATCGCTGTCTCTAACTTGCA[TAM]-3'	0.3 pmol/µL 0.3 pmol/µL 0.3 pmol/µL	1.88
SOAT1	Assay ID: Hs00922322_ml	Dilution 1:20	1.87
CYP2W1	Assay ID: Hs00908617_m1	Dilution 1:20	2.01
CYP11B1	Assay ID: Hs01596404_m1	Dilution 1:20	1.95
STAR	Assay ID: Hs00264912_m1	Dilution 1:20	1.97

Final concentration represents the concentration in the total reaction volume (12.5µl) used for each sample. *B-actin*, *GUSB*, *RRM1*, *SOAT1*, and *CYP2W1* are commercially available primers (Applied Biosystems, Alphen a/d Rijn, the Netherlands), therefore the accession numbers are depicted instead of the sequences. These primers should be diluted 20x from stock in the reaction volume. *B-actin*, Beta-actin; *GUSB*, glucuronidase beta; *HPRT1*, hypoxanthine-guanine phosphoribosyl transferase 1; *RRM1*, ribonucleotide reductase large subunit; *SOAT1*, Sterol-O-Acyl-Transferase 1; Forward, forward primer; reverse, reverse primer; EF, efficiency factor.



Supplementary Figure 1. mRNA expression of *CYP11B1*, and *StAR* in cells that were plated to obtain primary cultures for non-responders (NR), partial responders (PR) and responders (R). *B-actin*, Beta-actin; *GUSB*, glucuronidase beta; *HPRT1*, hypoxanthine-guanine phosphoribosyl transferase 1.



CHAPTER 8

MDR1 inhibition increases sensitivity to doxorubicin and etoposide in adrenocortical cancer

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ABSTRACT

Chemotherapy for adrenocortical carcinoma (ACC) has limited efficacy and is accompanied by severe toxicity. This lack of effectiveness has been associated with high tumoral levels of the multidrug resistance (MDR) pump P-glycoprotein (P-gp), encoded by the MDR1 gene. In this study, effects of P-gp inhibition on sensitivity of ACC cells to cytotoxic drugs were evaluated.

MDR1 mRNA and P-gp expression were determined in human adrenal tissues and cell lines. H295R, HAC15, and SW13 cells were treated with mitotane, doxorubicin, etoposide, cisplatin, and streptozotocin, with or without the P-glycoprotein inhibitors verapamil and tariquidar. Cell growth and surviving fraction of colonies were assessed.

MDR1 mRNA and P-gp protein expression were lower in ACCs compared to adrenocortical adenomas ($P < 0.0001$; $P < 0.01$, respectively). MDR1 and P-gp expression were positively correlated in ACC ($P < 0.0001$, $\rho = 0.723$). Mitotane, doxorubicin, cisplatin, and etoposide dose-dependently inhibited cell growth in H295R, HAC15, and SW13. Tariquidar, and in H295R also verapamil, increased the response of HAC15 and H295R to doxorubicin (6.3 and 7.5 fold EC_{50} decrease in H295R, respectively; all $P < 0.0001$). Sensitivity to etoposide was increased in H295R and HAC15 by verapamil and tariquidar (all $P < 0.0001$). Findings were confirmed when assessing colony formation.

We show that cytotoxic drugs, except streptozotocin, used for ACC treatment, inhibit ACC cell growth and colony formation at clinically achievable concentrations. P-gp inhibition increases sensitivity to doxorubicin and etoposide, suggesting that MDR1 is involved in sensitivity to these drugs and could be a potential target for cytotoxic treatment improvement in ACC.

INTRODUCTION

Adrenocortical carcinoma (ACC) is a highly malignant disease with limited treatment options (1). The only curative treatment is tumor-directed surgery (R0 resection) in case of local disease. The high recurrence rates (30-50%) after surgery, especially in high-risk patients, provide the rationale for adjuvant treatment with mitotane as standard of care (2-8). In case of progression of advanced disease, mitotane can be combined with cytotoxic drugs. In the first randomized controlled trial in ACC, the FIRM-act trial, mitotane in combination with etoposide, doxorubicin, and cisplatin (M-EDP) resulted in a longer median progression-free survival compared to patients receiving mitotane and streptozotocin (9). However, the median overall survival for this regimen was still only 14.8 months, but is now considered the treatment of choice (9). These data suggest that the resistance to chemotherapy is still a major impediment of successful systemic treatment of ACC patients. The limited efficacy of chemotherapy in ACC has been associated with the presence of high levels of P-glycoprotein (P-gp) in normal and tumoral adrenal tissue, an enzyme which actively pumps a variety of substrates like chemotherapy, out of the cell (10, 11). P-gp is also expressed in several other types of cancer, like colon-, kidney-, liver-, and pancreas tumors (12). Data on the role of mitotane as P-gp inhibitor are equivocal. Mitotane has *in vitro* shown to inhibit P-gp activity, encoded by the multidrug resistance protein 1 (MDR1) gene, suggesting that this could reverse the chemoresistant character of ACC (13, 14). In contrast, Theile and colleagues demonstrated induction of P-gp, but also MRP2 (MDR1 related protein 2) and breast cancer resistance protein (BCRP), under mitotane treatment *in vitro* (15). A phase II trial in 35 ACC patients investigating mitotane as a P-gp antagonist in combination with doxorubicin, etoposide, and vincristine, showed no *in vivo* P-gp inhibition by mitotane (16). The response rate to the chemotherapeutic drugs in combination with mitotane was 22%, a percentage difficult to interpret due to the lack of comparison in this study with mitotane or chemotherapy monotherapy (16).

Several P-gp inhibitors have been described in literature. Verapamil is a first generation P-gp inhibitor and substrate (competitive inhibitor) first described in 1981 (11, 17). Although verapamil was able to enhance drug accumulation *in vitro*, its clinical application is somewhat limited due to the challenging balance between the required doses to inhibit P-gp and potential verapamil associated toxicity (18, 19). Tariquidar (XR9576), a non-transporter P-gp inhibitor, belongs to the third generation P-gp inhibitors, described as most selective and potent inhibitors obtained by design (20). Tariquidar inhibits the basal ATPase activity associated with P-gp (21), and has shown to inhibit P-gp activity in ACC patients *in vivo* (22). Tariquidar also inhibits BCRP, another frequently studied ATP-binding cassette (ABC) protein associated with

decreased accumulation of anticancer drugs (23). The specificity depends upon the concentration and the relative density and capacity of P-gp versus BCRP. To the best of our knowledge, the effect of tariquidar on efficacy of cytotoxic drugs has not yet been tested *in vitro* for treatment of ACC.

The aim of the present study is to further explore the potential value of inhibiting P-gp activity. We therefore assessed *MDR1* mRNA and P-glycoprotein expression in normal adrenals (NAs), adrenocortical adenomas (ACAs) and ACCs. Thereby, we evaluated the effects of the current clinically used chemotherapeutic drugs, in combination with verapamil, and tariquidar, on the growth of human adrenocortical cancer cells.

MATERIALS AND METHODS

Adrenocortical tissues

Adrenocortical tissues were obtained between June 1994 and August 2016 at the Department of Surgery, Erasmus MC, Rotterdam. Directly after resection, adrenal tissues were embedded in Tissue-Tek and stored at -80 °C. Diagnosis was confirmed using the Weiss score or Van Slooten index (24, 25), dependent on the year of diagnosis. Normal adrenals (NAs) were collected during nephrectomy and confirmed by the pathologist as being normal. Patient and tumor characteristics were obtained from electronic patient records. The study was approved by the Medical Ethics Committee of the Erasmus University Medical Center and informed consent was obtained from all patients.

Real-time quantitative PCR

MDR1 mRNA expression was measured in cell lines and adrenocortical tissues. RNA isolation, cDNA synthesis and RT-PCR were performed as previously described, but using other primers (Supplementary Table 1; Sigma-Aldrich, Zwijndrecht, the Netherlands) (26). Three housekeeping genes were used to normalize mRNA levels using the Vandesompele method: hypoxanthine-guanine phosphoribosyl transferase 1 (*HPRT1*; Sigma-Aldrich, Zwijndrecht, the Netherlands), Beta-actin (*B-actin*; Thermo Fisher Scientific), and glucuronidase beta (*GUSB*; Thermo Fisher Scientific, Breda, the Netherlands) (27).

P-glycoprotein immunohistochemistry

Two tissue micro arrays (TMA) comprising specimens from NAs, ACAs and ACCs were constructed, as previously described using the automated TMA constructor (ATA-27 Beecher Instruments, Sun Prairie, WI) available at Erasmus MC, Department of Pathology (28). For an optimal representation, an expert pathologist identified three areas per specimen in Haematoxylin and eosin stained slides. Cores with a diameter of

1 mm were extracted from 'donor' blocks and brought into the 'recipient' paraffin block at predefined coordinates. In addition, formalin-fixed paraffin-embedded whole sections were used from 3 cases not included in the TMA. Five µm sections were cut and used for immunohistochemistry (IHC). Sections were deparaffinized, rehydrated, and heated for 20 minutes in TRIS-EDTA buffer (pH 9.0). Endogenous peroxidase was blocked for 15 minutes in hydrogen peroxide/PBS 3% solution in the dark. Afterwards, sections were washed and incubated with the rabbit monoclonal anti-P-glycoprotein antibody (dilution 1:500; ab170904; Abcam, Cambridge, England), overnight at 4 °C. After several wash steps, sections were incubated for 30 minutes with the second antibody horseradish peroxidase Rabbit/Mouse, where after bound antibody was visualized with freshly prepared DAB+ Chromogen 100 µl (both Dako Detection System). Slides were counterstained with haematoxylin, and coverslipped. Sections were independently and blinded for the tissue type scored by two investigators (SGC, LJH) using a semi-quantitative well-established Immunoreactivity Score (IRS), calculated by the product of the percentage positive cells (4, >80%; 3, >51-80%; 2, >10%; 1, 0) and intensity of staining (3, strong; 2, moderate; 1, mild; 0, no staining) (29).

Cell lines and culture conditions

Three available human ACC cell lines were used: H295R, HAC15 and SW13, respectively obtained from the American Type Culture Collection (Manassas, VA, USA), ECACC (Salisbury, Wiltshire, UK) and from Dr. W. Rainey (as a kind gift). Short tandem repeat (str) profiling using a Powerplex Kit (Promega, Leiden, the Netherlands) of NCI-H295R and SW13 gave results consistent with the ATCC database. HAC15 showed a genetic profile identical to H295R, which is consistent with a previous report by Rainey et al. (30). Culture conditions were described in detail previously (31). Mitotane, etoposide, and verapamil stock solutions were diluted in absolute ethanol (EtOH), whereas doxorubicin, cisplatin, and streptozotocin were diluted in H_2O. Tariquidar was diluted in 100% DMSO. All compounds were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands), except tariquidar, which was obtained from Tocris Biosciences (Abingdon, United Kingdom). To assess the optimal concentration of verapamil and tariquidar for the combination experiments with cytotoxic drugs, a dose-response curve was obtained for verapamil and tariquidar on cell growth in all three cell lines. After trypsinization, cells were plated at a concentration of 100,000, 75,000, and 20,000 cells per well for H295R, HAC15, and SW13, in order to obtain 80% confluency at the end of the experiment. The doubling times of H295R, HAC15, and SW13 are 30 hours, 49 hours, and 22 hours, respectively (data not shown). The next day, incubations were started in quadruplicate and control cells were vehicle treated. Medium and compounds were refreshed after 3 days. All cell culture experiments were carried out at least twice in quadruplicate.

Cell proliferation assay

Dose-dependent effects of mitotane, doxorubicin, cisplatin, etoposide, and streptozotocin, with or without the P-gp inhibitors, were assessed on cell amount after 7 days of treatment. After 7 days of cell culture experiment, media were removed and plates were stored at -20°C until DNA measurement. Measurement of total DNA was performed with the bisbenzimide fluorescent dye (Hoechst 33258, Sigma-Aldrich, Zwijndrecht, the Netherlands) as previously described (32).

Colony forming assay

The colony forming assay (CFA) is considered the gold standard for measurement of effects of cytotoxic agents on cancer cells *in vitro*. We determined the effects of cytotoxic drugs, with and without verapamil or tariquidar, on colony formation in H295R, HAC15, and SW13. Effects on surviving fraction and colony size were assessed at cell line specific concentrations of cytotoxic drugs that appeared to be effective in cell growth inhibition at monolayer culture (EC_{50} values after 7 days). Concentrations were adjusted when too strong or no effects were observed on colony formation. Final concentrations were 5, 10, and 25 μM for mitotane and 0.01, 0.01, and 0.05 μM for doxorubicin in H295R, HAC15 and SW13, respectively. For etoposide, 0.1 μM was used in all cell lines, whereas a concentration of 1 μM was used for cisplatin. For verapamil and tariquidar, 10 μM and 1 μM were used, respectively. Plates were coated with 1ml Poly-L-lysine (10 μg/ml), where after 3,000, 1,500, or 500 cells were plated in a 6 wells plate for H295R, HAC15, and SW13, respectively. After 1 day, drug treatment was initiated. Media were removed after 3 days and refreshed without treatment. After 2 weeks, the cells were washed and fixed. Cells were colored with Haematoxylin, and amounts and sizes of colonies were measured using MultiImage TM Light Cabinet (Alpha Innotech). Surviving fraction was calculated as previously described (33).

Statistical analysis

For statistical analysis, Graphpad Prism 6.0 (Graphpad Software, San Diego, CA) and SPSS Statistics 21 (SPSS 21.0; SPSS Inc., Chicaco, Il) were used. Kruskal-Wallis, followed by Dunn's multiple comparisons test, or the Mann Whitney U test when applicable was used to assess differences in *MDR1* mRNA and P-gp expression levels between different adrenal tissues. Correlations were tested using Spearman's rank coefficient. Non-linear regression curve fitting program was used to calculate the EC_{50} values on cell growth in the ACC cell lines. For analysis of the combined effect of P-gp inhibitors and cytotoxic drugs on cell growth and colony formation, verapamil or tariquidar monotherapy was set as control. One-way ANOVA with Tukey's multiple comparisons test was used to assess differences in colony formation between monotherapy and combination of cytotoxic drugs with P-gp inhibitors. Values of $P < 0.05$ were considered significant. Data are presented as mean ± SEM, unless specified otherwise.

RESULTS

Patient characteristics

Ten NAs, obtained during nephrectomy, 16 ACAs, and 42 ACCs were included. *MDR1* mRNA expression was assessed in 58 adrenal specimens (8 NAs, 13 ACAs, 37 ACCs), while P-gp protein expression, as determined by immunohistochemistry, was analyzed in 59 tissues (7 NAs, 14 ACAs, 38 ACCs). Four of the 42 ACC were recurrent cases, and 2 patients received mitotane presurgery. There were no patients, both primary and recurrent cases, who received chemotherapy before surgery. Patient and tumor characteristics are listed in Table 1.

Table 1. Patient and tumor characteristics of patients included in this study

	Adrenocortical carcinomas *n* = 42	Adrenocortical adenomas *n* = 16
Mean age at diagnosis (yrs, median, IQR)	52 (42 - 65)	45 (38 – 56)
Mean follow-up (months, median, IQR)	18.5 (8.0 – 89)	31 (13 - 55)
Male (%)	20 (48%)	3 (19%)
Mean tumor size (cm, median, IQR)	14 (8.0 – 19)	2.70 (1.6 – 4.8)
Secretion		
Androgens	9 (21%)	1 (6%)
Glucocorticoids	20 (48%)	6 (38%)
Mineralocorticoids	2 (5%)	5 (31%)
Precursors	5 (12%)	0 (0%)
Estradiol	4 (10%)	0 (0%)
Non secreting	15 (36%)	4 (25%)
Weiss score (median, IQR)	6.0 (5.0 – 7.0), *n* = 35	0.00 (0.0 – 0.8)
VanSlooten Index (median, IQR)	21 (18 – 25), *n* = 36	1.6 (1.6 – 1.6)
ENSAT		
I	2 (5%)	14 (88%)
II	18 (47%)	2 (12%)
III	3 (8%)	0 (0%)
IV	15 (40%)	0 (0%)

Van Slooten Index and Weiss Score were not available for all patients, dependent on the year of diagnosis.
ENSAT, European Network for the Study of Adrenal Tumors; IQR, interquartile range; Yrs, years.

MDR1 mRNA and protein expression in human adrenocortical carcinoma cell lines and tissues

MDR1 mRNA expression was higher in H295R cells compared to HAC15 ($P < 0.05$; Fig. 1A). *MDR1* mRNA expression could not be detected in SW13 cells. *MDR1* mRNA expression was lower in ACCs compared to ACAs and normal adrenals, with a 7.7 fold lower median expression level in ACC compared to ACA ($P < 0.0001$; $P < 0.01$, respectively; Fig. 1B).

P-glycoprotein expression, as determined by immunohistochemistry, was high in almost all adrenal tissues, with a mean IRS of 9.8 (range 0 – 12; Fig. 1C). Staining was primarily located in the cell membrane (Fig. 1E). P-gp expression was significantly lower in ACCs compared to ACAs ($P < 0.01$; Fig. 1C), which seems to be primarily driven by a subgroup of ACC patients with a P-gp expression below the median. *MDR1* mRNA expression was positively correlated with P-gp expression in the whole group of patients ($P < 0.0001$, $\rho = 0.723$), but also in the group of ACCs separately ($P < 0.0001$, $\rho = 0.6978$; Fig. 1D). No correlations were found between *MDR1* mRNA or P-gp expression and the Van Slooten index or the Weiss score. Expression levels were also comparable between different ENSAT stages. *MDR1* mRNA expression was negatively correlated with the maximum tumor diameter in ACCs ($P = 0.002$, $\rho = -0.461$). Furthermore, P-gp expression was significantly higher in cortisol-producing ACC compared to other ACC ($P = 0.048$, $n = 38$).

Effects of cytotoxic drugs on human adrenocortical carcinoma cell lines

H295R, HAC15, and SW13 demonstrated a dose-dependent inhibitory response to mitotane (EC_{50} range: 2.63 – 12.3 μM), doxorubicin (EC_{50} range: 0.0110 – 0.0608 μM), etoposide (EC_{50} range: 0.175 - 0.708 μM), and cisplatin (EC_{50} range: 0.579 – 1.14 μM), but not to streptozotocin (Fig. 2; Table 2).

Effects of the P-gp inhibitors verapamil and tariquidar on the response of human adrenocortical carcinoma cell lines to cytotoxic drugs

First, we examined the possible effects of verapamil and tariquidar alone on cell growth in H295R, HAC15, and SW13. To avoid cytotoxicity, a concentration of 10 μM verapamil and 1 μM tariquidar was chosen for combination experiments with cytotoxic drugs. Verapamil and tariquidar in these concentrations only minimally affected cell growth in HAC15 with respectively 11% and 9.7% ($P < 0.001$), whereas no effects were observed in H295R and SW13 on cell growth. Subsequently, we analyzed whether both P-gp inhibitors could sensitize ACC cells reflected by a decrease in the EC_{50} value of the cytotoxic drugs currently used for treatment of ACC (Fig. 3, Table 2).

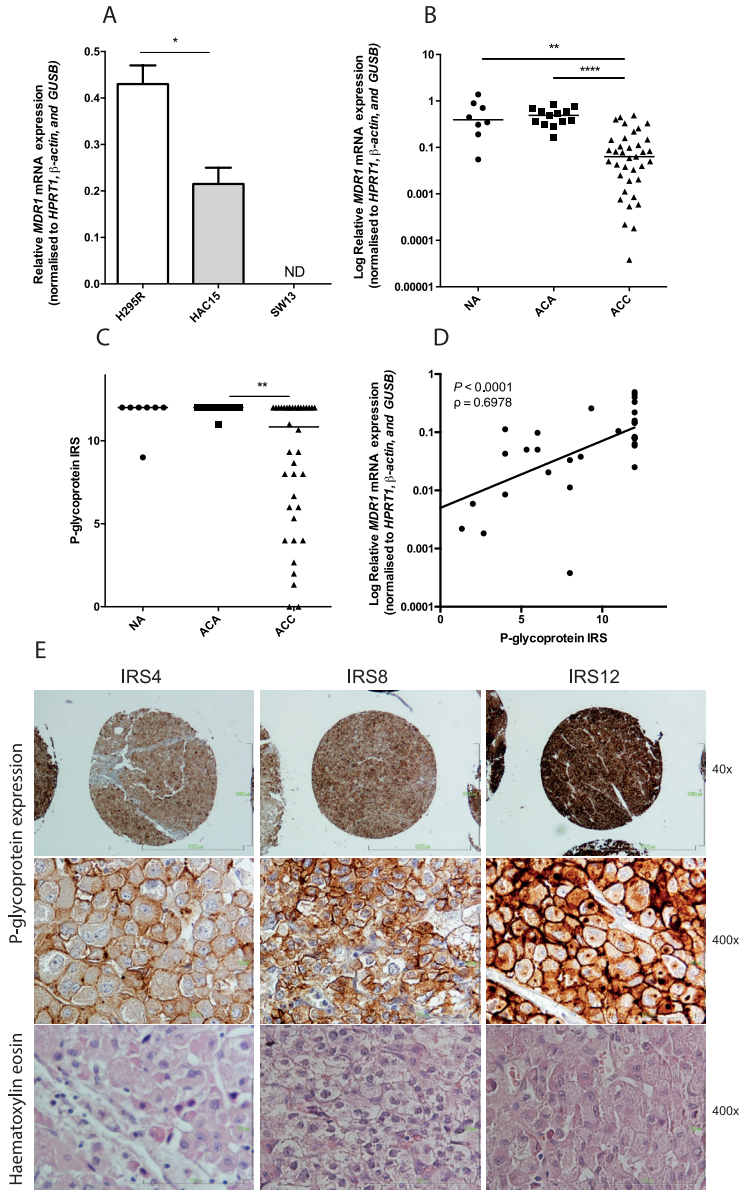

Figure 1. *MDR1* mRNA expression in adrenal cell lines (A) and adrenal tissues (B), and P-glycoprotein expression (C) in human adrenocortical tissues. (D) Correlation between *MDR1* mRNA expression and P-glycoprotein expression in ACCs. (E) Representative example of P-gp immunohistochemical staining in adrenocortical carcinoma as determined by immunohistochemistry. Sections were blinded and independently evaluated by two investigators. Microscopic magnification 40x, 200x, and 400x. Lines represent medians. ρ represents Spearman's rank correlation coefficient. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. NA, normal adrenals; ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; IRS, Immunoreactivity Score; ND, not detectable.

Table 2. EC_{50} values of mitotane, doxorubicin, cisplatin, and etoposide on cell growth in human adrenocortical cell lines with or without the P-gp inhibitors verapamil or tariquidar

		MIT
H295R	Monotherapy	2.96 (2.47 – 3.56)
	+ Verapamil	2.70 (2.11 – 3.44)
	+ Tariquidar	**2.45 (1.64 – 3.66) ***
HAC15	Monotherapy	2.63 (3.03 – 4.13)
	+ Verapamil	3.45 (2.56 – 4.65) *
	+ Tariquidar	2.65 (1.77 – 3.96)
SW13	Monotherapy	12.3 (9.09 – 16.7)
	+ Verapamil	9.57 (5.85 – 15.6)
	+ Tariquidar	**9.25 (6.53 – 13.1) ***

EC_{50} values are presented in micromolar (μM, 95% CI). Controls represent vehicle control for monotherapy of cytotoxic drugs, and treatment with verapamil (10 μM) or tariquidar (1 μM) for combination therapy.

When tariquidar was added to different doses of mitotane, sensitivity to mitotane only modestly increased in H295R and SW13, with 1.2- and 1.3-fold lower EC_{50} values, respectively (both $P < 0.05$ vs mitotane monotherapy). Verapamil, however, decreased the response to mitotane in HAC15, but not in H295R and SW13 ($P < 0.05$ vs mitotane monotherapy). Verapamil and tariquidar both increased the response of H295R cells to doxorubicin, as shown by a 7.5- and 6.3-fold decrease in EC_{50} value compared to doxorubicin monotherapy, respectively (both $P < 0.0001$ vs doxorubicin monotherapy). In HAC15, sensitivity to doxorubicin only increased when tariquidar was used, as shown by a 4-fold decreased EC_{50} ($P < 0.0001$ vs doxorubicin monotherapy). Sensitivity to doxorobucin did not change in SW13 cells with addition of P-gp inhibitors. Sensitivity to etoposide increased when the compound was combined with verapamil or tariquidar, as indicated by a 3.7- and 9.2-fold significant decrease in EC_{50} value in H295R and a 3.6- and 6.1-times decrease in HAC15, respectively. In both H295R and HAC15 cells, tariquidar more strongly sensitized cells to etoposide compared to verapamil (both $P < 0.01$ vs etoposide monotherapy). In SW13, effects of etoposide were only slightly potentiated by verapamil (2.0 fold decrease in EC_{50}; $P < 0.0001$). In all three ACC cell lines, tariquidar counteracted the effect of cisplatin on cell growth (range increase EC_{50} 2.6 – 3.4 fold; all $P < 0.0001$ vs cisplatin monotherapy). In HAC15 and SW13, verapamil increased sensitivity to cisplatin ($P < 0.05$, $P < 0.0001$, respectively).

DOX	ETO	CIS
0.0575 (0.0415 – 0.0795)	0.674 (0.565 – 0.803)	0.579 (0.460 – 0.728)
0.00765 (0.00596 – 0.00982) ****	**0.181 (0.124 – 0.265) ******	0.676 (0.556 – 0.822)
0.00914 (0.00582 – 0.0144) ****	**0.0732 (0.0596 – 0.0900) ******	1.48 (1.12 – 1.95) ****
0.0608 (0.0464 – 0.0796)	0.708 (0.576 – 0.870)	1.10 (0.907 – 1.322)
0.0435 (0.0293 – 0.0647)	**0.194 (0.146 – 0.259) ******	**0.855 (0.668 – 1.10) ***
0.0152 (0.0103 – 0.0225) ****	**0.116 (0.0836 – 0.161) ******	3.73 (3.17 – 4.39) ****
0.0110 (0.00694 – 0.0175)	0.175 (0.112 – 0.275)	1.14 (0.897 – 1.44)
0.0130 (0.00873 – 0.0194)	**0.116 (0.0768 – 0.174) ****	**0.731 (0.564 – 0.949) ******
0.0109 (0.00720 – 0.0165)	0.169 (0.0926 – 0.308)	3.51 (1.96 – 6.31) ****

P-values compare EC_{50} value of combination therapy with P-gp inhibitors verapamil or tariquidar to monotherapy of either mitotane (MIT), doxorubicin (DOX), etoposide (ETO), or cisplatin (CIS). EC_{50} values depicted in bold are significantly lower compared to monotherapy. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Effects of cytotoxic drugs, with and without P-gp inhibitors, on colony formation in human adrenocortical carcinoma cell lines

The effects of the compounds, with and without P-gp inhibitors, on colony formation appeared to primarily be cytotoxic, as stated by an effect on the fraction of colonies rather than an effect on colony size (Fig. 4). Effects on the size of colonies are presented in Supplementary Fig. 1. In H295R, mitotane in combination with tariquidar resulted in a less strong effect on surviving fraction of colonies compared to mitotane alone ($P < 0.01$ vs mitotane monotherapy). In HAC15, verapamil counteracted whereas tariquidar potentiated the effect of mitotane (Fig. 4B; $P < 0.0001$, $P < 0.05$, respectively). Both verapamil and tariquidar strongly potentiated the effects of doxorubicin in H295R and HAC15 (Fig. 4, D and E; all $P < 0.0001$ vs doxorubicin monotherapy). P-gp inhibition did not alter cytotoxic effects of mitotane and doxorubicin in SW13 cells. In H295R, the effect of etoposide on surviving fraction of colonies was enhanced when combined with verapamil or tariquidar (Fig. 4G, both $P < 0.05$ vs etoposide monotherapy), whereas this effect was in HAC15 only observed in combination with tariquidar (Fig. 4H, $P < 0.01$ vs etoposide monotherapy). In SW13, verapamil potentiated the effect of etoposide (Fig. 4I, $P < 0.01$). In H295R, the effect of cisplatin was slightly counteracted by tariquidar ($P < 0.001$ vs cisplatin monotherapy), whereas no effects were seen on sensitivity to cisplatin in HAC15 and SW13 (Fig. 4, J-L).

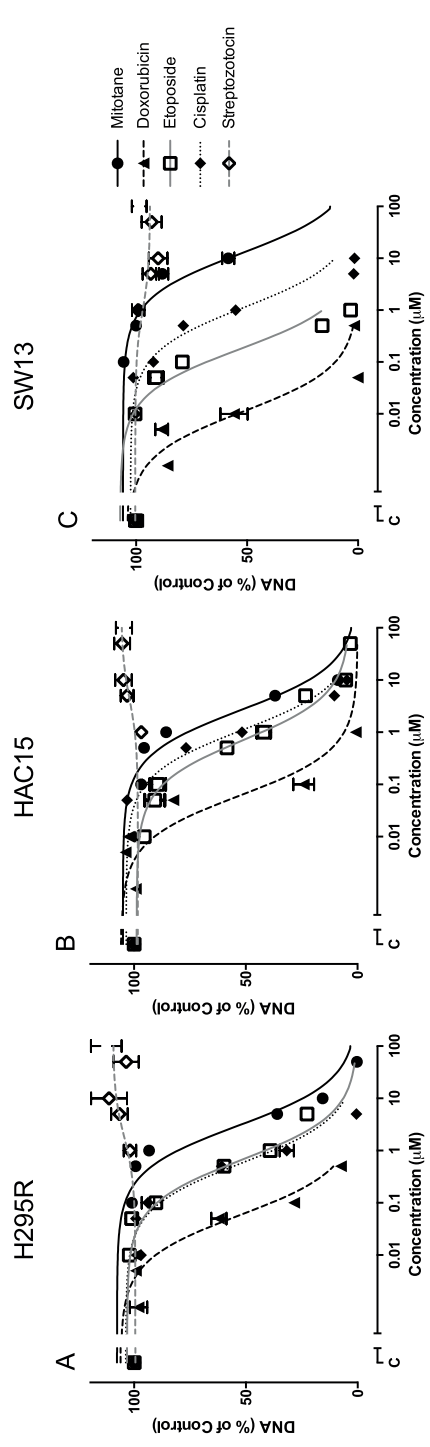

Figure 2. Dose-response curves of mitotane, doxorubicin, etoposide, cisplatin, and streptozotocin on total DNA measurement, as a measure of cell amount, in H295R (left panel), HAC15 (middle panel), and SW13 (right panel) after 7 days of treatment. Values represent mean ± SEM and are shown as percentage of control.

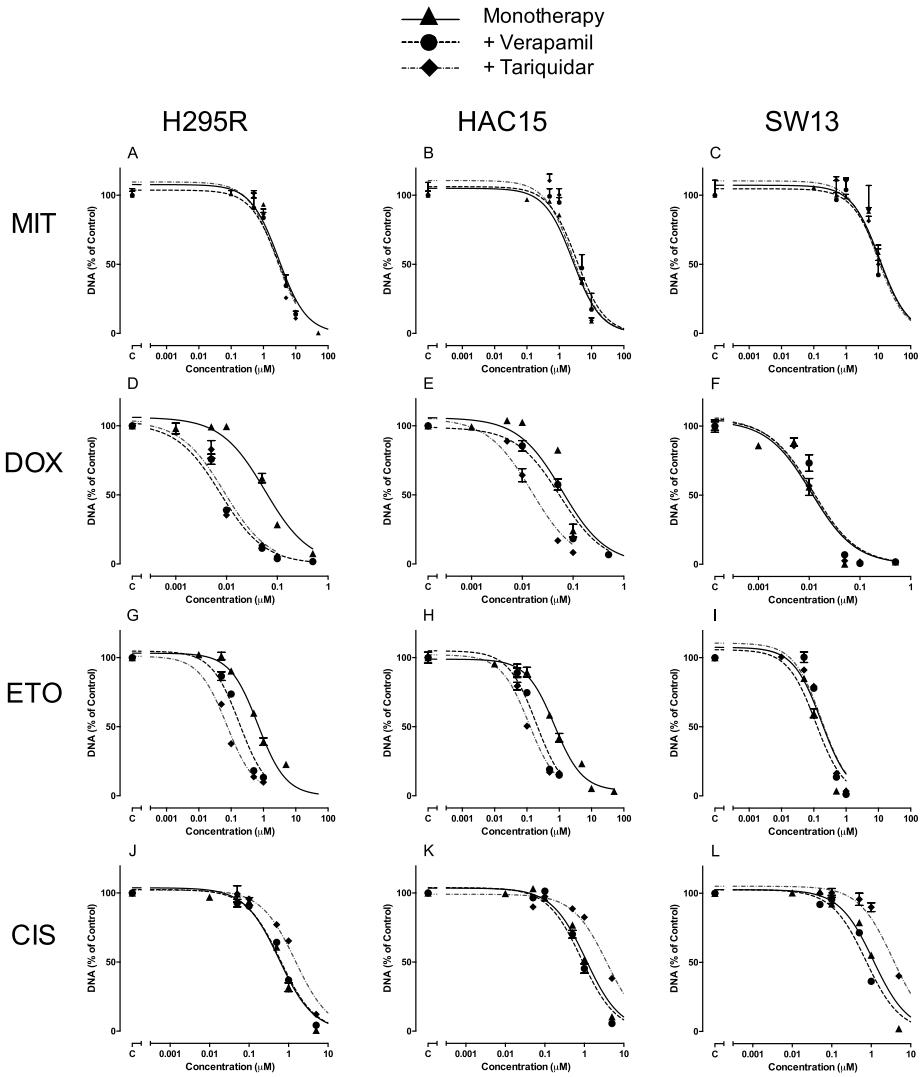

Figure 3. Effects of mitotane (MIT), doxorubicin (DOX), etoposide (ETO), and cisplatin (CIS), with or without the P-gp inhibitors verapamil (10 μM) or tariquidar (1 μM) on cell growth in 3 human ACC cell lines H295R (left panel), HAC15 (middle panel), and SW13 (right panel) after 7 days of treatment. Grey solid lines represent the effect of monotherapy. Dotted lines represent the combination of verapamil (black dotted lines) or tariquidar (grey dotted lines) with cytotoxic therapy. Data are presented as percentage of vehicle treated control. When combination of cytotoxic drugs with verapamil (+V) or tariquidar (+T) were examined, the control was set as verapamil (10 μM) or tariquidar (1 μM) monotherapy, respectively. Values represent mean ± SEM and are shown as percentage of control.

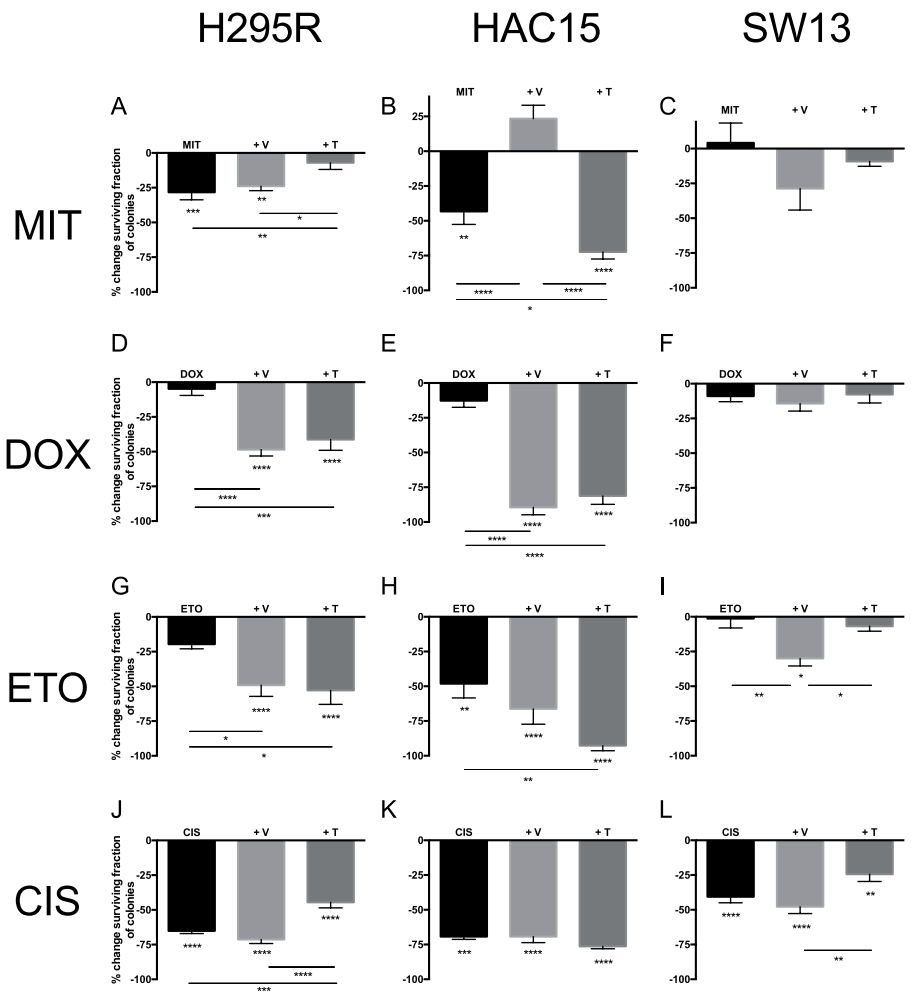

Figure 4. Effects of cytotoxic drugs with or without the P-gp inhibitors verapamil (V) or tariquidar (T) on the surviving fraction of colonies in H295R (left panel), HAC15 (middle panel), and SW13 (right panel). Data are presented as percentage change compared to vehicle treated control. When combination of cytotoxic drugs with verapamil (+V) or tariquidar (+T) were examined, the control was set as verapamil (10 μM) or tariquidar (1 μM) monotherapy, respectively. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs vehicle treated control or as stated by the lines. MIT, mitotane; DOX, doxorubicin; ETO, etoposide; CIS, cisplatin. Values represent mean ± SEM.

DISCUSSION

In this study, we show that P-glycoprotein is highly expressed in normal and neoplastic adrenal tissues, with lower levels in ACC compared to ACA and NA. Previously, it has been shown that verapamil might increase sensitivity to doxorubicin in ACC (14). This is the first study investigating the effects of verapamil and the clinically more promising P-gp inhibitor tariquidar on a panel of the most important clinically used chemotherapeutic drugs for ACC. We demonstrate that inhibition of P-glycoprotein in human ACC cells *in vitro* can increase sensitivity to specific cytotoxic drugs, used to treat ACC *in vivo*.

Chemotherapeutic drugs have demonstrated disappointing results in ACC patients so far. Targeting P-glycoprotein expression would potentially increase tumor cell exposure to cytotoxic agents and thereby improve efficacy of chemotherapy. In this study, we confirm the previously reported high P-glycoprotein expression in the majority of ACC cases (10, 34, 35). *MDR1* mRNA expression and P-glycoprotein expression are positively correlated in ACCs and lower in ACCs compared to ACAs. The lower expression in ACCs compared to ACAs might potentially be the result of involvement of the *MDR1* gene in the pathogenesis of ACC, as P-gp silencing is also an early event in colorectal carcinogenesis (36). Dedifferentiation of ACCs could be another explanation of lower *MDR1* mRNA and P-gp expression, although no correlation was found with histopathology. ACCs appeared to have a more variable P-gp expression compared to ACAs and NAs, and the hypothesis is that ACC patients with high P-gp expression might be more resistant to chemotherapeutic drugs and these patients may particularly benefit from simultaneous treatment with P-gp inhibitors. Most importantly, the majority of patients seem to have P-gp expression comparable with normal adrenals, which expression is thought to be high relative to other normal tissues (37-39). Furthermore, these ACC cases have *MDR1* mRNA expression comparable with the expression in the cell lines that have been used as a model.

Previously, the antitumoral effects of the M-EDP regimen were already demonstrated in NCI-H295R cells *in vitro* and in xenografts (40). In this study, we report the *in vitro* effects, in pharmacological concentrations, of currently used cytotoxic drugs in ACC separately. Surprisingly, streptozotocin did not inhibit growth of ACC cells *in vitro*. The first rationale for streptozotocin in the treatment of ACC was based on increased concentration of streptozotocin in the mice adrenal cortex after iv injection (41). In the FIRM-act trial however, streptozotocin in combination with mitotane appeared to be less effective compared to the M-EDP regimen (9), which turns out to be concordant with our *in vitro* results. We demonstrate that the other drugs used to treat ACC (mitotane, cisplatin,

etoposide, and doxorubicin) effectively inhibited cell growth in ACC cell lines, all with EC_{50} values within the range of achievable plasma concentrations after administration in humans (42-45). An important consideration is that plasma concentrations do not necessarily represent intratumoral concentrations.

The findings on cell growth inhibition in ACC cell lines were confirmed by performing a colony forming assay, which predominantly revealed comparable results. There is a clear difference between both assays; the CFA provides information on the long-term effects (two weeks without treatment) of a short-term (3 days) treatment with cytotoxic drugs on colony growth, whereas the cell growth assay studies the direct effect of cytotoxic drugs after 7 days of treatment. This difference could explain inequalities in effect of compounds. In H295R and HAC15, especially an increased response to doxorubicin and etoposide was found under P-gp inhibition both on cell growth and on the surviving fraction of colonies. Increased sensitivity can be explained by the fact that doxorubicin and etoposide are substrates of P-gp (11). In a study investigating the effects of mitotane on drug elimination pathways, it was suggested that mitotane retained its antineoplastic efficacy even in tumors overexpressing drug transporters, due to lack of substrate characteristics (15). Another explanation of lack of increased sensitivity to mitotane could be potential P-gp inhibition by itself, although the role of mitotane in P-gp inhibition is debatable (13-15). In line with this finding, only subtle differences in mitotane sensitivity were observed under P-gp inhibition on cell growth in ACC cells in our study. P-gp inhibition also only minimally affected cytotoxic effects of mitotane on colony formation in H295R and SW13. In HAC15 however, verapamil counteracted the effect of mitotane on colony formation, corresponding with the effect in monolayer culture on cell growth. The minimal or even absence of enhanced cisplatin effects after co-treatment with verapamil in H295R, HAC15, and SW13 could be explained by the fact that cisplatin is not a substrate of P-gp, but of several MDR1-related proteins (MRPs) (46). Decreased sensitivity to cisplatin was particularly observed with respect to cell growth when cisplatin was combined with tariquidar, and was confirmed in H295R in the colony forming assay. This attenuated sensitivity could potentially be explained by a compensatory increase in drug efflux by other transporters. Compensatory upregulation of other transporters has been demonstrated in mutant animals with deficiency of a specific drug efflux transporter (47). This in turn would indicate that it could be required to inhibit multiple drug transporters to reach optimal efficacy, which would also be reasonable in case there is expression of other ABC transporters in ACC as well. This could be subject to further investigation in ACC. In SW13, effects of P-gp inhibitors in general were much less pronounced, which is concordant with the undetectable *MDR1* mRNA expression in SW13 cells, in contrast to H295R and HAC15.

In preclinical studies, the question always arises whether the obtained findings can be translated to clinical practice. The present study uses ACC cell lines. Considering the limited availability of ACC cell line models, a subclone of H295R was also used (HAC15), aiming to obtain a better representation of the heterogeneity of ACC. Further research could focus on primary cultures. Finally, tariquidar in combination with mitotane, doxorubicin and etoposide could be tested in ACC patients with disease progression under the conventional M-EDP regimen. Tariquidar is well tolerated and a peak serum concentration of 2.3 μM can be reached (48, 49). In the present study, a lower concentration (1 μM) was used and appeared to significantly increase sensitivity to doxorubicin and etoposide. Tariquidar *in vivo* already demonstrated to induce inhibition of P-gp in patients with ACC as determined by increased accumulation of ^{99m}Tc-sestamibi in ACC lesions and blockade of rhodamine efflux in CD56+ mononuclear cells (22). Where verapamil is often used as a positive control for P-gp inhibition in preclinical studies, tariquidar and other third generation P-gp inhibitors are currently evaluated in several clinical trials in other types of cancer. The fact that this preclinical study combines currently clinically used chemotherapeutic drugs with a P-gp inhibitor that is already investigated in patients with ACC, could lead to a faster introduction in clinical trials aiming to improve sensitivity to chemotherapy in ACC. A recent review summarized the efficacy of clinical trials using third generation P-gp inhibitors until now, revealing however disappointing results in general (50). Several challenges have been encountered such as the limited fraction of tumors expressing the MDR1 gene. P-gp is for instance expressed in less than 50 % of breast cancers (51). No preselection based on P-gp expression was made in previous clinical trials. In ACCs however, we show that P-gp is expressed in 95% of cases, of which 78% of all cases with an IRS of at least 6. This observation provides a rationale for targeting this transporter in selected patients, which is associated with the limitation that P-gp inhibition might not be effective in a subgroup of ACC patients. An important consideration, however, is that P-gp expression can be upregulated following chemotherapy (52, 53), so baseline P-gp expression levels may not be predictive for clinical outcome of combined treatment with chemotherapy and P-gp inhibition. The large variability of P-gp expression observed in the present ACC panel could not be caused by upregulation, since no patients were pretreated with chemotherapy, and only two patients received mitotane presurgery. Thereby, we have to acknowledge that P-gp is only one of the multitude of mechanisms that can lead to drug resistance, and P-gp is part of a family of ABC transporters (11).

In conclusion, we show that cytotoxic drugs, except streptozotocin, used for treatment of ACC inhibit cell growth in ACC cell lines. P-glycoprotein is expressed in ACC, expression level is in the majority of cases comparable with the high expression in normal adrenals, and inhibition of P-gp increases sensitivity to particularly doxorubicin and etoposide. This study thereby marks the relevance of carefully selecting compounds as well as patients when designing clinical trials with P-gp inhibitors in patients with ACC. These findings suggest that the MDR1 gene plays a role in sensitivity of ACC cells to these cytotoxic drugs and could be a potential target for improvement of efficacy of cytotoxic drugs in ACCs.

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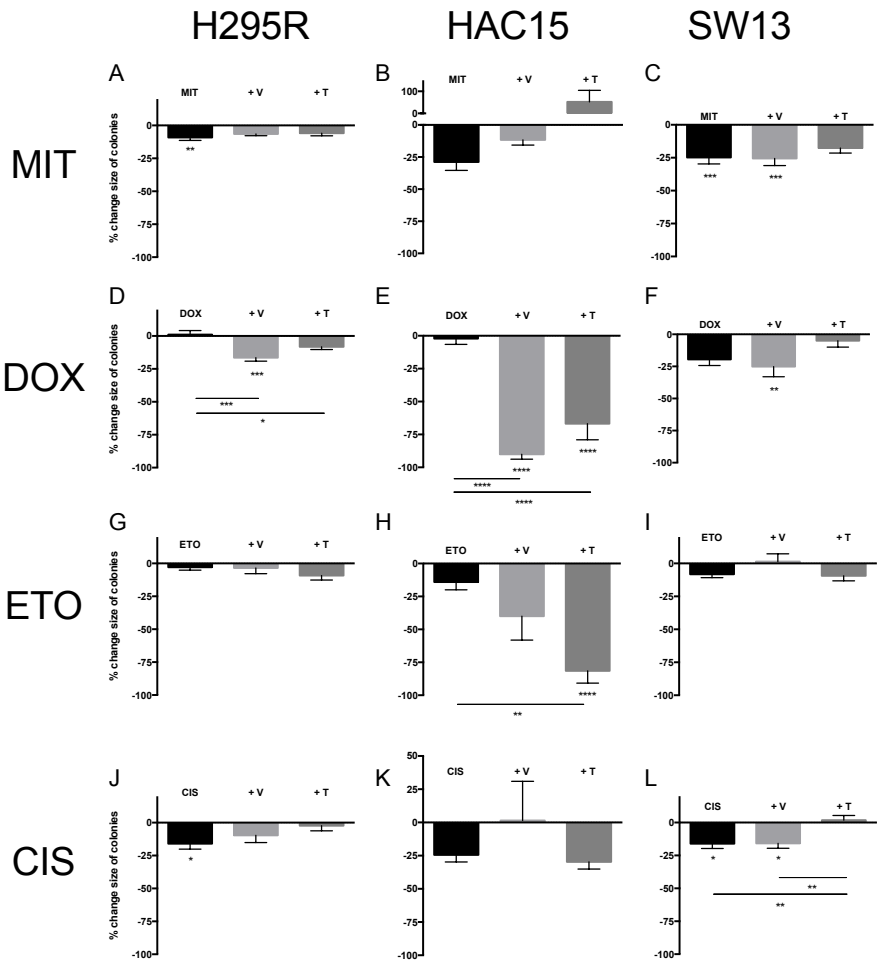
SUPPLEMENTARY MATERIAL

Supplementary Table 1. Primers and probes used for real time quantitative PCR

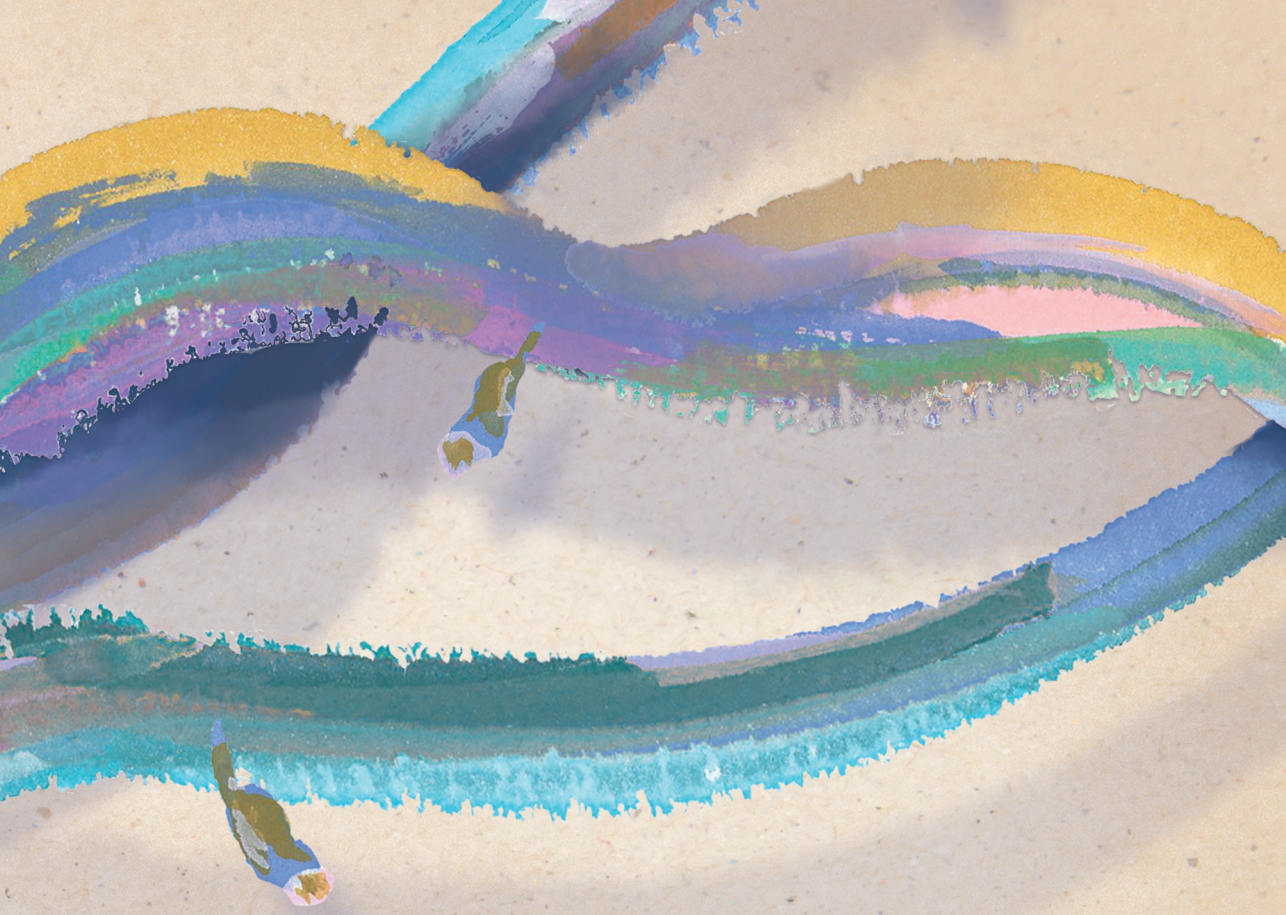
Gene	PCR primers (5'-3')
HPRT1	Forward: 5'-CACTGGCAAAACAATGCAGACT-3' Reverse: 5'-GTCTGGCTTATATCCAACACTTCGT-3' Probe: 5'-[6FAM]CAAGCTTGCGACCTTGACCATCTTTGGA[TAM]-3'
B-actin	Assay ID: Hs01060665_g1; NM001101.3
GUSB	Assay ID: Hs00939627_m1; NM00181.3
MDR1	Forward: 5'-GGAAGCCAATGCCTATGACTTTA-3' Reverse: 5'-TCAACTGGGCCCCTCTCTCTCTCC- 3' Probe: 5'-[6FAM]ATGAAACTGCCTCATAAATTTGACACCCTGG[TAM]-3'

B-actin and GUSB are commercially available primers (Thermo Fisher Scientific), therefore the accession numbers are depicted instead of the sequences. These primers should be diluted 20x from stock in the reaction volume. *B-actin*, Beta-actin; EF, efficiency factor; *GUSB*, glucuronidase beta; *HPRT1*, hypoxanthine-guanine phosphoribosyl transferase 1; MDR1, multidrug resistance protein 1.

	Amount (nmol/l) added in the total reaction volume (12.5 µl) used for each sample	EF
	500	
	500	1.91
	100	
	Dilution 1:20	1.90
	Dilution 1:20	1.90
	300	
	300	1.94
	100	



Supplementary Figure 1. Effects of cytotoxic drugs with or without the P-gp inhibitors verapamil (V) or tariquidar (T) on the colony size in H295R (left panel), HAC15 (middle panel), and SW13 (right panel). Data are presented as percentage change compared to vehicle treated control. When combination of cytotoxic drugs with verapamil (+V) or tariquidar (+T) were examined, the control was set as verapamil (10 μM) or tariquidar (1 μM) monotherapy, respectively. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs vehicle treated control or as stated by the lines. MIT, mitotane; DOX, doxorubicin; ETO, etoposide; CIS, cisplatin. Values represent mean ± SEM.



CHAPTER 9

Inhibition of human adrenocortical cancer cell growth by temozolomide in vitro and the role of the MGMT gene

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ABSTRACT

Context: Treatment of patients with adrenocortical carcinomas (ACC) with mitotane and/or chemotherapy is often associated with toxicity and poor tumor response. New therapeutic options are urgently needed.

Objective: To evaluate the therapeutic possibilities of temozolomide (TMZ) in ACC cells and to assess the potential predictive role of the DNA repair gene O6-Methylguanine-DNA methyltransferase (*MGMT)* in adrenocortical tumors.

Methods: Three human ACC cell lines and eight primary ACC cultures were used to assess effects of TMZ *in vitro*. In the cell lines, 11 normal adrenals, 16 adrenocortical adenomas, and 29 ACC, *MGMT* promoter methylation and expression were determined.

Results: IC_{50} values of TMZ on cell growth were 390 μM, 380 μM and 440 μM for H295R, HAC15, and SW13, respectively. TMZ induced apoptosis, and provoked cytotoxic and cytostatic effects by reducing the surviving fraction of ACC colonies and the colony size. TMZ thereby induced cell cycle arrests in ACC cell lines. TMZ and mitotane both inhibited growth of ACC cells cultured as 3-dimensional spheroids. TMZ inhibited cell amount in five of eight primary ACC cultures and induced apoptosis in seven of eight primary ACC cultures. In ACC cell lines and adrenal tissues, *MGMT* promoter methylation was low. In ACCs, methylation was inversely correlated with *MGMT* mRNA expression. MGMT protein expression was not correlated with *MGMT* methylation.

Conclusions: For the first time, we show the therapeutic potential of temozolomide for ACC, offering an urgently needed potential alternative for patients not responding to mitotane alone or with etoposide, doxorubicine, and cisplatin. (Pre-)clinical studies are warranted to assess efficacy *in vivo*.

INTRODUCTION

Adrenocortical carcinoma (ACC) includes a diverse group of tumors, with a generally poor prognosis (1, 2). Frequently patients present with advanced or metastasized tumors, in which mitotane (o,p'DDD) is the standard therapy. However, mitotane is effective in only a subset of these patients (25-30% response) and often manifests with severe toxicity (3-6). In case of progression, mitotane can be combined with cytotoxic drugs like etoposide, doxorubicine and cisplatin (7). The median overall survival for this regimen was still only 14.8 months (7). Several targeted therapies have been proposed and clinically tested, but to date with discouraging results (6). Therefore, better therapeutic options are urgently needed.

Temozolomide (TMZ), a DNA-alkylating agent, is used as cytostatic drug incorporated in the standard care for patients with malignant gliomas (8). TMZ is an oral formulation of the first metabolite of dacarbazine, but less toxic. TMZ has shown efficacy in 17 of 25 patients with poorly differentiated endocrine carcinomas, and in various other tumors (9, 10). Cytotoxicity and antiproliferative activity is primarily thought to act by alkylation of specific sites on especially the O^6 position of guanine, which mispairs with thymine during the next DNA replication cycle (11). The methyl group in O^6-methylguanine can be removed by the O6-methylguanine-DNA methyltransferase (*MGMT*) gene, leading to impaired efficacy of TMZ, one of the possible explanations for drug resistance (12). Epigenetic marks regulating *MGMT* expression are now used as a predictive marker for response to TMZ in glioblastoma patients (13).

In this study we investigated the therapeutic possibilities of TMZ in ACCs, by investigating the *in vitro* effects of TMZ on three ACC cell lines and 8 primary ACC cultures. We also determined *MGMT* methylation and expression and the potential predictive role of the *MGMT* gene in adrenal tumors.

MATERIALS AND METHODS

Adrenocortical tissues

Adrenocortical tissues were obtained between May 1995 and October 2015 at the Department of Surgery, Erasmus MC, Rotterdam. Directly after resection, adrenal tissues were embedded in Tissue-Tek and stored at -80 °C. For eight ACCs, a tissue part was used to obtain primary cultures. Diagnosis was confirmed using the Weiss score or Van Slooten index (14, 15). Patient and tumor characteristics were obtained from electronic patient records. The study was conducted under guidelines that were approved by the Medical Ethics Committee of the Erasmus MC. Informed consent was obtained from all patients.

Cell culture and compounds

Three available human ACC cell lines were used: H295R, HAC15 and SW13, obtained from the American Type Culture Collection (Manassas, VA), ECACC (Salisbury, Wiltshire, UK) and from Dr. W. Rainey (as a kind gift), respectively. Short tandem repeat (str) profiling using a Powerplex Kit (Promega, Leiden, the Netherlands) of NCI-H295R and SW13 gave results consistent with the ATCC database, confirming the identity of both cell lines. Str profiling of HAC15 showed a genetic profile identical to H295R, which is consistent with a previous report by Wang and Rainey that HAC15 is a clone of H295R (16). Cells were cultured as previously described (17). TMZ, mitotane, and the demethylating drug 5'-AZA-2'-deoxycytidine (AZA) stock solutions (10 mM), prepared in 100% dimethylsulfoxide, absolute EtOH, and H_2O, respectively (Sigma-Aldrich, Zwijndrecht, the Netherlands), were stored at -20°C. After trypsinization, cells were plated at the appropriate density to obtain 80% confluency at the end of the experiment. The next day, incubations were started in quadruplicate. Control cells were vehicle treated. Cell culture experiments were carried out at least twice, except primary cultures, due to limited number of cells obtained from the specimens. Primary cultures were obtained as previously described (18). Cortisol was measured in the supernatant of cortisol-producing ACCs using a chemiluminescence immunoassay system (Immulite 2000XPi).

DNA amount (as measure of cell amount) and apoptosis measurement

Effects of TMZ (10–1000 μM) and/or mitotane (1–50 μM), on cell growth in ACC cell lines were assessed as previously described (19). In primary cultures, DNA amounts were measured using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific, MA), an ultra-sensitive method for DNA measurement. Apoptosis was assessed using the Cell Death Detection ELISAPlus kit (Roche Diagnostics, Almere, the Netherlands).

Colony Forming Assay (CFA)

The CFA is the gold standard for measurement of effects of cytotoxic agents on cancer cells *in vitro*. Effects on colony size and surviving fraction of colonies were assessed at 250 μM (H295R and HAC15) or 500 μM (SW13) TMZ. Plates were coated with 1ml Poly-L-lysine (10 μg/ml), where after 1500, 1250, or 250 cells were plated for H295R, HAC15, and SW13, respectively. After 1 day, drug treatment was initiated. Media were removed after 1 or 3 days and refreshed without TMZ. When colonies contained at least 50 cells (3, 4, and 2 weeks for H295R, HAC15, and SW13, respectively), the cells were washed and fixed. Cells were colored with Haematoxylin, and amounts and sizes of colonies were measured using MultiImage TM Light Cabinet (Alpha Innotech). Surviving fraction was calculated as previously described (20).

Cell cycle analysis

For cell cycle analysis, ACC cells were treated with 125-1000 μM TMZ. After 3 and 7 days, cells were harvested, washed with NaCl, fixed with ice-cold 70% EtOH, and stored at -20 °C until analysis. Analyses were performed using the Muse® Cell Cycle Assay Kit utilizing Muse™ Cell Analyzer (Merck Millipore, Amsterdam, the Netherlands).

Three-dimensional (3D) multicellular spheroid cultures

For 3D spheroid cultures, 10 000 cells (H295R and HAC15), or 1 000 cells (SW13) were plated in 24-well culture plates with cell-repellent Surface (Greiner Bio-One, Alphen a/d Rijn, the Netherlands). The plates were rotated at 100 rpm for 72 hours. Seven days after plating, treatments were initiated (TMZ and mitotane 1-1000 μM). Photomicrographs were taken at 50x magnification at day 0, 3, and 7 from treatment initiation. Image J software (National Institutes of Health, Maryland) was used to measure the pixel number of the area occupied by the spheroid. Spheroids were only measured when a clear outer boundary of the spheroid was visible. Growth rate was used to compare control and treated spheroids.

Bisulfite conversion and pyrosequencing

DNA isolation from ACC cells (control, 1 μM AZA treated) and adrenal tissues, bisulfite conversion, PCR (hybrid temperature 57 °C), and pyrosequencing, were performed as previously described (21), but with the use of the *MGMT* primer. The primer was designed based on previous studies using Pyromark Assay Design (22, 23) (Supplementary Table 1). High and low methylated DNA (Qiagen, Benelux) was used as internal control.

MGMT mRNA expression analysis

MGMT mRNA expression was assessed in ACC cells (control, TMZ 125 μM and/or mitotane 5 μM, AZA 1 μM), and in adrenal tissues. RNA isolation, cDNA synthesis and RT-PCR were performed as previously described, but using other primers (Supplementary Table 2; Sigma-Aldrich, Zwijndrecht, the Netherlands) (21). Three housekeeping genes were used: hypoxanthine-guanine phosphoribosyl transferase 1 (*HPRT*; Sigma-Aldrich, Zwijndrecht, the Netherlands), Beta-actin (*B-actin*; Thermo Fisher Scientific, MA), and glucuronidase beta (*GUSB*; Thermo Fisher Scientific, MA). Reliable housekeeping genes were determined for cell culture experiments and relative *MGMT* expression was calculated using the comparative CT method $2^{-\Delta\Delta C}{}_{t}$. For accurate RT-PCR expression profiling in adrenal tissues, *HPRT*, *GUSB*, and *B-actin* were determined to normalize mRNA levels using the method of Vandesompele et al (24).

MGMT immunohistochemistry

Two tissue micro arrays (TMA) comprising specimens from seven normal adrenals (NA), 15 ACAs and 23 ACCs were constructed, as previously described using the automated TMA constructor (ATA-27 Beecher Instruments, Sun Prairie, WI) available at Erasmus MC, Department of Pathology (25). For an optimal representation, an expert pathologist identified three areas per specimen in Haematoxylin and eosin stained slides. Cores with a diameter of 1 mm were extracted from 'donor' block and brought into the 'recipient' paraffin block at predefined coordinates. In addition, formalin-fixed paraffin-embedded whole sections were used from three cases not included in the TMA. Five-micrometer sections were cut and used for immunohistochemistry (IHC) as previously described (26), with the adjustments that TRIS-EDTA buffer (pH 6.0) and the Mouse monoclonal MGMT antibody (MT 3.1 dilution 1:20; Thermo Fisher Scientific, MA) were used. Sections were independently and blinded for the tissue type scored by two investigators (SGC, LJH) using a semiquantitative, well-established immunoreactivity score (IRS), calculated by the product of the percentage positive cells (4, >80%; 3, >51-80%; 2, >10%; 1, 0) and intensity of staining (3, strong; 2, moderate; 1, mild; 0, no staining) (27).

Statistical analysis

Graphpad Prism version 3.0 (Graphpad Software) and SPSS Statistics version 21 (SPSS 21.0; SPSS Inc.) were used. For cell culture experiments, the ANOVA test (followed by a Tukey's test) or Student's *t* test was used for comparisons among treatment groups. A Kruskal-Wallis test or ANOVA, when applicable, was used to assess differences between adrenal tissues. Correlations were tested using Spearman's rank coefficient. Values of $P < 0.05$ were considered significant. Data are indicated as mean ± SEM, unless specified otherwise.

RESULTS

Patient characteristics

Eleven NA, obtained during nephrectomy, 16 ACAs, and 29 ACCs were enrolled. *MGMT* mRNA expression and promoter methylation were assessed in 42 adrenal specimens (seven NAs, 12 ACAs, 23 ACCs), whereas MGMT immunohistochemistry was performed in 46 tissues (seven NAs, 13 ACAs, 26 ACCs). Patient and tumor characteristics are listed in Table 1.

Table 1. Patient and tumor characteristics of patients included in this study

	Adrenocortical carcinomas *n* = 29	Adrenocortical adenomas *n* = 16
Mean age at diagnosis (yr)	53 yr (range 9-82)	46 yr (range 26-61)
Mean follow-up (months)	39 (range 3-187)	34 (range 1-83)
Male (%)	11 (38%)	3 (19%)
Mean tumor size cm (SD)	14.45 (SD 5.7)	3.17 (SD 1.95)
Secretion		
Androgens	9 (31%)	1 (6%)
Glucocorticoids	15 (52%)	6 (38%)
Mineralocorticoids	0 (0%)	5 (31%)
Precursors	3 (10%)	0 (0%)
Estradiol	4 (14%)	0 (0%)
Non secreting	12 (41%)	4 (25%)
Weiss score (SD)	6.0 (SD 1.31)	0.25 (SD 0.45)
VanSlooten Index (VSI)	21.1 (SD 4.8) *n*=26	1.56 (SD 0.869)
ENSAT		
I	0 (0%)	14 (88%)
II	15 (52%)	2 (13%)
III	3 (10%)	0 (0%)
IV	11 (37%)	0 (0%)

VanSlooten Index was not available for all patients, dependent on the year of diagnosis. Yr, year; SD, standard deviation; ENSAT, European Network for the Study of Adrenal Tumors.

Effects of temozolomide on ACC cell lines

Cell growth and apoptosis

TMZ strongly inhibited cell growth, measured as DNA amount per well, in H295R, HAC15, and SW13 (Fig. 1, A-C), in a time- and dose-dependent manner. IC_{50} values were lower for H295R (386 μM; 95% CI 255-583) and HAC15 (379 μM; 239-601), compared to SW13 (435 μM; 276-686; $P = 0.0007$ and $P = 0.0011$, respectively). Maximum inhibition of cell growth was 82% in H295R, 80% in HAC15, and 97% in SW13, which was significantly higher in SW13 ($P < 0.0004$). In H295R and SW13, there was a dose-dependent induction of apoptosis after TMZ, with 220% and 1169% induction at 1000 μM TMZ (Fig. 1, D and F; both $P < 0.001$ vs control), respectively. A less strong induction of apoptosis (61%) was present in HAC15 (Fig. 1E).

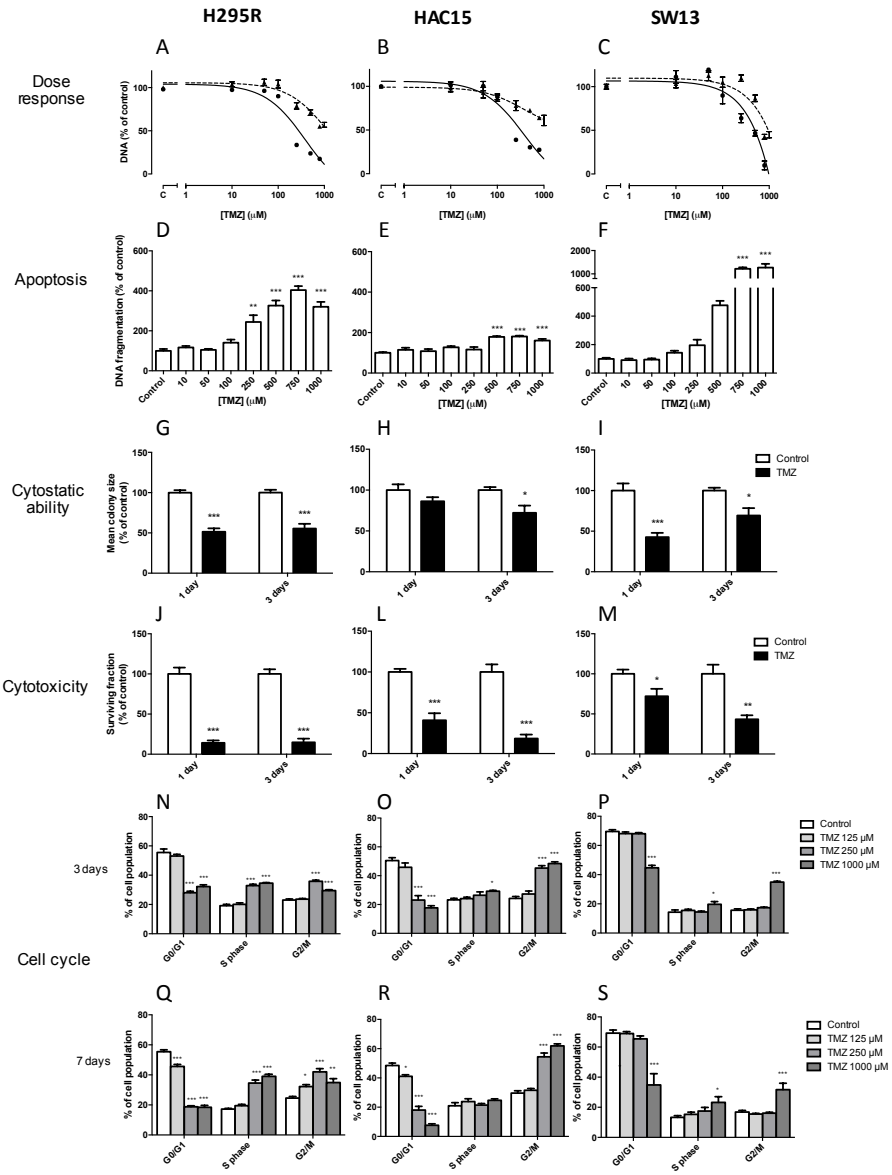

Figure 1. Effects of TMZ treatment on H295R, HAC15, and SW13 cells on cell amount expressed as DNA content (A–C), apoptosis expressed as DNA fragmentation (D–F), cytostatic ability expressed as mean colony size (G–I), cytotoxicity expressed as surviving fraction of colonies (J–L), and effect on cell cycle analysis after 3 (M–O) and 7 days (P–R) of treatment with TMZ. In panels A–C, dotted and solid lines represent 3 and 7 days of treatment, respectively, and panel C on the y-axis represents the vehicle-treated control. Apoptosis was assessed after 3 days. For the colony- forming assay, 250 μM was used for H295R and HAC15 and 500 μM TMZ for SW13. Values represent mean ± SEM and are shown as a percentage of control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control. C, control.

Colony-forming Assay

In H295R and SW13, the mean colony size significantly decreased after 1 day (both $P < 0.001$) and 3 days (Fig. 1, G and I; H295R, $P < 0.001$; SW13, $P < 0.05$) of TMZ treatment. In HAC15, we only observed an effect on colony size after 3 days of treatment (Fig. 1H; $P < 0.05$). Cytotoxicity analysis showed a significantly lower surviving fraction of colonies after 1 and 3 days of TMZ in all cell lines (Fig. 1, J-M; $P < 0.05$).

Cell cycle analysis

Cell cycle analysis showed an accumulation of H295R and HAC15 cells in the G_2/M phase after TMZ treatment in a dose-dependent manner (both $P < 0.001$), with maximum increase of 42% in H295R and 109% in HAC15. In H295R, the G_2/M phase accumulation was associated with the gradual appearance of cells in the S phase ($P < 0.001$) and a decrease of cells in the G_0/G_1 phase ($P < 0.001$). In HAC15, we observed a time- and dose-dependent decrease of cells in the G_0/G_1 phase ($P<0.001$), but without an increased S phase population. In SW13, treatment with TMZ caused a minimal accumulation of cells in the S phase ($P < 0.05$) and G_2/M phase ($P < 0.001$), and a minimal decrease in the G_0/G_1 phase (Fig. 1, P and S; $P < 0.001$).

Three-dimensional multicellular spheroids

SW13 cells did not form 3D multicellular spheroids and the effect of the drugs could thus not be studied in this cell line. Visually, TMZ and mitotane seemed to exert inhibition of spheroid growth differently, since only the mitotane-treated spheroids lacked a clear outer boundary in the highest treatment concentrations (Fig. 2). It was therefore not possible to reliably measure the spheroids treated with the highest concentrations of mitotane (H295R 10-100 μM; HAC15 50-100 μM) (Fig. 2B). A dose-dependent inhibitory effect was seen on growth rate of the spheroids by both TMZ and mitotane. Already at 250 μM, spheroid growth rate was inhibited by 91 and 45% in H295R and HAC15, respectively. TMZ 1000 μM decreased the initial size (size at start of drug treatment) of the spheroids in H295R after 7 days ($P < 0.05$) (Fig. 2).

Effects of combination of TMZ and mitotane on cell number and *MGMT* mRNA expression

Combination therapy of TMZ and mitotane showed no additive effect on cell growth (Fig. 3). In the lowest concentration of TMZ, where no effect on cell amount was seen in monotherapy, we observed the expected effect of mitotane (Fig. 3, A-C; $P < 0.05$). The same trend was seen when a low dose of mitotane was given with TMZ (Fig. 3, D-F; $P < 0.001$). When we compared the effects of mono- or combination therapy of TMZ and mitotane on ACC cells in the other concentrations, no differences were observed. There was no effect on *MGMT* mRNA expression when H295R cells were treated with 125 μM TMZ, 5 μM mitotane, or the combination (data not shown).

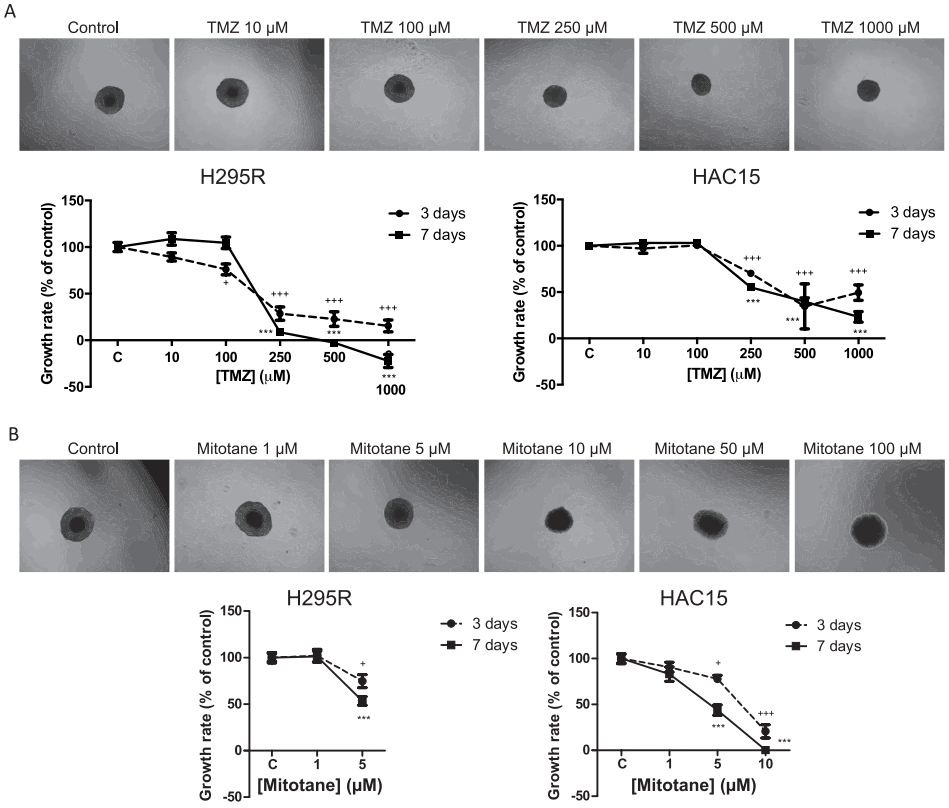

Figure 2. Effect of TMZ and mitotane on ACC cells cultured as 3D multicellular spheroids. Effects of different concentrations of TMZ (A) and mitotane (B) on H295R (left panel) and HAC15 (right panel) spheroid growth rate compared with control. Photographs are taken after 7 days of treatment. After mitotane treatment, it was not possible to measure the spheroids in the highest concentrations (H295R, 10, 50, and 100 µM; HAC15, 50 and 100 µM) because there was no clear outer boundary of the spheroids visible. Negative numbers indicate shrinking of the spheroid. Values represent mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control. C, control.

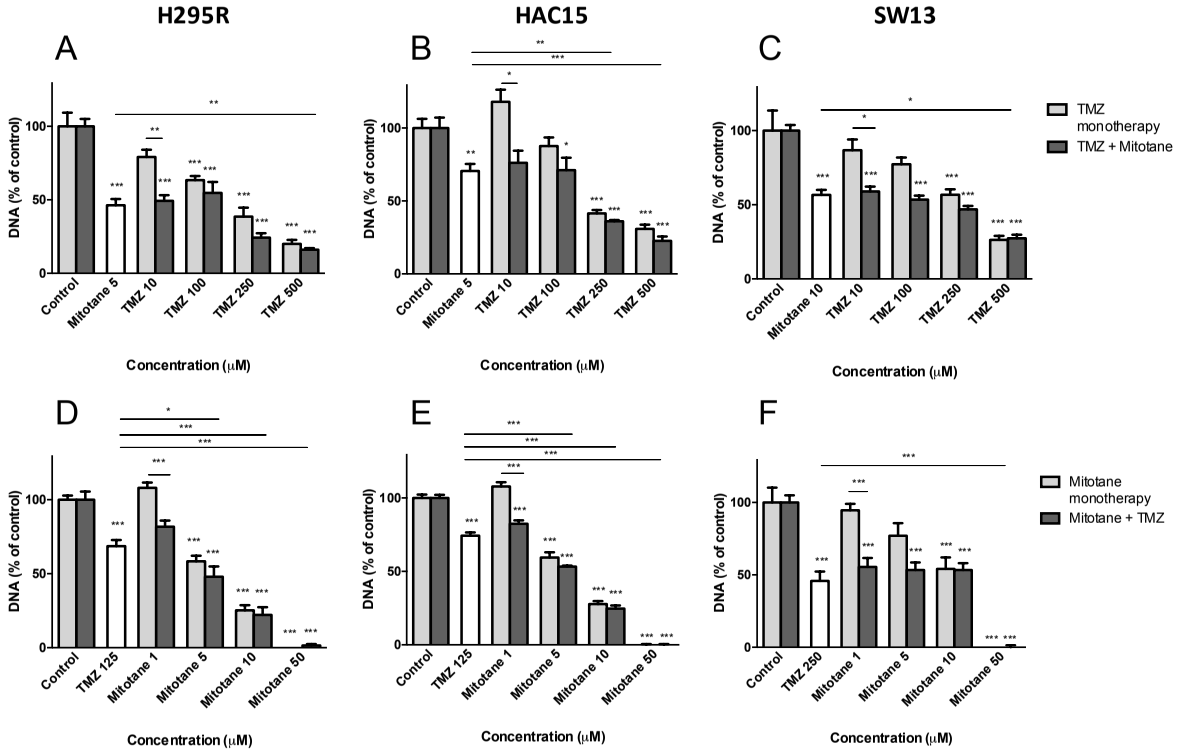

Figure 3. Combined effect of TMZ and mitotane on cell number in H295R, HAC15, and SW13. Effect of different concentrations of TMZ with or without a fixed concentration of mitotane (5 or 10 μM) in H295R (A), HAC15 (B), and SW13 (C) is shown. Effect of different concentrations of mitotane with or without a fixed concentration of TMZ (125 or 250 μM) in H295R (D), HAC15 (E), and SW13 (F) is also shown. Cell number is depicted as total DNA amounts. Values are represented as mean ± SEM and are shown as a percentage of control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs own control or as stated by the lines.

Effects of TMZ on primary cultures of adrenocortical carcinomas

The effect of TMZ *in vitro* was evaluated in eight primary ACC cultures (Supplementary Table 3). A statistically significant inhibitory effect on cell amount was seen in five of the eight primary ACC cultures after 7 days of TMZ treatment, varying from 29% to 83% inhibition (Fig. 4, A-E; primary cultures numbers 1, 2, 4, 6, 7). Apoptosis induction was seen in the same five primary ACC cultures, ranging from 37 to 716% increase (Fig. 4, F-J). In two other primary cultures, apoptosis was also induced with 42 and 127% at TMZ 1000 μM. TMZ inhibited cortisol production (corrected for cell amount) by 97% in one of three primary cultures of cortisol-producing ACCs (number 5). In one (number 4) of the six primary ACC cultures (numbers 1-4, 6, 7) in which TMZ and mitotane were combined, an additive inhibitory effect on cell amount was observed (Supplementary Fig. 1D). Primary culture 1 responded to the combined treatment only with mitotane (50 μM) and TMZ (250 μM), but not to the monotherapies (Supplementary Fig. 1B). In 3/6 primary ACC cultures, mitotane 50 μM strongly inhibited cell number, limiting the possibility to investigate additive effects of TMZ. 1 of these 6 primary cultures did not respond to either TMZ or mitotane (number 3).

MGMT promoter methylation status and mRNA expression in ACC cell lines and adrenal tissues

Mean *MGMT* promoter methylation percentages were low and amounted 2.9% ± 0.03%, 2.8% ± 0.09% and 2.4% ± 0.41% for H295R, HAC15, and SW13, respectively (Supplementary Fig. 2). *MGMT* mRNA expression was significantly higher in SW13 compared with H295R and HAC15 (both $P < 0.001$). Treatment with AZA decreased *MGMT* methylation on average with 24%, while *MGMT* mRNA expression only slightly decreased in H295R (Supplementary Fig. 2; $P < 0.05$).

MGMT promoter methylation was low in NAs (2.5% ± 1.6%), ACAs (2.0% ± 0.89%), and ACCs (5.4% ± 8.8%), with no statistically significant differences between groups (all mean ± SD; Fig. 5A). *MGMT* mRNA expression was lower in ACCs compared to ACAs (Fig. 5C; $P < 0.05$). In ACCs, an inverse relationship of *MGMT* methylation and mRNA expression was found (Fig. 5B; $\rho = -0.7174$, $P < 0.0001$), although within a small range of methylation. Mean MGMT IRS was respectively 7.2 ± 3.2, 6.4 ± 2.8, and 5.4 ± 2.4 for NAs, ACAs, and ACCs (all mean ± SD), with no significant differences between groups (Fig. 5, D and E). MGMT protein expression was not correlated with *MGMT* methylation and mRNA expression ($n = 20$) in ACCs ($\rho = -0.157$, $P = 0.509$; $\rho = -0.024$, $P = 0.919$; respectively). *MGMT* mRNA expression was inversely correlated with the Van Slooten Index and the Weiss score (Fig. 6, A and B; $\rho = -0.4574$, $P = 0.043$; $\rho = -0.4522$, $P = 0.027$, respectively), and was lower in tumors with ENSAT stage IV versus ENSAT stage II (Fig. 6C; $P < 0.05$).

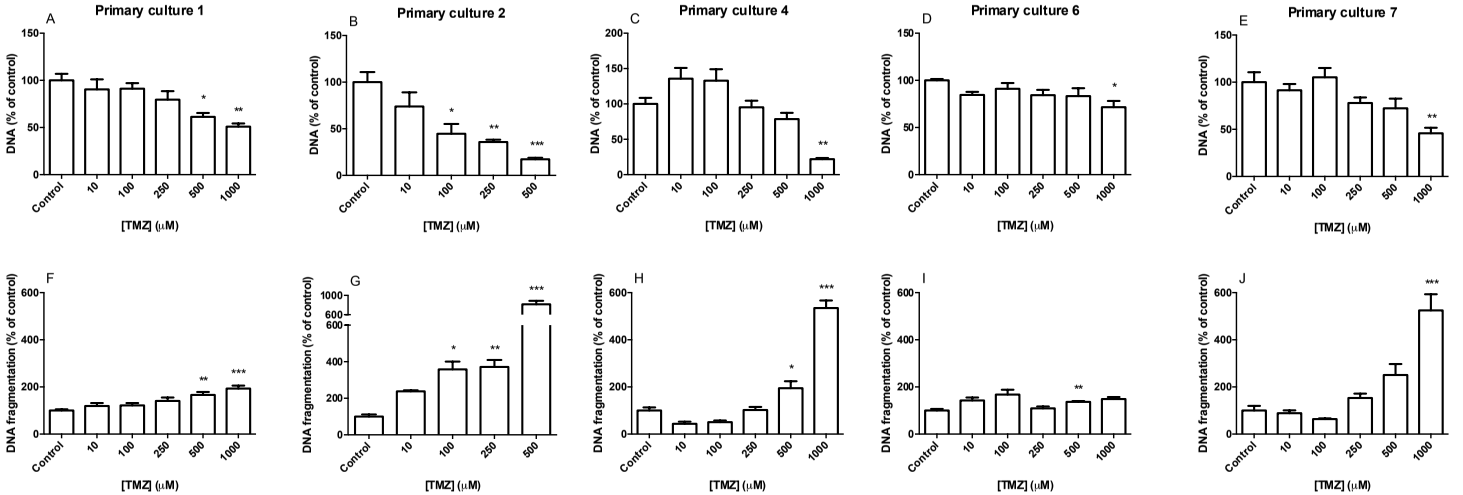

Figure 4. Effects of TMZ on five of the eight primary ACC cultures that responded to TMZ by an inhibition of cell number (A–E) after 7 days of treatment and an induction of apoptosis (F–J). Cell number is depicted as amount of DNA and apoptosis as amount of DNA fragmentation. Two additional primary cultures showed induction of apoptosis but no inhibitory effect on cell number. One primary culture did not respond to TMZ by either a change in cell amount or apoptosis. Values represent mean ± SEM and are shown as percentage of control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control.

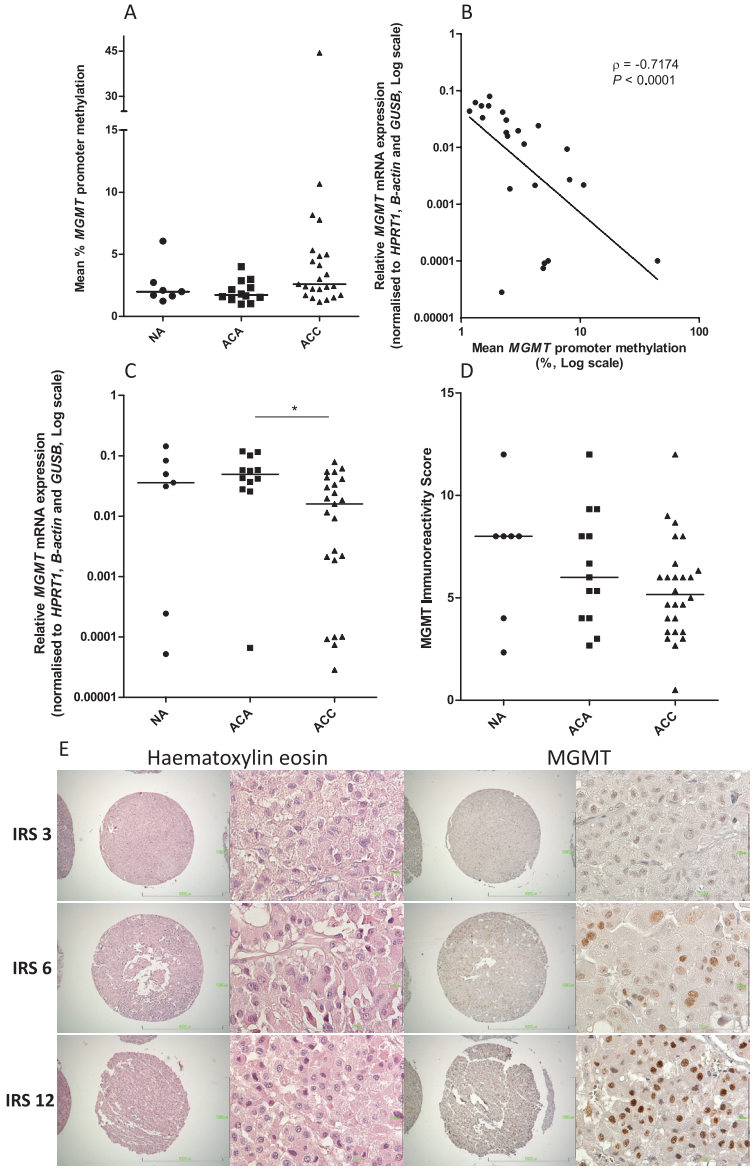

Figure 5. Overview of involvement of the *MGMT* gene in the adrenal with promoter methylation status of the *MGMT* gene (A), *MGMT* mRNA (B), and MGMT protein expression (C) in adrenocortical tissues. (D) Correlation of *MGMT* promoter methylation and mRNA expression in ACCs. (E) Representative example of MGMT immunohistochemical staining in adrenocortical carcinomas as determined by immunohistochemistry. Antibodies against MGMT stain tumor cell nuclei. Sections were blinded and independently evaluated by two investigators (microscopic magnification, x40 and x400). Lines represent medians. ρ represents Spearman's rank correlation coefficient. ** $P < 0.01$. NA, normal adrenals; ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; IRS, Immunoreactivity Score; MGMT, O6-Methylguanine-DNA methyltransferase.

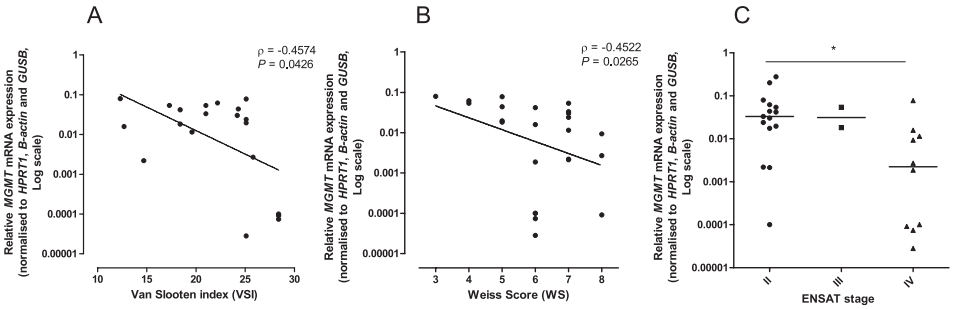

Figure 6. Correlation of *MGMT* mRNA expression with the Van Slooten Index (A), Weiss score (B), and the ENSAT stage (C) in ACCs. ρ, Spearman correlation coefficient. * $P < 0.05$.

In primary ACC cultures, a trend was observed towards a higher *MGMT* methylation in the cultures that responded to TMZ, compared to the three primary cultures that did not show a decrease in cell amount (mean 4.6% ± 1.0% vs 2.0% ± 0.30%; both mean ± SD; $P = 0.0571$). No correlation was observed with MGMT mRNA and protein expression.

DISCUSSION

Due to lack of effective therapies for patients with ACC, current research focuses on discovering new therapeutic targets. So far, chemotherapeutic drugs are effective in only a small subset of patients and targeted therapies showed disappointing results (6). In this study, we showed the first evidence for a potential novel treatment option of ACC patients with temozolomide.

We show that TMZ exerts potent antitumor responses on ACC cells *in vitro,* including a strong inhibition of cell growth, induction of apoptosis, cytotoxic and cytostatic effects, as well as a cell cycle arrest. Concentrations of TMZ that can be achieved in the plasma of patients with advanced cancers is approximately 25-55 μM (28-30). However, a relevant consideration is that these represent plasma concentrations, and not intratumoral concentrations. From studies of TMZ in glioblastoma and melanoma patients and cell lines, TMZ is known to cause a G_2/M arrest and to minimally induce apoptosis (31, 32). The induction of apoptosis was variable in ACC cell lines, with the strongest induction in SW13. H295R and HAC15 appear to respond to a greater extent by undergoing cell cycle arrest.

One of the challenges of basic and translational ACC research is the limited number of available human cell lines. To circumvent this limitation and assess efficacy of TMZ in a more representative model from the ACC cells of origin, we also investigated inhibition

of cell amount and induction of apoptosis in primary ACC cultures. Inhibition of the cell amount was demonstrated in five of eight cultures, whereas apoptosis induction was seen in an even larger number of primary ACC cultures, i.e. seven of eight. Furthermore, we showed an efficacy of TMZ in 3D ACC spheroids, giving the opportunity to study effects of TMZ on ACC cells in a more (patho)physiologically relevant context, harboring important cell-cell and cell-matrix interactions and availability of drugs to the inner layer of the spheroid. Interestingly, we visually observed a different treatment effect on the spheroid by TMZ and mitotane, suggesting a different mechanism of action. However, we did not see an additive or synergistic effect when we tested the combined effect of TMZ and mitotane in ACC cell lines. It does seem that the two drugs not adversely affect each other. We ruled out a possible upregulation of *MGMT* expression by the combination of mitotane and TMZ. In contrast, in one primary ACC culture, an additive effect was observed when TMZ was added to mitotane. This issue remains to be further investigated.

One of the aims of this study was to investigate the potential role of the DNA repair gene *MGMT* in ACC, since research has shown that epigenetic silencing of MGMT sensitizes glioblastoma cells to TMZ. Comparison of the IC_{50} values of TMZ on ACC cell lines and glioma cell lines shows that the IC_{50} values of TMZ in the ACC cell lines are comparable with the least sensitive glioma cell lines (IC_{50} 380 – 440 μM vs 371 – 441.6 μM). These glioma cell lines also have low MGMT methylation (33). Importantly, we also found low methylation in almost all ACCs, with the majority of the ACCs harboring *MGMT* mRNA expression in the same range as ACC cell lines. This suggests that our cell lines are a representative model for ACCs regarding the *MGMT* gene. Additionally, we observed a trend towards a slightly higher *MGMT* methylation in the responsive primary ACC cultures, despite the fact that the methylation was below 5% in all tissues. This issue requires further investigation in a larger group. Although in a small range, a strong significant inverse correlation was found between methylation and *MGMT* mRNA expression in ACCs. The inverse correlation of *MGMT* mRNA expression with histopathology and ENSAT stage may indicate that silencing of *MGMT* plays a role in the development or progression of ACC, like in other types of cancer (34). MGMT protein expression was not correlated with *MGMT* promoter methylation, which might be explained by confounding factors in the presence of sampling bias, since DNA and RNA are isolated from frozen specimens and IHC is performed on paraffin-embedded sections. These fragments may originate from different parts of the tumor. Other potential explanations include tumor heterogeneity, or a variety of normal resident cells within the tumor. In addition to these possiblities, several studies in human glioblastomas also reported no correlation between MGMT immunohistochemistry and promoter methylation, and promoter methylation assessment is generally accepted as the method of choice for prediction (35, 36).

Future investigations in ACCs could focus on other potential predictive factors, like absence of a functional DNA mismatch repair system because in gliomas this is known to contribute to TMZ resistance as well (37). Furthermore, research can focus on the combination of TMZ with capecitabine, considering the *in vitro* synergism shown in neuroendocrine tumor carcinoid BON cells (38). Reasonably, because these results are *in vitro*, (pre)clinical studies are warranted to further evaluate the therapeutic possibilities of TMZ for treatment of ACC. However, since the safety, pharmacodynamics, and pharmacokinetic profile of TMZ is known in cancer patients, the clinical application is more easily accessible. In addition, in contrast to several other drugs, CYP450-mediated metabolism does not seem to contribute significantly to the plasma clearance of temozolomide (39). This might indicate that mitotane, which increases CYP3A4 expression (40), does not influence TMZ plasma levels. Thereby, TMZ does not need hepatic metabolism to be activated. These findings are important beneficial remarks for a potential clinical application of TMZ in ACC patients, since many patients will be (co-) treated with mitotane.

In conclusion, we hypothesize that, based on the low MGMT methylation and meanwhile the responsiveness to TMZ comparable with unresponsive glioma cell lines, efficacy of TMZ in ACC cells might be limited by low methylation of MGMT. We show for the first time the therapeutic potential of TMZ for ACC, potentially offering an urgently needed alternative for patients who do not respond to the conventional therapy with mitotane, etoposide, doxorubicine and cisplatin.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Primers used for PCR and pyrosequencing of the *MGMT* promoter

Accession no.	Nucleotide position	PCR primers (5'-3')
ENSG00000170430	-11 - 96	*Forw*: 5'-[Btn]GTTTAGGATATGTTGGGATAGT -3' *Rev*: 5'- AACCACCCAAACACTCACCAA -3'

Primers were designed using Pyromark Assay Design (Qiagen, Benelux). Forw, forward primer; Rev, reverse primer; [Btn], biotynilated.

Supplementary Table 2. Primers and probes used for real time quantitative PCR

Gene	PCR primers (5'-3')
HPRT1	Forward: 5'-CACTGGCAAAACAATGCAGACT-3' Reverse: 5'-GTCTGGCTTATATCCAACACTTCGT-3' Probe: 5'-[6FAM]CAAGCTTGCGACCTTGACCATCTTTGGA[TAM]-3'
B-actin	NM_001101.2
GUSB	NM_000181.1
MGMT	Forward: 5'-CCTGGCTGAATGCCTATTTC-3' Reverse: 5'-GATGAGGATGGGGACAGGATT -3' Probe: 5'-[6FAM] CGAGCAGTGGGAGGAGCAATGAGA[TAM]-3'

Concentration represents the amount (nmol/l) added in the total reaction volume (12.5µl) used for each sample. B-actin and GUSB are commercially available primers (Applied Biosystems, Alphen a/d Rijn, the Netherlands), therefore the accession numbers are depicted instead of the sequences.

Supplementary Table 3. Overview of clinical and tumor characteristics of patients of which a primary ACC culture was obtained

Patient no.	Sex	Weiss score	Van Slooten index	Specimen
1	M	5	16.80	Primary tumor
2	M	6	28.4	Primary tumor
3	F	1	9.60	Primary tumor
4	M	6	28.4	Primary tumor
5	F	6	25.1	Primary tumor
6	F	5	25.1	Metastasis right adrenal
7	M	8	28.4	Primary tumor
8	M	5	13.90	Primary tumor

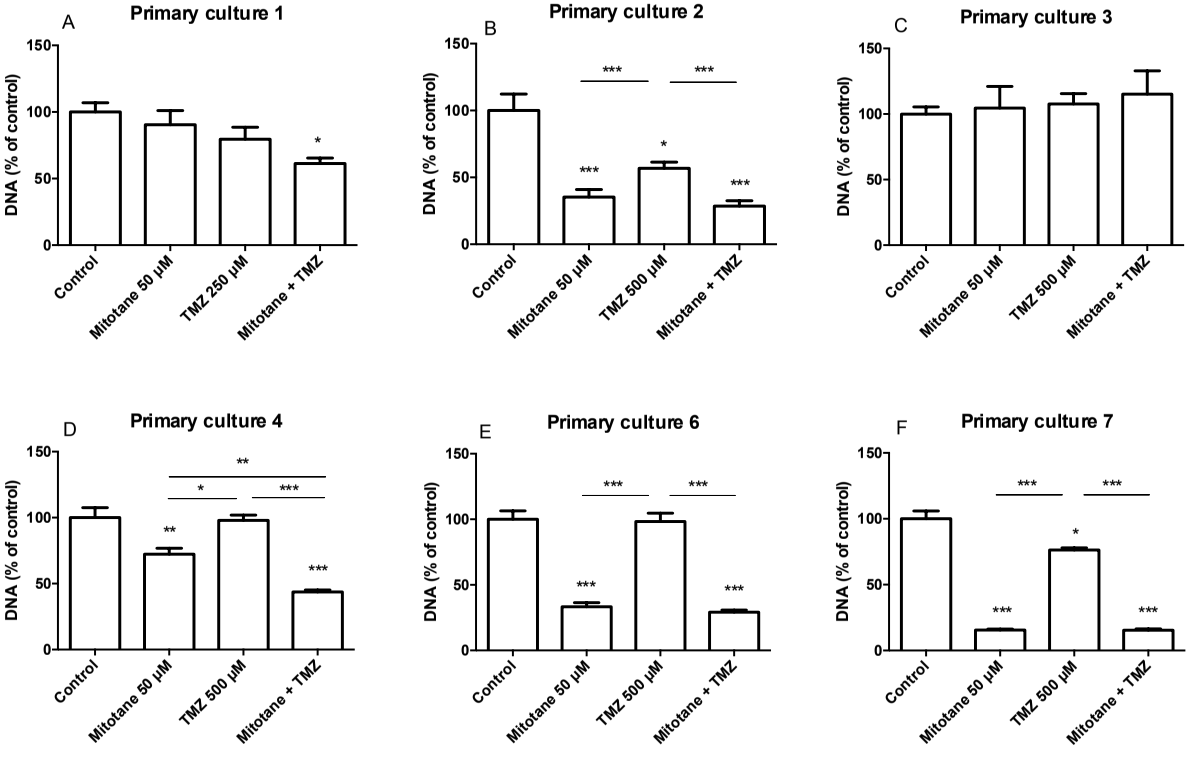
No, number; M, male; F, female; ENSAT, European Network for the Study of Adrenal Tumors; cm, centimeter.

Product length	Sequence primers (5'-3')
108	5'-CCAAACACTCACCAAAT-3'

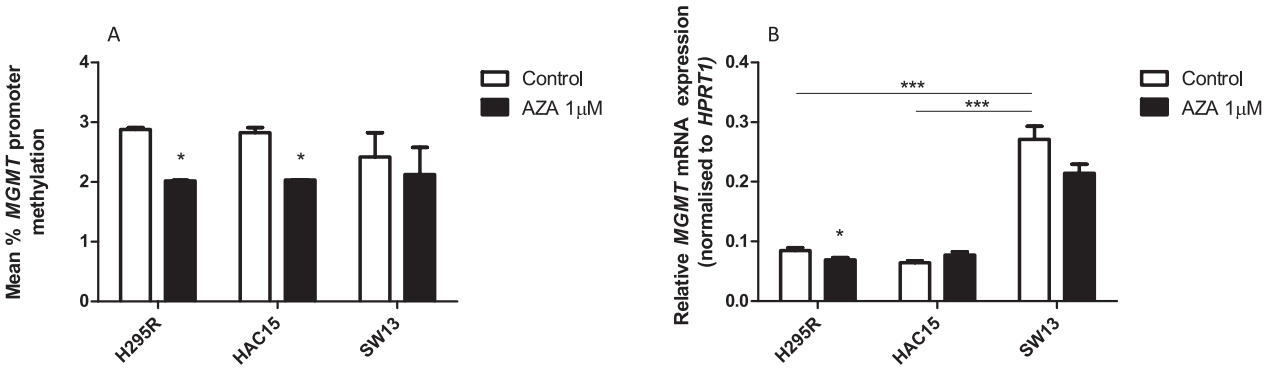
Concentration	EF
0.5 pmol 0.5 pmol 0.1 pmol	1.91
Dilution 1:20	1.91
Dilution 1:20	2
0.5 pmol 0.5 pmol 0.1 pmol	1.92

These primers should be diluted 20x from stock in the reaction volume. *HPRT1*, hypoxanthine-guanine phosphoribosyl transferase 1; *B-actin*, Beta-actin; *GUSB*, glucuronidase beta; EF, efficiency factor.

Tumor size (cm)	ENSAT stage	Hormonal secretion	Age at surgery (years)
16	II	Non secreting	76
19	IV	Non secreting	57
10	II	Non secreting	50
14	II	Non secreting	83
24	IV	Glucocorticoids and androgens	49
22	IV	Non secreting	55
16	IV	Glucocorticoids	55
6	II	Glucocorticoids	51

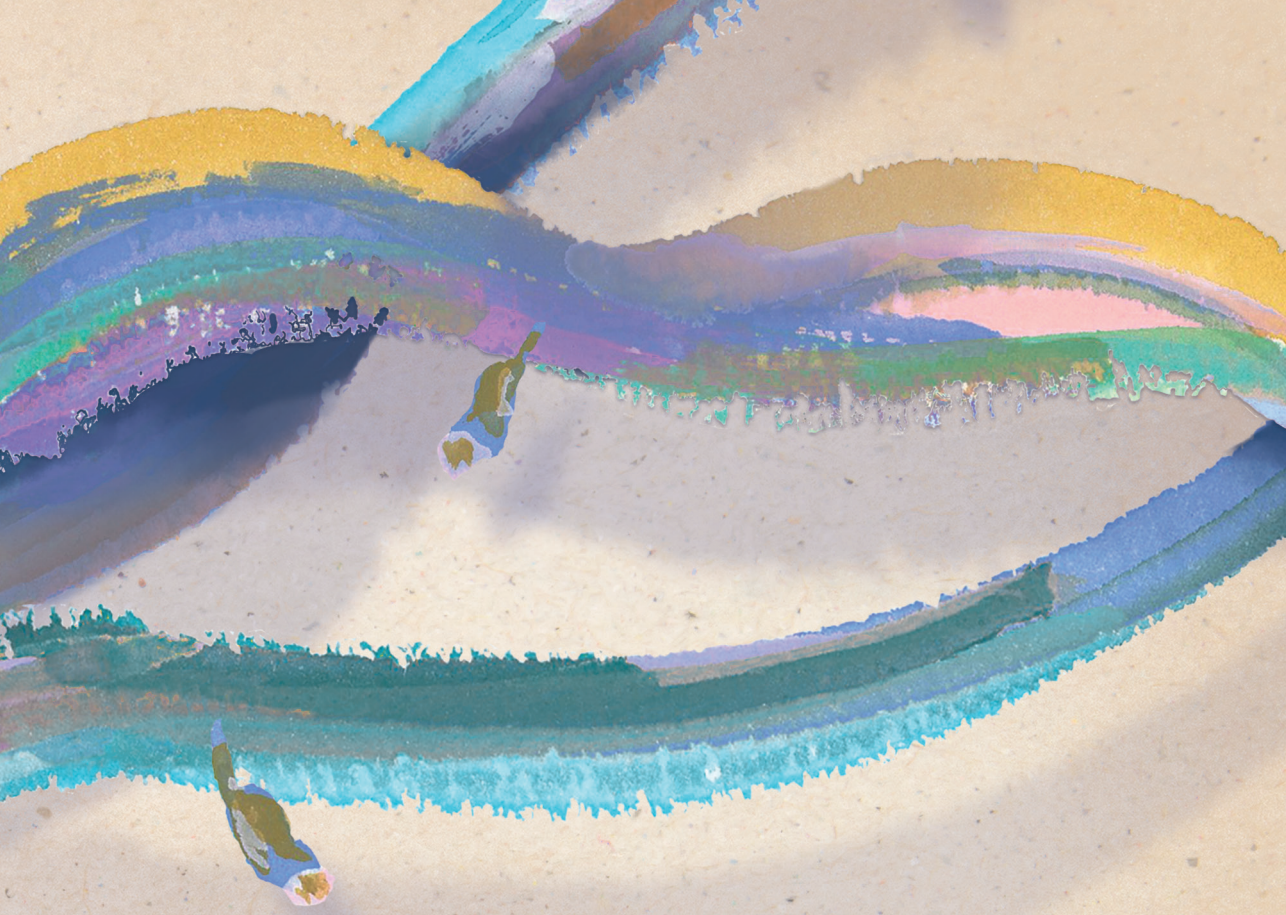


Supplementary Figure 1. Combined effects of mitotane 5 µM and temozolomide (TMZ) 250 or 500 µM in 6 primary ACC cultures on cell number depicted as total DNA amount (upper row) and apoptosis depicted as DNA fragmentation (bottom row). Values are represented as mean ± SEM and are shown as percentage of control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control or as depicted in the graph.



Supplementary Figure 2. *MGMT* promoter methylation (A) and mRNA expression (B) in vehicle treated cells and after 7 days of treatment with the demethylating drug 5'-AZA-2'-deoxycytidine (AZA) 1 μM in H295R, HAC15, and SW13. *MGMT* mRNA expression is normalized to Hypoxanthine-guanine phosphoribosyl transferase 1 (*HPRT1*). Values represent mean ± SEM and are shown as percentage of control. * p<0.05, *** p<0.001 versus control.

PART III



CHAPTER 10

General Discussion

PARTLY BASED ON:

Cushing's syndrome: an update on current pharmacotherapy and future directions
Creemers SG, Hofland LJ, Lamberts SW, Feelders RA
Expert Opin Pharmacother, 2015, 16(12):1829-44

Future directions in the diagnosis and medical treatment of adrenocortical carcinoma
Creemers SG, Hofland LJ, Korpershoek E, Franssen GJ, van Kemenade FJ, de Herder WW, Feelders RA
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Adrenocortical Carcinoma
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In this general discussion, the implications of the main findings of this thesis are discussed in the light of the current literature, and the current diagnostic and therapeutic strategies for patients with Cushing's syndrome (CS) and adrenocortical carcinoma (ACC). Furthermore, strategies for future research are explored.

PART I CUSHING'S SYNDROME

Despite the fact that surgery is the first treatment approach in all types of CS, medical treatment is indicated in several settings. In the past decades, much progress has been made in the field of medical treatment options. Steroidogenesis inhibitors are the mainstay of medical treatment of CS. The use of steroidogenesis inhibitors has been re-evaluated due to safety concerns, particularly regarding the hepatotoxicity of ketoconazole. In this light, there is a potential for novel steroidogenesis inhibitors to enter clinical practice, which is the main focus of the first part of this thesis.

In vitro effects of novel steroidogenesis inhibitors

Currently, one of the most frequently used steroidogenesis inhibitors for the treatment of CS is the racemic form of ketoconazole. In **Chapter 2**, the effects of levoketoconazole (COR-003), the single 2S,4R enantiomer of ketoconazole, are evaluated on human adrenocortical steroidogenesis *in vitro* and compared to those of racemic ketoconazole. Preclinical studies already suggested that levoketoconazole might be more potent compared to racemic ketoconazole, has a lower hepatic metabolism and less hepatotoxic effects (1, 2). Initial results of a prospective, open-label, phase III maintenance-of-benefit study investigating levoketoconazol demonstrated normalization of urinary free cortisol (UFC) levels in 38% (n/N = 40/94) of CS patients after a 6 months maintenance phase (3).

We show that, both for levoketoconazole and racemic ketoconazole, the sensitivity for direct inhibition of cortisol production *in vitro* is highly variable between different adrenal tissue specimens. Therefore, the ultimate response to steroid synthesis inhibitors might not only be determined by pharmacokinetics and plasma drug concentrations, but also by adrenal tissue sensitivity to these compounds. Differences in sensitivity to steroid synthesis inhibitors on the cellular level will be discussed in the following section. We show that levoketoconazole inhibits cortisol production more potently compared to racemic ketoconazole in HAC15 cells, both in the basal and adrenocorticotropic hormone (ACTH)-stimulated condition. In primary human adrenocortical cultures, there also seems to be a trend towards stronger inhibition of cortisol production by levoketoconazole, although differences are subtle. The usual dosage of ketoconazole for the management of CS is 400 – 1600 mg daily (4). Several

reasons opt for lower necessary dosages of levoketoconazole to normalize UFC levels in patients with CS, i.e. the potential higher potency of levoketoconazole and the expected higher serum concentration of levoketoconazole after oral administration, as has been shown before in rats (1). Further results of the phase III trial need to elucidate the required dosages of levoketconazole to obtain biochemical remission. In the clinical trial that is currently being conducted, concentrations up to 1200 mg levoketoconazole will be administered (NCT03277690).

Moderate and asymptomatic increases in liver enzymes during treatment with racemic ketoconazole are common (~10-20% of cases), which rates may slightly vary in literature (5-7). Severe hepatotoxicity, which usually starts with an acute hepatitis-like picture, is rare (6, 8, 9). Hepatic toxicity may occur less frequently during treatment with levoketoconazole because of lower therapeutic plasma concentrations and consequently less liver exposure. Besides, in a microsome preparation from rat liver, a weaker inhibitory effect of levoketoconazole compared to 2R,4S-ketoconazole was found on hepatic CYP7A, of which decreased activity may lead to functional cholestasis (2). The initial results of a phase III study reported reversible elevation of alanine aminotransferase >3x the upper limit of normal (ULN) in 11% of patients, of which 3% were >5x ULN (3). However, a direct comparison with the prevalence after treatment with racemic ketoconazole is yet difficult to make due to differences between studies. Another adverse effect, limiting the use of ketoconazole in male patients, is hypogonadism and gynaecomastia, caused by inhibition of various steps of steroidogenesis in the gonads (10-12). The effect of levoketoconazole on gonadal steroidogenesis has not yet been investigated. Further research could compare the effects of both compounds on gonadal steroidogenesis in Leydig cells *in vitro*, and in clinical trials by measuring the levels of testosterone and comparing the occurrence of gynaecomastia and hypogonadism in male patients. Continuing on extra-adrenal effects, inhibitory effects of ketoconazole on ACTH secretion by pituitary adenomas have been described before (13, 14). This effect is thought to be an explanation of a less profound increase in ACTH levels in patients with a corticotroph pituitary adenoma treated with ketoconazole for a longer period (5). We show that levoketoconazole inhibits cell number as well as ACTH secretion in mouse corticotroph AtT20 cells, with no clear differences with racemic ketoconazole. We also show that levoketoconazole inhibits cell number in one of the two primary corticotroph pituitary adenoma cultures, which was not observed for racemic ketoconazole.

Taken together, levoketoconazole might be slightly more potent compared to racemic ketoconazole, and together with the previously proposed potential advantages, levoketoconazole appears a promising novel treatment option for patients with CS. Prospective clinical trials are needed to investigate the clinical efficacy, the

optimal dose and uptitration, the tolerability, pharmacokinetics, and safety profile of levoketoconazole for patients with CS. Currently, a double-blind, placebo-controlled, randomized phase III withdrawal trial is ongoing (NCT03277690). To finally address the question whether levoketoconazole is a better alternative to racemic ketoconazole, a comparative prospective clinical trial should be conducted that compares levoketoconazole to racemic ketoconazole in patients with CS, focusing on efficacy, safety and tolerability.

Efficacy of the other frequently used steroidogenesis inhibitor, metyrapone, varies largely between studies, that are only retrospective. Besides, long-term effects are relatively unknown (7, 15). Osilodrostat (LCI699) is a compound with a comparable mechanism of action as metyrapone. It is a potent inhibitor of CYP11B2 (aldosterone synthase), and at higher doses it also inhibits CYP11B1 (11β-hydroxylase). Osilodrostat has already been evaluated in an open-label phase II study in patients with CS (16). In this study, the overall response rate, defined as normalization of UFC or ≥ 50% decrease of baseline UFC, was 78.9% (15/19) at 22 weeks (16). All patients were treated with osilodrostat in a dose escalation protocol according to the sequence of 10, 20, 40 and 60 mg/day until UFC was equal or below the upper limit of normal. Initial results of a phase III study investigating the effects of osilodrostat showed that 53% (n = 72) of patients had normalized UFC without up-titration at week 12 (17). These patients were then randomized to either continuation of osilodrostat or placebo. Eighty-six percent of the patients randomized to continue osilodrostat treatment for 8 weeks showed maintained normalized UFC compared to 29% in the placebo group (17). Treatment was generally well tolerated. However, effects on other adrenal steroids and efficacy compared to metyrapone and ketoconazole has not yet been fairly investigated. Therefore, in **Chapter 3**, the effects of osilodrostat on *in vitro* steroidogenesis were compared to those of metyrapone and ketoconazole. As for the effect of levoketoconazole and racemic ketoconazole, we again show that sensitivity to steroidogenesis inhibitors is highly variable in human adrenal patient tissues, which finding will be discussed in the next section. We show that osilodrostat is a potent inhibitor of cortisol production in HAC15 cells and primary human adrenocortical cultures. Osilodrostat inhibited cortisol production significantly more potent compared to ketoconazole. A slightly increased potency of osilodrostat compared to metyrapone was only found in HAC15 cells, but not confirmed in the primary adrenocortical cultures. Osilodrostat did inhibit aldosterone production more potently compared to metyrapone, as well as to ketoconazole. As it comes to the effects on the steroid profile, the effects of osilodrostat and metyrapone appeared to be largely similar, with in general a strong suppression of corticosterone and cortisol, an accumulation of 11-deoxycortisol, and only subtle effects on adrenal androgens. As expected, and based on its mechanism of action, ketoconazole had a distinctive effect on the steroid profile, as a result of inhibition of multiple steroidogenic enzymes.

Two groups of side effects known to be associated with metyrapone treatment are androgen related effects in female patients, and mineralocorticoid effects, due to increased concentrations of steroid precursors. Based on our *in vitro* study, androgen-related side effects in patients might be expected in a similar way for osilodrostat as for metyrapone. As is observed for metyrapone, osilodrostat inhibits androgen production only modestly, and less potent compared to the glucocorticoids and mineralocorticoids in the adrenal. *In vivo*, a (compensatory) ACTH stimulation due to a diminished negative feedback effect of lower cortisol concentrations can be expected, resulting in increased adrenal androgen production due to blockade of the other steroidogenic enzymes. This could explain the increased testosterone levels in 4 of the 14 female patients in the phase II clinical study (16), and the development of acne and hirsutism in 9% of patients based on initial results of the phase III study investigating osilodrostat (17). Further clinical trials have to elucidate whether the androgen-related side effects are different between metyrapone and osilodrostat. Due to the fact that we did not measure 11-deoxycorticosterone, we cannot give a clear statement about the expected mineralocorticoid-related side effects. Considering the comparable effects of both compounds on adrenal steroidogenesis, these effects might be expected highly similar. In the phase II study, 11-deoxycorticosterone levels indeed also increased under treatment with osilodrostat, which is postulated to have offset the blood pressure-lowering effect of inhibition on aldosterone synthesis (16). Besides, initial results of the phase III study showed hypokalemia, hypertension, and oedema in 13, 17, and 9% of patients, respectively (17). Further research needs to elucidate these findings.

Interestingly, clinically there appears to be a large difference in the required dose in Cushing's disease (CD) patients between osilodrostat and metyrapone. The median metyrapone dose, which is administered in CD patients to normalize UFC, is 1375 mg (range 500 – 3500 mg) per day (15), whereas doses of 4 to 100 mg daily osilodrostat were administered in the proof-of-concept study in CD patients (18). Initial results of the phase III study investigating osilodrostat demonstrated the use of a mean dose of approximately 8 to 15 mg per day in CD patients (17). Based on the similarities between osilodrostat and metyrapone observed in our *in vitro* study, we hypothesize that the clinically observed difference in required dose is driven by pharmacokinetic rather than pharmacodynamic differences. Indeed, metyrapone is suggested to have a shorter half-life compared to metyrapone (~2 vs ~4-5 hours) (18-20). Hypothetically, the longer half-life might result in more stable plasma levels resulting in higher efficacy *in vivo*. No solid comparison of plasma levels reached after oral administration between both compounds has been made yet. Considering the lower administered dose, there is a rationale to expect less side effects of osilodrostat. For example, it could be hypothesized that gastrointestinal upset is reduced for osilodrostat compared to metyrapone. This has, however, not been shown

in the clinical trials until now. From the proof-of-concept study in CD patients treated with osilodrostat, it also appears that responders need highly variable dosages (range 4 to 100 mg/day, factor 25 difference), with corresponding plasma levels (0.34 – 204 ng/mL) (18). For metyrapone, the range to normalize UFC in CD patients has been demonstrated to be only about 500 to 3500 mg (factor 7 difference) daily for normalization of UFC (15). Besides, hypocortisolism related adverse events were reported in 32 to 51% (n/N = 6/19 and n/N = 70/137) of the patients treated with osilodrostat (16, 17), compared to 7% of CD patients in a retrospective study on metyrapone treatment (15). These differences suggest that osilodrostat might have more diverse pharmacokinetic profiles between patients, stressing the need for careful uptitration of osilodrostat in patients in order to prevent adrenal insufficiency.

In conclusion, osilodrostat might eventually become a better alternative to metyrapone, particularly if osilodrostat indeed appears to have a superior pharmacokinetic profile. Prospective clinical trials have to further evaluate the feasibility and efficacy of osilodrostat in patients with CS, taking into account the potential side effects and safety profile. Therefore, another phase III trial studying the efficacy and safety of osilodrostat in patients with CS after 48 weeks was recently initiated (NCT02697734). As ultimate proof, a comparison study should be performed randomizing patients to either osilodrostat or metyrapone, to elucidate whether osilodrostat is more efficacious, safe, and tolerable, in CS patients compared to metyrapone.

Variable sensitivity of human adrenal (neoplastic) tissues to steroidogenesis inhibitors

For all steroidogenesis inhibitors that have been studied in this thesis, large differences in sensitivity were observed for inhibition of cortisol between primary adrenal cultures, as demonstrated by variances in the half maximal inhibitory concentration (IC_{50}). Although we aimed to make a difference between the tissue types, i.e. adrenocortical adenomas (ACA), adrenal hyperplasias and adrenocortical carcinomas (ACC), due to the small sample size, findings have to be interpreted with caution. In both studies, the lowest sensitivity was found in primary cultures of ACA. Both studies were partly based on the same primary cultures, so a solid conclusion cannot be made yet, since the different adrenal patient tissues followed the same order in sensitivity with the different steroidogenesis inhibitors. To date, only a single-nucleotide polymorphism (SNP) in the CYP17A1 gene has been shown to be associated with the response to ketoconazole and metyrapone in CS patients (21). Considering the small sample size and individual dose titration schemes in this study, these results have to be interpreted with caution and confirmed in larger populations. To investigate a direct relationship of this SNP with the sensitivity to steroidogenesis inhibitors, the presence of this SNP could be analyzed in the adrenal patient tissues of the primary cultures and correlated with the IC_{50} values. Additional

hypothetical explanations of variable *in vitro* sensitivity could include differences in basal enzyme levels between adrenal neoplastic tissues, differences in intracellular breakdown of drugs, genetic abnormalities, or cell-dependent differences in uptake. Further research may focus on elucidating this issue in an attempt to make a step towards understanding which CS patients will specifically respond to steroidogenesis inhibitors.

Combination therapy in Cushing's syndrome

Until now, no medical therapy has revealed efficacy in all CS patients, making combination therapy necessary in a subset of patients. Several other rationales exist for combination therapy in CS; it might be necessary in refractory cases, in moderate to severe hypercortisolism, and in case rapid control is desirable. Combining drugs could allow for lower doses with potentially fewer side effects. The rationale behind combination therapy in CD patients is that targeting the somatostatin receptor with a somatostatin analogue and the dopamine D2 receptor with a dopamine agonist simultaneously may have additive or synergistic effects in decreasing ACTH secretion (22). In a clinical trial investigating sequential combination therapy, subsequent addition of cabergoline and ketoconazole to pasireotide according to UFC levels, resulted in normalization of UFC in 88% of patients (23). The combination of different steroidogenesis inhibitors has also been studied and appeared to be effective, especially in patients with severe hypercortisolism (24, 25). There are no studies with a direct comparison between ketoconazole and metyrapone. The most important challenge in combination treatment is sufficient dose titration of the separate compounds. In the future, when levoketoconazole and osilodrostat are suggested to be a better alternative to ketoconazole and metyrapone, research could focus on the potential of using the combination of these novel compounds and the combination of the individual compounds with pituitary-targeting drugs for treatment of CS and CD.

Concluding remarks and future directions

In all patients with CS, treatment should aim to completely normalize cortisol production. Medical therapy is an important option for patients with CS in order to reverse morbidity and prevent mortality in patients who cannot be cured by surgery. Recently, prospective trials have been initiated with two 'new generation' steroid synthesis inhibitors, i.e. levoketoconazole and osilodrostat. The insights in these steroidogenesis inhibitors as presented in this thesis provide a better understanding in the effects of the compounds on adrenal steroid production, also in relation to the current clinically used steroidogenesis inhibitors (Fig. 1). In general, the choice of the agent for treatment of CS should be individually tailored, taking into account the efficacy, onset of action, and side effects. In the current knowledge for long-term treatment, ketoconazole might be a better choice in women with CS, to avoid hirsutism and worsening of acne. Further research should investigate the potential of levoketoconazole as a more safe and efficacious alternative to

the currently used racemic form of ketoconazole. Metyrapone, on the other hand, could be the rational first choice in young men, considering the side-effects hypogonadism and gynaecomastia of ketoconazole. Osilodrostat appears to have very similar effects to metyrapone on *in vitro* steroidogenesis, but is thought to potenially have favorable pharmacokinetics. Further clinical trials need to be performed to elucidate the efficacy and safety of osilodrostat in CS patients, also compared to metyrapone. In conclusion, the number of medical treatment options for CS is increasing and clinical trials are being conducted to evaluate the clinical potential for treatment of CS. Clinical trials should furthermore focus on the optimal order and combination of available drugs as well as the long-term efficacy and safety for the various forms of CS.

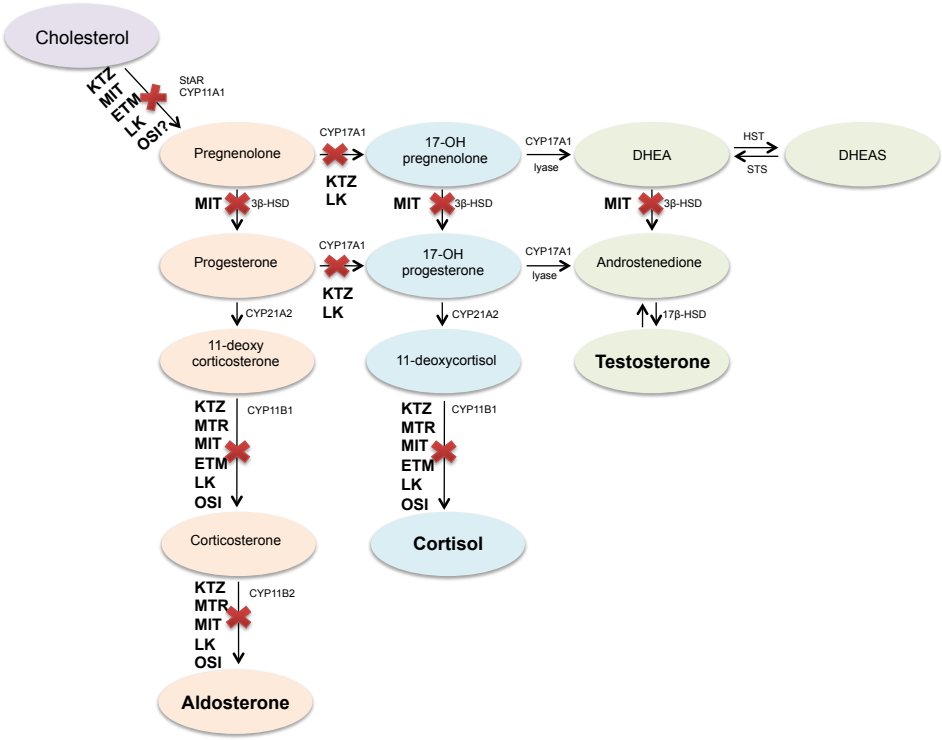

Figure 1. Overview of adrenocortical steroidogenesis and the specific pathways inhibited by steroidogenesis inhibitors. CYP, cytochrome P450 enzyme; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulphate; ETM, etomidate; HSD, hydroxysteroid dehydrogenase; HST, hydroxysteroid sulfotransferase; KTZ, ketoconazole; LK, levoketoconazole; MIT, mitotane, MTR, metyrapone; OH, hydrox; OSI, osilodrostat; STS, sulfotransferase.

PART II ADRENOCORTICAL CARCINOMA

Novel insights into diagnosis of adrenocortical carcinoma

Discrimination of adrenocortical carcinoma (ACC) from adrenocortical adenoma (ACA) is challenging, but comes with important clinical consequences, considering the choices for adjuvant treatment, intensity and duration of follow-up, and prognosis stratification. In case of a resected tumor, the Weiss score (WS) is the golden standard for pathological diagnosis of ACC (26, 27). High interobserver variability, and intratumoral heterogeneity makes the re-appraisal by an expert adrenal pathologist mandatory (26, 28). Thereby, the WS is difficult to apply in ACC variants and pediatric adrenocortical tumors, and the reliability of the WS is challenged in borderline cases, where a WS of 2 can still be associated with an ACC (29, 30). Therefore, there is an unmet need for an objective method to determine whether an adrenal tumor is benign or malignant. In **Chapter 4** and **5**, we propose a novel objective diagnostic tool by determination of methylation patterns of IGF2 regulatory regions. The European Network for the Study of Adrenal Tumors (ENS@T) consortium was used to perform an external multicenter validation study. The methylation marker is based on a frequently found alteration in ACC, i.e. increased IGF2 expression (31, 32). First, we confirmed in ACC cell lines that the *IGF2* gene is regulated by DNA methylation in ACC. Second, the IGF2 regulatory regions with the highest discriminative value were identified and included in the IGF2 methylation score. The hypothesis of the proposed score is that deregulation of the *IGF2* gene compared to normal adrenals, leading to either increased *IGF2* expression or not, is an important event in ACC. An important challenge in research focusing on novel diagnostic tools for malignancy of adrenocortical tumors is the fact that a definite diagnosis can only be made in case of local invasive tumor growth or metastases, which may occur late during follow-up (33, 34). On the other hand, there are also ACC, potentially due to adjuvant treatment, which will neither recur nor metastasize. The IGF2 methylation score seems to predict the diagnosis on the basis of the WS with high diagnostic accuracy. The performance of the IGF2 methylation score was however slightly lower in the multicenter validation study described in **Chapter 5** (sensitivity 85%, specificity 84%) compared to the study presented in **Chapter 4** (sensitivity 96%, specificity 94%), which could be due to differences in patients and tumors that were included in both studies. The multicenter multinational design may have resulted in larger heterogeneity of adrenocortical tumors compared to the first study. Besides, referral policies might be slightly different between the participating centers resulting in dissimilarities in selection of patients with adrenocortical tumors. The multicenter aspect does, however, largely increases the generalizability of our findings. In **Chapter 5**, also correlations with clinical outcomes in ACC were made. Although only the WS was independently associated with the occurrence of metastases during follow-up,

this could be affected by the limited statistical power due to a small sample size. Another important consideration is the retrospective design of the study. It has been demonstrated in two large series of ACC that in approximately 10% of cases, the diagnosis of ACC had to be revised by a reference pathologist (28, 35). Part of these cases included a change from ACC to ACA or vice versa. This indicates that initial pathological diagnoses might have been changed during follow-up or at a revision, resulting in overestimation of the predictive value of the Weiss score.

The methylation score is not designed nor proposed to replace the currently used WS. The most important advantages are that it is easy to apply, objective, and has minimal costs. Furthermore, although the diagnostic accuracy of the IGF2 methylation score is already high when applying one single cutoff value, we believe that the methylation score is especially useful when the value is below or above a proposed grey zone, eventually assuring a higher diagnostic accuracy. It is concluded that in centers with less experienced pathologists, or in centers where a second pathologist is not available for a review, the methylation score could provide extra guidance to multidisciplinary teams on decisions regarding postoperative strategies, i.e. the decision whether or not to give adjuvant treatment with mitotane after adrenalectomy. Besides, in case of a WS of 2 or 3, a high methylation score could opt for more intense follow-up, since it considers an objective and independent measure. As the diagnostic accuracy of the IGF2 methylation score increased when it was combined with tumor size, further research could investigate the combination of the IGF2 methylation score with imaging characteristics, other clinical data or image analyses from histopathology, to further improve the diagnostic accuracy. From a clinical point of view, the value of the IGF2 methylation score, also in relation to the WS, needs to be further investigated in a prospective study in order to accurately correlate the IGF2 methylation score with important clinical outcomes. This is particularly important for adrenocortical tumors with a WS of 2 or 3, the clinically most relevant group. In order to increase the clinical applicability, the performance of the IGF2 methylation score could furthermore be validated in DNA isolated from paraffin embedded adrenocortical tumors.

Monitoring tumor dynamics

In ACC, accurate prognostic and predictive biomarkers are lacking, raising attention towards novel approaches. Circulating cell-free tumor DNA (ctDNA) is a promising method to evaluate tumor dynamics, since it is a non-invasive method that overcomes the problems related to tumor heterogeneity and accessibility (36, 37). It thereby has a short half-life of approximately 2 hours (38). In patients with localized breast or colon cancer, ctDNA analysis has been shown to identify minimal residual disease shortly after completion of local therapy (39, 40). For genotyping, analyzing ctDNA might be

more appealing compared to circulating tumor cells, since plasma can be collected and analyzed without prior enrichment and without the technical challenges associated with isolation of a rare cell population (41). In **Chapter 6**, we determined the potential value of detecting ctDNA in a case series of patients with ACC. The identification of ctDNA is based on the detection of tumor-specific aberrations, which can be both genetic and epigenetic, in circulating cell-free DNA (cfDNA) (42). Epigenetic aberrations often include hypermethylation of the promoter region of tumor suppressor genes. The IGF2 methylation score, as described in **Chapter 4** and **5** of this thesis, could hypothetically serve as an ACC specific aberration. The challenge as it comes to the detection of these epigenetic aberrations in cfDNA is that the methylation in non-tumoral DNA has a theoretical level of 50%, since *IGF2* is an imprinted gene. The varying fraction of cfDNA derived from the tumor (0.01% to 90%) will drive the DNA methylation towards the methylation in healthy DNA and makes this for now an inconvenient approach (38). As a proof of concept for the presence of ctDNA in patients with ACC, we therefore considered in our approach the mutations, found by next-generation sequencing (NGS), in the primary tumor as a marker for tumoral DNA. This, in turn, limits the technique only to primary tumors with a mutation detected by NGS. In ACC, this definitely should be considered as a limiting factor, since mutations have not been found in all ACC (43). Mutations were found in the primary tumor in three of the six patients included in the pilot study. In these three patients, cfDNA was isolated, and mutations comparable with the primary tumor were found in one of these three patients. As expected, the mutation frequencies were lower in the cfDNA compared to the primary tumor DNA, because only ctDNA includes mutations and the fraction of ctDNA will be influenced by tumor heterogeneity and the cfDNA amount released by apoptotic cells from healthy or inflamed tissues unrelated to the tumor. The three mutations did follow the same order of relative presence in the cfDNA as the primary tumor. The three mutations were found at two time moments in the cfDNA, two weeks before surgery and 6 months postsurgery, with the lowest frequencies at the second time point. Mutation detection in leucocytes served as a negative control. Although not all patients may have ctDNA in the plasma, research should focus on more sensitive methods that are potentially necessary to detect tumor-specific mutations in cfDNA among the many thousands of non-tumoral DNA fragments. Several modified targeted sequencing approaches have been developed using molecular barcodes (unique identifiers), in order to reduce the background error rate of sequencing and detect one mutant allele in 10,000 DNA template molecules (44). Thereby, allele-specific PCR, and digital droplet PCR, are frequently used (42). The clinical application in ACC of the latter two will probably be limited, since only a small number of genomic positions can be analyzed and therefore a separate assay needs to be designed for each individual patient based on the mutation in the primary tumor. When ctDNA is investigated in a larger group of ACC patients, background noise can be quantified in

order to set a true threshold for detection of mutations. Research has already shown that mutations at low frequencies (from 0.5%) might be clinically relevant in patients with metastatic solid tumors, as they found high concordance between mutations in tumor DNA and cfDNA (45). In a more recent report from Garinet et al., our results in ACC were confirmed (46). Mutations in cfDNA were found in two of the eight ACC patients by using both high-depth NGS and digital droplet PCR. Both patients had metastatic disease with high tumor burden, and ctDNA paralleled the tumor evolution (46).

In this thesis, we provide a rationale for further research into the identification of ctDNA in ACC, although this release seems to be variable between patients. Whether this detection could eventually function as a marker for tumor burden, response to treatment, or prognosis stratification has to be investigated in further studies. Besides, ctDNA might add pivotal molecular information on mechanisms underlying treatment response, considering that parts of the tumor with cells that do not respond to systemic therapies will remain present in the body and release ctDNA in the circulation during treatment. Several factors, like tumor burden and progression, systemic therapies, tumor characteristics, presence of circulating tumor cells, the detection technique, and the ratio of ctDNA versus cfDNA, might impact ctDNA detection and deserve further investigation in ACC.

Advances in understanding mitotane action in ACC

Complete R0 resection of ACC is the keystone and only curative treatment modality for patients with ACC. However, many patients present with large tumors and already at an advanced disease stage (47). Systemic therapies are necessary in these patients, but also in patients presenting with recurrent disease, which occurs frequently (30-50%) (48-50). Currently, in order to reduce these high recurrence rates, adjuvant mitotane treatment is recommended after adrenalectomy, particularly in ACC patients with features that are associated with a high recurrence risk (stage III, Ki67 > 10%, R1 or Rx resection) (26). Response rates to systemic therapies are generally very low, which makes new therapeutic strategies urgently needed.

Mitotane is the only approved drug for treatment of ACC, but is associated with severe toxicity, even in patients with subtherapeutic plasma concentrations of mitotane. Besides achieving the therapeutic plasma concentration of 14-20 mg/L (51-53), additional factors are urgently needed to identify subgroups of patients who will respond to mitotane. In **Chapter 7**, we determined the direct antitumor effects of mitotane in a unique panel of primary human ACC cultures and made a correlation of *in vitro* mitotane sensitivity with previously proposed predictive factors, as well as clinical and tumor characteristics. Corresponding with *in vivo* data, one-third of ACC patients was classified as responder *in vitro*, and sensitivity appears to be highly variable between tumors from individual

patients. These *in vitro* findings suggest that *in vivo* differences might also be the result of differences in direct antitumor effects, rather than only pharmacokinetics. Further research should focus on the correlation of the effects of mitotane in primary ACC cultures and the clinical effects of this drug in patients. When *in vitro* and *in vivo* effects appear to be highly correlated, the mitotane effect on primary cultures postsurgically may eventually serve as a predictor for mitotane response in patients and contribute to the decision to start therapy with mitotane. Mitotane is a more potent inhibitor of cortisol production compared to its effect on cell number, suggesting that monitoring steroid production is not an adequate marker of treatment response. Tumoral cortisol production has previously been identified as a negative prognostic factor (54). Thereby, mitotane is thought to predominantly increase disease-free survival in patients with cortisol-secreting ACC, although data have been inconsistent (55, 56). Explanations for increased benefit of mitotane treatment include improvement of Cushing's syndrome comorbidities, and less immunosuppressive effects of cortisol. The better response to mitotane *in vitro* in cortisol-producing ACC compared to non-cortisol-producing ACC, supports the hypothesis of an increased direct antitumor effect of mitotane in cortisol-producing, compared to non-cortisol-producing ACC. One of the explanations could be involvement of het SOAT1 gene, since this has recently been identified as an important prerequisite for mitotane action (57). SOAT1 expression is thought to be higher in cortisol-producing ACC compared to ACC that do not secrete cortisol. In this *in vitro* study however, we did not find a correlation of the previously proposed predictive factors RRM1, SOAT1 and CYP2W1 with sensitivity to mitotane *in vitro*, which might be caused by lack of power or differences between *in vitro* and *in vivo* sensitivity. We also provide a potentially more reliable SOAT1 antibody for immunohistochemistry, which was correlated with *SOAT1* mRNA expression and specifically stained the adrenal cortex, in contrast to the polyclonal antibody that was previously used in ACC (57).

In conclusion, we show that the direct effects of mitotane on human ACC cultures are highly variable, reflecting clinical practice. Furthermore, we provide evidence that cortisol secretion by ACC is associated with enhanced mitotane sensitivity due to increased direct antitumor effects of mitotane. The role of RRM1, SOAT1, and CYP2W1 expression in mitotane sensitivity remains to be elucidated, and when studying these factors in a larger panel of ACC, we recommend taking into account the potential advantages of the monoclonal SOAT1 antibody.

Chemotherapy in adrenocortical carcinoma

In patients who progress under mitotane, in advanced ACC not qualified for local treatment, or in case of a recurrence within 6 months of surgery/loco-regional therapy, mitotane with etoposide, doxorubicin, and cisplatin (M-EDP) is recommended (26).

Since the first randomized controlled trial in ACC, the FIRM-act trial, this regimen is the golden chemotherapeutic standard for late-stage disease ACC patients (26). This treatment regimen is however associated with serious adverse events, like bone marrow toxicity, infection, cardiovascular or thromboembolic events, neurotoxicity, and general health detoriation (26). M-EDP appeared to be superior to mitotane with streptozotocin, with a median progression-free survival of 5.0 vs 2.1 months, respectively. However, the overall survival of the M-EDP regimen was still only 14.8 months (58). Only a few other large trials have been conducted studying chemotherapy in ACC, all showing relatively disappointing results. The unresponsiveness of ACC to chemotherapy has previously been associated with the multi-drug resistance (*MDR1*) gene product, P-glycoprotein (P-gp) (59, 60). P-gp is highly expressed in the normal adrenal gland, as it is thought to function as a biological barrier by extruding toxic substances and permits adrenal cells to handle their high steroid environment (61). In **Chapter 8**, we show that the median expression of P-gp is lower in ACC compared to ACA and normal adrenals. Most importantly, however, the majority of ACC cases appear to have P-gp expression levels similar to normal adrenals. We hypothesize that particularly in these patients, inhibition of P-gp might be effective. We furthermore analyzed all chemotherapeutic agents of the M-EDP regimen *in vitro*, with and without the P-gp inhibitors verapamil and tariquidar. Two different techniques were used: the cell growth assay, in which direct effects after 7 days of treatment can be studied, and the colony forming assay, which reflects the long-term effects (2 weeks without treatment) of a short-term (3 days) period of treatment. We show that inhibiting P-gp especially increases sensitivity to doxorubicin and etoposide, two known substrates of the P-glycoprotein pump (60). In contrast, the sensitivity to cisplatin decreased when it was combined with P-gp inhibitors. In general, the role of P-gp inhibitors in clinical practice is controversial. Tariquidar, a third generation P-gp inhibitor, has shown minimal interference with the pharmacokinetics of cytotoxic agents and appears to be effective in our study at a concentration that can be reached in plasma of patients (62, 63). It therefore holds, compared to verapamil, the most promise in clinical trials. On top of that, tariquidar has already shown to inhibit P-gp *in vivo* in patients with ACC (64). By combining chemotherapeutic drugs with P-gp inhibitors, the dose of the cytotoxic agents could potentially be decreased, resulting in improved tolerance.

We show that P-gp is a potential target for improving chemotherapeutic treatment in ACC, although several issues need to be addressed before applying this concept in patients with ACC. First, performing similar experiments in primary ACC cultures gives the opportunity to correlate basal P-gp expression levels and P-gp inhibition, with efficacy to chemotherapeutic agents. As such, we can confirm the relevance of selecting patients with high P-gp expression to potentially increase success rates of combination therapy. Decreased sensitivity to cisplatin in combination with tariquidar

thereby marks the importance of carefully selecting compounds, since the compounds may have counteracting effects and unnecessary side effects can be prevented. At present, no study provides evidence of additional value of the M-EDP regimen over a selection of these chemotherapeutic agents, like only mitotane, etoposide, and doxorubicin (M-ED). A case series or pilot trial using these three compounds in combination with tariquidar, preferably in ACC patients with proven high tumoral P-gp expression levels, should eventually show whether this regimen results in tumor response, and increased tolerance. On the basis of these findings, a larger clinical study could be performed when there are signs of response of ACC to tariquidar in combination with the M-ED regimen.

In an attempt to further improve the chemotherapeutic options for patients with ACC and explore compounds that have not been studied before in ACC, we evaluated in **Chapter 9** the effects of temozolomide (TMZ) on ACC cells *in vitro*. The main benefit of this compound is that it is already used in clinical practice in glioblastoma patients, increasing its clinical potential. TMZ is an alkylating prodrug, delivering a methyl group to purine bases of DNA (65). A good response to TMZ requires a low expression level of O6-methylguanine DNA methyltransferase (MGMT), a functional mismatch repair (MMR) complex and DNA base excision repair (BER) (65). Glioblastoma can be resistant to TMZ, either inherent or acquired during treatment as a result of selective pressure. The use of TMZ might be considered according to the *MGMT* promoter methylation status (66). Also other factors, like P-gp expression and non-coding RNAs, have been implicated in TMZ sensitivity (65). TMZ has thereby shown efficacy in 17 of 25 patients with poorly differentiated endocrine carcinomas (mainly gastro-intestinal), and in various other tumors (67, 68). In the study described in **Chapter 9**, we show that TMZ has a dose-dependent antitumor effect on ACC cells, primarily by inducing the expected cell-cycle arrest and apoptosis. The growth inhibition of multicellular three-dimensional ACC spheroid cultures and primary ACC cultures shows efficacy of TMZ in a more (patho)physiologically relevant context. An important consideration is that the effects observed in this study occurred at higher concentrations than the concentrations that have been achieved in plasma of patients treated with TMZ (69-71). Plasma concentrations of TMZ do, however, not necessarily represent intratumoral concentrations. A study in glioma patients using positron-emission tomography data to detect TMZ distribution *in vivo*, showed that the TMZ peak concentration and total drug exposure was about two times higher in glioma tumors compared to normal brain and plasma (72). This was potentially attributed to breakdown of the blood-brain barrier and to increased intratumoral angiogenesis (72). The normal adrenal cortex is one of the most highly vascularized tissues in humans, with a single layer of endocrine cells surrounding each sinusoidal capillary (73). Adrenocortical carcinomas have been shown to have a similar or even higher vascularization compared to normal adrenals (74). Besides, vascular endothelial growth factor (VEGF) and the VEGF-receptor

are overexpressed in ACC (75, 76), altogether suggesting high vascularization in ACC and potential high uptake of TMZ. In addition, TMZ undergoes spontaneous hydrolysis at a physiological pH to its active metabolite 3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MTIC), which biotransformation may be variable based on differences in pH (77). The pH in ACC is unknown and could also be different between tumors. Consequently, this limits the ability of plasma concentrations to adequately reflect TMZ levels at target tissue sites. The measured plasma concentrations of TMZ thereby do not take into account the fraction of already activated TMZ, MTIC, in the blood.

The sensitivity of ACC cells is comparable with the least sensitive glioma cell lines, which have an active *MGMT* gene by hypomethylation of the *MGMT* promoter (78). Using pyrosequencing, the threshold for defining a hypo- or hypermethylated *MGMT* promoter varies between 2.68% and 45%, dependent on the study and the exact CpGs studied (79). Many studies also report on the difficulties of dichotomizing the pyrosequencing data and setting the correct threshold. The majority of glioblastomas have an MGMT hypomethylated phenotype (80). In our ACC panel, methylation percentages are low, and further studies need to investigate the potential predictive value for TMZ sensitivity, since the methylation percentage range which has been found in ACC, already appears to be associated with different clinical outcomes in glioblastoma patients. The primary ACC cultures that have been investigated all had an MGMT methylation below 5%. Increased sensitivity might be expected for ACC with a higher percentage of MGMT methylation. Since there are almost no options when patients do not respond to mitotane or the M-EDP regimen, all other therapies should be carefully considered. Preliminary data of an Italian multicenter retrospective study in twenty-six ACC patients showed a clinical benefit, defined as partial response or disease stabilization, of TMZ treatment in ten patients (38.5%) (81). Although the median progression-free survival (3.5 months) and overall survival (7 months) were limited, we have to acknowledge that these patients were progressive under the conventional M-EDP regimen. Toxicities were limited to grade G1-2, confirming TMZ safety and tolerability. It will be important to assess the *MGMT* gene status in the tumors of these patients. Prospective trials are needed to further assess the therapeutic potential of TMZ, with or without potential compounds increasing sensitivity to TMZ, as described in the following section.

Several ways could be investigated to increase the sensitivity to TMZ in ACC. In a slow-growing neuroendocrine tumor cell line, a synergistic cell kill was found when capecitabine was administered prior to TMZ (82). Capecitabine, which is converted to the active form 5-FU, depletes MGMT expression levels, thereby enhancing the alkylating effect of TMZ (83). Another explanation is reduction of the thymidine pool by inhibition of thymidylate synthase by capecitabine (82), attenuating the mismatch repair process by

TMZ, and leading to breaks in the DNA and stimulation of apoptosis (82). By performing combination experiments and using isobolograms, the potential synergistic effect of the CAPTEM regimen could be investigated *in vitro* in ACC (84). This combination regimen could increase the sensitivity and prevent resistance to either one of the single agents. Furthermore, by combination therapy, total accumulation of chemotherapy drugs could be increased, without increasing overall toxicity. For example, in the CAPTEM regimen, TMZ is only administered for 5 days per treatment month, which can reduce bone marrow toxicity. Another focus of research could include the role of inhibition of P-gp on TMZ sensitivity, as described in **Chapter 8** with other chemotherapeutic compounds. TMZ has been shown to be a substrate of P-gp and TMZ resistance was reversed by the use of three different P-gp inhibitors in glioblastome cells *in vitro* (85). Thereby, MGMT inhibition is an interesting therapeutic approach to circumvent TMZ resistance. Several agents have the ability to decrease MGMT expression and increase TMZ sensitivity, like O(6)-benzylguanine (O(6)-BG) and lomeguatrib, which are now being tested in clinical trials and preclinical studies, respectively (65, 86, 87). Preclinical studies in ACC could focus on the potential of increasing sensitivity in ACC using these compounds.

Concluding remarks and future directions

Due to its rarity, collaborative efforts, like the European Network of the Study of Adrenal Tumors, have emerged and have made important contributions in the research field of ACC. Continued collaborations and large consortia will be necessary to eventually improve the diagnostic and therapeutic strategy for patients with ACC. Large molecular studies until now mainly identified subgroups of ACC patients with different clinical outcomes. These studies necessitate further validation of the most promising abnormalities in order to be able to extrapolate these large data to the individual patient. In this thesis, we provide novel insights into the diagnosis of adrenocortical carcinoma by targeting one of the frequently found abnormalities, i.e. increased IGF2 expression, offering a potential additive tool to the subjective and difficult to apply Weiss Score. Prospective trials are needed to further investigate the discriminative value of the IGF2 methylation score. The use of liquid biopsies may possibly contribute to a more personalized medicine approach for this rare and aggressive malignancy, and may in the future provide novel insights into sensitivity to systemic therapies or molecular drivers of ACC. In order to improve the therapeutic potential of compounds in ACC, the heterogeneity inherent to ACC drives the need for patient selection, based on tissue expression levels, genotype, tumor and patient characteristics. This thesis contributes to the further understanding of the effects of mitotane on human ACC, which will hopefully eventually lead to a thought-out selection of patients that will respond to mitotane. Several compounds now under development for treatment of ACC make specific use of the characteristics of the adrenal cortex and adrenocortical tumors, ensuring specific targeting of the adrenal cortex. Examples of

these are liposomal forms of drugs, considering that 75% of the cholesterol in the adrenal comes from plasma lipoproteins, targeting the steroidogenic pathway, and targeting P-gp expression, as demonstrated in this thesis. We provide evidence that in the majority of ACC patients, targeting P-gp might enhance sensitivity to specific chemotherapeutic drugs already used in clinic. However, the low incidence of ACC challenges a biomarker-based strategy in clinical trials, again emphasizing the need for international collaborations in order to make progress in the standard of care protocol of patients with ACC. The fact that the first large international and multicenter collaborative studies have been conducted recently, gives hope for the future as it comes to the recruitment of ACC patients for new clinical trials. When designing clinical trials, it is crucial to search for well-considered (combinations of) therapies, taking into account effects of drugs on cellular processes, pharmacokinetics and -dynamics, side effects, interactions between compounds, and selecting patients with the highest probability of response, with as final goal making substantial progress in the clinical management of ACC.

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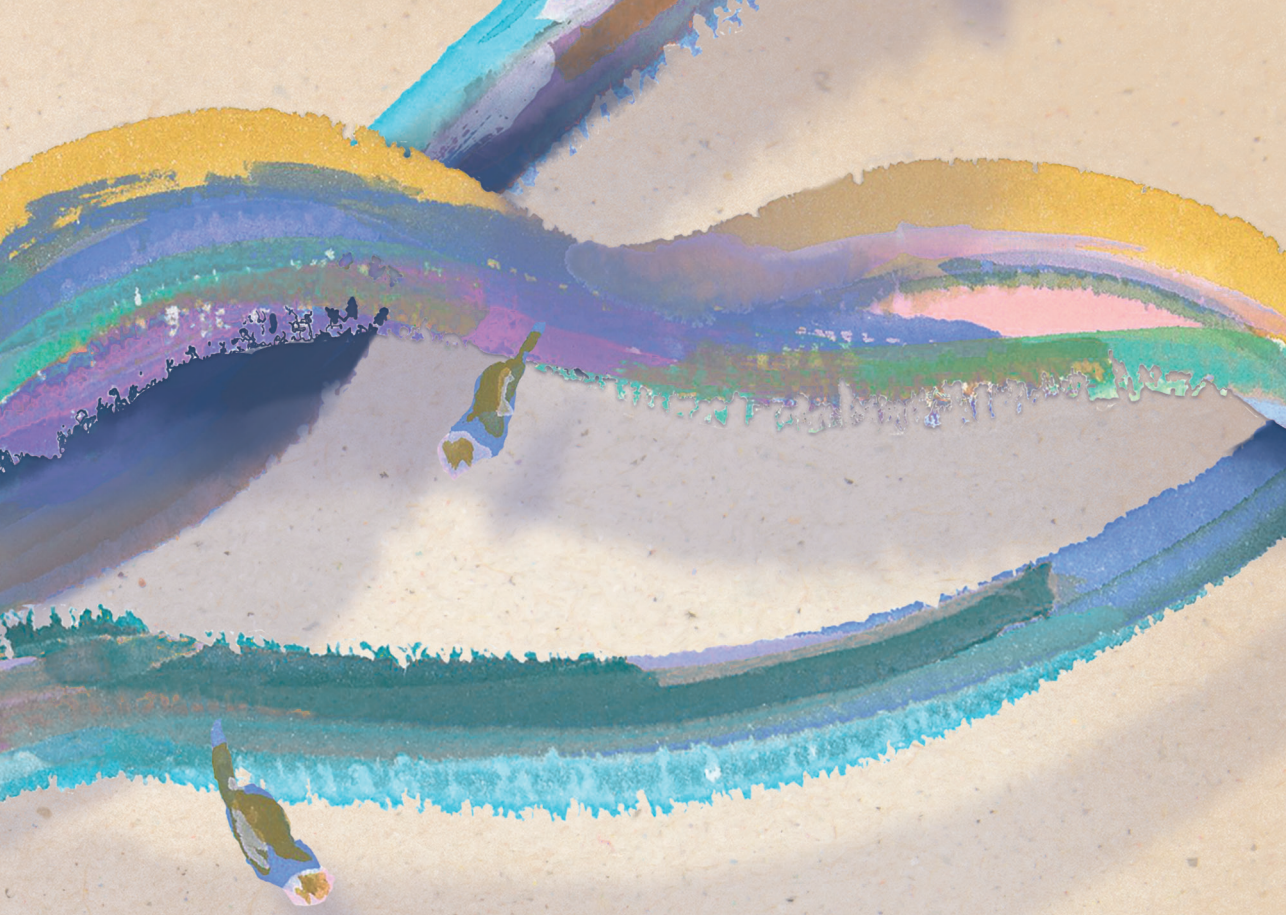


CHAPTER 11

Summary/Samenvatting

PART I CUSHING'S SYNDROME

Cushing's syndrome (CS) is characterized by chronic exposure to excess cortisol levels and is, when untreated, associated with an increased morbidity and mortality. CS can be caused by a pituitary adenoma, ectopic adrenocorticotropic hormone (ACTH) production, or primary adrenal disease. Several pharmacotherapeutic targets have been identified, i.e. pituitary targeting drugs, adrenocortical steroidogenesis inhibitors and glucocorticoid receptor blockers. The mechanism of action, efficacy and side-effects are described in **Chapter 1**. Finally in this chapter, the aims of the first part of this thesis, i.e. to examine the effects of two novel adrenal steroidogenesis inhibitors *in vitro*, are described.

Ketoconazole is a steroidogenesis inhibitor, consisting of a 50/50 racemic mixture of two enantiomers (2S,4R and 2R,4S). In **Chapter 2,** the effects of the novel drug levoketoconazole, a purified 2S,4R enantiomer of ketoconazole, were studied on adrenocortical steroidogenesis, as well as on ACTH secretion by pituitary corticotroph adenoma cells. We show that for both levoketoconazole and racemic ketoconazole, sensitivity to the steroidogenesis inhibitory effects of both drugs varies largely between adrenal patient tissues. Levoketoconazole is a slightly more potent inhibitor of cortisol production and the steroid profile *in vitro* compared to racemic ketoconazole. Levoketoconazole might also have effects on pituitary adenomas, as defined by a decrease in cell number and inhibition of ACTH secretion. Previous research has already demonstrated that levoketoconazole might have a favorable safety profile and a lower hepatic metabolism. Together with these potential advantages, levoketoconazole is a promising novel drug for treatment of CS. Clinical trials are needed to assess efficacy and safety of levoketoconazole *in vivo* in patients with CS.

Chapter 3 describes a comparison between osilodrostat (LCI699), a potent aldosterone synthase and 11β-hydroxylase inhibitor, and metyrapone and ketoconazole on human adrenocortical cells and pituitary adenoma cells. All three steroidogenesis inhibitors inhibited cortisol production with a large variability in sensitivity between different adrenal patient tissues. We show that osilodrostat inhibits cortisol production more potently compared to ketoconazole, whereas differences with metyrapone were not evident. Inhibition of aldosterone was significantly stronger by osilodrostat compared to both other compounds. Effects of osilodrostat and metyrapone on the steroid profile were highly comparable. No pituitary-directed effects of osilodrostat were observed. It is concluded that osilodrostat is a promising medical treatment option for CS, in which *in vivo* differences with metyrapone appear to be potentially driven by pharmacokinetic differences and not by differences in the direct effects on adrenocortical steroidogenesis.

PART II ADRENOCORTICAL CARCINOMA

The second part of this thesis focuses on adrenocortical carcinoma (ACC), a rare malignancy with a poor prognosis. The Weiss score (WS), consisting of nine histopathological parameters, is currently considered as the "gold standard" for ACC diagnosis. Regarding therapy, successful tumor-directed surgery is the only curative therapy. In patients with high risk of recurrence after resection, adjuvant mitotane treatment is recommended. In case of progression of advanced disease, mitotane can be combined with etoposide, doxorubicin, and cisplatin. However, systemic therapies are accompanied by severe side-effects and have limited efficacy. **Chapter 1** provides an overview of the current standard of care and elaborates on the recent novel findings in the field of diagnosis and treatment of ACC. Lastly, the general aims and outline of the second part of this thesis are described.

Discrimination of ACC from adrenocortical adenoma (ACA) is currently challenging, and based on histopathological grounds. There is an unmet need for molecular markers that can confirm malignancy of adrenocortical tumors. **Chapter 4** describes a potential new objective diagnostic tool based on the most frequently found aberration in ACC, i.e. increased IGF2 expression. DNA methylation percentages were assessed in several IGF2 regulatory regions. In two independent Dutch cohorts, we show that a combined standard deviation score of three different IGF2 regulatory regions (IGF2 methylation score) can identify ACCs with a sensitivity of 89-96% and specificity of 92-100%. By performing this study, we introduce a new concept, which might lead to the first objective diagnostic tool to identify malignant adrenocortical tumors.

In **Chapter 5**, the diagnostic value of the IGF2 methylation score was validated in a retrospective multicenter European Network for the Study of Adrenal Tumors (ENSAT) study. The IGF2 methylation score and tumor size were independently associated with the pathological diagnosis of ACC. In multivariate analysis, only the WS was predictive for the development of metastases during follow-up. We furthermore propose a grey zone in which the discriminative value of the IGF2 methylation score is less accurate and present clinically useful cutoff values for determination of malignancy. Considering the known limitations in clinical applicability of the WS, the objective IGF2 methylation score could provide extra guidance to multidisciplinary teams on decisions regarding postoperative strategies in patients with adrenocortical tumors. Prospective studies are needed to validate these findings.

Markers for response to systemic therapies and prognosis stratification are limited in ACC. In other types of cancer, circulating cell-free tumor DNA (ctDNA), characterized by the presence of tumor-specific aberrations in circulating cell free DNA (cfDNA), has proven to be effective in tracking tumor dynamics. **Chapter 6** describes a proof-of-concept study, in which we demonstrate that it is possible to identify ctDNA in ACC patients, using the tumor-specific

mutations as marker. The amount of ctDNA release appears to vary among ACC patients. We provide a basis for further research and furthermore discuss that other approaches might be required to detect tumor response and monitor tumor dynamics.

For a better understanding of the effects of mitotane on ACC, we describe in **Chapter 7** the effects of mitotane in 32 primary human ACC cultures. Mitotane inhibited cortisol production at significantly lower concentration compared to the effect on cell number. We show that inhibitory effects of mitotane on cell number in human ACC *in vitro* are highly variable, reflecting clinical practice. No association was found between *in vitro* mitotane sensitivity and RRM1, SOAT1, and CYP2W1, three previously proposed predictive factors for mitotane sensitivity *in vivo*. The percentage cortisol-producing ACC gradually increased with increasing response to mitotane, indicating that cortisol secretion by ACC might be associated with enhanced mitotane sensitivity due to increased direct antitumor effects of mitotane..

In an attempt to improve the chemotherapeutic sensitivity in ACC, the role of P-glycoprotein, encoded by the multidrug resistance (MDR1) gene, was studied in **Chapter 8**. P-glycoprotein is a protein that pumps substrates, like chemotherapy, out of the cells. The majority of ACC has a level of P-glycoprotein expression comparable with normal adrenals. We show that in the ACC cell lines H295R and HAC15, sensitivity to doxorubicin and etoposide can be increased when co-treated with either verapamil or tariquidar, two known P-gp inhibitors. Our findings were confirmed using a colony formation assay. It is concluded that MDR1 is involved in sensitivity to specific cytotoxic drugs and could be a potential target for improvement of cytotoxic treatment in ACC.

Conventional therapy of patients with ACC is often accompanied by severe toxicity and tumor progression, so novel therapeutic options are urgently needed. **Chapter 9** shows the effects of temozolomide (TMZ), a DNA-alkylating agent, on ACC cells *in vitro*. TMZ is incorporated in the standard care protocol for patients with malignant gliomas. In this study, we show that TMZ exerts several antitumor responses in 3 ACC cell lines, like a decrease in cell growth, induction of apoptosis, cytotoxic and cytostatic effects, as well as a cell cycle arrest. Furthermore, TMZ reduced the cell amount in 5 out of 8 primary ACC cultures. We showed efficacy of TMZ against three-dimensional spheroids of ACC cells, giving the opportunity to study effects of TMZ on ACC cells in a more (patho)physiologically relevant context. Based on analysis of the *MGMT* gene status in ACC cell lines and adrenocortical tissues, we hypothesized that efficacy of TMZ in ACC cells might be limited by low methylation of the *MGMT* gene. Further (pre) clinical studies are warranted to assess efficacy in ACC.

Chapter 10 contains a general discussion in which the research described in this thesis is reviewed in a broader perspective and suggestions for future directions are provided.

DEEL 1 HET SYNDROOM VAN CUSHING

Het syndroom van Cushing wordt gekarakteriseerd door chronische blootstelling aan te hoge cortisol waardes en is onbehandeld geassocieerd met verhoogde morbiditeit en mortaliteit. Het syndroom van Cushing kan veroorzaakt worden door een hypofyse adenoom, ectopische adrenocorticotroop hormoon (ACTH) productie of door een primaire bijnier aandoening. Verschillende farmacologische aangrijpingspunten zijn reeds geïdentificeerd: de hypofyse, de steroidgenese in de bijnier en de glucocorticoïd receptor op weefsel niveau. **Hoofdstuk 1** van dit proefschrift beschrijft het werkingsmechanisme, de effectiviteit en de bijwerkingen van farmacotherapie voor het syndroom van Cushing. Het hoofdstuk wordt afgesloten met de doelstellingen van het eerste deel van dit proefschrift, namelijk het onderzoeken van de effecten van twee nieuwe steroidgenese remmers *in vitro*.

Ketoconazol is een steroidgenese remmer, bestaande uit een racemisch (50:50) mengsel van twee enantiomeren (2S,4R en 2R,4S). In **hoofdstuk 2** worden de effecten van het nieuwe geneesmiddel levoketoconazol (COR-003), de 2S,4R enantiomeer van ketoconazol, op steroidgenese in de bijnier en op ACTH secretie door hypofyse adenoom cellen onderzocht. Voor zowel levoketoconazol als racemisch ketoconazol varieert de gevoeligheid voor inhibitie van cortisol sterk tussen bijnier weefsels afkomstig van verschillende patiënten. Levoketoconazol blijkt een krachtigere remmer van de *in vitro* cortisol productie en van het steroid profiel, vergeleken met racemisch ketoconazol. Levoketoconazol kan daarnaast een ACTH en celgroei remmend effect hebben op hypofyse adenoom cellen. Eerder onderzoek heeft reeds aangetoond dat levoketoconazol mogelijk veiliger is en een lager lever metabolisme heeft. Samen met deze potentiële voordelen is levoketoconazol een veelbelovende nieuwe behandeling voor patiënten met het syndroom van Cushing. Klinische studies zijn nodig om de effectiviteit en veiligheid in patiënten met het syndroom van Cushing te onderzoeken.

Hoofdstuk 3 beschrijft een vergelijking van de effecten van osilodrostat (LCI699), een potente aldosteronsynthase en 11β-hydroxylase remmer, met metyrapon en ketoconazol op de humane bijnier steroidgenese en hypofyse adenoom cellen. Bijnier weefsels afkomstig van verschillende patiënten laten een sterke variatie zien in gevoeligheid voor cortisol remming door osildorostat, metyrapon en ketoconazol. Osilodrostat is een krachterigere remmer van cortisol productie *in vitro* vergeleken met ketoconazol, maar er werd geen duidelijk verschil gevonden met metyrapon. Aldosteron afgifte werd significant sterker geremd door osilodrostat vergeleken met de twee andere geneesmiddelen. De effecten van osilodrostat en metyrapon op het steroid profiel waren zeer vergelijkbaar. Er werden geen directe effecten van osilodrostat op hypofyse adenomen gevonden. Er wordt

geconcludeerd dat osilodrostat een veelbelovende nieuwe behandelings optie is voor het syndroom van Cushing, waarbij klinische verschillen met metyrapon waarschijnlijk gedreven worden door verschillen in farmacokinetiek en niet door verschillen in het directe effect op de bijnier steroidgenese.

DEEL 2 BIJNIERSCHORSCARCINOOM

Het tweede deel van dit proefschrift richt zich op het bijnierschorscarcinoom, een zeldzame maligniteit met een slechte prognose. De Weiss score (WS), bestaande uit 9 pathologische parameters, is de huidige gouden standaard voor diagnose van het bijnierschorscarcinoom. Betreffende de behandeling is operatieve verwijdering van de tumor de enige kans op genezing. Voor patiënten met een hoog risico op recidief na resectie wordt adjuvante behandeling met mitotaan geadviseerd. In het geval van progressie van gevorderde ziekte kan mitotaan gecombineerd worden met etoposide, doxorubicine en cisplatine. Systemische behandelingen gaan echter vaak gepaard met ernstige bijwerkingen en hebben een beperkte effectiviteit. In **hoofdstuk 1** wordt een overzicht van de huidige zorg van patiënten met bijnierschorscarcinoom gegeven en wordt ingegaan op de recente nieuwe ontwikkelingen omtrent de diagnose en behandeling van deze groep patiënten. Tenslotte worden de algemene doelen van het tweede deel van dit proefschrift beschreven.

Onderscheid tussen bijnierschorscarcinomen en bijnierschorsadenomen kan moeilijk zijn en dit onderscheid wordt momenteel gebaseerd op histopathologie. Er is een onvervulde behoefte aan moleculaire markers die maligniteit van bijnierschorstumoren kunnen bevestigen. In **hoofdstuk 4** wordt een potentiële nieuwe diagnostische test beschreven, die is gebaseerd op verhoogde IGF2 expressie, de meest voorkomende afwijking in bijnierschorscarcinomen. DNA methylatie percentages werden bepaald in verschillende IGF2 regulerende gebieden. In twee onafhankelijke Nederlandse cohorten kon een gecombineerde standaard deviatie score van drie IGF2 regulerende gebieden (IGF2 methylatie score) bijnierschorscarcinomen identificeren met een sensitiviteit van 89-96% en een specificiteit van 92-100%. In deze studie wordt een nieuw concept geïntroduceerd, die mogelijk kan leiden tot de eerste objectieve diagnostische test voor het aantonen van maligne bijnierschorstumoren.

Hoofdstuk 5 beschrijft een retrospectieve multicenter European Network for the Study of Adrenal Tumors (ENSAT) studie, waarin de IGF2 methylatie score zoals gepresenteerd in **hoofdstuk 4** wordt gevalideerd. De IGF2 methylatie score en de tumorgrootte waren onafhankelijk geassocieerd met de pathologische diagnose van het

bijnierschorscarcinoom. In multivariate analyse was alleen de WS voorspellend voor de ontwikkeling van metastasen. In dit hoofdstuk stellen we een grijs gebied voor waarin de discriminerende waarde van de IGF2 methylatie score minder accuraat is en we presenteren klinisch bruikbare cutoff waardes voor het vaststellen van maligniteit. Gezien de bekende beperkingen in de klinische toepasbaarheid van de WS zou de objectieve IGF2 methylatie score extra richting kunnen geven aan multidisciplinaire teams voor de beslissing omtrent postoperatieve strategieën in patiënten met bijnierschorstumoren. Prospectieve studies zijn nodig om deze bevindingen te valideren.

Markers voor de respons op systemische behandelingen en prognose stratificatie zijn beperkt beschikbaar voor patiënten met het bijnierschorscarcinoom. In andere type maligniteiten is het aantonen van circulerend cel-vrij tumor DNA (ctDNA), gekarakteriseerd door de aanwezigheid van tumor-specifieke alteraties in circulerend cel-vrij DNA (cfDNA), effectief gebleken in het monitoren van tumor dynamiek. **Hoofdstuk 6** beschrijft een proof-of-concept studie, waarin we laten zien dat het mogelijk is om ctDNA, gekarakteriseerd door tumor-specifieke mutaties, aan te tonen in patiënten met bijnierschorscarcinoom. De mate waarin ctDNA wordt uitgescheiden lijkt te verschillen tussen patiënten. Deze proof-of-concept studie vormt een basis voor verder onderzoek en laat zien dat er mogelijk andere benaderingen nodig zijn om de respons op systemische behandelingen vast te stellen en de tumor dynamiek te monitoren.

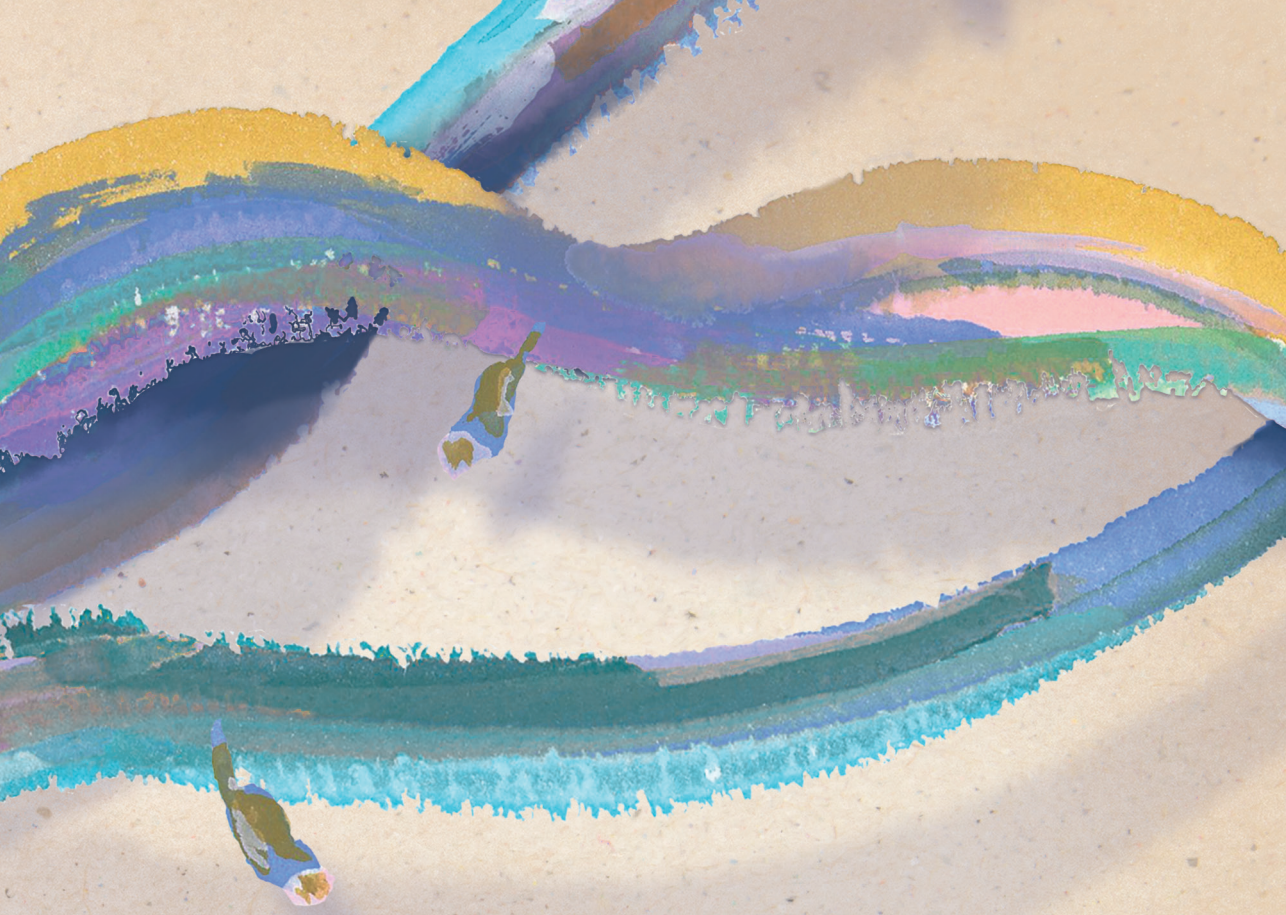
Voor een beter begrip van de werking van mitotaan op het bijnierschorscarcinoom, worden in **Hoofdstuk 7** de effecten van mitotaan in 32 humane primaire bijnierschorscarcinoom kweken beschreven. Mitotaan remde de productie van cortisol bij een aanzienlijk lagere concentratie vergeleken met het effect op celgroei. We laten zien dat de directe remmende effecten van mitotaan op de cel groei in humane primaire bijnierschorscarcinoom kweken zeer variabel zijn, wat de klinische praktijk reflecteert. Er werd geen associatie gevonden tussen *in vitro* mitotaan gevoeligheid en expressie van RRM1, SOAT1 en CYP2W1, drie eerder gepresenteerde voorspellende factoren *in vivo*. Het percentage cortisol producerende bijnierschorscarcinomen nam toe bij een toenemende mitotaan gevoeligheid *in vitro*. Dit suggereert dat cortisol productie door bijnierschorscarcinomen mogelijk geassocieerd is met een hogere mitotaan gevoeligheid als gevolg van een toename in het directe antitumor effect van mitotaan.

In een poging om de gevoeligheid voor chemotherapie in patiënten met bijnierschorscarcinomen te verhogen werd de rol van P-glycoproteïne, gecodeerd door het multidrug resistance (MDR1) gen, bestudeerd in **hoofdstuk 8**. P-glycoproteïne is een transporteiwit dat substraten, zoals chemotherapy, uit de cel pompt. Het grootste deel van de bijnierschorscarcinomen heeft een P-glycoproteïne expressie die vergelijkbaar is met

de expressie in normale bijnieren. De gevoeligheid voor doxorubicine en etoposide kon verhoogd worden door het toevoegen van verapamil of tariquidar, twee P-glycoproteïne remmers. Onze bevindingen werden bevestigd in een kolonievormende test. Er wordt geconcludeerd dat MDR1 betrokken is bij gevoeligheid voor specifieke cytotoxische geneesmiddelen en een doelwit zou kunnen zijn voor het verbeteren van effectiviteit van bepaalde vormen van chemotherapie in bijnierschorscarcinoom patiënten.

Conventionele systemische behandeling van patiënten met bijnierschorscarcinoom gaat vaak gepaard met ernstige toxiciteit en tumor progressie. Derhalve zijn nieuwe therapeutische mogelijkheden dringend nodig. **Hoofdstuk 9** laat de effecten van temozolomide (TMZ), een DNA-alkylerend geneesmiddel, op bijnierschorscarcinoom cellen *in vitro* zien. Temozolomide wordt reeds gebruikt in de behandeling van patiënten met maligne gliomen. In deze studie wordt aangetoond dat TMZ verschillende antitumor effecten heeft in 3 bijnierschorscarcinoom cellijnen, zoals het remmen van celgroei, inductie van apoptose, cytotoxische en cytostatische effecten, en een celcyclus arrest. Ook remt TMZ het celaantal in 5 van de 8 primaire bijnierschorscarcinoom kweken. We laten bovendien effectiviteit zien tegen bijnierschorscarcinoom cellen gekweekt als driedimensionale sferoïden, waardoor effecten van TMZ in een meer (patho)fysiologisch relevante context kunnen worden bestudeerd. Gebaseerd op analyse van de status van het MGMT gen in bijnierschorscarcinoom cellen en bijnierweefsels werd verondersteld dat werkzaamheid van TMZ in bijnierschorscarcinoom cellen mogelijk wordt beperkt door hypomethylatie van het MGMT gen. Aanvullende (pre-)klinische studies zijn nodig om de werkzaamheid in bijnierschorscarcinomen verder te onderzoeken.

Hoofdstuk 10 bevat een algemene discussie waarin de bevindingen uit dit proefschrift worden bediscussieerd in een breder perspectief en waarin suggesties en aanbevelingen voor toekomstig onderzoek worden gedaan.



APPENDIX

List of publications
Curriculum vitae
PhD portfolio
Acknowledgments / Dankwoord

LIST OF PUBLICATIONS

1. **SG Creemers**, LJ Hofland, SW Lamberts, RA Feelders. Cushing's syndrome: an update on current pharmacotherapy and future directions. *Expert Opin Pharmacother*, 2015, 16(12):1829-44
2. **SG Creemers**, LJ Hofland, E Korpershoek, GJH Franssen, FJ van Kemenade, WW de Herder, RA Feelders. Future directions in the diagnosis and medical treatment of adrenocortical carcinoma. *Endocr Relat Cancer*, 2016, 23(1):R43-69
3. **SG Creemers**, PM van Koetsveld, FJ van Kemenade, TG Papathomas, GJH Franssen, F Dogan, EMW Eekhoff, P van der Valk, WW de Herder, JAMJL Janssen, RA Feelders, LJ Hofland. Methylation of IGF2 regulatory regions to diagnose adrenocortical carcinomas. *Endocr Relat Cancer*, 2016, 23(9):727-37
4. **SG Creemers**, PM van Koetsveld, ESR van den Dungen, E Korpershoek, FJ van Kemenade, GJH Franssen, WW de Herder, RA Feelders, LJ Hofland. Inhibition of human adrenocortical cancer cell growth by temozolomide *in vitro* and the role of the MGMT gene. *J Clin Endocrinol Metab*, 2016, 101(12):4574-4584
5. **SG Creemers***, E Korpershoek*, PN Atmodimedjo, WNM Dinjens, PM van Koetsveld, RA Feelders, LJ Hofland. Identification of mutations in cell-free circulating tumor DNA in adrenocortical carcinoma: a case series. *J Clin Endocrinol Metab*, 2017, 102(10):3611-3615
6. **SG Creemers**, LJ Hofland, RA Feelders. Adrenocortical Carcinoma. Chapter in book: Management of Adrenal Masses in Children and Adults, *Springer,* 2017, ISBN 978-3-319-44136-8
7. **SG Creemers**, PM van Koetsveld, WW de Herder, F Dogan, GJH Franssen, RA Feelders, LJ Hofland. MDR1 inhibition increases sensitivity to doxorubicin and etoposide in adrenocortical cancer. *Endocr Relat Cancer, Epub ahead of print, DOI:* 10.1530/ERC-18-0500
8. PM van Koetsveld*, **SG Creemers***, F Dogan, GJH Franssen, WW de Herder, RA Feelders, LJ Hofland. The efficacy of mitotane in human primary adrenocortical carcinoma cultures. *Submitted for publication*
9. **SG Creemers**, RA Feelders, FH de Jong, GJH Franssen, YB de Rijke, PM van Koetsveld, LJ Hofland. Osilodrostat is a potential novel steroidogenesis inhibitor for the treatment of Cushing's syndrome: an in vitro study. *Submitted for publication*
10. **SG Creemers**, RA Feelders, N Valdes, CL Ronchi, M Volante, BM van Hemel, M Luconi, MHT Ettaieb, M Mannelli, MD Chiara, M Fassnacht, M Papotti, MN Kerstens, G Nesi, HR Haak, FJ van Kemenade, LJ Hofland. The IGF2 methylation score as an objective marker for adrenocortical carcinoma: validation study of the European Network for the Study of Adrenal Tumors (ENSAT). *Submitted for publication*

PUBLICATIONS NOT BELONGING TO THIS THESIS

1. MH van der Linden, **SG Creemers**, R Pieters. Diagnosis and management of neonatal leukaemia. *Semin Fetal Neonatal Med,* 2012, 17(4):192-195

** shared first authorship*

CURRICULUM VITAE

Sara Gerdine Creemers was born on November 4, 1991, in Gouda, the Netherlands. During her last two years of high school she already developed an extraordinary interest in research, and she was selected to participate in a pre-university program 'the Junior Med School' at the Erasmus Medical Center. She graduated from the St.-Antoniuscollege in Gouda (Gymnasium, cum laude), and started Medical School at the Erasmus University Rotterdam in 2009. She took part in the Erasmus MC Honours Class during the first years of Medical School, an extra program to get acquainted with the cutting edges between Medicine, Science and Society. During Medical School, she was also selected to start with a program to obtain her Master of Science degree in Clinical Research at the Netherlands Institute for Health Sciences on top of the regular medical curriculum. During this Master of Science program, she attended a summer program at the Johns Hopkins Bloomberg School of Public Health in Baltimore. Her masters' graduation research entitled 'The Methylation Pattern Of *IGF2* Regulatory Regions as a Novel Biomarker to Distinguish Adrenocortical Carcinomas from Adenomas' was awarded as 'outstanding' in 2014, after which she got appointed as a PhD candidate at the Laboratory of Neuro-Endocrinology of the Department of Internal Medicine of the Erasmus Medical Center, under the supervision of Prof.dr. L.J Hofland and dr. R.A. Feelders. She interrupted Medical School and worked full-time on her PhD thesis from September 2014 until December 2016, after which she started clinical rotations and in the meantime finished her thesis. She presented her research at several (inter)national meetings, received 5 grants, 3 poster awards and one prize for best basic paper of the year 2016 from the Dutch Endocrine Society. In her future career as medical doctor, she hopes to follow her passion and combine scientific research with clinical practice.

ERASMUS MC PHD PORTFOLIO

Name PhD student:	Sara Gerdine Creemers
Erasmus MC department:	Internal Medicine, Division of Endocrinology
Research School:	Molecular Medicine
PhD period:	2014 – 2018
Promotor:	Prof.dr. L. J. Hofland
Co-promotor:	dr. R. A. Feelders

	Year	Workload (ECTS)
1. PhD training		
General academic and research skills		
Research Integrity	2016	0.5
Molmed Course Basic and Translational Oncology	2015	2.2
Scientific Writing in English for Publication	2013	2
Molmed Couse and Workshop Basic and Translational Endocrinology	2013	2.2
Meetings, seminars and workshops		
Presentation at Quarterly Internal Medicine Research Meeting	2016	0.4
Adrenal Center, Erasmus MC, Rotterdam, the Netherlands	2014 - 2016	0.5
Dutch Adrenal Network Meeting, the Netherlands	2014 - 2016	0.5
Science Days Internal Medicine, Antwerp, the Netherlands	2014	1
Weekly attendance of journal clubs and department seminars	2013 - 2016	2
(Inter)national presentations		
Levoketoconazole, the single 2S,4R enantiomer of ketoconazole, is a potential novel steroid synthesis inhibitor for medical treatment of Cushing's Syndrome		
ENS@T Scientific Meeting, Florence, Italy, **Oral**	2018	2
The IGF2 methylations score as an objective marker for adrenocortical carcinoma: a European Network for the Study of Adrenal Tumors (ENSAT) validation study		
ENS@T Scientific Meeting, Florence, Italy, **Oral**	2018	2
Novel insights in the diagnosis and treatment of patients with adrenocortical tumors		

	Year	Workload (ECTS)
Science Days Internal Medicine Erasmus MC, Antwerp, Belgium, **Oral** *LCI699 is a potent inhibitor of cortisol production in vitro*	2017	2
Science Days Internal Medicine Erasmus MC, Antwerp, Belgium, **Poster**	2017	1
European Congress of Endocrinology, Munich, Germany, **Guided Poster**	2016	1
ENS@T Scientific Meeting, Birmingham, England, **Poster** *Identification of mutations in cell-free circulating tumor DNA in adrenocortical carcinoma: a case report*	2016	1
ENS@T Scientific Meeting, Birmingham, England **Poster** *The methylation pattern Of IGF2 regulatory regions as a novel biomarker to distinguish adrenocortical carcinomas from adenomas*	2016	1
Dutch Adrenal Network, Academic Medical Center Amsterdam, the Netherlands, **Oral**	2016	2
ENS@T Scientific Meeting, Munich, Germany, **Poster**	2015	1
Endo Retreat, Erasmus MC, Rotterdam, the Netherlands, **Oral**	2015	2
Annual Meeting of the Endocrine Society, San Diego, the United States, **Poster**	2015	1
Dutch Endocrine Meeting, Noordwijkerhout, the Netherlands, **Oral**	2015	2
Science Days Internal Medicine Erasmus MC, Antwerp, Belgium, **Oral** *Therapeutic potentials of temozolomide on human adrenocortical cancer cells in vitro and the role of the O6-Methylguanine-DNA Methyltransferase gene*	2015	2
Annual Meeting of the Endocrine Society, Boston, the United States, **Poster**	2016	1
Dutch Endocrine Meeting, Noordwijkerhout, the Netherlands, **Oral**	2016	2
Science Days Internal Medicine Erasmus MC, Antwerp, Belgium, **Poster**	2016	1
ENS@T Scientific Meeting, Munich, Germany, **Poster** *Epigenetic regulation of IGF2 and H19 expression in adrenocortical cancer*	2015	1
Dutch Endocrine Meeting, Noordwijkerhout, the Netherlands, **Oral**	2014	2

	Year	Workload (ECTS)
2. Teaching activities		
Lecture at Summer Programme of 1st year Junior Med School Students	2015, 2016	1
Education Endocrinology for 1st year Medical Students, Erasmus MC, Rotterdam Subjects: Hypercortisolism and Diabetes Mellitus	2014-2016	2.5
Supervising bachelor thesis Biomedical Sciences: E. Eckinci *The role of temozolomide as a potential new therapeutic option for adrenocortical carcinoma*	2014	5
3. Awards and prizes		
Hippocrates Study Fund Prize, Hippocrates Studiefonds	2018	
NVE-Ipsen Prize for best Dutch Basic Endocrinology Paper 2016, Dutch Endocrine Society	2017	
ENS@T Poster Prize, European Network for the Study of Adrenal Tumors (ENS@T) Meeting	2016	
Basic Science Meeting Grant, European Society of Endocrinology	2016	
NVE Goodlife Healthcare Travel grand, Dutch Endocrine Society	2016	
Best Poster Award, Science Days Internal Medicine Erasmus MC, Antwerp, Belgium	2016	
Winner of Presidential Poster Competition, 97th Annual Meeting of the Endocrine Society	2015	
NVE Goodlife Healthcare Travel grand, Dutch Endocrine Society	2015	
Early Career Award, The Endocrine Society	2015	
Grants received to visit conferences from Stichting Erasmus Trustfonds	2014 - 2016	

DANKWOORD

En dan ben ik ineens aanbeland bij het laatste, waarschijnlijk meest gelezen, onderdeel van dit boekje. Dit proefschrift was er niet geweest zonder de hulp van velen!

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