Adrenocortical carcinoma

Adrenocortical Carcinoma:
an orphan disease with many faces

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Aan mijn ouders,

Tonny Moos & Ben Nejib Ettaieb

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General introduction

Anatomy

The adrenal glands are endocrine glands that produce a variety of hormones. The human body has two adrenal glands, there is one localized on the superior medial aspect of the upper pole of each kidney in the retroperitoneum.

The adrenal sizes vary in normal subjects, measuring about 4cm long and 3cm thick, and are surrounded by adipose tissue (1). A normal adrenal gland in an adult weighs approximately 4 to 6 grams. The left adrenal is larger and flatter than the right adrenal gland. The right adrenal gland is pyramid-shaped ("witch's hat") and lies above the upper pole of the right kidney, between the liver and the diaphragm. The left adrenal gland is semilunar-shaped and is found between the kidney and aorta, near the tail of the pancreas and the splenic artery.

Each adrenal gland holds two functionally distinct endocrine units that are developmentally, structurally and functionally different. The outer part is called the adrenal cortex, composing 80 to 90 percent of the volume of a normal gland, and the inner part the adrenal medulla, constituting 10 to 20 percent of the volume of a normal gland. The cortex develops from mesoderm whereas the medulla develops from the neural crest.

The adrenal cortex is divided into three distinct functional zones producing steroids:

- An outer zone, 15% of the cortex, called zona glomerulosa secreting mineralocorticoids (aldosterone), which regulate sodium and potassium homeostasis.
- A middle zone, 75% of the cortex, called zona fasciculata secreting glucocorticoids (most importantly, cortisol)
- An inner zone, 10% of the cortex, called zona reticularis secreting sex steroids (primarily androgens).

The adrenal medulla consists of so called chromaffin cells, because they stain brown with chromium salts, secreting catecholamines: epinephrine and norepinephrine, which regulate many cardiovascular and metabolic processes (2).

Adrenal gland tumors

Adrenal tumors (tumor is Latin for swelling) or masses of the adrenal gland are common. Adrenal tumors include benign and malignant adrenocortical tumors and benign and malignant tumors of the adrenal medulla named pheochromocytomas (PCC).

Adrenal masses are often referred to as adrenal incidentaloma since they are serendipitously discovered by radiologic examination. Incidentalomas are defined as adrenal masses of one centimeter or more in diameter (3). They are found in up to 10% of the imaging done for other reasons, being more frequent in older patients. In autopsy studies, the prevalence of incidentalomas is two percent, ranging from one to nine percent. The prevalence is higher in obese, diabetic, and hypertensive patients. (4,5)

Adrenocortical carcinoma

Adrenocortical carcinoma (ACC) is a rare aggressive neoplasm with poor prognosis. ACC has an annual incidence of 0.5 -2.0 patient per million people per year (6-9). ACC accounts for 0.02% to 0.2% of adult cancers and about 0.2 % of all cancer deaths in the United State (7,10-12). ACC has a bimodal age distribution with a peak <5 years of age and a second peak during the fourth to fifth decades of adult life, but the tumor can appear at any age. One point three percent of all childhood cancers are ACCs, suggesting a higher relative incidence early in life. Interestingly, the incidence of pediatric ACC is 10–15 times higher in children in southern Brazil, which is related to an inherited germline p53 mutation(13,14).

Adrenocortical carcinoma is slightly more common in women than men with a ratio of 1.5:1 (15). Studies show that the left adrenal is more frequently affected.

Pathogenesis and genetics

ACC pathogenesis is heterogeneous and still incompletely understood. Most of the progress in understanding its pathogenesis and identifying genes involved in ACC comes from the study of (rare) familial diseases. Although most cases of ACC are sporadic with no identifiable risk factor it can be a part of hereditary tumor syndromes such as:

- Multiple endocrine neoplasia type 1 (MEN1): mutations in the MENIN gene on chromosome 11q13. Approximately 10% of MEN1 patients have distinct adrenal tumors, and of these, up to 14% are malignant (12,16).
- Li–Fraumeni syndrome (LFS): Mutation of tumor suppressor protein TP53, located on 17p13.Three to 10% of LFS associated cancers are ACCs. (17)
- Lynch syndrome: mutations in genes involved in DNA mismatch repair genes MSH2, MSH6, PMS2, MLH1, and TACSD1/EPCAM. The prevalence of Lynch syndrome in patients with ACC has been described approximately 3 (18).
- Beckwith–Wiedeman syndrome (BWS): alterations of DNA methylation of the 11p15 locus, which harbors the coding regions for Insulin-growth-factor 2(IGF2), the cell cycle regulator CDKN1C, and the non-translated RNA, H19 (19). Up to 15% of all malignancies in BWS are ACC (12,20).
- Familial adenomatous polyposis coli (FAP): inactivating germline mutation of the tumor suppressor gene adenomatous polyposis coli (APC) gene, which encodes for a downstream regulator of the Wnt/β-catenin pathway (21). So far, six cases of ACC have been described in FAP patients (22).
- Werner syndrome: a premature aging disease caused by the mutation in the *WRN* gene (23).
- Neurofibromatosis type 1 (NF1): mutation in the NF1 gene that encodes neurofibromin. ACC has been reported in four patients with neurofibromatosis type 1 (NF1) (22).

The familial cancer susceptibility syndromes described above established that there are three major dysfunctional molecular pathways in ACC: the IGF pathway, the Wnt pathway and TP53 (24).

IGF pathway

The IGF pathway is involved in the development and in the maintenance of adrenocortical functions. The pathway consists of ligands (IGF-1 and IGF-2), receptors,

binding proteins and binding protein proteases. The main role of IGF-2 lies in fetal development and growth, whereas IGF-1 mainly acts postnatal.

Chromosome locus 11p15, contains the genes cyclin-dependent kinase inhibitor (CDKN1C), IGF2 and H19, structurally organized in a cluster. Normally, these genes are expressed monoallelically, in a parent-of-origin-specific manner. IGF2 is maternally imprinted, therefore only the paternal allele is expressed. The paternal alleles of CDKN1C and H19 are silenced by imprinting, thus only the maternal alleles are expressed (24,25). CDKN1 acts as a negative regulator of cell proliferation, H19 however acts as a transcriptional repressor of IGF2.

IGF2 is highly overexpressed in majority of ACCs, whereas the expression H19 and/or CDKN1C is decreased. Overexpression of IGF2 is a result of loss of the maternal allele – loss of heterozygosity (LOH) - and duplication of the paternal allele, called paternal isodisomy. Also, by loss of imprinting (LOI) because of demethylation of the maternal allele (26,27). IGF2 regulates the growth and apoptosis of cells, and interact with insulin-like growth factor 1 receptor (IGF1R). The latter is also found to be over-expressed in ACC. Activation of IGF-1R results in stimulation of downstream signaling pathways including the mitogen-activated protein kinase (MAPK) and phosphoinositol-3-kinase (PI3-AKT) pathway, leading to increased cell proliferation and survival (28,29).

IGF2 overexpression by LOH is associated with poorer outcome (30).

Wnt pathway

The Wnt/β-catenin pathway is active during normal embryonic development and has been shown to play an important role in organ development. Dysregulation of Wnt signaling has been found in a variety of cancers, but it was first identified and intensively researched in FAP patients. Research has shown that is also plays and important role in adrenocortical tumorigenesis. It are the activating mutations of the Catenin Beta 1 gene (CTNNB1), and deletions in the Zinc And Ring Finger 3 (ZNFR3) gene that leads to activation of the Wnt/β-catenin pathway in ACC. Consequently this activation of the Wnt pathway induces cell proliferation and resistance to apoptosis.

Furthermore, the somatic mutation of the CTNNB1 is an independent predictor of less favorable survival in ACC. (31)

TP53

Germline inactivating TP53 mutations are very frequent in pediatric ACCs, but rare in adult patients with ACC, explaining the relative increase in incidence in childhood.

The prevalence of germline TP53 mutations in children diagnosed with ACC ranges from 50–97%. Somatic TP53 mutations contrary are common in adult patients, being described in 25–70% of samples (32).

TP53, known as the 'guardian of the genome', is located on chromosome 17p13 and its main functions are halting the cell cycle and/or inducing apoptosis in response to DNA damage. Unable to do so when mutated propagation of a corrupted genome can result in malignancy (33).

Epigenetics

Genetic studies aimed at adrenal tumors have identified multiple molecular alterations as factors in ACC carcinogenesis. Increasing evidence suggests that DNA methylation, the addition of methyl group to the cytosine pyrimidine ring or adenine purine ring of the DNA molecule without changing the DNA sequence, in addition to genetic modification causes altered patterns of gene expression resulting in tumorigenesis. In normal cells, DNA methylation is responsible for selective regulation of gene expression. The process of DNA methylation may occur throughout the genome in C phosphate-G (CpG) dinucleotides. The genome overall has a low CpG content, except for areas in gene promoter regions. These CpG dinucleotides clusters in gene promoter regions are called CpG islands. Methylation of CpG islands in a gene promoter region mechanically blocks transcription by creating a barrier. An increased prevalence of CpG island methylation has been proposed to be involved in carcinogenesis of several tumors and has also been identified in adrenocortical adenoma and carcinoma (24).

Genetic screening

Currently there is no genetic screening for ACC where most ACCs are sporadic but it is important to consider the occurrence of ACC as part of a syndrome. Actually, Chompret criteria advocated for *TP53* genetic testing for all patients diagnosed with ACC regardless of age at diagnosis or family history (34).

Aside from genetic predisposition, no risk factors have been confirmed. Smoking in men and contraceptive use in women, have been suggested as risk factors for ACC (35).

Clinical presentation

Early diagnosis of ACC is very challenging because of the rarity of the disease and the lack of distinct and specific alarm symptoms. Most physicians are unlikely to encounter the disease and as a result are unfamiliar with diagnosing and treating ACC.

Often patients present with nonspecific complaints that are related to the tumor mass effect or endocrine disturbances.

Adrenocortical carcinoma can grow up to more than 20 centimeters. The increase in mass can cause abdominal or flank pain or other abdominal discomfort e.g. nausea, vomiting or fullness (6,36,37). Thirty percent of patients present with abdominal mass effects.

Most patients, 40% to 60%, present with symptoms of steroid hormone excess (glucocorticoids, mineralocorticoids, androgens).

When there is an excess of hormone secretion, ACC is referred to as functional. Depending on which hormones are over-secreted, different clinical consequences manifest: Cushing syndrome, virilization syndrome, feminization syndrome or a mixed Cushing-virilization syndrome. ACC is nonfunctional when the tumor does not secrete excessive hormones or in insufficient quantities to have clinical consequences.

In these 'functioning' ACCs, hypercortisolism, or Cushing syndrome, is the most common presentation of hormone excess (50% to 80%) with patients showing profound muscle atrophy, severe hypertension and diabetes mellitus (12). Women can present with signs and symptoms of androgen secreting ACC - acne, hirsutism and virilization (deepening of the voice, male pattern baldness and oligoamenorrhea) while men can present with estrogen-secreting ACC leading to feminization - gynecomastia, loss of libido and testicular atrophy. It should be noted that androgen-secreting tumors in men and estrogen secreting tumors in women, may not result in clinically significant syndromes (10).

Aldosterone-producing ACC is rare (Conn syndrome), symptoms are hypertension with hypokalemia. However, severe hypokalemia is also caused by elevated cortisol secretion.

Nonspecific symptoms of malignancy, such as fever, weight loss or general malaise affect only a small minority of ACC patients.

Diagnosis

The ACC working group of the European Network for the Study of Adrenal Tumors (ENSAT) has proposed standards for the diagnostic procedures (37). It is of most

importance that each step of the management strategy should be decided in the setting of a multidisciplinary team including different expertise (endocrinology, radiology, pathology, surgery, oncology), in expert centers.

Adrenal biopsy is discommended, because of increased risk of (needle-track) metastases, in the diagnostic work-up of patients with suspected ACC. Unless, there is evidence of metastatic disease that interferes with surgery and histopathologic diagnosis is required to optimize oncological management.

Imaging

There are currently three main imaging techniques available for the differentiation of malignant and benign adrenal tumors: Computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET) with 18F-2-deoxy-d-glucose (FDG) (mostly combined with CT; FDG-PET/CT). Endoscopic ultrasonography (EUS) has no major role in the diagnostic procedures of ACC (38). These imaging methods cannot determine the exact identity of the adrenal mass but (imaging) criteria have been defined to differentiate between benign and malignant. Adrenocortical carcinomas are mostly inhomogeneous and irregular. Tumor size is considered to be an important marker for malignancy. Masses of more than 6 cm in diameter are considered malignant until proven otherwise (15). Local invasion or tumor extending in to the inferior vena cava, as well as lymph node or other metastases are signs of malignancy and often found in advanced ACC.

Hounsfield units (HU) measured with non-contrast CT provides additional information in the diagnosis of adrenal tumors. Hounsfield unit is a relative quantitative measurement of radio density used by radiologists in the interpretation of CT images. Most benign adenomas are lipid-rich and have low HU whereas ACC are characterized by low fat content and hence higher HU density. A threshold value of 10 HU is used to distinguish adrenal masses, where a low density/ attenuation value ≤10HU is considered as benign. In fact, the ACC working group of ENSAT states that only non-contrast CT is sufficiently reliable to rule out ACC when the adrenal lesion is homogenous and has low CT density ≤10 HU (37). Additional delayed contrast-enhanced CT is used, analyzing washout of contrast medium, especially to discriminate between lipid-poor adenoma and ACC. A wash out <50% and a delayed attenuation of >35 HU (on 10-15 minutes delayed contrast CT) are suspicious for malignancy (6,39-41).

Magnetic resonance imaging lesions with the dynamic gadolinium enhanced- and chemical shift technique is similar effective in characterizing adrenal lesions. Adrenocortical carcinomas are isointense to the liver on T1-weighted images but are inhomogeneous and show an increase intensity in T2-weighted sequences. When

using gadolinium, adenomas show mild enhancement with quick washout, whereas malignant tumors show strong enhancement and slower washout. MRI is considered to be superior to CT in assessing the extent of vascular invasion, especially into the inferior vena cava.

With FDG-PET, a high uptake of FDG demonstrates increased glucose metabolism and indicates malignancy, but the FDG-PET cannot differentiate reliably ACC from a metastasis from other tumor or even a pheochromocytoma (42). PET imaging has been proposed as possibly the best second line test to assess indeterminate masses by unenhanced CT (37).

Imaging from chest to pelvic is done to evaluate for metastases. It is mandatory to systematically and rapidly evaluate for metastases, before initiation of any anti-tumor treatment. Thoraco-abdomino-pelvic imaging will cover the vast majority of metastatic locations, which most often are lung and liver (37).

Hormonal work up

Hormonal analyses is crucial because of various reasons: 1) to assess if there is hormone excess 2) to establish the adrenocortical origin of a tumor (pheochromocytoma has to be excluded prior to an invasive procedure where it requires a specific perioperative management) 3) to suggest malignancy (androgen or estrogen production) 4) to establish suitable tumor markers for follow-up and surveillance and 5) to optimize the perioperative management (e.g. glucocorticoid replacement therapy). A comprehensive work-up as advised by ENSAT is presented in the table below (15,37,43).

Glucocorticoid excess (*Minimum three out of four tests)*	
	Dexamethasone suppression test: 1mg
	Excretion of free urinary cortisol (24h urine)
	Late-night salivary cortisol (23:00)
	Basal adrenocorticotropic hormone (ACTH)
Sexual steroids and steroid precursors	
	Dehydroepiandrosterone sulphate (DHEA-S)
	17-OH-progesterone (serum)
	Androstenedione (serum)
	Testosterone (serum, only in woman)
	17-beta-estradiol (serum, only in men and postmenopausal women)
	11-Deoxycortisol
Mineralocorticoid excess	
	Potassium (serum)
	Aldosterone/renin ratio (only in patients with arterial hypertension and/or hypokalemia)
Exclusion of pheochromocytoma	
	Meta- and Normetanephrines (plasma and urine)

Urinary steroid profile

Urine steroid metabolomics (in 24h urine samples) are proofing to be a significant alternative diagnostic tool for discriminating between benign and malignant adrenal lesions. Tetrahydro-11-deoxycortisol (THS) levels have been shown to be able discriminate between ACC and benign adrenal mass with high specificity (44-48).

Pathology

Adrenocortical carcinoma diagnosis should be confirmed by histopathology, preferably by and experienced pathologist. Different diagnostic scores have been introduced for the diagnosis of carcinoma versus adenoma. The Van Slooten Index (VSI) is a weighted system based on seven histopathological parameters(regressive changes, loss of normal structure, nuclear atypia, nuclear hyperchromasia, abnormal nucleoli, mitotic activity, capsular and/or vascular invasion), each combined with a numerical value. A score of eight or higher corresponds to a high probability of malignancy(49). The Weiss score system is recommended and most used (50). This classification is based on a combination of nine histologic criteria. The nuclear grade

uses the criteria of Fuhrman et al(51): grade 1) round nuclei, homogenous, small size, no nucleoli; grade 2) nuclei slightly irregular, more voluminous, conspicuous nucleoli at x400; grade 3) irregular nuclei, voluminous nucleoli at x100; grade 4 idem grade 3 with monstrous cells with very irregular nuclei.

The presence of three or more Weiss criteria highly correlates with malignancy, whereas scores between zero and two defines the adrenal adenoma (52). A score of two or three may be considered borderline malignant (tumors with a score of three and an benign clinical course have been noted) and should be treated cautiously where they have uncertain malignant potential. The guideline advices to use additional classification systems and/or the addition of reticulin stain assessment ((37,53).

The Weiss system		
Histological criteria	Weight of criteria	
	0	1
Nuclear grade	1 and 2	3 and 4
Mitotic rate	≤ 5 for 50 fields x400	≥6 for 50 fields x400
Atypical mitotic figures	No	Yes
Eosinophilic tumor cell cytoplasm/Clear cells	>25%	≤25%
Diffuse architecture	≤33% surface	>33% surface
Necrosis	No	Yes
Invasion of venous structures	No	Yes
Invasion of sinusoidal structures	No	Yes
Capsular invasion	No	Yes

Immunohistochemistry

There are two important immunohistochemistry criteria that can help differentiate carcinomas from adenomas: The steroidogenic factor 1 (SF-1), a transcription factor expressed primarily in the hypothalamus, pituitary, and steroidogenic organs like adrenal glands, testes, and ovaries. Besides discriminating between adenoma and carcinoma it also has been suggested as prognostic factor for ACC (54).

The cellular proliferation marker, Ki-67, is a protein involved in different phases of the cell cycle. It is a key marker associated with proliferating cancer cells and a poor prognosis (55,56).

Upcoming

Of upcoming interest is a so called liquid biopsy, which is a minimal invasive test using a sample of blood. This can be used to look for cancer cells from a tumor that are circulating in the blood or for pieces of DNA from tumor cells that are in the blood. Circulating tumor cells (CTC) have been found in the blood of ACC patients. It was shown to support differential diagnosis between benign and malignant adrenocortical tumors but it needs to be confirmed in a larger study cohort (57).

Circulating cell-free DNA (cfDNA) describes DNA that is freely circulating in the bloodstream, but is not necessarily of tumor origin. It was found that in cancer patients increased amounts of cfDNA circulates which is caused by additional circulating tumor DNA (ctDNA). In a pilot study cfDNA was found in ACC patients but there were difficulties identifying ctDNA (58). Another study was able to find ctDNA only in a subset of ACC patients (59).

Staging

In 2004, the International Union Against Cancer (UICC) and the World Health Organization published the first staging classification based on the Tumor, Node, Metastasis (TNM) criteria for ACC. In 2008, an analysis based on the German ACC Registry revealed several shortcomings with this classification. Therefore, the European Network for the Study of Adrenal Tumors (ENSAT) developed a revised staging system(60). This staging system defines stage I (T1N0M0) and stage II (T2N0M0) as strictly localized tumors with a size of ≤5 cm or >5cm, respectively. Stage III tumors are characterized by infiltration of surrounding tissue, positive regional lymph nodes or a tumor thrombus in the renal vein and/or vena cava (T1-2N1M0 or T3-4N0-1M0). Stage IV is defined by the presence of distant metastasis (T1-4N0-1M1).

In 2015 Libe et al. suggested a modified ENSAT (mENSAT) staging system. It defined stage III as T3–4N0M0 and stage IVa, IVb, IVc according to the number of tumor-involved organs (including the primary tumor and the 'N' as 'organ'): 2, 3 or >3, respectively (61). It has not been implanted in European Society of Endocrinology Clinical Practice Guidelines yet.

Treatment

Despite a lot of effort to improve care for patients with ACC, treatment still has limited opportunities.

The only potentially curative treatment for localized ACC is a radical surgical resection (R0). Open adrenalectomy by an experienced surgeon is recommended. There is

growing support that for tumors without any evidence of local invasion laparoscopic adrenalectomy could be considered if the surgeon has sufficient experience in these types of surgery and respects the principles of oncologic surgery (37,62,63).

Even after complete resection, recurrence rates are high with reported percentages of up to 91% (64,65). Recurrence free survival is worse after a surgery with microscopically positive margins (R1) or macroscopically positive margins (R2) (66), stressing the importance of a carefully planned and executed initial surgical treatment. Therefore the guidelines suggest that if the first surgery was suboptimal and R2, to discuss repeat surgery in a multidisciplinary expert team (37).

Surgery is also important in case of recurrence (67,68). For selected patients a metastasectomy has shown beneficial (69-71). In the setting of metastatic disease at primary presentation, the role of aggressive surgery is considered an individualized decision.

Mitotane

Currently, the only FDA approved adjuvant treatment of ACC is the adrenocorticolitic agent mitotane. Mitotane or o,p'-DDD (1-(o-chlorophenyl)-1-(p-chlorophenyl)-2,2-dichloroethane), is an isomer of Dichlorodiphenyldichloroethane (DDD), an analogue of the insecticide dichlorodiphenyltrichloroethane (DDT).

In 1948 Mitotane* was shown to produce adrenal atrophy in dogs by inhibiting 11β-hydroxylation and cholesterol chain cleavage in the mitochondria of steroidogenic cells therefore blocking cortisol synthesis (72,73). These observations led to the discovery of the potential value of mitotane in the treatment of adrenal cortical carcinoma and in fact it has been used for treating advanced ACC since the sixties(74). Although the antitumor activity of mitotane is well-recognized, the exact mechanism for its cytotoxic activity is poorly understood. In addition to disruption of mitochondria (75,76), endoplasmatic reticulum stress was identified as a key molecular pathway activated by mitotane with sterol-O-acyl-transferase 1(SOAT1) as key molecular target. Mitotane inhibits SOAT1 which triggers lipid-mediated endoplasmic reticulum stress and apoptosis in adrenocortical carcinoma cells (77).

Mitotane is difficult to manage and treatment is unsatisfactory at multiple levels. Mitotane is commercially available only as tablets of 500mg under the brand name Lysodren®. Intestinal absorption of mitotane varies significantly and is best when taken with fatty substances (e.g. chocolate, milk) (78). When absorbed mitotane is distributed in the body and metabolized in the liver, 25% is recovered in the urine. Because mitotane is lipophilic, the drug reaches highest concentrations in adipose tissue but can also be detected in the brain, adrenals, liver, bile and serum (79).

A close correlation of mitotane plasma levels with both efficacy and adverse effects are known, therefore plasma concentrations between 14 and 20 mg/L are aimed at (80,81). Reaching and maintaining these therapeutic levels is complicated. Mitotane has a low bio-availability, about 35%–40% of the drug is absorbed from the gastrointestinal tract and stored in adipose tissue (82). The half-life of mitotane is extensive, elimination ranges from 18 to 159 days with a median of 53 days (78,83). The large distribution volume and long half-life of mitotane impede the ability to predict mitotane plasma. Furthermore, there is a high inter-individual variability in the pharmacokinetics (PK) of mitotane (84). Currently, the dosage tapering is highly expert-based. It may take up to several months to reach target plasma levels, months in which the cancer is unrestrained.

Two dosage regimens have been suggested to reach therapeutic levels. A low dose regimen which builds up in 14 days to 3 grams a day and a high dose regimen which builds up to 6 grams a day (85). The daily dosage is often divided into two or three doses, because i.e. 6 grams of mitotane is 12 tablets Lysodren®. The dose is adjusted until it reaches an 'optimal' dose that gives the best results, mitotane levels predict treatment response, without causing unacceptable side effects, frequently monitoring plasma trough levels (at least 12 hours after the last dosage) accordingly(80,86).

Side effects are dose-dependent: plasma levels above 20mg/l may be associated with severe side effects and bring no further therapeutic advantage. However, if a patient tolerates high mitotane levels it can be considered to continue treatment. Gastrointestinal disturbances occur in about 80% of the patients and consist of anorexia, nausea or vomiting, and in some cases diarrhea. It has been reported that gastrointestinal symptoms are particularly evident at the beginning of treatment with a threshold of 5mg/l, and relapse at any increment in mitotane dose (87,88).

The central nervous system is affected in 40% of the patients with a reported threshold of 15mg/l. These effects consist primarily of depression as manifested by lethargy and somnolence (25%) and dizziness or vertigo (15%). At high doses and after prolonged utilization, brain function impairment can occur which is reversible after treatment is terminated. Skin toxicity has been observed in about 15% of patients. In women ovarian cysts may develop and gynecomastia in men. Moreover, mitotane exerts a complex effect on the endocrine system that may require multiple hormone replacement therapy in which glucocorticoid replacement is obligatory (87-89).

Mitotane is given in adjuvant setting in those patients without macroscopic residual tumor after surgery with a perceived high risk of recurrence. Adjuvant treatment is given for at least 2 years (37,65,90). Mitotane is also given for recurrent disease and advanced disease possibly in combination with intravenous chemotherapy.

* In 1948 these first observations in eleven dogs were done with technical grade DDD. Nichols and Hennigar [195] (92) and Cueto and Brown [1958] (93) worked with technical DDD and its fractions and identified o,p'-DDD (mitotane) as the active fraction (88).

Overall, mitotane leads to stable disease or partial remissions in 30% of cases (12,91).

Chemotherapy

Several cytotoxic agents have been studied in ACC. The FIRM-ACT trial was the first ever conducted randomized controlled trial in ACC that confirmed the efficacy of chemotherapy and the superiority of etoposide, doxorubicin, cisplatin, mitotane (EDP-M) over streptozotocin, mitotane. Patients in the EDP–M group had a significantly higher response rate than those in the streptozotocin–mitotane group and longer median progression-free survival (5.0 months vs. 2.1 months) (94). EDP-M is the first therapy of choice in metastasized disease, mitotane mono therapy can be considered with less aggressive slow growing tumors.

Figure 1 shows different drugs that are currently used or previously studied in ACC with their corresponding cellular targets and pathways involved. Figure 2 shows response rates from studies with systemic therapies in ACC. Unfortunately, the advanced molecular understanding of ACC has not yielded a major therapeutic breakthrough yet and hope remains to identify better treatments compared to the currently used options.

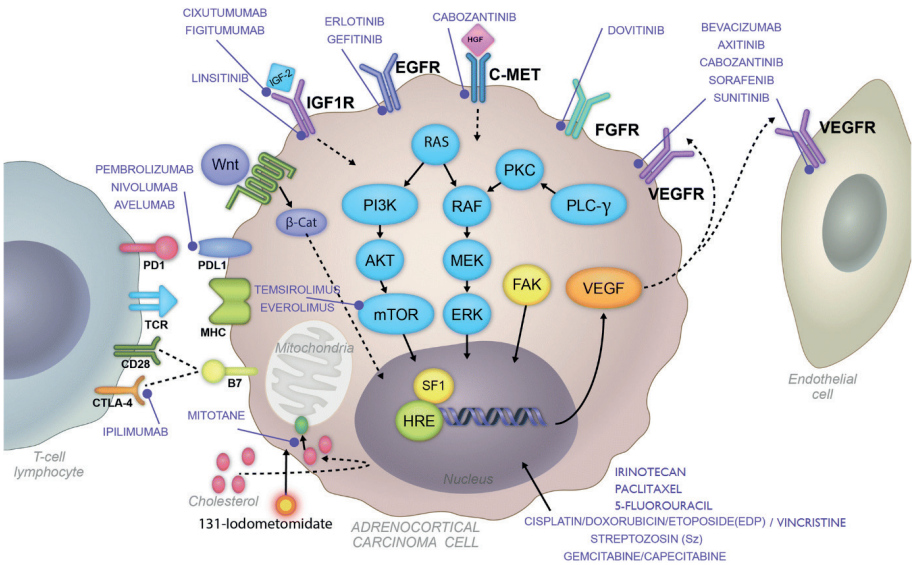

Figure 1 | Variable pathways and therapeutic targets in ACC and drugs studied or currently used. Adapted with permission from Springer Nature © Jasim and Habra, Current Oncology Reports 2019 (95)

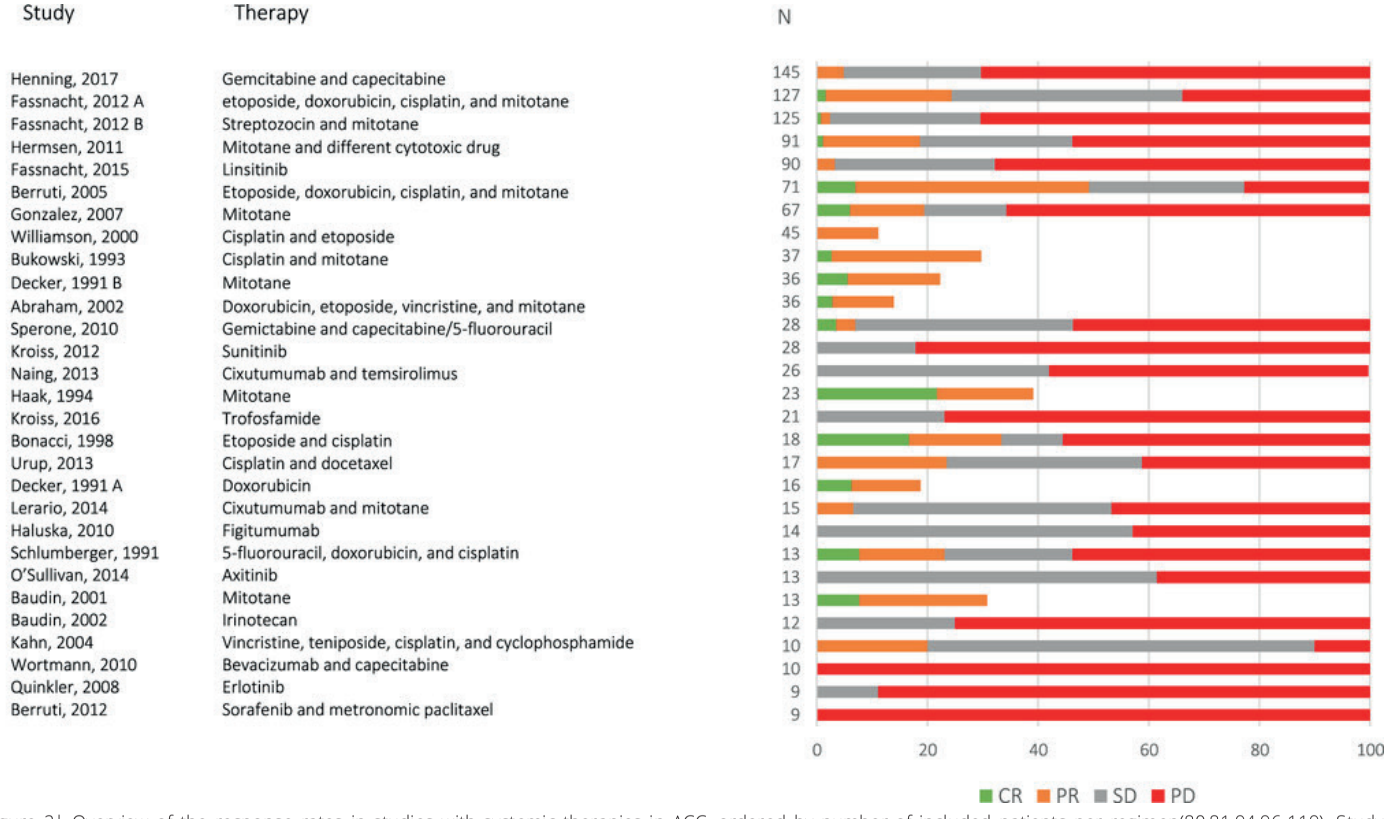

Figure 2| Overview of the response rates in studies with systemic therapies in ACC, ordered by number of included patients per regimen(80,81,94,96-119). Study protocols, patient cohorts and characteristics as well as outcome measurements are quite different precluding direct comparison. CR: complete response; PR: partial response; SD, stable disease; PD: progressive disease. Not all studies reported stable disease or progression, thus these columns do not sum up to 100%. Permission obtained from Bioscientifica Ltd© Fassnacht, M. et al. European Journal of Endocrinology 2018 (37).

Immunotherapy

Immunotherapy is aimed at programmed cell death-1 (PD-1), an immune-checkpoint receptor expressed by T cells, and programmed cell death ligand-1 and -2 (PD-L1 and PD-L2), expressed in the tumor microenvironment of various cancers. Recent research has shown that an estimated 11% of ACCs express PD-L1 on tumor cell membranes, and 70% of tumor-infiltrating monocytes are PD-L1-positive (120). Immunotherapy has positively changed the prognosis significantly for patients with melanoma, renal cell carcinoma and lung cancer. Logically, multiple clinical trials are currently exploring the role of immunotherapy in ACC. So far results that have been published show mixed response, and there is a hypothesis that concomitant mitotane may be beneficial (121-124).

Other

Radiotherapy is used as a palliative therapy especially in symptomatic bone and brain metastases (125) and has been opted as adjuvant therapy of the tumor bed to reduce risk of local recurrence (126).

Data on data on loco-regional therapeutic options as hyperthermic intraperitoneal chemotherapy (HIPEC) (127) and transcatheter arterial chemoembolization (TACE) (128) is limited but show potential in selected patients with recurrent ACC confined to the peritoneal cavity or liver metastases respectively.

Radiofrequency ablation (RFA) was described in 8 ACC patients in which 15 lesions were ablated: 8 out of the 15 ablated tumors showed decreased tumor size (129) and is suggested suitable in cases with limited number of liver lesions (e. g. ≤ 3) with a diameter of less than 3 cm.

Metformin, the anti-diabetic drug, has been associated with a decreased cancer prevalence and mortality in some solid tumors. It was shown to inhibit cell growth in an ACC experimental cell line (130). Peixoto et al described a case of female patient who achieved stable disease with mitotane, cisplatin, doxorubicin, and etoposide as first-line therapy, but then had an objective response to oral metformin that lasted 9 months(131) validating further research.

Prognosis

Median overall survival of ACC patients is about 3-4 years, the five-years overall survival being below 40%. However, the prognosis is very heterogeneous and difficult to predict in clinical practice.

Great variability in clinical course is observed, ranging from tumors with an indolent

behavior and patients with extreme long survival (132) to aggressive tumors with prompt fatal outcome. This heterogeneity in survival makes it complicated to tailor treatment strategies for an individual patient. Therefore, a lot of effort has been made to identify prognostic markers for survival

The extent of the disease at the time of diagnosis is considered a major determinant of survival. This can be assayed by the ENSAT staging score with a five-year stage-dependent survival of 66–82% for stage I, 58–64% for stage II, 24–50% for stage III, and 0–17% for stage IV(133). Approximately 30% of patients are diagnosed with metastases at primary presentation (15). Within the group of patients with locally advanced disease survival mainly seem influenced by positive lymph nodes (N1) or vena cava invasion and could have a similar prognosis to those with stage IV disease (61,134). The number of involved organs in patients with stage IV ACC has been reported to influence prognosis, a high number of involved organs means worse prognosis (61,135).

Several studies showed that patients with cortisol-secreting ACC have a decreased overall survival. It has been hypothesized that excess cortisol poses multiple clinical challenges related to other comorbidities, such as hypertension, hyperglycemia, hypokalemia, bone loss, hypercoagulability and immune suppression (136-139).

Furthermore, several studies also claim that age, the Weiss score (a Weiss-score greater than six has been found to correspond with poor prognosis) index and the resection (R) status may impact prognosis of ACC patients (61,140,141).

The Ki67 has consistently been proven to be associated with prognosis. A malignant tumor with a Ki67 index under 10% is associated with a relatively good prognosis; an index over 20% is associated with a detrimental course of disease (142).

Molecular markers have been of increasing interest for prognostication of ACC. Pan-genomic studies have been performed in ACC, showing distinct molecular subtypes with clinically relevant differences in outcome (143-145).

In particular two subgroups of ACCs, strongly correlated with survival, were identified by clustering of mRNA expression (145). The aggressive, high mutation rate, cluster C1A is associated with a poor outcome, which has been characterized by a specific transcriptomic signature, a methylome signature of hypermethylation on CpG islands called CIMP (146), a high number of chromosome alterations called *noisy*, and an accumulation of mutations among a limited number of recurrent driver genes (143,147). The C1A ACCs can be classified into three subgroups according to methylation profile: CIMP-high, CIMP-low and non-CIMP (143,146).

Conversely, another patient subgroup has been associated with a better outcome, the indolent C1B transcriptomic, generally non-CIMP, cluster (143,144).

Aims and outline of this thesis

To date, the European Society of Endocrinology Clinical Practice Guidelines Clinical recommends taking into account the factors tumor stage, resection status, Ki67 index, autonomous cortisol

secretion and the patient's general condition when assessing prognosis for patients with ACC (37). The acronym 'GRAS' has been put forward: Grading (G), the R status (R), Age (A) and Symptoms (S), defined as tumor- or hormone-related symptoms at diagnosis to correspond to these parameters(61,148). Still, using these parameters survival varies among patients complicating therapeutic decision-making. In order to gain insight into factors affecting prognosis, we analyzed the influence of different time patterns of metastases are associated with the outcome of patients with ACC in **chapter 2**. In addition, we aimed to study the effect of the number of metastases and affected organs on survival.

A prediction model combining the available prognostic parameters that have been individually associated with survival could be of significant additional value, and may be more useful in clinical practice. Therefore in **chapter 3** a clinical prediction model for ACC-specific mortality is presented.

Mitotane is the only FDA approved drug treatment for ACC. At present, no available pharmacological options have been proven better than mitotane. Despite 60 years of use, many of its pharmacological properties, such as exact pharmacodynamics and activation in humans, remain not fully understood.

There is a large inter-patient variability in reaching and maintaining therapeutic concentrations, without a clear relationship between the mitotane dose and the serum concentration. The variability in mitotane requirement that cannot be explained by the clinical factors, in other words the residual variability, may very well be explained by pharmacogenetic differences between patients. Identifying these differences and designing a pharmacokinetic model in which such pharmacogenetic differences are taken into account could allow a more individualized elegant treatment of ACC with mitotane.

Recent study has shown considerably weak correlations between weight (both total weight and Lean Body Mass) and the volume of distribution in central compartment (V1) of mitotane. Other covariates (age, gender, height, BodySurfaceArea) displayed correlations in the same order of magnitude (84). These findings have led to the hypothesis that differences in genetic constitution, are of significant importance. Indeed, recent research states that patients with a certain genotype of the CYP2B6 gene show higher mitotane plasma concentrations at three months of treatment, which supports this hypothesis (149). Mitotane concentrations were not influenced by the polymorphisms of the ABCB1 gene. Other genetic polymorphisms need further exploration. In **chapter 4** pharmacogenetic factors or single nucleotide

polymorphisms are investigated using the Drug Metabolizing Enzymes and Transporters (DMET) Platform**.** Furthermore a population pharmacokinetic (PopPK) model is described, allowing optimal treatment schedules for individual patients.

ACC management remains challenging because of the heterogeneous and often unpredictable nature of this disease. Advances in molecular analysis have enhanced the molecular understanding of ACC. Unfortunately, this has not yielded a major therapeutic breakthrough and EDP-M remains the main systemic therapy option for many patients. However, emerging genomic techniques may be able to identify cancer dependencies and select priority drug candidates in the near future. In **chapter 5** a view is presented on the future of ACC treatment that is expected to be brighter than it has been so far. Furthermore, ACC diagnostics and prognostics is discussed since significant changes in these fields are expected contributing to new treatment strategies.

Epigenetic regulatory mechanisms have emerged as clinically relevant diagnostic and prognostic factors in ACC. DNA methylation levels and CIMP-phenotypes have shown to be able to stratify ACC prognostic subgroups. The therapeutic potential of epigenetic alterations in ACC is unclear and should be further investigated. In **chapter 6** an overview of new insights in the evolving field of epigenetic studies on adrenal tumors is provided.

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Part I

Clinical prediction

Chapter 02

Synchronous versus Metachronous Metastases in Adrenocortical Carcinoma: an analysis of the Dutch Adrenal Network

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Abstract

Adrenal Cortical Carcinoma (ACC) is a rare malignancy with an incidence of 1.0 per million per year in the Netherlands. Median survival varies according to the European Network for the Study of Adrenal Tumours (ENS@T) tumour stage. It is unknown whether time until development of metastases is of influence on prognosis. To assess this, data were retrospectively obtained from centres of the Dutch Adrenal Network. Patients who presented with ACC between January 1, 2004 and October 31, 2013 were included. Date of detection of metastases, number of metastases and affected organs were registered. One hundred sixty patients were included in the analysis. Synchronous metastases were defined as diagnosis of metastasis ≤6 months after the initial diagnosis of ACC. Overall survival rate was calculated from the date of diagnosis of metastasis until death from any cause.

At first presentation, 50 patients (31 %) had ACC with metastases (ENS@T stage IV). Another 67 (42 %) developed metastases during follow-up. Amongst the 117 patients with metastases, 84 (72 %) patients had synchronous metastases and 33 (28 %) developed metachronous metastases. Diagnosis of synchronous metastases ($p = 0.046$), more than one affected organ ($p < 0.001$) and four or more metastases ($p < 0.001$) were found to be associated with reduced overall survival. Limitations included retrospective design and limited details regarding pathological data.

We conclude that synchronous metastases of ACC are associated with a poorer prognosis compared to metachronous metastases of ACC. The clinical characteristics associated with prognosis in this study support the view to refine the prognostic classification for patients with stage IV ACC.

Introduction

Adrenal Cortical Carcinoma (ACC) is a rare malignancy with an incidence of 1.0 per million in the Netherlands [1]. Median survival varies according to tumour stage, from 159 to 5months for patients with ENS@T stage I–II and ENS@T stage IV, respectively [1]. ACC usually presents with symptoms caused by hormone production or local symptoms due to tumour growth.

Radical surgical resection of locoregional tumour lesions is a mainstay of the curative treatment for ACC. Nevertheless, 75–85% of patients who underwent radical resection showed recurrence of local disease often with concurrent distant metastases [2, 3]. Options for palliative treatment are limited.

Approximately, 30 % of patients already present with metastases at the time of diagnosis [4]. The most common sites for metastases are the lungs (~40 %), liver (~40 %), skeleton (~30 %) and lymph nodes (~25 %) [5-7].

For various malignancies, it has been described that the distinction between synchronous and metachronous metastases has a prognostic value [8, 9]. It is currently unknown, however, whether these different time patterns of metastases are associated with the outcome of patients with ACC. In addition, the influence of the number of metastases and affected organs on prognosis in patients with ACC is not well known. The number of involved organs in patients with stage IVACC has been suggested to be useful for the prediction of outcome [10].

The primary objective of the present retrospective study was to investigate if there was a difference in survival of ACC between patients with synchronous metastases and patients with metachronous metastases. In addition, we aimed to study the effect of the number of metastases and affected organs on survival.

Methods

Data were retrospectively obtained from the nine centres of the Dutch Adrenal Network (DAN). The DAN includes eight Dutch university hospitals (Erasmus Medical Centre Rotterdam, Leiden University Medical Centre, VU Medical Centre Amsterdam, Radboud University Medical Centre Nijmegen, University Medical Centre Groningen, Academic Medical Centre Amsterdam, University Medical Centre Utrecht and Maastricht University Medical Centre) and Máxima Medical Centre (MMC). It has initiated the registration of clinical data on ACC in a national database, e.g. for research purposes and the improvement of patient care [11, 12].

Patients who presented with ACC between January 1, 2004 and October 31, 2013 were included. Start and end of the observation period coincided with the founding

year of the DAN and the latest update of the DAN database, respectively.

In order to make the inclusion as complete as possible, hospital pathology departments, whom all participate in a nationwide network system (PALGA), were asked to compose a lists of all patients diagnosed with any adrenal tumour. Furthermore, the Dutch hospital diagnosis registry system, the Diagnosis Treatment Combination (DTC) system, was screened for patients labelled with an adrenal disease DTC code.

Inclusion criteria were age ≥18 years and confirmed diagnosis of ACC, either histological (Weiss, Van Slooten, Ki67) or by a combination of clinical analysis, hormonal analysis (e.g. urinary steroid profiling, dexamethasone suppression test) and imaging data e.g. computed tomography (CT).

Patient records were assessed to obtain retrospective information on patient characteristics, metastases, radiology imaging (CT, Magnetic Resonance Imaging and positron emission tomography and treatment). Comorbidity was classified according to the Charlson Comorbidity Index (CCI) [13, 14].

Definitions

The primary endpoint was overall survival (OS). OS was calculated from the date of first diagnosis of metastases to death from any cause. One-year survival and 2-year survival were also calculated from the date of diagnosis of metastases. Conditional survival derived from the concept of conditional probability was calculated using the traditional Kaplan-Meier.

The mathematical definition used can be expressed as follows [15-18]:

$$CS(t|s) = \frac{S(s+t)}{S(s)}$$

Metastases were defined as synchronous when found <6 months after the initial diagnosis of ACC [8, 9, 19].

Consequently, 'metachronous metastases' was defined as metastases diagnosed ≥6 months after the initial diagnosis of ACC.

The number of metastases was classified as 'limited' if there were one to three metastases and as 'extended' if there were four or more metastases [20]. A distinction was made between metastases found in only one organ/lymph node or in more than one organ/lymph node localization (discriminated organs: liver, lung, bone, lymph nodes under diaphragm, lymph nodes above diaphragm and elsewhere). Multiple metastases found in one organ were registered as one affected organ in the analysis of number of affected organs.

Diagnostic delay was calculated from the first hospital visit for ACC-related symptoms or signs until the moment the diagnosis of ACC were confirmed.

The effect of resection of the primary tumour on overall survival was assessed amongst patients with synchronous metastases. In addition, to further investigate the impact of ENS@Tstage at primary diagnosis, its impact on survival was analysed within the group of synchronous metastases. The IBM SPSS Statistics 22 was used for analysis. T tests and Pearson chi-square tests were used to analyse patient characteristics. Univariate Cox regression was used to analyse the effect of synchronous metastases vs. metachronous metastases, one or more involved organs, number of metastases and different involved organs. Those factors found to be significant were analysed in a multivariate Cox-regression analysis. Cox regression was used to evaluate the effect of different treatments on 1-year survival and 2-year survival as well as overall survival. Survival was calculated using the Kaplan-Meier method and applied log-rank test to assess differences in survival rates.

Results

One hundred ninety-two patients were identified with adrenal tumour disease upon screening of provided PALGA and DTC patient lists. Adrenal metastases of other primary malignant origin, adenomas and pheochromocytomas were excluded at first screening. Finally, a total of 160 patients from the DAN centres fulfilled the inclusion criteria. Thirty-two patients were excluded: 30 patients were not diagnosed in the time period set for this study, 1 patient had an ectopic ACC and 1 patient's file was incomplete and data not retrievable.

Patient characteristics are summarised in Table 1. The ratio female to male patients was 1.6:1. Most patients in this study had stage II disease at diagnosis (36 %) followed by stage IV disease (31 %). In 153 patients, ACC was confirmed based on histology of biopsy or tumour resection material. There were 7 patients in whom the diagnosis was solely based on the combination of clinical presentation, hormonal and imaging analysis and therefore treated as ACC (patients were not operated nor was there a biopsy performed). Median duration of follow-up was 17 months (range 0–118 months). Three patients in remission were lost to follow-up because they were referred back to their treating physician in a non-DAN hospital, and follow-up data were not available. During follow-up,

87 patients died as a direct result of ACC, 4 as a result of treatment toxicity and 2 patients died due to other causes (1 patient died of a pulmonary infection and the other patient died as a result of melanoma). At the end of follow-up, 23 patients were alive with disease and 44 patients were alive without evidence of disease.

Table 1: Clinical characteristics of study population

Sex	**N (% of total 160)**		
Male	61 (38%)		
Female	99 (62%)		
Age (years)			
Median	55 (range: 19-89)		
Follow up (months)			
Median	17(range: 0-118)		
Survival (months)			
Median	31 (range 0-118)		
ENS@T Stage at Diagnosis			**Survival** Median
Unknown	2 (1%)		
I	9 (6%)		.*
II	58 (36%)		62 months
III	41 (26%)		32 months
IV	50 (31%)		7 months
Metastases			
None	43 (27%)		
Synchronous metastases	84 (53%)		
Metachronous metastases	33 (21%)		
	At the time of first metastases	*Any time during follow up*	
Liver	43 (27%)	65 (41%)	
Lung	58 (36%)	79 (49%)	
Bone	8 (5%)	21 (13%)	
Lymph nodes under diaphragm	32 (20%)	52 (33%)	
Lymph nodes above diaphragm	11 (7%)	27 (17%)	
Other location	16 (10%)	47 (29%)	

* Could not be calculated

Survival

Of the 160 included patients, 117 (73 %) were diagnosed with metastases during the follow-up period of this study: 84 patients had synchronous metastases and 33 developed metachronous metastases. Figure 1 shows the Kaplan-Meier curve of the time interval between initial diagnosis and detection of the first metastasis.

At 2-year survival, 28 (33 %) of the patients with synchronous metastases and 19 (58 %) of the patients with metachronous metastases were still alive (p = 0.036).

At the end of follow-up of this study, 16 patients (19 %) with synchronous and 13 patients (39 %) with metachronous metastases were still alive. Median overall survival after diagnosis of the first metastases for patients with synchronous or metachronous metastases was 12 and 29 months, respectively (p = 0.046; Fig. 2). The 1-year conditional survival for ACC patients with synchronous and metachronous metastases is presented in figure 3.

Upon first diagnosis of metastatic disease, 40 patients (34 %) had one to three metastases. The other 77 patients (66 %) had four or more metastases. Median survival was 44 months for patients with 1–3 metastases and 7 months for patients with ≥4 metastases (p < 0.001). Overall survival for these two groups was significantly different with 50 and 12 %, respectively (p < 0.001). Also, the number of affected organs was of influence on median survival. In 75 patients with only one affected organ, median survival was 24 months; in 42 patients with two or more involved organs, median survival was 4 months. Overall survival percentages were 33 and 10 %, respectively (p < 0.001) (Fig. 2). When analysing overall survival in a selected group of patients with ≥2 affected organs (n = 42) by the factor synchronous vs. metachronous metastases, a log-rank p value of 0.006 was found.

The results of synchronous disease, ≥2 affected organs and ≥4 metastases on 1-, 2-year and overall survival in univariate and multivariate analysis are shown in Table 2.

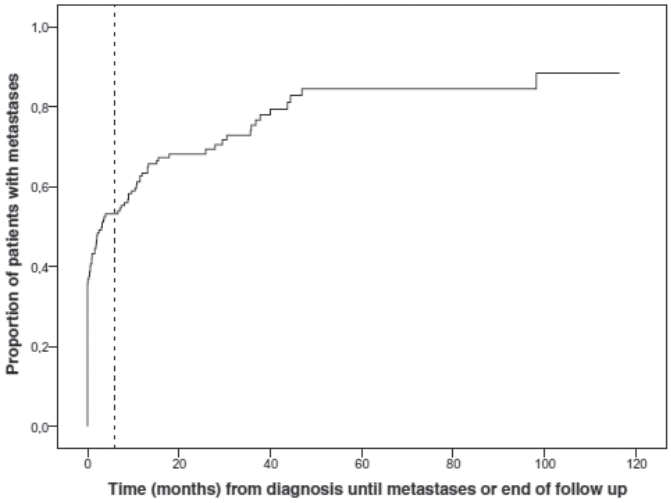
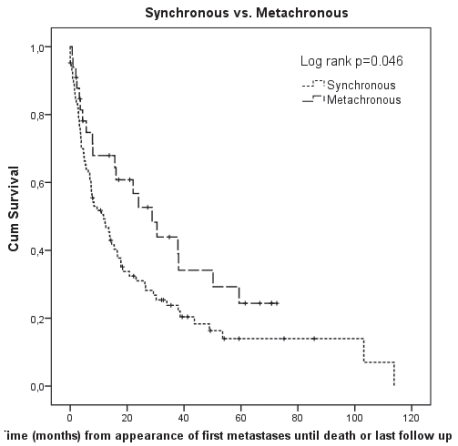
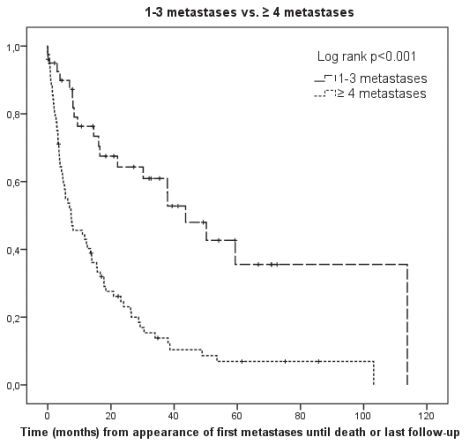
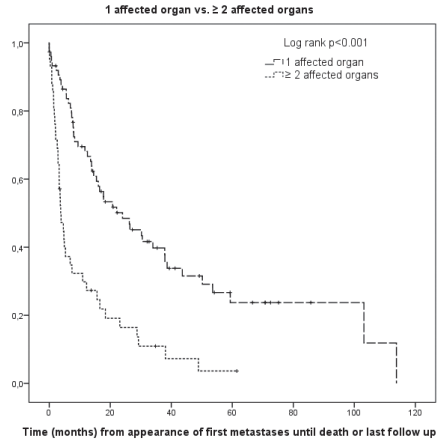


Fig. 1 Kaplan-Meier curve of the time in days between diagnosis of adrenocortical carcinoma and the diagnosis of the first metastases (n = 160). The cutoff (6 months) between synchronous and metachronous metastases is marked by the *dotted line*.







Number at Risk							
Time in Months	0	20	40	60	80	100	120
Synchronous	84	30	21	18	18	16	16
Metachronous	33	21	15	13	13	13	13

Number at Risk							
Time in Months	0	20	40	60	80	100	120
1-3 metastases	40	28	24	21	21	21	20
≥ 4 metastases	77	23	12	10	10	10	9

Number at Risk							
Time in Months	0	20	40	60	80	100	120
1-3 metastases	75	42	31	27	27	27	25
≥ 4 metastases	42	9	5	4	4	4	4

Fig. 2 Overall survival in months for a synchronous (*n* = 84) vs. metachronous (*n* = 33) metastases; b 1–3metastases (*n* = 40) vs. ≥4metastases (*n* = 77); c 1 affected organ (*n* = 75) vs. ≥2 (*n* = 42) affected organs.

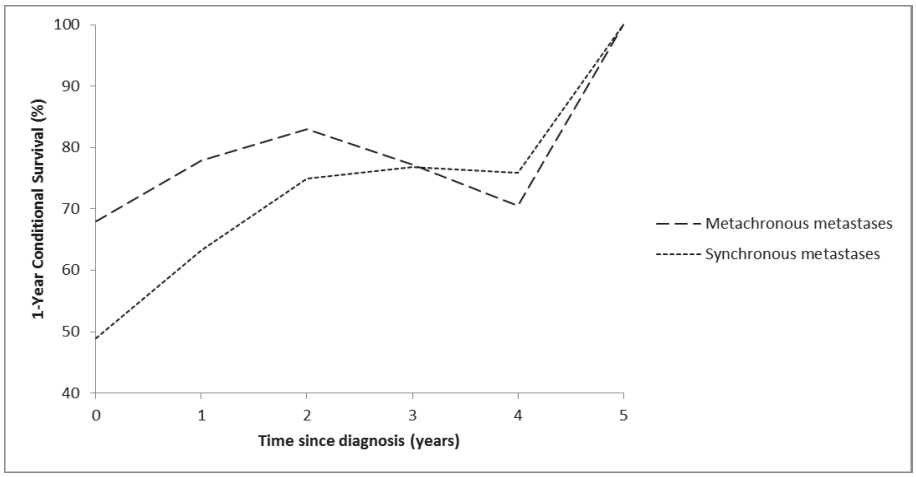


Fig. 3 One-year conditional survival CS (1 | s) in ACC patients with synchronous (N = 84) and metachronous (N = 33) metastases.

Affected Organs and Therapy

Overall survival between subgroups with only one affected organ system (i.e., metastasis only in the lung and liver of lymph nodes) was not different.

Of the patients with only lung metastases (n = 27), 44 % received chemotherapy (50 % EDP, 42 % Streptozotocin, 8 % Cisplatin), 17 % had a metastasectomy and 89 % received mitotane. For the patients with only liver metastases (n=20), 45 % received chemotherapy (78 % EDP, 11 % Streptozotocin, 11 % Cisplatin), 45 % had a metastasectomy, 30 % received radiofrequency ablation (RFA) and 75 % received mitotane. In the group of patients with only lymph nodes under the diaphragm (n = 14), 36 % received chemotherapy (80 % EDP, 20 % Streptozotocin), 57 % had a metastasectomy and 79 % were treated with mitotane.

One-year, 2-year and overall survival was not different between patients who received chemotherapy and those who did not in patients with only liver metastases (n = 20), only lung metastases (n = 27) or only metastases in the lymph nodes under the diaphragm (n = 14). The small number of patients treated with radiotherapy precludes separate analysis of its potential benefit. Mitotane had a significant impact on one-year (p = 0.017), two-year (p = 0.007) and overall survival (p = 0.007) in the group of patients with only metastases in the lymph nodes under the diaphragm in comparison to those who did not receive mitotane in this group. In other subgroup analysis, mitotane showed no significant impact on survival in our population.

RFA was performed in 6 patients with only liver metastases, and none of these patients died within 2 years (p = 0.046). Overall survival, however, of this subgroup was not different from the whole group of patients with liver metastases only (p = 0.158).

Metastasectomy appeared to have a significant impact for patients with lung metastases only on overall survival (p = 0.012) in comparison to patients with lung metastases only who did not undergo a metastasectomy.

Patients with Synchronous Metastases

Amongst the patients with synchronous metastases of ACC (n = 84), 50 patients (60 %) were ENS@T stage IV ACC at primary diagnosis, 22 stage III (26 %) and 12 stage II (14 %). Median time interval until diagnosis of metastasis was 0, mean time 17 days (range 0–119 days). Age and CCI did not differ significantly between patients who presented with stage IV at initial diagnosis or stage II–III. Patients with stage IV disease at initial diagnosis had significantly more affected organs compared to patients who developed metastases within 6 months after primary diagnosis (p = 0.006). Characteristics of these two subgroups are summarised in Table 3. Diagnostic delay (time in days until diagnosis of ACC was confirmed) did not differ significantly between these subgroups (p = 0.060).

The 1-year, 2-year and overall survival rates of patients who were stage IV at first presentation (n = 50) and of those who developed stage IV within 6 months after the initial primary diagnosis (n = 34) were significantly different (Table 3). Median survival was 7 and 23 months, respectively (p = 0.003).

Sixty-seven of the synchronous metastasized patients did undergo an adrenalectomy for their primary tumour, whereas in 17 patients the primary tumour was not resected. All but 1 of the patients who did not undergo an adrenalectomy (n = 17) had stage IV disease at the moment of diagnosis of ACC.

Patients with only one synchronous affected organ were more frequently submitted to a surgical resection of the primary adrenal tumour (p = 0.023). Of the 67 patients undergoing an adrenalectomy, 22 patients also had a metastasectomy. In contrast, of the 17 patients with synchronous metastases not undergoing an adrenalectomy, only 2 patients had a diagnostic metastasectomy to confirm ACC and determine the therapeutic strategy.

Median survival of patients with synchronous metastases who did or did not undergo adrenalectomy was 16.6 (range 0–113.8 months) and 1.8 months (range 0–16.5 months), respectively (p < 0.001) (Fig. 4). This result did not change after correction for number of affected organs and CCI.

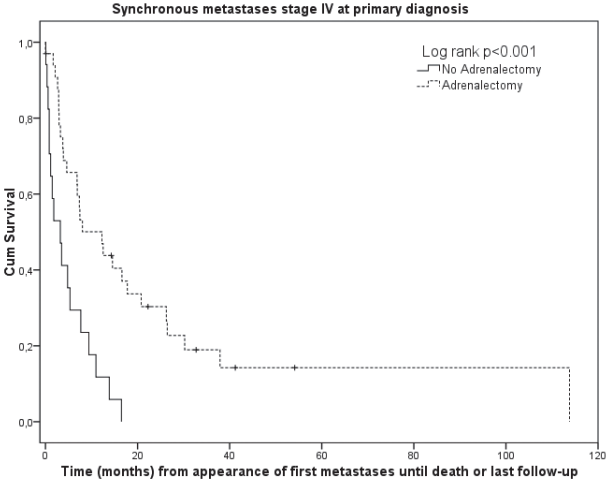
Table 2 Univariate and multivariate analyses of 1-, 2-year and overall survival for synchronous metastases, ≥2 affected organs and ≥4 metastases.

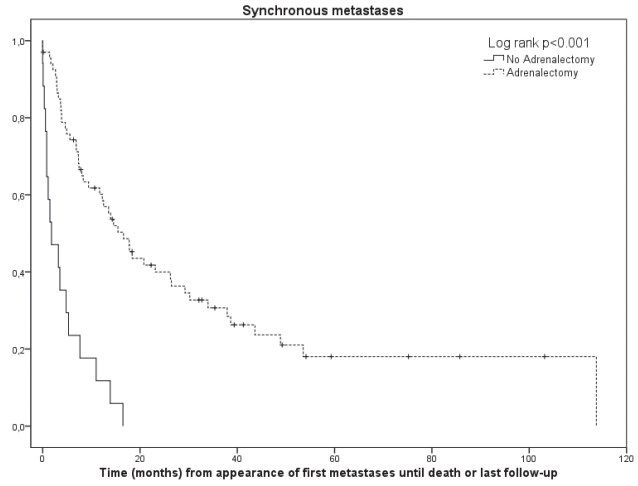
	1-year survival			2-year survival			Overall survival		
	HR	95% CI	p	HR	95%CI	p	HR	95%CI	p
Univariate analysis									
Synchronous metastases	.94	.87-1.01	0.096	1.86	1.03-3.34	0.039	1.66	1.02-2.75	0.048
≥2 affected organs	3.52	2.025-6.134	<0.001	2.95	1.83-4.74	<0.001	2.82	1.82-4.35	<0.001
≥4 metastases	3.30	1.61-6.78	0.001	3.38	1.85-6.2	<0.001	3.26	1.95-5.45	<0.001
Multivariate analysis									
Synchronous metastases	.94	.87-1.01	.101	1.85	1.02-2.36	0.044	1.66	.99-2.8	0.055
≥2 affected organs	2.80	1.46-5.35	0.002	2.19	1.27-3.77	0.005	2.07	1.2-3.42	0.005
≥4 metastases	1.80	.76-4.09	0.189	2.15	1.08-4.27	0.029	2.19	1.22-3.94	0.009

Table 3 Patient characteristics of patients with synchronous metastases: stage IV at diagnosis vs. metastases ≤6 months but not at diagnosis

	Stage IV at diagnosis (n=50)		**Metastases ≤6 months, but not at diagnosis (n=34)**		
Sex (ns)					
Male	16	32%	Stage II	Stage III	50%
			3	14	
Female	34	68%	9	8	40%
Age (ns)					
Mean	53	(range: 24-77)	51		(range: 21-81)
Charlson Comorbidity Index (ns)					
Mean	1.8		1.4		
Survival (months, p = 0.003)					
Median	7	(range: 0-114)	23		(range: 0-103)
Number of metastatic organs (p = 0.006)					
1	25	50%	27		79%
≥2	25	50%	7		21%
Number of metastases (ns)					
1-3 metastases	14	28%	11		33%
4+ metastases	36	72%	23		67%
Adrenalectomy (p = 0.001)					
Yes	34	68%	33		97%
No	16	32%	1		3%
Diagnostic delay (months, ns)					
Median	1,3	(range 0-11)	2,1		Range (0-33)

Significant differences between the two groups are indicated with a *p* value *ns* not significant





Number at Risk																								
Time in Months	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100	105	110	115
No Adrenalectomy	17	6	3	1	0																			
Adrenalectomy stage IV	33	21	16	12	10	8	6	4	3	2	2	1	1	1	1	1	1	1	1	1	1	1	1	0
Adrenalectomy all	67	50	39	31	25	22	19	15	11	9	7	5	4	4	4	4	3	3	2	2	2	1	1	0

Fig. 4a Overall survival in ACC patients with synchronous metastases and stage IVat primary diagnosis treated with adrenalectomy (n = 50; 34 underwent an adrenalectomy, 16 did not) p < 0.001. b Overall survival in all ACC patients with synchronous metastases (n = 84; 67 underwent an adrenalectomy, 17 did not) p < 0.001

Discussion

In different types of cancer, the moment metastases occur has been shown to have a significant impact on survival. Synchronous metastases, defined as metastases originating within 6 months after diagnosis of the primary tumour, are associated with a significant negative effect on prognosis in the colon [21] and lung cancer [22].

A trend towards better survival in ACC patients with metachronous metastases compared to synchronous metastases has previously been described [10]. To our knowledge, this is the first study to describe that timing of metastases is associated with overall survival in ACC. Late synchronous metastases, i.e. development of stage IV disease within 6 months after stage II–III at primary diagnosis, were associated with improved survival compared to patients that present with stage IV disease.

The number of affected organs has been considered a prognostic factor for survival in metastatic ACC [10, 23]. Our results confirm that the number of affected organs is indeed a prognostic factor.

Earlier studies evaluating the relationship between the number of metastatic lesions and survival have been inconclusive: In a cohort of 124 patients with ACC, Asssie et al. identified the presence of more than 5 metastatic lesions or the involvement of more than two organs as predictive factors for survival [10]. In the study by den Winkel et al., number of metastases, synchronous vs. metachronous metastatic disease and lymph node involvement did not significantly influence survival in a small cohort of 24 patients [24]. Our study shows a significant negative effect of having ≥4 metastases on survival, suggesting that an increasing number of metastases is prognostically unfavourable. Moreover, our data suggest that adrenal cancer spread to the lymph nodes affects survival to the same extent as hematogenic metastases. These results support a modified ENS@T classification observed heterogeneity in survival of patients with stage IVACC [25].

Although it has been suggested that oligometastatic disease is associated with more favourable behaviour than extended metastatic disease, this has not resulted in a tailored treatment approach in ACC [26]. The low efficacy of current treatment options for ACC [27] raises the question whether patients with limited metastatic disease might benefit from a different approach.

Recent research shows that a radical adrenalectomy and metastasectomy in patients with synchronous, limited metastatic disease, resulted in a more favourable prognosis [28]. We found that a metastasectomy for ACC patients with lung metastases was associated with significant better overall survival. It has been suggested that patients with locally recurrent ACC also benefit from surgical treatment, but we did not examine the impact of local adrenal recurrence on prognosis [29]. Previous studies demonstrated a better prognosis in patients with a time to local recurrence longer

than 1 year, and those in whom a complete resection of the local recurrence was performed [30].

In this study, adrenalectomy had a strong influence on survival, even in the presence of metastases. Patients with only one synchronous affected organ more frequently underwent an adrenalectomy than patients with two or more affected organs. Of notice, we were unable to find any other difference in clinical characteristics between patients who did undergo an adrenalectomy and those who did not. We had expected that patients with a bad performance status at disease presentation would have been less likely to have undergone surgical resection.

The improved survival that is associated with surgical intervention [31, 32] might overrule decision-making based on performance status and stage of disease in this group to a certain extent.

We found that patients who presented with stage IV disease at diagnosis had a much shorter survival than patients with stage II–III disease at diagnosis who developed late synchronous metastases. Thus, even within the group of synchronous metastases, a difference in survival was observed between early and late metastases. Future analysis should focus on identifying this specific subgroup of patients with ACC which exhibits less aggressive behaviour, who may benefit from adrenalectomy[33].

Our study is limited because of its retrospective nature. There were missing data, especially pathological data. The present study, however, was performed in a relatively large and well-defined cohort of adrenocortical cancer patients that reliably reflects the Dutch ACC population as a whole [1].

Current treatment options in ACC are limited, and new therapeutic options are anticipated[34]. Metachronous metastases are associated with better overall survival. Local therapy directed at metastasis should be considered in patients with limited metachronous disease. Otherwise, mitotane in combination with chemotherapy according to the FIRM-ACT protocol is indicated in case of more extended metachronous or synchronous metastases [34], although the latter patient group often has a bad prognosis despite systemic therapy [27]. The role of other local therapies already implemented in other malignancies has not been thoroughly investigated in ACC.

Stereotactic radiotherapy for lung metastases could be a promising alternative to surgical intervention. Radiofrequency ablation (RFA) for liver metastases, as a local-regional treatment with relatively little morbidity, should be further investigated as a therapeutic option in the treatment of selected cases of liver metastases in ACC.

In conclusion, synchronous metastases of ACC are associated with a worse prognosis compared to metachronous metastases of ACC and patients with late synchronous

metastases have a better prognosis than those with metastases at initial diagnosis. Further refinement of ENSAT stage IV disease should be considered, as these prognostic differences could be taken into account when determining the optimal treatment for a patient with ACC. Number of affected organs, number of metastases and timing of development of metastases influence survival and could potentially be considered in therapeutic decision making.

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Chapter 03

Development and Internal Validation of a Multivariable Prediction Model for Adrenocortical-Carcinoma-Specific Mortality

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Simple Summary

Adrenocortical carcinoma is a rare and aggressive cancer. Great variability in clinical course is observed, ranging from patients with extreme long survival to aggressive tumors with prompt fatal outcome. This heterogeneity in survival makes it complicated to tailor treatment strategies for an individual patient. Therefore we sought to identify prognostic factors associated with ACC specific mortality. We analyzed the data of 160 ACC patients and developed a clinical prediction model including age, modified European Network for the Study of Adrenal Tumors (mENSAT) stage, and radical resection. This easy-to-use prediction model for ACC-specific mortality has the potential to guide clinical decision making if externally validated.

Abstract

Adrenocortical carcinoma (ACC) has an incidence of about 1.0 per million per year. In general, survival of patients with ACC is limited. Predicting survival outcome at time of diagnosis is a clinical challenge. The aim of this study was to develop and internally validate a clinical prediction model for ACC-specific mortality. Data for this retrospective cohort study were obtained from the nine centers of the Dutch Adrenal Network (DAN). Patients who presented with ACC between 1 January 2004 and 31 October 2013 were included. We used multivariable Cox proportional hazards regression to compute the coefficients for the prediction model. Backward stepwise elimination was performed to derive a more parsimonious model. The performance of the initial prediction model was quantified by measures of model fit, discriminative ability, and calibration. We undertook an internal validation step to counteract the possible overfitting of our model. A total of 160 patients were included in the cohort. The median survival time was 35 months, and interquartile range (IQR) 50.7 months. The multivariable modeling yielded a prediction model that included age, modified European Network for the Study of Adrenal Tumors (mENSAT) stage, and radical resection. The c-statistic was 0.77 (95% Confidence Interval: 0.72, 0.81), indicating good predictive performance. We developed a clinical prediction model for ACC-specific mortality. ACC mortality can be estimated using a relatively simple clinical prediction model with good discriminative ability and calibration.

Introduction

Adrenocortical carcinoma (ACC) is a rare malignancy with an annual incidence of about 1.0 per million individuals [1]. With an overall one-year survival rate of 60% and a five-year survival rate of 32% [1], the disease has a poor prognosis in general. Nonetheless, patients who survive for 12–28 years have been documented [2]. The exact reason for the long survival in these patients is unknown. Several factors may be responsible for these differences in survival, e.g., tumor type, lifestyle, and genetic variability. Predicting survival outcome at the time of diagnosis is difficult in clinical practice. Some clinical variables show prognostic potential: the Weiss score is the internationally acknowledged pathologic scoring system to differentiate between malignant and benign tumors. The Weiss score is a nine-point scoring system, in which a score of three or higher corresponds to a high probability of malignancy [3,4]. A Weiss score greater than six has been found to correspond to poor prognosis [5].

Another histological weighted scoring system is the Van Slooten Index (VSI). A score of eight or higher corresponds to a high probability of malignancy [6]. Unfortunately, neither of these pathological features are sufficiently specific for an estimation of prognosis in ACC.

The Ki67 index, an immunohistochemical marker, has been suggested as an additional prognostic parameter. The Ki67 index is an estimate of the percentage of tumor proliferation. A Ki67 index over 5% is suggestive of malignancy [7]. A malignant tumor with a Ki67 index under 10% is associated with a relatively good prognosis; an index over 20% is associated with an undesirable course of disease [5,8]. However, the Ki67 scoring assessment varies greatly, and both inter- and intra-observer variations cause significant limitations to its clinical utility [9]. Additionally, the clinically relevant cutoff values that are suggested to be associated with prognosis are debatable.

Other molecular or genomic prognostic markers that can clearly distinguish between low-risk and high-risk ACC tumors are being increasingly investigated [10], but their clinical value as prognostic tools has not yet been determined in large prospective series and therefore they are not part of the current clinical guidelines [11].

In 2004, the International Union Against Cancer (UICC) and the World Health Organization published the first staging classification based on the Tumor, Node, Metastasis (TNM) criteria for ACC [12]. Due to shortcomings, the European Network for the Study of Adrenal Tumors (ENSAT) developed a revised staging system [13]. The ENSAT staging system is currently recommended for estimating the prognosis of a patient with ACC [5,12]. However, the ENSAT stage is still associated with considerable heterogeneity, as reflected by a five-year stage-dependent survival of 66–82% for stage I, 58–64% for stage II, 24–50% for stage III, and 0–17% for stage IV [14]. A prediction model combining multiple parameters that have been individually associated with survival could be of

significant additional value, and may be more useful in clinical practice. A clinical prediction model can inform patients and their physicians of the patients' probability of a specified outcome and help them with associated decision making. Previous prediction models were developed for specific subgroups of ACC patients: recurrence-free (RFS) and overall survival (OS) after curative resection of ACC [15], OS of ACC patients after surgery [16], or lacking essential (histologic) predictors in the model development [17–19]. Therefore, the aim of this study is to develop and internally validate a multivariable, generally applicable clinical prediction model for ACC-specific mortality.

Results

A total of 160 patients were included in the cohort. One patient was omitted from the analysis, as no outcome measures were available. Patients were followed for a median period of 33 months (1st and 3rd quartile: 11.0–61.5). A total of 108 (67.9%) patients died during the course of follow-up. The median survival time was 35.6 months (range 0.7–145.4 months). The characteristics of all patients included in this study are shown in Table 1. Figure 1 shows the Kaplan–Meier curve of the total cohort, for a total follow-up time of 60 months.

Table 1. Patient characteristics (N = 160) *.

	Original Data	**Imputed Data**
	N (% of total) (Range)	N (% of total) (Range)
Male	61 (38)	61 (38)
Female	99 (62)	99 (62)
Age at diagnosis	55 (19–89)	55 (19–89)
ENSAT Stage		
I	9 (6)	9 (6)
II	58 (36)	58 (36)
III	42 (26)	42 (26)
IV	51 (32)	51 (32)
mENSAT Stage		
I	9 (6)	9 (6)
II	58 (36)	58 (36)
III	30 (19)	30 (19)
IVa	36 (22)	36 (22)
IVb	17 (11)	17 (11)
IVc	10 (6)	10 (6)

	Original Data	Imputed Data
Resection status		
R0	74 (46,25)	74 (46)
R1/R2/Rx	61 (38,1)	68 (43)
No surgery	18 (11,25)	18 (11)
Missing	7 (4,4)	-
Ki67		
≤5	27 (17)	47 (29)
6–10	19 (12)	28 (18)
11–15	8 (5)	16 (10)
>15	30 (18,5)	69 (43)
Missing	76 (47,5)	-
Capsular and/or vascular invasion		
Yes	90 (56)	125 (78)
No	24 (15)	35 (22)
Missing	46 (29)	-
Hypercortisolism		
Yes	88 (55)	88 (55)
No	35 (22)	72 (45)
Missing	37 (23)	-
Complaints due to tumor mass		
Yes	78	78
No	13	82
Missing	69	-

* To prevent a loss of statistical precision and to decrease the likelihood of obtaining biased results, we imputed the available data (see methods for further explanation). See method section for definition of the predictor variables.

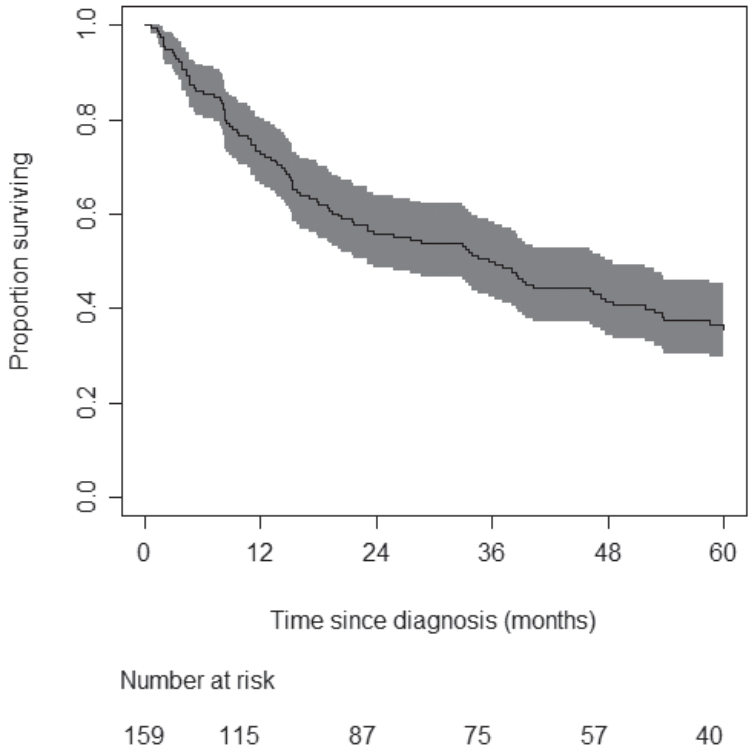


Figure 1. Kaplan–Meier curve, including 95% confidence band of the risk of adrenocortical carcinoma-related mortality. The numbers above the *x*-axis denote the number of patients still at risk at each point in time.

In the original database, before attempted revision, 48 (30%) out of 160 patients had a Ki67 score. Fifty-five of 160 (34%) tumor samples were retrieved for central revision of the Ki67. Of only 49 samples, the appropriate material was available for immunohistochemistry analysis and of these 49 samples 13 tumors already had a Ki67 record in the database (net: 49 − 13 = 36 new Ki67 data). Revision data were used for final analysis. The number of valid records increased from 48 (30%) in the original database to (48 + 36) 84 (52.5%) in the new database.

The correlation between ENSAT and mENSAT was high (rho = 0.93, $p < 0.001$). We selected mENSAT for further statistical modelling based on a stronger univariable association with survival. The c-statistic for mENSAT was 0.73, compared to 0.71 for ENSAT. The restricted cubic spline regression revealed no significant non-linear associations between continuous predictor variables and mortality. Because of the high proportion of missing values on the Ki67, the modelling procedure was performed both with and without Ki67.

Table 2 shows the coefficients of the predictor variables that were significantly associated with ACC-related mortality. In the model that was derived without the Ki67 index, cortisol and pathology positivity were also excluded during the backward elimination process because their *p*-values were too high ($p = 0.469$ and $p = 0.155$ upon exclusion, respectively). All predictors were risk factors for ACC-specific mortality, except for radical resection, which was protective. The scaled Schoenfeld residuals revealed that no predictor variables violated the proportional hazards assumption, indicating that the predictor variables are valid for the whole follow-up period.

Pathology was scored positive if venous invasion or capsular invasion were present according to the Weiss criteria in the pathology report or capsular and/or vascular invasion was scored yes according to the Van Slooten Index in the pathology report (see method section).

The c-index, which was computed to quantify the ability of the model to separate events of ACC-specific mortality from patients who did not experience the event, was 0.77 (95% CI: 0.73, 0.81) and 0.77 (95% CI: 0.72, 0.81) for the models including and excluding Ki67 in the selection process, respectively. Figure 2 shows the calibration plots of both models. The lines closely follow the ideal line of 45 degrees, indicating that both models are well-calibrated.

Table 2. Initial and internally validated coefficients of the prediction model for ACC-specific mortality.

Predictor	**Model with Ki67**			**Model without Ki67**		
	Coefficient	**HR (95% CI)**	**Shrunk Coefficient ***	**Coefficient**	**HR (95% CI)**	**Shrunk Coefficient ***
Age at diagnosis (year)	0.02	1.02 (1.01, 1.03)	0.02	0.02	1.02 (1.00, 1.03)	0.02
Pathology positive (yes)	0.52	1.68 (0.98, 2.89)	0.47			
mENSAT (stage)	0.61	1.84 (1.55, 2.18)	0.65	0.66	1.93 (1.64, 2.28)	0.63
Ki67 (%)	0.01	1,.01 (1.00, 1.03)	0.01			
Radical resection of tumor (yes)	−0.37	0.69 (0.46, 1.04)	−0.34	−0.46	0.63 (0.42, 0.94)	−0.44

* Internally validated (*shrunk*) coefficients were obtained by multiplying the coefficients by the shrinkage factor of 0.91 for the model with Ki67, and 0.95 for the model without Ki67.

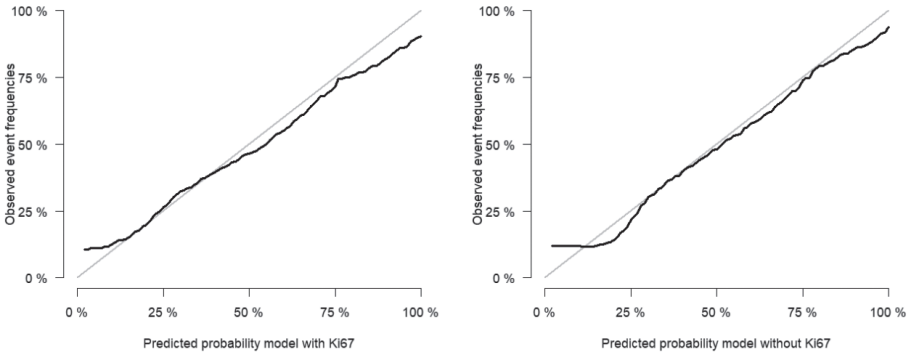
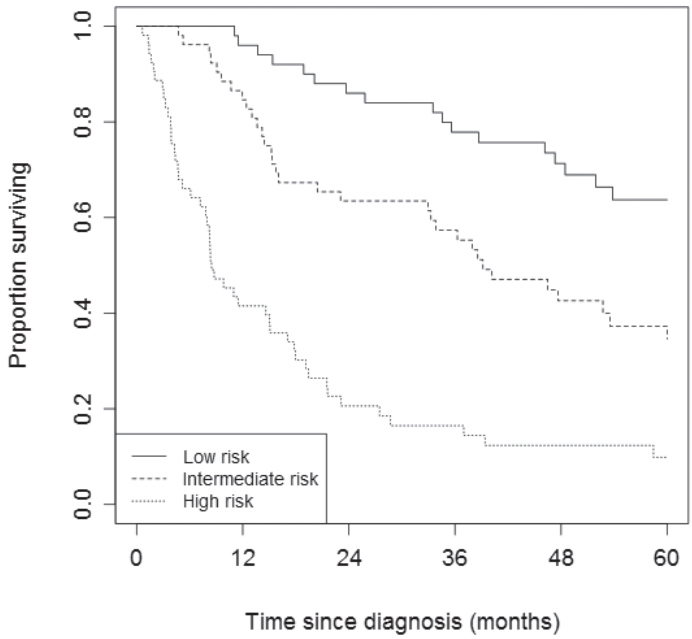


Figure 2. Months of follow-up time.

The internal validation yielded a shrinkage factor of 0.91 for the model with Ki67, and 0.95 for the model without Ki67 (Table 2). Hence, slightly more overfitting was present in the model that included Ki67. In addition, the internal validation yielded a measure of optimism of the c-index of only 0.01, indicating that the c-index as a measure of the model's ability to discriminate in future patients is estimated to be 0.76 and 0.76 for the two models, respectively (compared to the apparent ability to discriminate of 0.77 and 0.77, respectively). The shrinkage factors that are close to 1, together with the small measures of optimism, indicate that these models are not much overfitted. Figures 3 and 4 show the Kaplan–Meier curves for patients stratified by their risk score, for both models.



Number at risk

51	48	43	38	31	24	(RS < –0.58
52	44	33	28	19	13	(RS –0.58 to 0.44
53	22	10	8	6	2	(RS > 0.44)

Figure 3. Kaplan–Meier stratified by risk groups based on each individual's risk score (RS) of the model including Ki67.

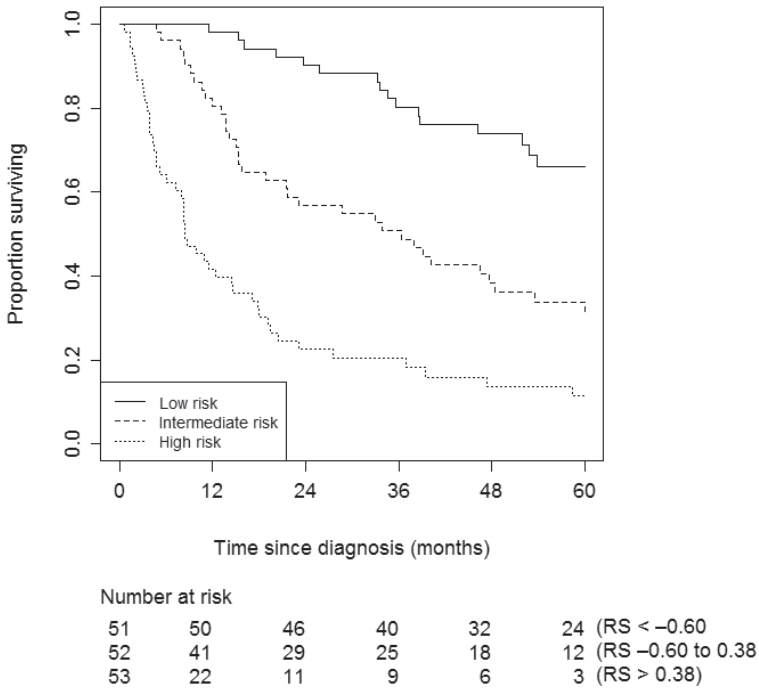


Figure 4. Kaplan–Meier stratified by risk groups based on each individual's risk score (RS) of the model without Ki67. The Risk Score can be computed as 0.02 * (age − 54.5) + 0.63 * (mENSAT − 3.2) − 0.44 * (radical resection of the tumor − 0.8).

Prediction for Future Patients

The prediction models presented in Table 2 can either be used to compute an individual risk score to determine risk category (Figures 3 and 4), or to compute an actual probability that an individual experiences ACC-specific mortality within one, two, or five years. How to do so is outlined below.

The risk score (RS) can be computed by multiplying the shrunk coefficients by the values of an individual patient minus the sample average for age (54.5), mENSAT (3.2), and radical resection (0.8). mENSAT takes on a score from 1–6, with mENSAT I-III scoring 1–3 points, and IVa-IVc scoring 4–6 points. The surgical resection is scored one if radical and zero if not (see method section), for example, using the model without Ki67: 0.02 * (age − 54.5) + 0.63 * (mENSAT − 3.2) − 0.44* (radical resection − 0.8) of the tumor (no = 0, yes = 1). A 64-year old patient with an mENSAT stage of 4a, and no radical resection of the tumor, would have a risk score of 0.02 * (64 − 54.5) + 0.63 * (4 − 3.2) − 0.44 * (0 − 0.8) = 1.046. Figure 4 shows that this individual would be classified as being at high risk of ACC-specific mortality.

The probability of ACC-specific mortality within one, two, or five years is computed by combining the Kaplan–Meier estimate at one, two, or five years and the sum of the shrunk coefficients to the centered values of an individual patient (i.e., the value minus the sample average) as $1 - S(t)^{exp\ (LP)}$: S(t) is the survival function in which t is time. S(t) for one, two, and five years is 0.73, 0.56, and 0.37, respectively (see Kaplan–Meier Figure 1). Exp(LP) stands for *e* raised to the power of the linear predictor (exp: exponential (function), *e* is the mathematical constant, lp: lineair predictor). The linear predictor can be computed as 0.02 * (age − 54.5) + 0.63 * (mENSAT − 3.2) − 0.44 * (radical resection of the tumor − 0.8). The 64-year old patient with an mENSAT stage of 4a, and no radical resection (no = 0) of the tumor, would have a two-year probability of ACC-specific mortality of $1 - 0.56^{exp(LP)}$. LP = 0.02 * (64 − 54.5) + 0.63 * (4 − 3.2) − 0.44 * (0 − 0.8) = 1.046; hence, the probability = $1 - 0.56^{exp(1.046)}$ = 0.81 = 81%. This can be confirmed in Figure 4, in which approximately 20% of the high-risk group survives past 24 months.

Discussion

In this collaborative study of the Dutch Adrenal Network, a model capable of predicting ACC-specific mortality was developed. An accurate prediction model could help to identify patients at greater risk of death, and support the decision making on early systemic therapy. Furthermore, a prediction model could support selection of a specific subgroup eligible for new therapeutic compounds.

Ki67 is a suggested prognostic marker in ACC [5,20]; therefore, we performed modelling both with and without Ki67. Both models were based on tumor stage defined by the mENSAT classification, age, and radical resection of the primary tumor. The model with Ki67 also relied on hormonal status and the pathology criteria capsular and/or vascular invasion. Although both models showed comparable discriminative ability and calibration, the model with Ki67 was partially based on imputed data because of missing data despite our efforts to revise the Ki67 index for all patients. Previous research has shown that pathology data are often incompletely described in ACC [21]. In addition, pathology reports are not standardized in ACC. Prognostic scoring systems based on the Weiss and Van Slooten criteria as well as the Ki-67 are to a certain extent subjective, as a reliable assessment largely depends on the expertise of the pathologist [22]. Furthermore, there is often significant inter-observer variability in the determination of the Ki67 [9]. Therefore, in order to improve the generalizability of our prediction model, we decided to use more reliable and easily accessible clinical variables such as age, mENSAT, and completeness of the resection.

This is the first time the mENSAT stage has been considered in a prediction model. Both ENSAT stage, number of affected organs, presence of metastasis, as well as

nodal status have shown to be correlated with survival [5,13,23–25]. The mENSAT stage combines those variables in one staging system, and considers the number of affected organs, including the primary tumor and lymph nodes [5]. The important difference compared with the ENSAT staging system currently used is the fact that T3-4N1M0 is considered stage IV instead of stage III. Lymph node positive disease has been demonstrated to be associated with a less favorable prognosis [26,27], and consequently, given the high recurrence rates for ENSAT stage III, a more prominent role for neo- and adjuvant therapy has been put forward [27]. We endorse using the mENSAT stage in clinical practice.

Estimating survival with the Kaplan–Meier analysis, which is commonly done, has its limitations. It is a nonparametric approach to survival outcomes, and it is able to show univariate relationships graphically or to compute survival factions at a certain time of follow-up. However, the Kaplan–Meier method and the log-rank test cannot be used for multivariate analysis. When looking at current data based on Kaplan Meier data, a stage IV patient could have zero percent change of 5-year survival or almost 20%. Most patients want to know if they have this small chance at 5-year survival or no chance at all when deciding on starting chemotherapy or mitotane. A clinical prediction model provides such tailored estimation on prognosis. In daily practice, a physician would like to estimate the prognosis tailored for a particular patient, underscoring the need for a reliable clinical prediction model.

In contrast to previously proposed prediction models for ACC, we included pathological data, imputed missing data to prevent a loss of statistical precision, and considered the most recently proposed mENSAT staging system [5]. Another strength of the present study is that our modelling was not based on a pre-selected group of patients with ACC, as our sample included all ENSAT stages. Kim et al. [24] developed a prediction model based on a multi-institutional group of patients treated in the United States who underwent surgery for ACC (Table 3). Notably, they excluded patients with metastatic disease at presentation as well as patients with a macroscopically nonradical resection (R2). Although they studied a relatively large cohort (n = 148), the external validity of their study is limited because up to 53% of patients with ACC may present with metastatic disease [21]. Even if their model was solely meant to predict RFS and OS after surgery, they still excluded patients. Surgery is being considered in patients with metastatic disease, and it has been shown that surgery may even improve the outcome in selected patients with stage IV disease, especially if an R0 resection can be achieved [28].

Table 3. Overview of current models for prognostication in patient with ACC.

Study	N			Outcome	Predictors	C Statistics
	Model development	Internal validation	External validation			
Zini et al., 2009 [19]	205	-	207	CSM and ACM in patients managed with either surgery or no surgery for ACC.	CSM/ACM: age, stage (localized, regional, and distant), and surgical status (surgery or no surgery).	-
Kim et al., 2016 [24]	148	Bootstrap validation with 200 resamplings.	-	RFS and OS after curative surgical resection of ACC.	RFS: tumor size (<12 or ≥12 cm), nodal status (N0, N1, or Nx), T stage (I/II or III/IV), cortisol-secreting tumor, and capsular invasion.	RFS: 0.74
					OS: tumor size (<12 or ≥12 cm), nodal status (N0, N1, or Nx), and resection margin (R0 or R1).	OS: 0.70
Li et al., 2018 [17]	751	Bootstrap validation with 200 resamplings.	-	OS and CSS in patients with ACC.	OS: age at diagnosis, year of diagnosis (1973–1987, 1988–2001, 2002–2015), histologic grade (I + II, III + IV, unknown), historic stage (localized, regional, distant, unknown), and chemotherapy (no/unknown, yes).	OS: 0.677
					CSS: age at diagnosis, year of diagnosis (1973–1987, 1988–2001, 2002–2015), historic stage (localized, regional, distant, and unknown), and chemotherapy (no/unknown, yes).	CSS: 0.672
Kong et al., 2019 [16]	404	318, and bootstrap validation with 1000 resamplings.	82 + 82 * = 164	OS in patients with ACC after surgery.	OS: age, T stage (T1-T4), N stage (N0, N1), M stage (M0, M1).	OS: 0.715

ACC: adrenocortical carcinoma; ACM: all-cause mortality; CSM: cancer-specific mortality; CSS: cancer-specific survival; OS: overall survival; RFS: recurrence-free survival. T, tumor. N, lymph node. M, metastasis. N0, no positive lymph nodes; N1, positive lymph node(s); Nx, not harvested. M0, no distant metastases; M1, presence of distant metastasis. Complete resection (R0); microscopically irradical (R1).* two external validation sets were used: the Cancer Genome Atlas set and a Chinese multicenter cohort dataset.

Li et al. presented a nomogram for overall survival (age, year of diagnosis); histologic grade (I + II, III + IV, and unknown); historic stage (localized, regional, distant, and unknown); chemotherapy (no/unknown or yes); and cancer-specific survival (CSS), including age, year of diagnosis, historic stage and chemotherapy [17]. Kong et al. did not include any histological criteria, but their nomogram included age and TNM stage (according to the 7th American Joint Committee on Cancer (AJCC) TNM staging) [16]. With the nomogram of Li et al., future use causes a problem where year of diagnosis can only be scored till 2015. In addition, their other predictors in that nomogram are not commonly used in clinical practice [17]. The same limitation applies to the nomogram by Kong et al., who use the AJCC TNM staging [16]. Age, however, is a predictor in both models.

It is interesting to note that although the work of Zini et al. [19] suffered from lack of detailed prognostic information, their prediction model included age, stage, and surgery status. In our analysis, we actually include a broad selection of potential predictor variables, and confirm age, stage, and completeness of tumor resection to be significant predictors for ACC specific mortality. So, even in the absence of detailed information, e.g., patients of whom no immunohistochemistry is available, it is possible to make an estimate of cancer-specific mortality.

The study by Kebebew et al. [29] was not designed to develop a prediction model, but their highly powered (n = 725) multivariable analysis for ACC mortality also showed that ACC stage, surgical resection, and tumor grade (localized, regional, or distant) were independent prognostic factors.

There is a lack of consensus regarding the cut-off point and combination of pathologic criteria that are associated with prognosis [20,30,31]. Consequently, there are limitations to the clinical use of pathology criteria for ACC prognosis. Perhaps in the near future, results of genome and transcriptome studies could be useful. The results of their potential prognostic value seem promising [10,32–34]. For example, hypermethylation of CpG islands, or as a pattern called CpG island methylator phenotype (CIMP), is associated with a poor prognosis in ACC [10,33,35]. At the moment, a clearly defined picture of CIMP in ACC is lacking, and although study results on CIMP patterns demonstrate a certain degree of overlap, there are some (methodological) inconsistencies. Furthermore, it is uncertain whether methylation status has the potential to become a prognostic factor on its own, or if it will be an additive to a clinical prediction model, as presented in this study. The latter might be expected considering the fact that DNA methylation is a dynamic process with potential fluctuations over time, and with differences between primary tumor and metastasis. Libbert et al. recently presented a COMBI score, integrating clinical predictors with number of somatic mutations, alterations in the Wnt/beta-catenin and p53/Rb pathways, and promoter region methylation pattern. Again, heterogeneity of

molecular analysis and definition of cut-off values used pose a problem, but it could be useful as a prognostic determinant in the future.

Individual biomarkers like *G0S2* [34] and *BUB1*, *PINK1* [36,37] might overcome the problem of heterogeneity with CIMP, but those biomarkers merely identify ACC with a poor prognosis.

Some limitations of our study merit further mentioning. The prediction model is only applicable to ACC patients of 18 years or older. The cohort we used had a relatively small sample size, which limited the number of predictors for consideration in our model. In view of the fact that the Netherlands currently holds 17 million inhabitants, the size of our study cohort is in agreement with the reported incidence of ACC. In our opinion, the prediction model presented here has the potential to provide a more individualized and practical estimate of the prognosis of ACC, compared to the currently used staging system. Although our model was internally validated using bootstrap validation, external validation is warranted before widespread implementation of the algorithm in a user-friendly prediction calculator.

Materials and Methods

Data for this retrospective cohort study were obtained from the nine centers of the Dutch Adrenal Network (DAN), as has been described earlier [23]. Patients of ≥18 years who presented with ACC between 1 January 2004 and 31 October 2013 were included. Follow-up data was investigated until 30 June 2016. Because of missing Ki67 data in the original database, an attempt was made to revise the Ki67 index, from 31 January 2018 until 2 April 2020.

Potential Predictor Variables

We identified potential predictor variables based on clinical reasoning, and on previously published risk factors for mortality. These variables were age at the time of diagnosis [38], sex, body mass index (BMI), TNM classification, hypercortisolism, complaints directly related to tumor mass, venous invasion, ENSAT stage, modified ENSAT (mENSAT) stage [5], radical resection of the tumor (R0 variable "yes"; Rx/R1/R2 variable "no") [20,26], and Ki67 index.

ENSAT stage was defined according to Fassnacht et al. [13]: stage I, tumor size ≤5 cm (T1N0M0); stage II, tumor size >5 cm (T2N0M0); stage III, tumor of any size with at least one of the following factors: tumor infiltration in surrounding tissue (T3), tumor invasion into adjacent organs, or venous tumor thrombus in the vena cava or renal vein (T4), positive lymph node (N1), but no distant metastasis (M0); and stage IV, the presence of distant metastases irrespective of tumor size or lymph node status (T1-

T4N0-N1M1). The mENSAT classification defines T3-4N0M0 as stage III (invasion of surrounding tissues/organs, or invasion of the renal vein or inferior vena cava), and both T3-4N1M0-1 and T3-4N0M1 as stage IV. Stage IV is then subcategorized into Stage IVa (two involved organs), IVb (three involved organs), and IVc (>three involved organs). The primary tumor and "N" are included as "organ" in the count of number of involved organs [5].

Pathology was scored positive if venous invasion or capsular invasion were present according to the Weiss criteria in the pathology report or capsular and/or vascular invasion was scored yes according to the Van Slooten Index in the pathology report.

Hypercortisolism was defined clinically if this was reported by the treating physician in the patients' file or biochemical with a cortisol level above the upper limit of normal as defined by the hospital laboratory where the patients' cortisol level was analyzed. Cortisol level was determined either in serum, saliva, 24-h urine, and/or during a dexamethasone suppression test.

Complaints due to tumor mass were scored yes if the patient had abdominal pain or back pain that could be due/directly related to the tumor mass.

Immunohistochemistry Revision Ki67

An attempt was made to revise the Ki67 index for all 160 patients. We were able to track down 55 tumor samples.

To assess the number of cells in cycle (preparing for cell division), selected blocks were stained at one location using the MIB-1 antibody (obtained from Dako, Glostrup, Denmark). All sections were stained in an immunostainer (Ventana Benchmark Ultra, from Roche, Tucson, Arizona, US) in 3 runs. Sections were developed using the Optiview 3,3-diaminobenzidine (DAB) system (also from Roche) as a second step and DAB as chromogen. To assess the number of stained cells, the area with the highest staining intensity was searched for, and that area was magnified using a 40x objective (hot spot method). All cells and all positive cells were counted, and the percentage of positive cells was calculated. A cell was deemed positive when the nucleus was no longer blue. Additionally, faint staining was included as positivity. As a control for the staining effectivity, a tissue microarray was mounted on each individual stained slide.

Unfortunately, information on the Ki67 analytic process used for the other tumor samples in the database is not available because of historical data.

Model Development

We chose to impute our data to prevent a loss of statistical precision and to decrease

the likelihood of obtaining biased results [39]. Imputation was performed using stochastic regression imputation with fully conditional specification. Predictive mean matching was used to draw the values to be imputed.

Baseline characteristics of the participants were described using means and standard deviations or absolute numbers and percentages. The overall survival of our cohort was estimated using the Kaplan–Meier method. Median follow-up time was computed, including the first and third quartiles. The median survival time was estimated including 95% confidence interval (CI) around the median.

We computed a correlation matrix to assess correlations between predictor variables. If variables were highly correlated (Pearson's correlation coefficient, or rho, >0.8), we chose to include only the variable that was deemed more convenient to implement in a clinical prediction tool.

Multivariable Cox proportional hazards regression was used to estimate the coefficients in the prediction model. Backward stepwise elimination was performed to derive a more parsimonious model. We used the Akaike Information Criterion as the rule for deleting variables from the model, which corresponds to a more liberal alpha of 0.157 [40]. The correlation between the scaled Schoenfeld residuals and time was computed for each variable to test the proportional hazards assumption [41].

The performance of the initial prediction model was quantified by measures of discriminative ability and calibration. Discriminative ability is expressed as the c-index, which can take on any value between 0.5 (no discriminative ability) and 1 (perfect discriminative ability), and is an estimate of the probability that of any two randomly chosen patients, the one with the higher prognostic score will outlive the one with the lower prognostic score [41]. Calibration was assessed by visual inspection of the calibration plot. The calibration plot shows the agreement between predicted probabilities and pseudo-observed event status at a follow-up time of two years.

Internal Validation of the Model

As a rule of thumb, it is suggested that for each potential predictor variable in the model, 10 events should be observed to prevent overfitting. An overfitted model would perform well in the development data, but poorly when applied to new patients. Often, such an overfitted model would produce too extreme predictions. Since our dataset was relatively small, an internal validation step was performed to counteract the possible overfitting of our model to the data. We used standard bootstrapping techniques (B = 1000) to obtain optimism-corrected measures of performance (the c-index) and a shrinkage factor. The shrinkage factor is calculated as the optimism-corrected calibration slope. It is a constant between 0 and 1, and

the regression coefficients are then shrunk by multiplying by this number. These penalized regression coefficients produce fewer extreme predictions, and hence counteract the effect of overfitting.

Finally, we stratified all patients into three groups based on their predicted risk score (i.e., low, medium, and high risk) for both the model with and the model without the Ki67 index.

Conclusions

In conclusion, we have developed and internally validated an easy-to-use prediction model for ACC-specific mortality; this model is essentially based on age, mENSAT stage, and completeness of tumor resection.

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Part II

Treatment strategies

Chapter 04

Population pharmacokinetic and pharmacogenetic analysis of mitotane in patients with adrenocortical carcinoma: towards individualized dosing

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Background and Objectives

Mitotane is the only approved treatment of patients with adrenocortical carcinoma (ACC). A better explanation of the variability in pharmacokinetics (PK) of mitotane and optimization and individualization of mitotane treatment are desirable for patients. This study aims to develop a population pharmacokinetic (PopPK) model to characterize and predict the PK profiles of mitotane in patients with ACC, as well as to explore the effect of genetic variation on mitotane clearance. Ultimately, we aim to facilitate mitotane dose optimization and individualization for patients with ACC.

Methods

Mitotane concentrations data and dosing data were collected retrospectively from the medical records of patients with ACC taking mitotane orally and participating in the Dutch Adrenal Network. PopPK modelling analysis was performed with NONMEM (version 7.4.1). Genotypes of drug enzymes and transporters, patients' demographic information, and clinical characteristics were investigated as covariates. Subsequently, simulations were performed for optimizing treatment regimens.

Results

A two-compartment model with first-order absorption and elimination best described the PK data of mitotane collected from 48 patients. Lean body weight (LBW), genotypes of *CYP2C19*2* (rs4244285), *SLCO1B3* 699A>G (rs7311358), and *SLCO1B1* 571T>C (rs4149057) were identified to affect mitotane clearance (CL/F) significantly, which decreased the coefficient of variation (CV%) of random individual variability of CL/F from 67.0% to 43.0%. Fat amount (i.e. body weight – LBW) was identified to affect the central distribution volume significantly. Simulation results indicated that determining the starting dose with the developed model is beneficial to shorten the period to reach the therapeutic target and limit the risk of toxicity. A regimen that can effectively maintain mitotane concentration within 14-20 mg/L was established.

Conclusions

A two-compartment PopPK model well characterized mitotane PK profiles in patients with ACC. Enzyme CYP2C19 and transporter SLCO1B1 and SLCO1B3 may play roles in mitotane disposition. The developed model is beneficial to optimize mitotane treatment schedules and to individualize the initial dose for patients with ACC. Further validation of these finding is still required.

1. Introduction

Adrenocortical carcinoma (ACC) is a rare endocrine malignancy (1 per million per year) with a poor prognosis and limited treatment options[1]. Mitotane, a highly lipophilic compound, is the only approved treatment of ACC by the Food and Drug Administration and the European Medicine Agency[1]. Mitotane is developed as an orally administered treatment and its absorption is improved by concomitant intake of fat-rich food[2]. The bioavailability of mitotane is around 35 - 40%[3]. Mitotane has a high volume of distribution and the primary distribution site is fat[3, 4]. The half-life of mitotane elimination ranges from 18 to 159 days with a median of 53 days[2, 3].

The efficacy and toxicity of mitotane are related to the plasma concentration[1, 3]. In order to ensure efficacy and avoid increased toxicity, mitotane plasma concentration should be between the therapeutic range of 14-20 mg/L, which requires therapeutic drug monitoring (TDM)[1].

However, due to the large distribution volume and long half-life of mitotane, a long-time interval (around 3-5 months[1]) is usually required for patients to reach the effective concentration[3], which limits the clinical utility of mitotane. The inability to reliably predict mitotane plasma concentrations may result in a prolonged time to reach the target value, hence causing a significant delay in tumor treatment, or may give rise to drug toxicity. In addition, it has been demonstrated that only half of the patients who received a high-dose regimen for 3 months achieved the target[5], suggesting a demand for individualized treatment and a presence of high inter-individual variability in the pharmacokinetics (PK) of mitotane. Currently, the dosage titration is largely expert-based making it prone to errors. Therefore, a tool enabling mitotane concentration prediction and an optimized treatment regimen for individual patients, which shortens the period required to reach the target concentration while limiting the toxicity, would be desirable for patients with ACC.

Population PK (PopPK) modelling approach with mixed-effect models enables a quantitative characterization and prediction of drug PK profiles for both the study population and individuals [6]. The development of a PopPK model of mitotane would be beneficial for the characterization and understanding of mitotane PK, as well as for the optimization and personalization of mitotane treatment. Up until now, two studies have performed PopPK modelling analysis on mitotane in patients with ACC[3, 7]. One-compartment models were developed in these two studies. One assuming a self-induced clearance and the body mass index (BMI) was identified to be a covariate of mitotane distribution volume[3]. The other identified the effects of triglyceride and high density lipoprotein on mitotane clearance[7]. Another model-based PK study of mitotane developed a three-compartment model and showed weak correlations of age, gender, body weight, height, and body surface area with model parameters[8].

In order to further elucidate the variability of mitotane PK, it would be beneficial to explore the effect of pharmacogenetic polymorphisms[8]. Although the exact PK pathway of mitotane and the enzymes involved in mitotane metabolism remain unknown[9], two studies suggested possible roles for CYP2B6 and CYP2C9[10, 11]. One study demonstrated that the genotype of *CYP2B6*6* (rs3745274) was significantly correlated with mitotane plasma concentrations at 3 and 6 months after initiation of treatment[10]. The other study showed that one patient with high mitotane concentration was an CYP2C9 intermediate metabolizer[11]. Further analysis of the relationship between genes encoding for pharmacokinetic enzymes and transporters and mitotane PK profiles, and incorporating these variables into a PopPK model may allow better explanation of mitotane PK variability.

In the current study, a PopPK analysis was performed for mitotane in patients with ACC utilizing the retrospectively collected PK data. The effect of genes encoding drug absorption, distribution, metabolism, and elimination (ADME), patients' demographic information, and clinical characteristics on mitotane PK were investigated as covariates. We aimed to develop a PopPK model to describe and predict the PK of mitotane in patients with ACC, as well as to explore the effect of genetic variation on mitotane clearance. Moreover, we intended to better explain mitotane PK variability with the developed model and to facilitate treatment optimization and individualization for patients with ACC.

2. Methods

2.1 Patients

Forty nine adult patients diagnosed with ACC (≥18 years old), who were enrolled in Dutch Adrenal Network registry, had been treated with mitotane, with consent, and with available mitotane dosing information as well as concentration data were included in this PopPK analysis. One patient was eventually excluded because of the missing starting dosing information.

The study was approved by the Medical Ethical Committee of the Máxima Medical Center Veldhoven (2015), and the approval for inclusion of patients in other institutes was obtained from their local boards. All required informed consents were obtained from all patients. All procedures performed in this study were in accordance with the ethical standards of the institutional medical ethical committee and the 1964 Helsinki Declaration.

2.2 Pharmacokinetics data

Data on mitotane plasma concentrations, including concentrations from routine TDM, data sampled during one treatment interval, and data collected after treatment

discontinuation, as well as all mitotane dosing data were collected retrospectively from patients medical records. Patients administrated mitotane orally and were advised to take mitotane with fat-rich food. The concomitant medication information was not included in the current analysis since the data was not complete. The mitotane plasma concentrations were determined by a validated gas-chromatography/mass spectrometry assay at the Department of Clinical Pharmacy and Toxicology of Leiden University Medical Center (LUMC) [12]. The lower limit of quantification (LLOQ) was 2 mg/L. In addition, patients' demographic information, including age, gender, body weight (WT) and height (HT) at the start of treatment were collected. Furthermore, levels of serum aspartate transaminase (ASAT), alanine transaminase (ALAT), gamma-glutamyl transferase (gGT), total cholesterol, and estimated glomerular filtration rate (GFR) (recorded as 0 if the result was 60 mL/min/1.73 m^2 and otherwise 1) were also collected in our analysis.

Lean body weight (LBW) and fat amount (FAT) were also calculated for each patient. LBW was estimated with the *Boer* formula[13] and FAT was obtained by subtracting LBW from WT.

2.3 Genotyping method

DNA of included patients was isolated from EDTA blood samples using Maxwell (Promega, Leiden, the Netherlands) or Magnapure compact (Roche, Almere, the Netherlands). Genotyping of patients was performed with Drug Metabolizing Enzymes and Transporters (DMET™) plus array (Affymetrix UK ltd, High Wycombe, United Kingdom), which contains 1936 genetic variants (1931 single nucleotide

polymorphisms (SNPs) and 5 copy number variations (CNVs)) of ADME-related enzymes and transporters[14], according to manufacturers' protocol. The method was described in detail previously[15, 16].

A pre-set selection was performed using the DMET™ console software that generates fully annotated marker reports based on a translation file as recommended by Affymetrics®[17]. The reports include commonly recognized, haplotype-based allele calls commonly cited in Medline reference studies[18-20]. The DMET™ Plus allele translation software produces a comprehensive genotyping report containing pharmacogenomic reference data on all probes. This step leads to a selection of 959 SNPs from the total of 1931 SNPs present on the DMET™ platform. Subsequently, the SNPs that deviated from Hardy-Weinberg equilibrium ($p<0.0001$), with call rate below 97%, or with a minor allele frequency (MAF) < 0.1, as well as tri-allelic SNPs and SNPs of genes located on X chromosome were excluded from further analysis.

2.4 Population PK model development

Based on the obtained mitotane concentration data, a non-linear mixed-effects model was developed. Parameters were estimated by using the first order conditional estimation method with interaction (FOCEI) implemented in NONMEM software, version 7.4.1 (ICON Development Solutions). One-, two- and three-compartment models, with first-order absorption and first-order elimination, were explored as the structural model. Data points below LLOQ were omitted since they only contributed to 3.6% of the observations[21, 22].

Since the majority of collected data were trough concentrations and data concerning the absorption phase was limited, absorption rate constant (KA) was first estimated based on a sub-dataset containing data of the patients who contributed multiple data points during one treatment interval at steady state. The estimate of KA was then fixed to analyze the full dataset. Inter-occasion variability (IOV) was incorporated on apparent systematic clearance (CL/F) and every 200 days of treatment was defined as an occasion. In addition, to simplify the situation, all patients were assumed to receive a single dose once a day at 8:00 AM with the dose amount being equal to the total daily dose.

Further detailed description of the PopPK modelling methods is shown in **Online Resource 1**.

2.5 Identify potential correlated SNPs and covariate analysis

Since knowledge about the relationship between mitotane clearance and pharmacogenetic polymorphisms is limited, an exploratory analysis was first performed to find potential SNPs which were correlated with mitotane clearance. The estimates of random inter-individual variability (IIV) of CL/F () from the basic model and the genotyping results were utilized. For each SNP, when the number of patients in a minor homozygous group was less than 4, the results of these patients were combined with the corresponding heterozygote group for the association analysis assuming a dominant allele effect. Additionally, when the number of patients with genotype results of Zero Copy Number or Possible Rare Allele was less than 4 or when patients had NoCall results, the results were not included for statistical analysis. One-way ANOVA test and two sided t-test were performed with R (version 3.6.1; R Foundation for Statistical Computing, Vienna, Austria) to evaluate the difference of across genotype groups for each SNP. The selection of test method depended on the number of genotype groups of each SNP after the combination. The SNPs were considered to correlate with mitotane clearance if the p-value is lower than 0.05. The correction for multiple testing was not performed because of the exploratory characteristic of the current analysis.

The identified SNPs, as well as patients' demographic information and clinical characteristics were considered in covariate analysis. Stepwise covariate modelling (SCM) function implemented with Perl-Speaks-NONMEM (version 4.7.0) [23] was applied. Both a forward inclusion ($p< 0.05$) and a backward elimination process ($p<0.01$) were performed to identify significant covariates. More detailed description of the covariates analysis is shown in **Online Resource 1**.

2.6 Model evaluation

The predictability and stability of the final model was evaluated with goodness-of-fit (GOF) plots, prediction-corrected virtual predicted check (pcVPC)[24], and nonparametric bootstrap. Normalized prediction distribution error (NPDE) were also applied for evaluation. All figures were created with R. Detailed description of the evaluation methods is shown in **Online Resource 1.**

2.7 Simulations for treatment optimization

Based on the final model, simulations were performed to optimize mitotane dosing regimen and starting dose determination, in order to shorten the target reaching time while limiting the risk of toxicity. The simulation was performed for patients included in this study, as they are considered to be able to represent the corresponding adult patients population. The individual parameters of each patient were used to simulate the "real" mitotane concentrations (C_{sim_real}) under each regimen. The residual errors were not considered. Different strategies of adjusting the dose according to C_{sim_real} are shown in **Fig. 1.** All simulations were performed by R and the differential equations were solved using RxODE package (version 0.6-1)[25]. Detailed description of the regimens and simulation methods are shown in **Online Resource 1**.

On the basis of the simulated PK curves, for patients who originally reached the target, the mean and max time needed to reach the target (, the first day when C_{sim_real} 14mg/L), the mean percentage of days when C_{sim_real} were higher than 20mg/L in the first 200 days (), and the mean percentages of C_{sim_real} located outside the therapeutic window after reaching the target (), were calculated and compared across different strategies. represents a probability of causing toxicity in the early phase of treatment and represents the ability of maintaining the concentration within therapeutic window. Meanwhile, the median maximum and minimum $C_{sim\_real,}$ as well as the range of determined starting dose were also collected and evaluated. As an optimized regimen is expected to be able to ensure a quicker target reaching and well maintain the concentration within the therapeutic window while not causing much toxicity, the optimization target was defined as the mean 90 days (3 months), the mean 10%,

and the mean 15%.

Using the optimized regimen, a Shiny app was created based on the shiny package (version 1.4.0) and RxODE package in R in order to perform simulation for a random patient and to elucidate an option of providing treatment advice for a new patient based on the model. The detailed description is shown in **Online Resource 2.**

3. Results:

3.1 Patients and data

Data from 48 patients with ACC (21 males and 27 females) were included in the PopPK analysis. The characteristics of patients are summarized in **Table 1.** Patients received mitotane treatment between 2002-2017 and the median duration of treatment was 713.5 days (range from 90-2856 days). The total daily dosage ranged from 0.5g - 16g per day and was divided into one to four doses. Five (2 patients), six (1 patient), and eight (1 patient) daily dosages were also applied occasionally. Forty-one patients reached the concentration target during treatment, among whom 16 patients reached the target after 150 days. In total, 914 concentration data points were collected from patients' electronic hospital records, 33 of which were below the LLOQ. The time-course of collected mitotane concentrations was shown in **Fig. 2**. Nine patients contributed multiple sampling data within one treatment interval and 13 patients have more than one data point collected after treatment discontinuation. The median number of data points contributed by each patient was 16.5, ranging from 2 to 47.

3.2 The basic model

Based on the sub-dataset containing data from the 9 patients with multiple sampling data within one treatment interval, the KA was estimated as 22.1 (/day) and 15.0 (/day) under a one-compartment and a two-compartment model structure, respectively. A three-compartment model could not be identified since: 1) the time-course of mitotane concentration did not meet the characteristics of a three-compartment model; 2) when running the three-compartment model, the parameters were shown to be unidentifiable. The basic models were then developed by fitting the full dataset with fixed KA and incorporating IOV on CL/F. Relative standard error (RSE) of parameter estimates of both two model structures were all within the acceptable range (<30%). The objective function value (OFV) of the two-compartment model reduced 92.13 compared with that of the one-compartment model ($p<0.001$, degree of freedom=4), suggesting an improvement on the model fitness. Therefore, the two-compartment model was ultimately selected for describing mitotane PK profiles in patients with

ACC in this study. The model structure is shown in **Online Resource 1, Fig. S1**. The parameter estimates of the basic model were shown in **Table 2**. High percentage coefficient of variation (CV%) of IIV for all parameters were identified and the CV% of IIV for apparent distribution rate constant (Q/F) was even higher than 100%.

3.3 Pharmacogenetic analysis

For each patient, the genotyping results of the 959 SNPs from the DMET™ platform were obtained. A list of the these SNPs can be found in **Online Resource 3**. All SNPs were in Hardy-Weinberg equilibrium (p0.0001). The flow diagram of the genetic variants selection is shown in **Fig. 3**. Eventually, 172 SNPs were included for the further investigation.

Among the 172 SNPs, 55 SNPs had less than 4 patients belonging to the minor homozygous group. The NoCall result was reported in one patient in 19 SNPs and the Possible Rare Allele was reported in one patient in 1 SNP. The results of these patients were thus not included in the association analysis of corresponding SNPs. In contrast, the Zero Copy Number occurred in 3 SNPs in 8, 24, and 24 patients respectively. Thus, patients with Zero Copy Number were treated as a different genotype group in the association analysis of these 3 SNPs.

Finally, the result of the association test showed that 11 SNPs, as is shown in **Online Resource 1, Table. S1**, were potentially related to mitotane clearance (p0.05). Among these 11 SNPs, the genotyping results of *CYP2C18* 1154C>T (rs2281891) and *CYP2C19**2 (rs4244285) were shown to be 100% in linkage disequilibrium in our dataset, same as the genotyping results of *SLCO1B3* 334G>T (rs4149117), 699A>G (rs7311358), and 1557G>A (rs2053098) and that of the 3 SNPs located on *VKORC1* (283+124G>C, 174-136C>T, and -1639G>A). The results of the identified 11 SNPs were subsequently combined into the full dataset for the stepwise covariate analysis.

3.4 The final model

The parameter estimates of the final model are shown in **Table 2.** Genotypes of *CYP2C19*2* (rs4244285), *SLCO1B3* 699A>G (rs7311358), and *SLCO1B1* 571T>C (rs4149057), and LBW at the start of treatment with power relation were identified to have significant effect on the CL/F of mitotane (**Table 2**). Carrying 'A' variant in *CYP2C19*2* reduced the CL/F by 44.9%, and carrying 'G' variant in *SLCO1B3* 699A>G resulted in a 39.9% reduction of CL/F (**Table 2**). As for *SLCO1B1* 571T>C, the CL/F of patients carrying one 'C' variant decreased to 40.2% of that of wild type patients, and the CL/F of patients carrying two 'C' variants decreased to 30.2%. The distribution of derived from the basic model in each genotype group of above 3 SNPs was shown

in **Online Resource 1, Fig. S2**. In addition, FAT at the start of treatment with power relation was identified to influence the apparent distribution volume of central compartment (V_c/F) significantly. The inclusion of these covariates decreased the CV% of CL/F and V_c/F from 67.0% and 68.1% to 43.0% and 47.2%, respectively. Overall, the parameter estimates showed to be in good agreement with the bootstrap results (**Table 2**).

The GOF plots (**Fig. 4**) show that the individual predictions of the final model are in good accordance with the observations, while the population predictions are slightly deviated from the observations. The conditional weighted residual errors (CWRES) randomly distributed around zero without obvious trends over time or across population predictions. The pcVPC plot (**Fig. 5**) shows that the 5th, 50th, and 95th percentiles of prediction-corrected concentrations can be mostly adequately covered by the 95% CI of the corresponding percentiles of simulations, although a few large prediction-corrected concentrations present. The NPDE results is shown in **Online Resource 1, Fig. S3**.

3.5 Simulation results

The simulation results of different regimens in included patients who originally reached the target (N=41) are summarized in **Table 3.**

The previously suggested high-dose regimen (Regimen 1) resulted in the lowest but the highest . The C_{sim_real} can also not be well maintained within the therapeutic range.

As for the newly designed strategies, if all patients started with the same dosage (Regimen 2-2g, 2-4g and 2-6g), the increase in the starting dosage reduced but increased and weakened the ability of maintaining C_{sim_real} within the therapeutic range. When determining the starting dose individually (Regimen 3-77day, 3-98day and 3-119day), Regimen 3-98day fulfilled the optimization target and resulted in lower but higher and compared with Regimen 2-4g. The range of determined starting dose was in accordance with what is currently recommended [26] (**Table 3**).

Compared with Regimen 2-4g and 3-98day, increasing the dose reduction amount to 4g when C_{sim_real} > 20mg/L reduced the and , whereas setting 50% deduction when C_{sim_real} > 20mg/L reduced but increased the (Regimen 4 and 5). Both these changes did not affect . In contrast, when adjusting the dose change amount when C_{sim_real} 14 mg/L, the evaluated regimens did not provide better results (Regimen 6 and 7).

Regimen 8, where a constant starting dose determined by the model was applied, provided generally better results compared with starting with 4 g/per day for all patients (Regimen 2-4g) in terms of , , and the ability of maintaining concentration within the therapeutic range. The range of suggested starting dose (3 to 7g, median

5g) was slightly beyond the current recommended range but was considered to be acceptable. In comparison, when determining a constant dose using individual PK parameters (incorporating IIV estimates) (Regimen 9), the and max decreased. Although increased, it is still low enough. The suggested doses under Regimen 9 were relatively higher (3 to 10 g) since IIV was taken into account.

Overall, Regimen 2-4g, 3-98day, 4-(-4g), 4-(-50%), 5-(-4 g), 7-1, 8, and 9 fulfilled the optimization target. Individualized starting dose resulted in lower but higher compared with fixed starting dose. Regimen 3-98day and 5-(-4g) provided the lowest mean and regimen 5-(-4g) resulted in lower . Regimen 8 provided the lowest and Regimen 9 provided the lowest max and mean . Based on these results, Regimen 5-(-4g) and Regimen 8 were considered to be more beneficial, and Regimen 9 could also be applied considering the patients' tolerance to the level of dose increase.

The Shiny app was established based on the final model and treatment strategy 5-(-4g) was applied since this regimen provided the lowest mean . An reduced model where the effect of pharmacogenetic variation was not included was also built in to serve as an alternative option for patients when genotyping results are not available. The results are shown in **Online Resource 2**.

4. Discussion

In the current study, a two-compartment PopPK model was developed which adequately described the PK profile of mitotane in patients with ACC. The identified covariates explained 24% and 20.9% of random variability in mitotane clearance and distribution volume, respectively. As mitotane distributes in most body tissues and predominantly in the fat[1], the two-compartment model structure is considered to be also in line with the PK characteristics of mitotane, although wide 95%CI of parameter Q/F still indicate an uncertainty in the estimation. A three-compartment model structure, which has been applied previously on mitotane[8], could not be identified in this study as the time-course of mitotane concentration did not meet the characteristics of a three-compartment model and parameter estimates for the three-compartment model were found to be unidentifiable.

Because of the limited data in the absorption phase, KA was first estimated based on a sub-dataset and then fixed to analyze the full dataset. Precise KA estimation was unidentifiable if estimating based on the full dataset. The estimates of V_c/F and V_p/F are relatively large, which is in accordance with precious reports and the fact that mitotane distributes in many body tissues[1, 3]. The separate effects of LBW and FAT on mitotane distribution volumes were of interest in this study as they are more realistic covariates physiologically[3, 4]. As a result, FAT was identified to be a significant covariate on the V_c/F. The estimated half-life of mitotane in the included patients

ranged from 16.4 to 700.6 days with a median of 101.5 days. This range is wider than what was reported previously[1, 2]. This may be explained by the larger number of patients included in the current study than the original study[2]. Incorporating IOV on CL/F in the current study explained the intra-subject variability. The estimates of IOV indicate an overall increasing clearance during the first 500 days followed by a decrease thereafter (**Online Resource 1, Fig. S4**). This dynamic indicates that a self-induction in mitotane clearance, which has been suggested previously[3], may exist temporarily.

The current study for the first time explored and quantified the potential effect of pharmacogenetic variation on mitotane clearance in patients with ACC. Due to the lack of knowledge regarding the PK pathway of mitotane, a wide range of SNPs from DMET™ plus array were considered. However, because of the limited number of patients, it was decided to focus on the SNPs with known functionality by adopting a pre-set selection[17], although an exploratory analysis based on all genetic variants from DMET™ plus array was also performed. The flow diagram of the SNP selection and the 9 additional SNPs that are potentially correlated to mitotane clearance if the pre-set selection was not considered are shown in **Online Resource 1, Table S2 and Fig. S5**. Genes located on the X chromosome were excluded since only the general influence of gender on mitotane PK was considered.

Eventually, three SNPs, i.e. *CYP2C19*2* (rs4244285), *SLCO1B3* 699A>G (rs7311358), and *SLCO1B1* 571T>C (rs4149057), were included in the final model and were considered as the pharmacogenetic polymorphisms that should be considered for mitotane dose selection. This result also suggests that enzyme CYP2C19 and transporters SLCO1B3 and SLCO1B1 for drug uptake in the liver might be involved in mitotane PK pathways, but further confirmation is required.

In fact, *CYP2C19**2 was in 100% linkage disequilibrium with *CYP2C18* 1154C>T (rs2281891) in our dataset, same as *SLCO1B3* 699A>G with *SLCO1B3* 334G>T (rs4149117) and *SLCO1B3* 1557G>A (rs2053098). Comparable high linkage disequilibrium was also found in 1000 genomes CEU. Compared with *CYP2C18* 1154C>T for which no sufficient evidence has been found about the effect on the drug PK, the 'A' variant of *CYP2C19**2 is known to be a nonfunctioning variant and has been demonstrated to decrease the activity of CYP2C19[27, 28]. Similarly, the variants of *SLCO1B3* 699A>G with *SLCO1B3* 334G>T have been reported to be associated with the decrease of drug clearance and *SLCO1B3* 699A>G has stronger level of clinical annotations[29, 30]. Therefore, *CYP2C19*2* and *SLCO1B3* 699A>G were included in the final model.

*CYP2B6*6* , which has been reported to be related to mitotane plasma concentrations detected at 3 and 6 months[10], was not identified to have significant effect on mitotane clearance in the current study. Among the 5 SNPs located on *CYP2B6* which

were included in the association analysis, none of them was significantly related to mitotane clearance ($p>0.05$). This discrepancy may be due to the much longer observation period in the present study. One SNP located on *CYP2C9*, *CYP2C9*2* (rs1799853), was not identified to be significant either. However, the evidence of the involvement of CYP2C9 is in fact insufficient.

The predictability and stability of the final model were confirmed to be acceptable. In the pcVPC plot, a few prediction-corrected concentrations are inadequately covered by the simulations. A possible explanation is that the observations at corresponding time points are from a single patient and the population prediction of this patient is much smaller than real observations. The deviation of population predictions from observations can also be seen in the GOF plots. Patients' adherence and other unknown factors may also introduce additional bias. Identification of additional covariates, such as the effect of co-medication and food intake, might improve the population predictions.

Based on the final PopPK model, several mitotane treatment strategies were designed and evaluated by simulations. A regimen with bolus dose followed by maintenance dose was not considered as this regimen requires high dosage which is not tolerable for patients. Among the regimens that fulfilled the optimization target, applying individual starting dose determined by the model was demonstrated to shorten the time to achieve the therapeutic window compared with starting with fixed dose for all patients. Under the setting of individualized starting dose, the regimens with stepwise increasing dose at start required less time to reach the therapeutic target, while the one with constant starting dose demonstrated the lowest risk of having toxicity. The determined individual starting dose was also acceptable. In addition, the newly designed dose adjustment strategies were able to satisfactorily keep the mitotane concentrations within the therapeutic range. Therefore, determining the starting dose with the developed model is considered to be most beneficial in terms of shortening the time to reach the therapeutic target and limit the risk of toxicity. However, due to the fact that a shorter is normally paired with a higher , it is suggested to consider based on patients' condition whether the increased risk of having toxicity can be tolerated in order to gain the benefit of a shorter time to reach the therapeutic target when selecting a dosing regimen.

Obtaining individual parameters based on one (or more) TDM result with the PopPK model and determining the dose amount accordingly can also decrease the risk of toxicity while providing a satisfactory target reaching time, thus it is also a promising strategy. However, patients tolerance to the high level of dose increase need to be considered when applying this strategy. This method can also be useful to estimate an adequate dose for the drug concentration level maintenance after reaching the therapeutic window so that to decrease the frequency of dose adaptation.

Simulation results also indicate that in order to reduce the risk of having toxicity and effectively maintain mitotane concentration within the therapeutic range, a better strategy is to set the concentration boundary of dose decreasing at 18mg/L instead of 20 mg/L. This early dose adjustment takes the 7 days' time when the monitoring result is unknown and the dose is not adjusted into consideration. The concentration boundary of dose increasing needs to be 14 mg/L since it affects the adequacy of maintaining the plasma concentration above 14 mg/L. The frequency of TDM was set at once every 21 days as suggested by the guideline in the simulation. If TDM is performed less frequently, a larger dose change step will be required.

The current study has some limitations. Firstly, the small number of patients included in this study and the exploratory characteristic of this analysis may influence the power of covariate analysis especially for pharmacogenetic analysis. However, as the dataset consisted of concentrations on different occasions for each patient, which enabled differentiation between IIV and intra-subject variability (i.e. IOV) in clearance, the certainty of the possible genotype effect on clearance which is more likely to be covered by IIV since genotype is a constant factor in patients was increased. Nonetheless, further validation with an external dataset to replicate the findings is warranted to confirm the identified associations and to translate the findings into a clinical recommendation. However since the ACC is a very rare disease (1 per million per year), collection of another comparable or even larger dataset will be challenging. Therefore, an *in vitro* assay might be more feasible in future studies to substantiate the activity of the suggested enzymes in mitotane PK. Secondly, the model lacks a strong ability to accurately predict high concentrations (e.g, peak concentrations) due to the limited data input in the absorption and distribution phase. Furthermore, the accuracy of parameter estimates may be affected by our simplification of multiple daily dosing to a single dose. However, the prediction of mitotane trough concentrations and the suggestion of daily dose based on the model will not be significantly affected. Therefore we believe this model is still fit for the current application. Thirdly, the impact of co-administrated drugs and the food intake on mitotane PK was not was not taken into account in this study due to the lack of data.

In conclusion, the current study presents a two-compartment PopPK model which well characterizes mitotane PK profiles in patients with ACC. The polymorphisms of *CYP2C19*2* (rs4244285), *SLCO1B3* 699G>A (rs7311358), and *SLCO1B1* 571T>C (rs4149057) are identified to be correlated to mitotane PK. Further external or in vitro evaluation is suggested to confirm the results. Moreover, optimized mitotane treatment schedules for patients with ACC were identified by simulation and the developed model can be of help to individualize the initial dose. These strategies should be confirmed in a prospective study.

Table 1 Patient characteristics (N=48)

Characteristic	Value/Mean	SD	Range
Patient characteristics			
Number of patients (N)	48		
Gender, Male (N(%))	21 (43.8%)		
Age (years)[a]	52.0	12.1	22.6-76.8
Weight (kg)[a] (N=2 No record)	80.0	15.9	52.5-120
Height (cm)[a] (N=5 No record)	172	10.0	154-193
BMI (kg/m^2) (N=5 No record)	27.1	4.48	18.2-38.3
LBW (kg) [a] (N=5 No record)	55.8	10.0	39.7-78.5
ASAT (IU/L)[b] (N=1 No record)	45.15	35.3	16-185
ALAT (IU/L)[b] (N=1 No record)	42.68	35.6	9-197
GammaGT (IU/L)[b] (N=1 No record)	278.70	215.9	55-898
GFR, > 50% normal records (N(%)) (N=7 No record)	39 (95.1%)		
Cholesterol (mmol/L)[b] (N=11 No record)	6.54	1.56	3.6-11.6
Disease characteristics			
ENSAT I, patients N(%)	2 (4.2%)		
ENSAT II, patients N(%)	19 (39.6%)		
ENSAT III, patients N(%)	10 (20.8%)		
ENSAT IV, patients N(%)	17 (35.4%)		
Target reaching characteristics			
Patients reached the target (N)	41		
150 days (N(%))	16 (39.0%)		
90 days (N(%))	19 (46.3%)		
Target reaching time (days)	142	113.9	24 - 579
Duration of treatment (days)	742	553.2	90-2856

SD, standard deviation; LBW, lean body weight; ASAT, aspartate transaminase; ALAT, alanine transaminase; GGT, gamma-glutamyl transferase; GFR, glomerular filtration rate; ENSAT, European Network for the Study of Adrenal Tumors [a] at the start of treatment [b] mean record of each patient

Table 2 Parameter estimates of the basic model and the final model

	Basic model			Final model			Bootstrap	
Parameters	**Estimate (RSE%)**	**IIV (CV%) [shrinkage]**	**IOV[a] (CV%)**	**Estimate (RSE%)**	**IIV (CV%) [shrinkage]**	**IOV[c] (CV%)**	**Median**	**95% CI**
KA (/day)	15.0 fixed	-	-	15.0 fixed	-	-	15	-
CL/F (L/day)[a]	217 (11%)	67.0 [8%]	30.5	298 (13%)	43.0 [16%]	31.6	281.6	200.5-398.4
CL_SNP1 (GA/AA)	-	-	-	0.551 (15%)	-	-	0.573	0.385-0.881
CL_SNP2 (AG/GG)	-	-	-	0.601 (19%)	-	-	0.613	0.419-0.949
CL_SNP3 (CC)	-	-	-	0.753 (10%)	-	-	0.784	0.550-1.07
CL_SNP3 (TT)	-	-	-	2.49 (29%)	-	-	2.67	0.991-6.16
CL_LBW (power)	-	-	-	1.10 (16%)	-	-	1.07	0.205-2.13
V_c/F (L)[b]	4790 (20%)	68.1 [53%]	-	6210 (18%)	47.2 [55%]	-	6795	3281.0-10752
V_c_FAT (power)	-	-	-	1.22 (19%)	-	-	1.29	0.450-2.18
V_p/F (L)	19300 (13%)	76.9 [17%]	-	18100 (12%)	88.8 [15%]	-	17882	11341-25709
Q/F (/day)	1100 (21%)	102 [34%]	-	883 (20%)	97.3 [34%]	-	785.4	337.4-1502
Residual errors								
PRO (CV%)	16.6 (7%)	-	-	16.6 (6%)	-	-	16.8	14.2-18.9
ADD (mg/L)	0.931 (28%)	-	-	0.920 (17%)	-	-	0.871	0.373-1.384

SNP1: *CYP2C19*2* (rs4244285); SNP2: *SLCO1B3* 699A>G (rs7311358); SNP3: *SLCO1B1* 571T>C (rs4149057); LBW, lean body weight; FAT, fat amount; RSE, relative standard error; CV, coefficient of variation; IIV, inter-individual variability; IOV, inter-occasion variability; PRO, proportional residual error; ADD, additive residual error; CL/F, apparent system clearance; KA, absorption rate constant; V_c/F, apparent distribution volume of central compartment; V_p/F, apparent distribution volume of peripheral compartment; Q/F, apparent distribution rate constant; CI, confidence interval

[a] $CL/F = CL/F_t * CL\_SNP1 * CL\_SNP2 * CL\_SNP3 * (\frac{LBW}{56.6})^{CL\_LBW}$

[b] $V_c/F = V_c/F_t * (\frac{FAT}{23.6})^{Vc\_FAT}$

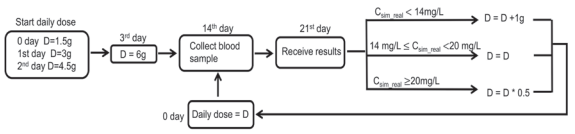
[c] Every 200 days of dosing was defined as an occasion

Table 3 Simulation results of different treatment regimens for included patients who originally reached the target (N=41)

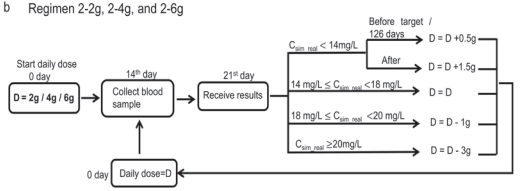
Regimen (Fig. 1)	Mean	Max	Mean	Mean	Median max / min C_{sim_real}	Starting dose range (g)	
1	54.22	125	23.6	18.35	22.3 / 13.11	-	
2-2g	133.98	236	4.16	12.6	20.65 / 13.14	2	
2-4g	89.8	182	7.01	13.15	20.90 / 13.20	4	*
2-6g	60.61	149	13.85	15.13	21.13 / 13.09	6	
3-77day	73	173	10.63	12.7	21.07 / 13.29	3.5 - 7	
3-98day	85.07	182	9.26	14.35	21.03 / 13.16	3 - 6	*
3-119day	97.9	191	6.44	12.22	20.96 / 13.21	2.5 - 5	
4-(-4g)	89.8	182	5.96	12.66	20.91 / 13.22	4	*
4-50%	89.8	182	8.82	12.37	20.91 / 13.22	4	*
5-(-4g)	85.07	182	7.92	13.01	20.84 / 13.14	3 - 6	*
5-50%	85.07	182	11.13	12.21	20.84 / 13.22	3 - 6	
6-1	91.12	194	6.61	13.37	20.84 / 12.91	4	
6-2	74.32	151	14.34	16.26	21.57 / 13.02	4	
7-1	86.12	194	8.52	14.69	21.03 / 12.96	3 - 6	*
7-2	80.27	160	14	15.53	21.46 / 12.87	2.5 - 5	
8	87.85	191	5.05	11.26	20.34 / 13.30	3.5 - 7	*
9	87.8	161	5.56	10.72	20.33 / 13.09	3 - 10	*

T_{target}, target reaching time (the day when simulated mitotane concentration 14mg/L);$P_{toxicity}$ percentage of days when simulated mitotane concentrations were higher than the upper limit of mitotane therapeutic window (20mg/L) in the first 200 days;$P_{o.window}$, percentages of simulated mitotane concentrations located outside the therapeutic window after reaching the target. *Follow the optimization target

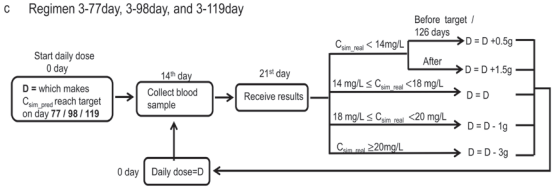
a Regimen 1



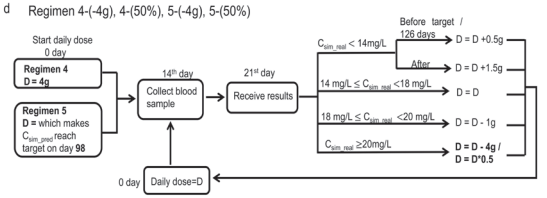
b Regimen 2-2g, 2-4g, and 2-6g



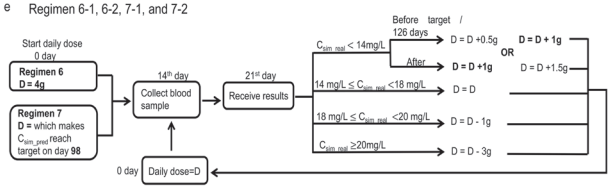
c Regimen 3-77day, 3-98day, and 3-119day



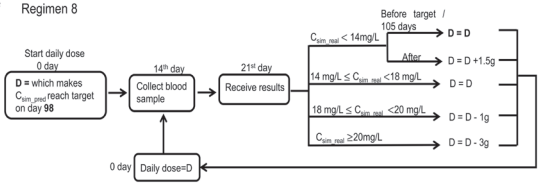
d Regimen 4-(-4g), 4-(50%), 5-(-4g), 5-(50%)



e Regimen 6-1, 6-2, 7-1, and 7-2



f Regimen 8



g Regimen 9

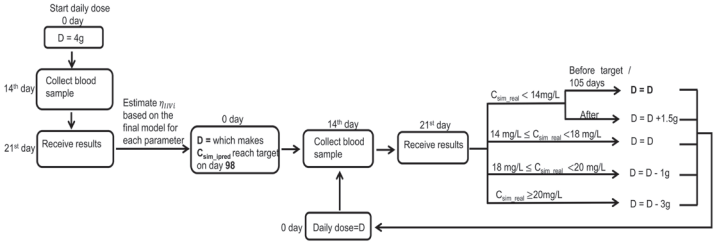


Fig. 1. Fig. 1. Designed treatment regimens that were evaluated by simulation: a) A previously reported dosing regimen (Regimen 1), where the dose started as 1.5g per day and increased up to 6g per day in 4 days and continued until next dose adjustment. The dosage was adjusted each time according to the monitored mitotane concentration level; b) Regimens where all patients started with 2g (Regimen 2-2g), 4g (Regimen 2-4g) or 6g (Regimen 2-6g) per day. Dosage increased by 0.5g every 21 days till the target was reached or 126 days if Csim_real < 14mg/L. Thereafter, the dosage increased by 1.5g if Csim_real < 14mg/L, remained unchanged if 14mg/L ≤Csim_real < 18 mg/L, decreased by 1g if 18 mg/L ≤Csim_real < 20 mg/L, and decreased by 3g if Csim_real ≥ 20 mg/L; c) Regimens where patients started with individualized dose which allowed Csim_pred on day 77 (Regimen 3-77day), 98 (Regimen 3-98day), or 119 (Regimen 3-119day) reach the target. The rest dose adjustment strategies were same as Regimen 2; d) Regimens where patients started with 4g per day (Regimen 4) or individualized dose (Regimen 5) and the dosage decreased by 4g or 50% if Csim_real ≥ 20 mg/L. The rest dose adjustment strategies were same as Regimen 2; e) Regimens where patients started with 4g per day (Regimen 6) or individualized dose (Regimen 7) and dosage increased by 1g after reaching target or 126 days if Csim_real < 14mg/L (Regimen 6-1 and 7-1), or increased by 1g till reaching target or 126 days if Csim_real < 14mg/L (Regimen 6-2 and 7-2). The rest dose adjustment strategies were same as Regimen 2; f) A regimen where patients started with individualized dose which remained unchanged till reaching target or 105 days if Csim_real < 14mg/L. The rest dose adjustment strategies was same as Regimen 2; g) A regimen where patients started with 4g per day for the first 21 days and the next dosage was determined as which allowed Csim_ipred on day 98 reach the target (Regimen 9). The rest dose adjustment strategies was same as Regimen 8. Csim_real, simulated "real" mitotane concentrations based on individual parameters; Csim_pred, model predictions based on patients characteristics; Csim_ipred, the model predictions considering the inter-individual variability (η_IIVi) estimated based on the first monitored concentration.

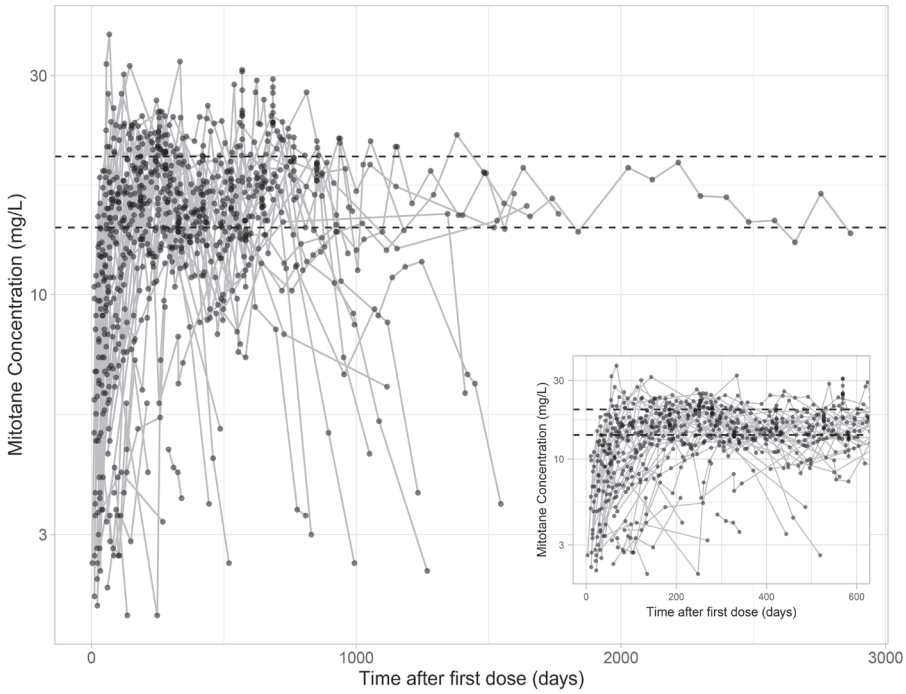


Fig. 2. Mitotane concentration-time curve collected from patients on logarithmic scale. Inserts show the data during the first 600 days of treatment.

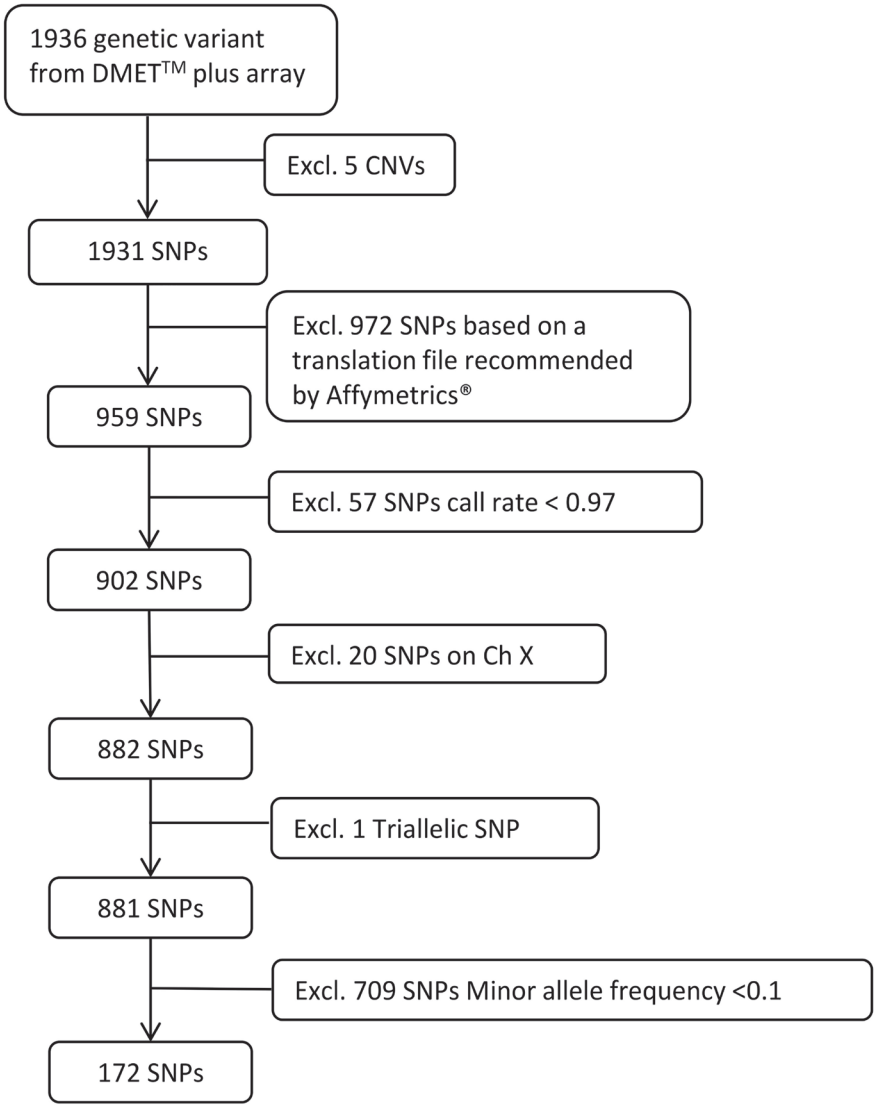


Fig. 3. Flow diagram of genetic variants selection. Excl. represents excluding, Ch X represents chromosome X, DMETTM represent Drug Metabolizing Enzymes and Transporters, CNVs represents copy number variations.

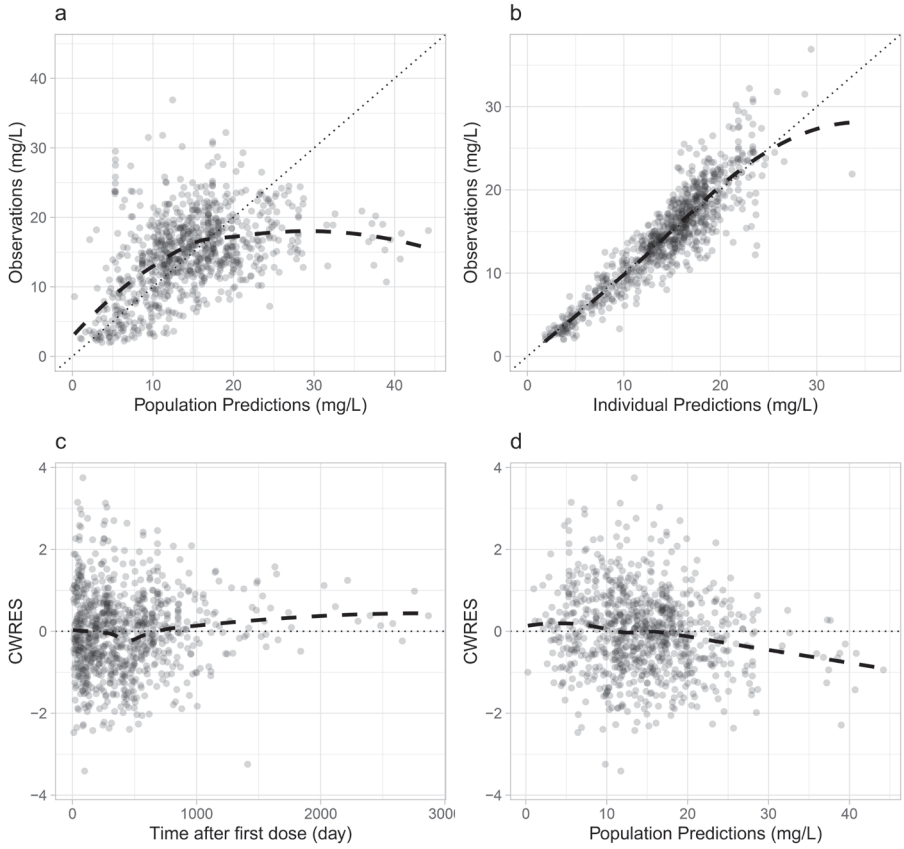


Fig. 4. Goodness-of-fit plots of the final population PK model of mitotane in patients with ACC, including observations versus population predictions (a) and individual predictions (b), and conditional weighted residual errors (CWRES) versus time (c) and populations predictions (d). The black dotted lines represent y=x (a, b) and y=0 (c, d). Black dashed lines represent corresponding loess regressions.

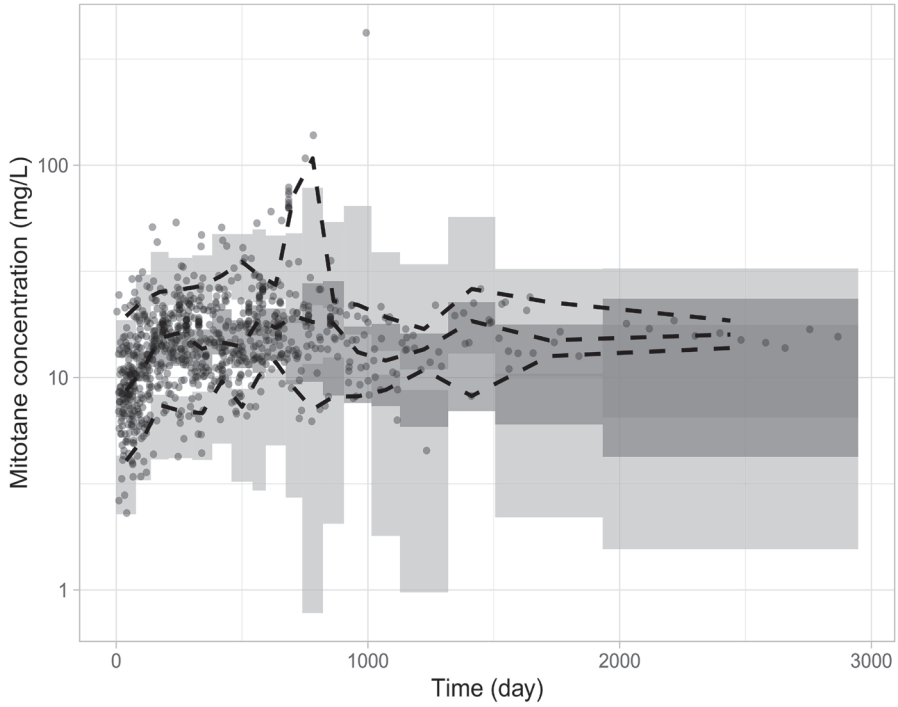


Fig. 5. Prediction corrected visual predictive check (pcVPC) plot of the final model on logarithmic scale. Black dashed lines represent 50th, 95th and 5th percentile of the observations, light grey shading areas represent 95% confidence interval of the 95th and 5th percentiles of the simulations respectively, and dark grey shading area represents 95% confidence interval of the 50th percentiles of the simulations.

Supplementary material caption

1. **Online Resource 1** Supplementary methods, figures and tables.

2. **Online Resource 2** Shiny app establishing method and results

3. **Online Resource 3** List of 959 SNPs from DMET™ array of which the genotyping results were obtained for each patient

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Online Resource 1 Supplementary methods, figures and tables.

Supplementary population PK analysis methods

One-, two- and three-compartment models, with first-order absorption and first-order elimination, were explored as the structural model. Relative standard error (RSE) of parameters, which represent the precision of parameter estimates, and the objective function value (OFV) were considered when evaluating the structural models. The one with acceptable RSE and lower OFV was selected as the final basic model structure.

Inter-individual variability (IIV) of parameters were estimated with Eq. 1, where P_i represents the parameter of ith individual and was assumed to be log-normally distributed, P_t represents typical value of the parameter, and η_{IIV} represents the random IIV which was assumed to be normally distributed with mean of 0 and variance of ω_1^2. In addition, inter-occasion variability (IOV), which reflects the intra-individual variability, of apparent systematic clearance (CL/F) was also included when analyzing the full dataset. As is shown in Eq.S1, η_{IOV} represents the random IOV. The distribution of η_{IOV} in each occasion was assumed to be similar and normally distributed with mean of 0 and variance of ω_2^2. In this study, every 200 days of treatment was defined as an occasion as the total observation periods of the patients were long.

The residual error was characterized with a combined proportional and additive model as is shown in Eq. S2, where Obs represents observations, $IPRED$ represents individual predictions, and ε_1 and ε_2 represent the proportional residual error and additive residual error respectively which were assumed to be normally distributed with mean of 0 and variance of $\sigma_1^2$and σ_2^2 , respectively.

$$Pi = Pt \cdot e^{\eta IIVi + \eta IOVj} \quad \text{Eq.S1}$$

$$Obs = IPRED \cdot (1 + \varepsilon_1) + \varepsilon_2 \quad \text{Eq.S2}$$

As for the covariate analysis, the identified SNPs, as well as patients' demographic information and clinical characteristics were considered. For continuous covariates, for each patient the mean values of all measurements during the monitoring period were taken. In case of missing continuous covariates, the corresponding median value of all patients was assigned. For patients who only missed HT but not

WT, LBW was calculated using real WT and imputed HT. For GFR, 0 (normal) was assigned if ≥ 50 % of the collected patient's records were 0 otherwise 1 was assigned. Patients who missed GFR measurements, 0 was assigned.

The effect of all above covariates on mitotane CL/F and the effect of WT, LBW, FAT,

and gender on apparent distribution volumes (V/F) were investigated using stepwise covariate modelling (SCM) function implemented with Perl-Speaks-NONMEM (version 4.7.0) [1]. Both a forward inclusion ($p <$

0.05) and a backward elimination process ($p < 0.01$) were performed to identify significant covariates. For SNPs that were in 100% linkage disequilibrium, if they were included during the SCM analysis, the more clinically relevant ones would be selected in the final model. The effects of continuous covariates were investigated with both linear relation (Eq.S3) and power relation (Eq.S4), where P_i represents the parameter of i th individual, P_t represents typical value of the parameter, and η_i represents the individual variability, θ_{cov} represents the estimate of covariate effect, COV_i represents the covariate value of ith individual, COV_m is the median value of the covariate. Categorical covariates were analyzed with Eq. S5, where θ_{cov} was set as 1 for reference category and was estimated for other categories.

$$P_i = P_t \cdot (1 \pm \theta_{COV} \cdot (COV_i - COV_m)) \cdot e^{\eta i} \quad \text{Eq.S3}$$

$$COVi\ \theta COV \cdot e\eta i \quad \text{Eq.S4}$$

$$Pi = Pt \cdot (COVm)$$

$$P_i = P_t \cdot \theta_{cov} \cdot e^{\eta i} \quad \text{Eq.S5}$$

Supplementary model evaluation methods

pcVPC was performed by 1000 times of simulation and the data points, 5th, 50th, and 95th percentiles of prediction-corrected observations were plotted together with 95% confidence intervals (CI) of 5th,

50th, and 95th percentiles of simulations. NPDE evaluation was performed with npde package (version 2.0) implemented in R statistics software based on 1000 times of simulations. The bootstrap was conducted by 1000 runs of bootstrap replicates sampled from original dataset with replacement, which was stratified on whether the subject contributed more than two data points after the end of treatment.

The median as well as 95% CI of parameters were derived and compared with original parameter estimates.

Supplementary simulation method

Based on the final model structure, simulations were performed to evaluate different designed treatment strategies and approaches of starting dose determination. Patients were assumed to receive treatment as long as their last mitotane

concentration monitoring time. The blood samples were assumed to be collected once every 2 weeks after knowing the result of the last sample, and the concentration of mitotane was assumed to be known 7 days after blood collection, which is in accordance with the optimal scenario in the clinical practice. The dose amount was subsequently adjusted accordingly.

As a comparison, a previous recommended 'high-dose' starting regimens, where the mitotane dose starts with 1.5g per day and increases up to 6g per day in 4 days, were simulated (**Regimen 1**)[2].

As for the newly designed regimens, the starting dose was 1) set as 2g, 4g, or 6g for all patients according to the guideline[3] **(Regimen 2, 4, and 6**) or 2) set individually considering patients characteristics with the help of the model (**Regimen 3, 5, 7, and 8**). As the expected time to reach the therapeutic target of mitotane is 3 to 5 months, the individually starting daily mitotane dose was estimated as the dose that allows the predicted mitotane concentrations on day 98 (C_{sim_pred98}) reach the therapeutic target. The C_{sim_pred98} was obtained by performing simulation under a regimen of 6g per day increasing by 0g (**Regimen 8**), 0.5g (**Regimen 2, 3, 4, 5, 6-1, and 7-1**), or 1g (**Regimen 6-2 and 7-2**) once every 21 days till the 98th day of treatment, with only typical parameter values and covariate effects considered. Given the linear PK feature of mitotane, the suggested starting daily dose ($Dose$) was therefore determined by Eqs. S6 and S7, where $\lfloor X \rfloor$ represents the least integer greater than or equal to

X, $\lfloor X \rfloor$ represents the greatest integer less than or equal to X. Determining the starting dose based on the C_{sim_pred} on day 77 and 119 were also used for comparison.

$$X = \frac{14\ \text{mg/L}}{{}^{C}sim\_(i)pred} \cdot 6\text{g} \qquad \text{Eq. S6}$$

$$\lfloor X \rfloor, \quad X - \lfloor X \rfloor > 0.650\ Dose = \{\lfloor X \rfloor + 0.5, \quad 0.350 \le X - \lfloor X \rfloor \le 0.650$$

$$\text{Eq. S7}\ \lfloor X \rfloor, \quad X - \lfloor X \rfloor < 0.350$$

Besides the above regimens, since individual parameters could be estimated after knowing one TDM result, **Regimen 9** was also designed and evaluated. In this strategy, patients were assumed to start with 4g per day until the first TDM result was obtained. C_{sim_real} of each patient on day 14 was simulated, based on which the η_{IIVi} and η_{IOV1} were estimated for each patient using NONMEM with the POSTHOC function. Subsequently, the next daily dose of each patient was determined with Eq. S6-S7 according to the individual C_{sim_pred98} ($C_{sim\_ipred98}$) under the daily dosing of 6g, based on the model incorporating η_{IIVi} as was suggested in a previous study[4]. The constant starting regimen was applied in this regimen.

In **Regimen 2 to 8**, the dose increasing amount when C_{sim_real} < 14 mg/L was set differently before and after the target was reached (starting and maintenance regimen), in order to limit the toxicity at start and maintain the mitotane trough concentration within the therapeutic range at a later phase. The combination of 0g/1.5g, 0.5g/1.5g, 0.5g/1g, and 1g/1.5g were simulated and evaluated. **Regimen 2 to 7** applied stepwise increasing starting regimen and **Regimen 8** applied constant starting regimen. A maximum number of days that follows the starting regimen was set as 126 (around 4 months) and 105 (98+7 days) for the stepwise increasing or constant starting regimens, respectively.

When C_{sim_real} reached 20 mg/L, a 50% dose reduction was suggested in **Regimen 1**. In comparison, both fixed dose amount reduction (3g or 4g) and 50% reduction were evaluated in the newly designed regimens (**Regimen 2 to 9)**. If a reduction resulted in a dose level lower than 0g, then 0g was applied. Besides, an additional concentration threshold of dose reduction, 18 mg/L, with 1g dose reduction was introduced in **Regimen 2 to 9,** since a 7-day period of no dose adjustment presented.

Reference

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Table S1 Potential SNPs out of the 959 SNPs that are correlated to mitotane clearance based on the association analysis.

Table S2 Additional potential SNPs that are correlated to mitotane clearance based on the association analysis, if the pre-set selection based on a translation file as recommended bybAffymetrics® was not considered.

Fig. S1. The population PK model structure of mitotane.

Fig. S2 The boxplots of estimated $\eta IIVi\_CL$ in each genotype group of SNP (a) CYP2C19*2 (rs4244285), (b) SLCO1B3 699A>G (rs7311358), and (c) SLCO1B1 571T>C (rs4149057).

Fig. S3. Normalized prediction distribution error (NPDE) results of the final population PK model of mitotane in patients with ACC.

Fig. S4. The estimates of inter-occasion variability (IOV) over time. Red dashed lines represent loess regression result.

Fig. S5 Flow diagram of the genetic variants selection if the pre-set selection based on a translation file as recommended by Affymetrics® was not considered.

Table S1 Potential SNPs out of the 959 SNPs that are correlated to mitotane clearance based on the association analysis

Gene		Common Name	dbSNP.RS.ID	P value
1	*CYP2C18*	CYP2C18_c.1154C>T(T385M)	rs2281891	0.020
2	*CYP2C19*	CYP2C19*2_19154G>A(P227P)	rs4244285	0.020
3	*SLCO1B3*	SLCO1B3_c.334G>T(A112S)	rs4149117	0.027
4	*SLCO1B3*	SLCO1B3_c.699A>G(I233M)	rs7311358	0.027
5	*SLCO1B3*	SLCO1B3_c.1557G>A(A519A)	rs2053098	0.027
6	*SLCO1B1*	SLCO1B1_c.571T>C(L191L)	rs4149057	0.020
7	*VKORC1*	VKORC1_c.*134G>A(3'UTR)	rs7294	0.050
8	*VKORC1*	VKORC1_c.283+124G>C	rs8050894	0.030
9	*VKORC1*	VKORC1_c.174-136C>T	rs9934438	0.030
10	*VKORC1*	VKORC1_c.-1639G>A(Promoter)	rs9923231	0.030
11	*UGT1A6*	UGT1A6_c.315A>G(L105L)	rs1105880	0.042

Table S2 Additional potential SNPs that are correlated to mitotane clearance based on the association analysis, if the pre-set selection based on a translation file as recommended by Affymetrics® was not considered.

Gene		Common Name	dbSNP.RS.ID	P value
1	*CA5P*	CA5P_A>G(rs11859842)	rs11859842	0.029
2	*SLC16A1*	SLC16A1_c.*1942T>C	rs9429505	0.0067
3	*CHST10*	CHST10_c.*381G>A	rs1530031	0.040
4	*CYP20A1*	CYP20A1_50767C>T(L346F)	rs1048013	0.014
5	*SLC22A13*	SLC22A13_c.*8336G>A	rs4679028	0.032
6	*UGT2A1*	UGT2A1_c.1305-109A>C	rs2288741	0.042
7	*ADH6*	ADH6_c.-930T>C	rs10002894	0.012
8	*ADH6*	ADH6_c.-2874T>C	rs6830685	0.012
9	*SLCO5A1*	SLCO5A1_c.97C>T(L33F)	rs3750266	0.015

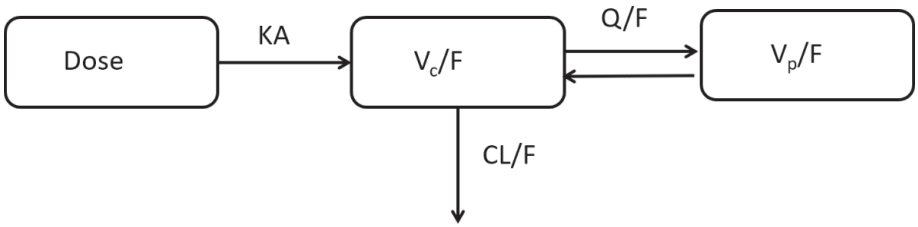


Fig. S1. The population PK model structure of mitotane. CL/F represents apparent system clearance, KA represents absorption rate constant, Vc/F represents apparent distribution volume of central compartment, Vp/F represents apparent distribution volume of peripheral compartment, Q/F represents apparent distribution rate constant.

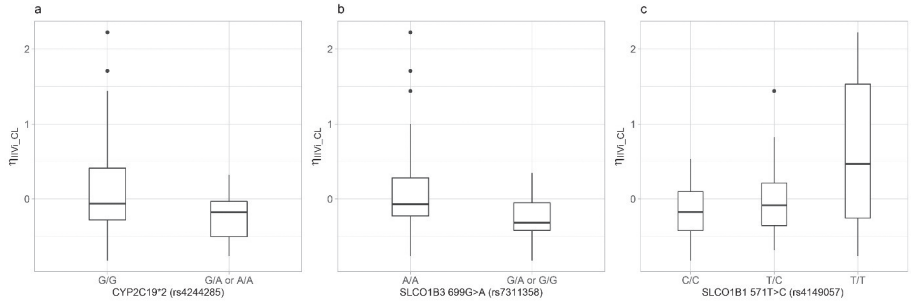


Fig. S2 The boxplots of estimated η_{IIV_CL} in each genotype group of SNP (a) CYP2C19*2 (rs4244285), (b) SLCO1B3 699A>G (rs7311358), and (c) SLCO1B1 571T>C (rs4149057)

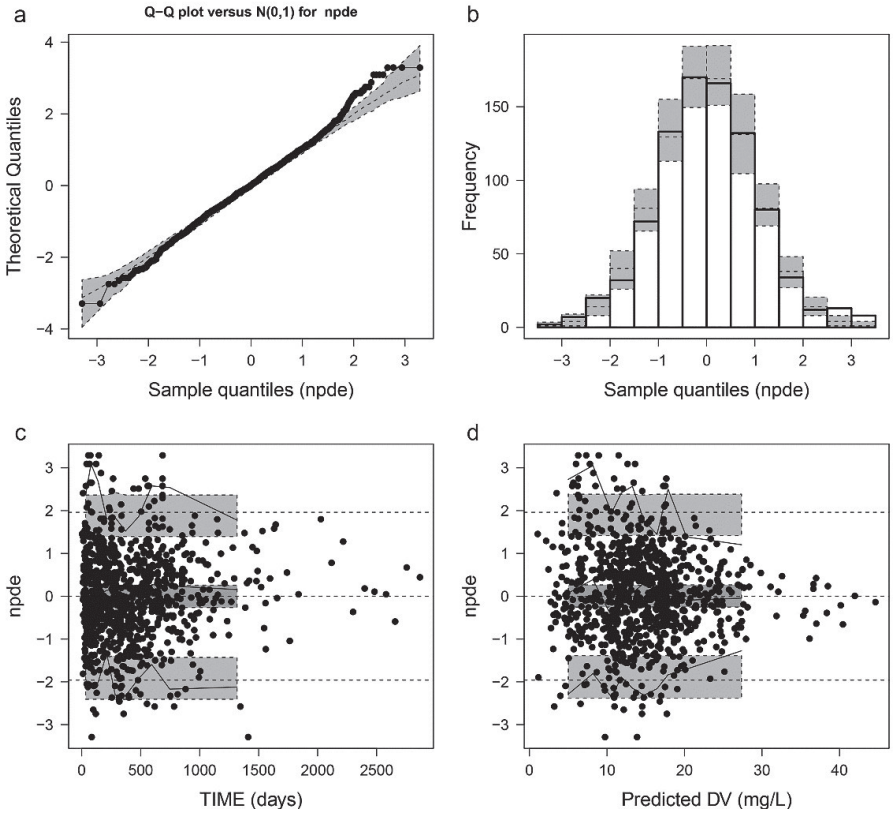


Fig. S3. Normalized prediction distribution error (NPDE) results of the final population PK model of mitotane in patients with ACC, including the quantile–quantile plot (a), the distribution histogram of NPDE (b), and the NPDE versus time (c) and population predictions (d). The NPDE results are shown to distribute around a mean of 0.03616 with a variance of 1.134.

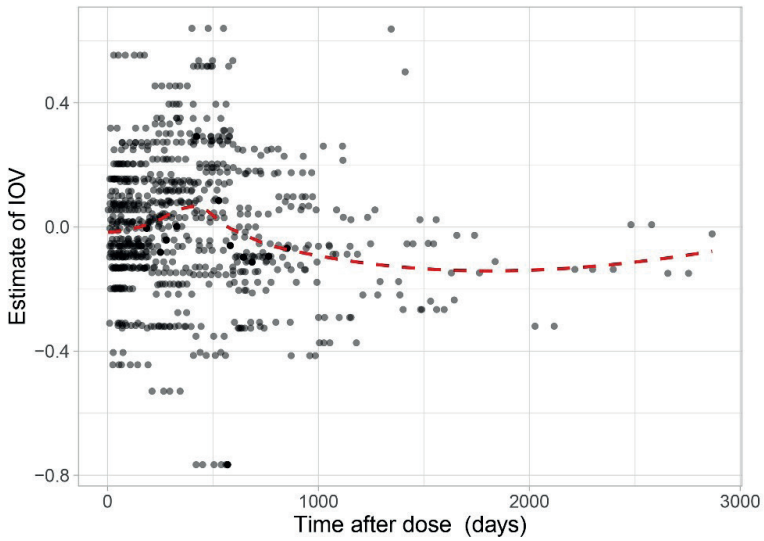


Fig. S4. The estimates of inter-occasion variability (IOV) over time. Red dashed lines represent loess regression result.

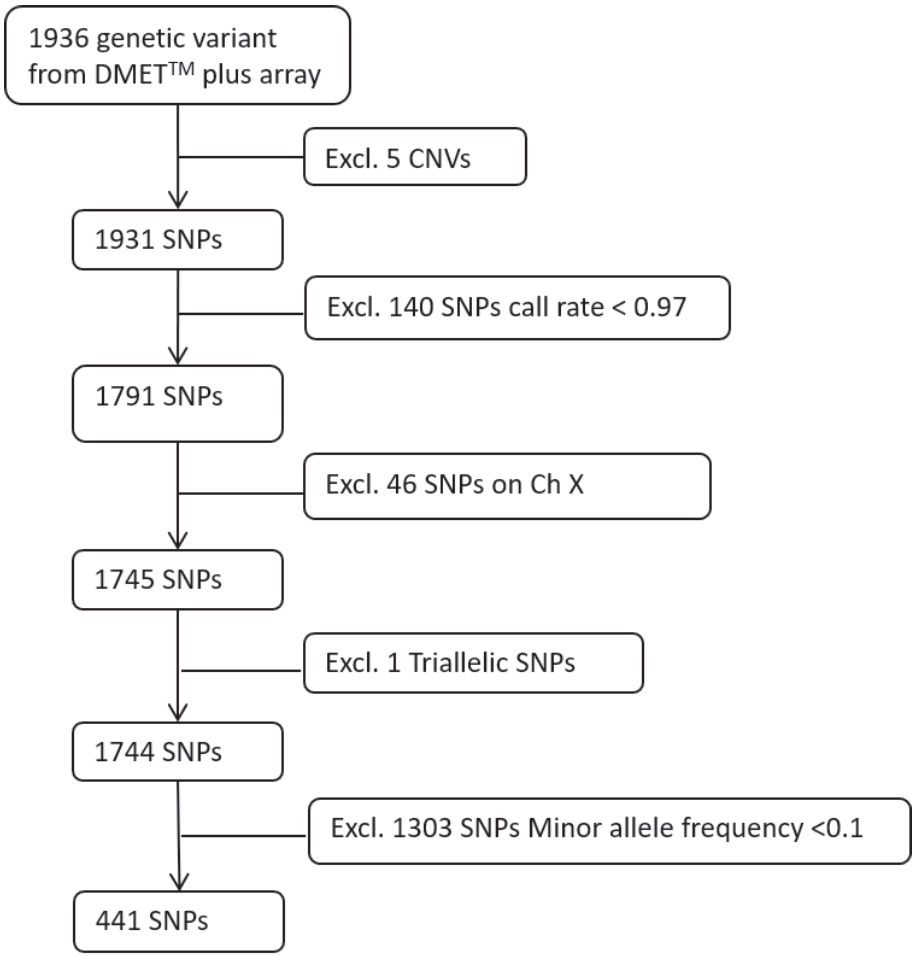


Fig. S5 Flow diagram of the genetic variants selection if the pre-set selection based on a translation file as recommended by Affymetrics® was not considered. Excl. represents excluding, Ch X represents chromosome X, DMET™ represent Drug Metabolizing Enzymes and Transporters, CNVs represents copy number variations.

Online Resource 2 Shiny app establishing method and results

Shiny app establishment

Based on the final mitotane population pharmacokinetic model, a Shiny app was established for the simulation for a random patient and to elucidate an example of the model application on guiding treatment for a new patient. Package shiny (version 1.4.0) and RxODE (version 0.6-1) in R statistics software (version 3.4.2; R Foundation for Statistical Computing, Vienna, Austria) were utilized. The R script can be found through: https://github.com/AnyueYin/Shiny-app-script-for-model-simulation--Population-PK-and-PG-analysis-of-mitotane. Patient gender, weight, and height, which were used to estimate lean body weight (LBW) and fat amount (FAT), as well as the results of three SNPs were in the input panel, based on which the starting dose was suggested. One hundred times of simulation under an optimized mitotane treatment regimen, Regimen 5-(-4g), were performed given the input information. The 90% prediction interval, 50th percentile of the predictions, target reaching time, and suggested starting dose were plotted in the output figure. The residual errors were not considered in the simulation.

Screen shots of the developed shiny app is shown in **Fig. S6**. The result shows that for a male patient with 85 kg weight and 180 cm height who carries G/G, A/A, and T/C for *CYP2C19*2* (rs4244285), *SLCO1B3* 699A>G (rs7311358), and *SLCO1B1* 571T>C (rs4149057), respectively, the 90% prediction interval can nicely locate within the therapeutic window of mitotane. The starting dose was suggested as 5.5g per day and the 50th percentile of the predictions reached the target on day 92. If the genotype result of *CYP2C19*2* (rs4244285) changed to G/A, the suggested starting dose became 4 g per day and the 50th percentile of the predictions reached the target on day 94.

In addition, a model with FAT effect on central distribution volume as the only covariate (**Table S3**) was also built in the Shiny app as an alternative option to allow dosing advice and concentration prediction for patients when genotyping results are not available (**Figure S6c**).

Table S3 Parameter estimates of the final model without genotyping results as covariates

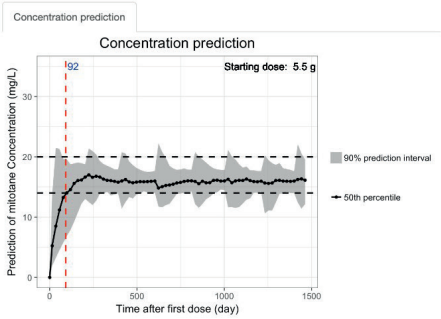
Parameters	Final model Estimate (RSE%)	IIV (CV%) [shrinkage]	IOV[a] (CV%)
KA (/day)	15.0 fixed	-	-
CL/F (L/day)	217 (9%)	66.3 [7%]	31.2
V_c/F (L)	8450 (16%)	63.5 [37%]	-
V_c_FAT (power)	1.12 (18%)	-	-
V_p/F (L)	15500 (15%)	80.4 [36%]	-
Q/F (/day)	609 (28%)	100.5 [38%]	-
Residual errors PRO (CV%)	16.7 (6%)	-	-
ADD (mg/L)	0.907 (16%)	-	-

FAT, fat amount; RSE, relative standard error; CV, coefficient of variation; IIV, inter-individual variability; IOV, inter-occasion variability; PRO, proportional residual error; ADD, additive residual error; CL/F, apparent system clearance; KA, absorption rate constant; V_c/F, apparent distribution volume of central compartment; V_p/F, apparent distribution volume of peripheral compartment; Q/F, apparent distribution rate constant; [a] Every 200 days of dosing was defined as an occasion.

Fig. S6. Screen shot of the shiny app established based on the final model. a) a male patient with 85 kg weight and 180 cm height who carries G/G, A/A, and T/C for *CYP2C19*2* (rs4244285), *SLCO1B3* 699A>G (rs7311358), and *SLCO1B1* 571T>C (rs4149057), respectively. b) a male patient with 85 kg weight and 180 cm height who carries G/A, A/A, and T/C for *CYP2C19*2* (rs4244285), *SLCO1B3* 699A>G (rs7311358), and *SLCO1B1* 571T>C (rs4149057), respectively. c) a male patient with 85 kg weight and 180 cm height whose genotyping results are unknown.

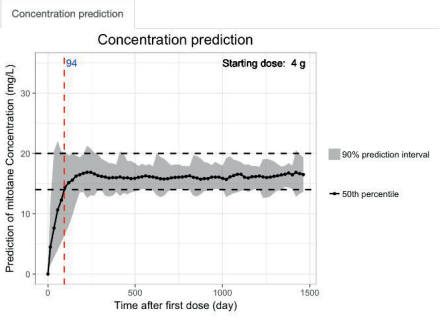
a **Mitotane**

Characteristics of patients:
Is genotyping results available?
YES
CYP2C19*2 (rs4244285)
G/G
SLCO1B3 669A>G (rs7311358)
A/A
SLCO1B1 571T>C (rs4149057)
T/C
Weight of patient (kg)
85
Height of patient (cm)
180
Gender of patient (F/M)
F
M
Plot



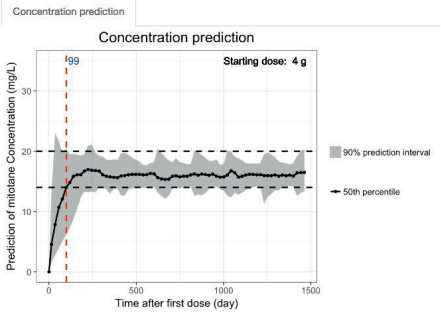
b **Mitotane**

Characteristics of patients:
Is genotyping results available?
YES
CYP2C19*2 (rs4244285)
G/A
SLCO1B3 669A>G (rs7311358)
A/A
SLCO1B1 571T>C (rs4149057)
T/C
Weight of patient (kg)
85
Height of patient (cm)
180
Gender of patient (F/M)
F
M
Plot



c **Mitotane**

Characteristics of patients:
Is genotyping results available?
NO
Weight of patient (kg)
85
Height of patient (cm)
180
Gender of patient (F/M)
F
M
Plot



Online Resource 3 List of 959 SNPs from DMETTM array of which the genotyping results were obtained for each patient

Gene	Summary Flag	Common Name	Probe Set ID	dbSNP RS ID	Chrom
ABCB1	N	ABCB1_c.-129T>C	AM_14633	rs3213619	Ch7:87230193
ABCB1	N	ABCB1_c.-1G>A	AM_14631	rs2214102	Ch7:87229501
ABCB1	F17L	ABCB1_c.49T>C(F17L)	AM_14630	rs28381804	Ch7:87229452
ABCB1	N21D	ABCB1_c.61A>G(N21D)	AM_14628	rs9282564	Ch7:87229440
ABCB1	N44S	ABCB1_c.131A>G(N44S)	AM_14627	rs1202183	Ch7:87214983
ABCB1	A80E	ABCB1_c.239C>A(A80E)	AM_14625	rs9282565	Ch7:87214875
ABCB1	N	ABCB1_c.287-25G>T	AM_14624	rs2235015	Ch7:87199564
ABCB1	G185V	ABCB1_c.554G>T(G185V)	AM_14622	rs1128501	Ch7:87195534
ABCB1	N	ABCB1_c.555A>T(G185G)	AM_14621	rs1128502	Ch7:87195533
ABCB1	N	ABCB1_c.729A>G(E243E)	AM_14620	rs2235022	Ch7:87190677
ABCB1	N	ABCB1_c.738G>A(A246A)	AM_14619	rs28381867	Ch7:87190668
ABCB1	N	ABCB1_c.IVS9-44A>G	AM_14617	rs10276036	Ch7:87180198
ABCB1	S400N	ABCB1_c.1199G>A(S400N)	AM_14616	rs2229109	Ch7:87179809
ABCB1	N	ABCB1_c.1236C>T(G412G)	AM_14612	rs1128503	Ch7:87179601
ABCB1	N	ABCB1_c.1350+44C>T	AM_14610	rs2032588	Ch7:87179443
ABCB1	N	ABCB1_c.1554+24C>T	AM_14609	rs2235033	Ch7:87179143
ABCB1	N	ABCB1_c.1662G>C(L554L)	AM_14607	rs2235012	Ch7:87178727
ABCB1	E566K	ABCB1_c.1696G>A(E566K)	AM_14606	rs28381902	Ch7:87178693
ABCB1	N	ABCB1_c.1725+38A>G	AM_14605	rs2235013	Ch7:87178626
ABCB1	R593C	ABCB1_c.1777C>T(R593C)	AM_14604	rs28381914	Ch7:87175289
ABCB1	N	ABCB1_c.1794C>T(I598I)	AM_14603	rs28381915	Ch7:87175272
ABCB1	A599T	ABCB1_c.1795G>A(A599T)	AM_14602	rs2235036	Ch7:87175271
ABCB1	V801M	ABCB1_c.2401G>A(V801M)	AM_14599	rs2235039	Ch7:87165854
ABCB1	N	ABCB1_c.2481+24G>A	AM_14598	rs2235040	Ch7:87165750
ABCB1	I829V	ABCB1_c.2485A>G(I829V)	AM_14596	rs2032581	Ch7:87160810
ABCB1	N	ABCB1_c.2505A>G(V835V)	AM_14595	rs28381966	Ch7:87160790
ABCB1	I836V	ABCB1_c.2506A>G(I836V)	AM_14594	rs28381967	Ch7:87160789
ABCB1	N	ABCB1_c.2650C>T(L884L)	AM_14593	rs9282563	Ch7:87160645
ABCB1	A893S, A893T	ABCB1_c.2677G>T>A (A893SorT)	AM_14592	rs2032582	Ch7:87160618, Ch7:87160618
ABCB1	A999T	ABCB1_c.2995G>A(A999T)	AM_14591	rs72552784	Ch7:87145914
ABCB1	N	ABCB1_c.3084G>A(P1028P)	AM_14590	rs2235044	Ch7:87145825
ABCB1	P1051A	ABCB1_c.3151C>G(P1051A)	AM_14589	rs28401798	Ch7:87144678
ABCB1	G1063A	ABCB1_c.3188G>C(G1063A)	AM_14588	rs2707944	Ch7:87144641
ABCB1	N	ABCB1_c.3189C>G(G1063G)	AM_14587	rs2707943	Ch7:87144640
ABCB1	S1141T	ABCB1_c.3421T>A(S1141T)	AM_14582	rs2229107	Ch7:87138659
ABCB1	N	ABCB1_c.3435C>T(I1145I)	AM_14581	rs1045642	Ch7:87138645
ABCB1	N	ABCB1_c.3747C>G(G1249G)	AM_14579	rs2235051	Ch7:87133655
ABCB1	V1251I	ABCB1_c.3751G>A(V1251I)	AM_14578	rs28364274	Ch7:87133651
ABCB1	N	ABCB1_c.*89A>T(3'UTR)	AM_14577	rs17064	Ch7:87133470
ABCB1	N	ABCB1_c.*193A>G(3'UTR)	AM_14575	rs3842	Ch7:87133366
ABCB4	N	ABCB4_c.-1921T>C	AM_14572	rs3747806	Ch7:87106702

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ABCB4	N	ABCB4_c.-1584C>T	AM_14570	rs4148805	Ch7:87106365
ABCB4	N	ABCB4_c.-1484T>C	AM_14569	rs4148806	Ch7:87106265
ABCB4	N	ABCB4_c.-1031C>T	AM_14568	rs4148807	Ch7:87105812
ABCB4	N	ABCB4_c.-1014A>G	AM_14567	rs4148808	Ch7:87105795
ABCB4	N	ABCB4_c.147C>T(S49S)	AM_14563	rs8187789	Ch7:87092213
ABCB4	N	ABCB4_c.175C>T(L59L)	AM_14562	rs2302387	Ch7:87092185
ABCB4	L73V	ABCB4_c.217C>G(L73V)	AM_14561	rs8187788	Ch7:87092143
ABCB4	N	ABCB4_c.240G>A(E80E)	AM_14560	rs8187787	Ch7:87092120
ABCB4	R144X	ABCB4_c.430C>T(R144X)	AM_14558	rs72552780	Ch7:87082366
ABCB4	N	ABCB4_c.504C>T(N168N)	AM_14556	rs1202283	Ch7:87082292
ABCB4	N	ABCB4_c.696C>T(A232A)	AM_14554	rs8187791	Ch7:87080951
ABCB4	N	ABCB4_c.711A>T(I237I)	AM_14553	rs2109505	Ch7:87079406
ABCB4	L238V	ABCB4_c.712C>G(L238V)	AM_14552	rs45596335	Ch7:87079405
ABCB4	E528D	ABCB4_c.1584G>C(E528D)	AM_14546	rs8187797	Ch7:87069130
ABCB4	Q636X	ABCB4_c.1906C>T(Q636X)	AM_14542	rs72552775	Ch7:87056224
ABCB4	R652G	ABCB4_c.1954A>G(R652G)	AM_14540	rs2230028	Ch7:87056176
ABCB4	W658X	ABCB4_c.1973G>A(W658X)	AM_14539	rs72552774	Ch7:87056157
ABCB4	N	ABCB4_c.2325G>C(T775T)	AM_14537	rs8187802	Ch7:87049383
ABCB4	R788Q	ABCB4_c.2363G>A(R788Q)	AM_14536	rs8187801	Ch7:87049345
ABCB4	N	ABCB4_c.2559T>G(G853G)	AM_14535	rs3761810	Ch7:87046751
ABCB4	N	ABCB4_c.2952A>G(A984A)	AM_14533	rs45574932	Ch7:87038681
ABCB4	N	ABCB4_c.3111T>C(N1037N)	AM_14532	rs8187808	Ch7:87037521
ABCB4	N	ABCB4_c.3144C>T(N1048N)	AM_14531	rs8187807	Ch7:87037488
ABCB4	A1100T	ABCB4_c.3298G>A(A1100T)	AM_14530	rs31655	Ch7:87035792
ABCB4	I1185V	ABCB4_c.3553A>G(I1185V)	AM_14529	rs8187811	Ch7:87032531
ABCB4	N	ABCB4_c.*509T>C	AM_14528	rs2097937	Ch7:87030903
ABCB7	N	ABCB7_c.457-2518A>G	AM_15425	rs5937939	ChX:74299007
ABCB7	N	ABCB7_c.457-1932A>T	AM_15424	rs4892538	ChX:74298421
ABCB7	R259K	ABCB7_c.776G>A(R259K)	AM_15423	rs1054913	ChX:74295279
ABCB7	L272P	ABCB7_c.815T>C(L272P)	AM_15422	rs1054914	ChX:74295240
ABCB7	A581V	ABCB7_c.1742C>T(A581V)	AM_15415	rs1340989	ChX:74284997
ABCB7	N	ABCB7_c.1743A>G(A581A)	AM_15414	rs1340990	ChX:74284996
ABCB11	N	ABCB11_c.-12519C>T	AM_12785	rs4148768	Ch2:169887154
ABCB11	N	ABCB11_c.-10013G>A	AM_12783	rs4668115	Ch2:169884648
ABCB11	N	ABCB11_c.-8583G>A	AM_12782	rs3770603	Ch2:169883218
ABCB11	N	ABCB11_c.-7449T>C	AM_12781	rs4148770	Ch2:169882084
ABCB11	N	ABCB11_c.-7056C>T	AM_12780	rs3770602	Ch2:169881691
ABCB11	N	ABCB11_c.-5543C>T	AM_12778	rs7602171	Ch2:169880178
ABCB11	N	ABCB11_c.-2450A>G	AM_12776	rs7578587	Ch2:169877085
ABCB11	N	ABCB11_c.-904C>A	AM_12775	rs3755163	Ch2:169875539
ABCB11	N	ABCB11_c.-899delCT	AM_12772	rs4148771	Ch2:169875534
ABCB11	N	ABCB11_c.-899delCT_alternate	AM_12773	rs4148771	Ch2:169875534, Ch2:169875534
ABCB11	N	ABCB11_c.-610C>A	AM_12771	rs3755162	Ch2:169875245
ABCB11	N	ABCB11_c.99-18T>C	AM_12767	rs4148776	Ch2:169870882

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ABCB11	N	ABCB11_c.108T>C(D36D)	AM_12766	rs3815675	Ch2:169870855
ABCB11	N	ABCB11_c.270T>C(F90F)	AM_12762	rs4148777	Ch2:169869901
ABCB11	G238V	ABCB11_c.713G>T(G238V)	AM_12749	rs72551306	Ch2:169850291
ABCB11	N	ABCB11_c.807T>C(Y269Y)	AM_12744	rs2287616	Ch2:169847412
ABCB11	R299K	ABCB11_c.896G>A(R299K)	AM_12742	rs2287617	Ch2:169847323
ABCB11	N	ABCB11_c.957A>G(G319G)	AM_12739	rs7563233	Ch2:169842746
ABCB11	A444V	ABCB11_c.1331C>T(A444V)	AM_12726	rs2287622	Ch2:169830328
ABCB11	D482G	ABCB11_c.1445A>G(D482G)	AM_12722	rs72549402	Ch2:169828550
ABCB11	R575X	ABCB11_c.1723C>T(R575X)	AM_12719	rs72549401	Ch2:169826641
ABCB11	N	ABCB11_c.2412A>G(A804A)	AM_12688	rs11568373	Ch2:169801403
ABCB11	G982R	ABCB11_c.2944G>A(G982R)	AM_12677	rs72549399	Ch2:169791806
ABCB11	N	ABCB11_c.3084A>G(A1028A)	AM_12674	rs497692	Ch2:169789016
ABCB11	R1057X	ABCB11_c.3169C>T(R1057X)	AM_12672	rs72549397	Ch2:169788931
ABCB11	R1090X	ABCB11_c.3268C>T(R1090X)	AM_12670	rs72549396	Ch2:169787318
ABCB11	R1153C	ABCB11_c.3457C>T(R1153C)	AM_12667	rs72549395	Ch2:169783827
ABCB11	E1186K	ABCB11_c.3556G>A(E1186K)	AM_12666	rs1521808	Ch2:169783728
ABCB11	R1268Q	ABCB11_c.3803G>A(R1268Q)	AM_12660	rs72549394	Ch2:169780295
ABCB11	N	ABCB11_c.*236G>A	AM_12658	rs473351	Ch2:169779896
ABCB11	N	ABCB11_c.*281T>G	AM_12657	rs3732038	Ch2:169779851
ABCB11	N	ABCB11_c.*368G>A	AM_12656	rs495714	Ch2:169779764
ABCB11	N	ABCB11_c.*420A>G	AM_12655	rs496550	Ch2:169779712
ABCC1	S92F	ABCC1_c.275C>T(S92F)	AM_10915	rs8187844	Ch16:16103682
ABCC1	R230Q	ABCC1_c.689G>A(R230Q)	AM_10917	rs8187848	Ch16:16130340
ABCC1	N	ABCC1_c.825T>C(V275V)	AM_10920	rs246221	Ch16:16138322
ABCC1	V353M	ABCC1_c.1057G>A(V353M)	AM_10922	rs8187852	Ch16:16139709
ABCC1	N	ABCC1_c.1068G>A(T356T)	AM_10924	rs8187853	Ch16:16139720
ABCC1	R433S	ABCC1_c.1299G>T(R433S)	AM_10925	rs60782127	Ch16:16142079
ABCC1	D526A, 526FS	ABCC1_c.1577A>CorCA (D526AorX)	AM_10926	rs72547522	Ch16:16150052, Ch16:16150052
ABCC1	N	ABCC1_c.1704C>T(Y568Y)	AM_10928	rs8187858	Ch16:16162039
ABCC1	N	ABCC1_c.1911C>T(D637D)	AM_10929	rs8187859	Ch16:16165585
ABCC1	N	ABCC1_c.2001C>T(S667S)	AM_10930	rs8187863	Ch16:16173221
ABCC1	N	ABCC1_c.2007C>T(P669P)	AM_10931	rs2301666	Ch16:16173227
ABCC1	R723Q	ABCC1_c.2168G>A(R723Q)	AM_10932	rs4148356	Ch16:16177275
ABCC1	N	ABCC1_c.3282G>A(P1094P)	AM_10936	rs4148377	Ch16:16215891
ABCC1	T1345M	ABCC1_c.4034C>T(T1345M)	AM_10939	rs8057331	Ch16:16230411
ABCC1	N	ABCC1_c.*866T>A	AM_10944	rs212090	Ch16:16236004
ABCC1	N	ABCC1_c.*1293G>A	AM_10945	rs4148380	Ch16:16236431
ABCC1	N	ABCC1_c.*1385G>T	AM_10946	rs8056298	Ch16:16236523
ABCC1	N	ABCC1_c.*1512T>C	AM_10947	rs212091	Ch16:16236650
ABCC2	N	ABCC2_c.-24C>T(5'UTR)	AM_10143	rs717620	Ch10:101542578
ABCC2	N	ABCC2_c.-23G>A(5'UTR)	AM_10144	rs17216156	Ch10:101542579
ABCC2	F39Y	ABCC2_c.116T>A(F39Y)	AM_10145	rs927344	Ch10:101544447
ABCC2	N	ABCC2_c.159A>G(K53K)	AM_10146	rs17222596	Ch10:101544490
ABCC2	M246L	ABCC2_c.736A>C(M246L)	AM_10147	rs17222744	Ch10:101556957

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ABCC2	D333G	ABCC2_c.998A>G(D333G)	AM_10149	rs17222674	Ch10:101559094
ABCC2	R353H	ABCC2_c.1058G>A(R353H)	AM_10150	rs7080681	Ch10:101560169
ABCC2	N	ABCC2_c.1219C>T(L407L)	AM_10151	rs17216198	Ch10:101563785
ABCC2	V417I	ABCC2_c.1249G>A(V417I)	AM_10152	rs2273697	Ch10:101563815
ABCC2	N	ABCC2_c.1434G>T>A(A478AorA)	AM_10153	rs4267009	Ch10:101564000, Ch10:101564000
ABCC2	T486I	ABCC2_c.1457C>T(T486I)	AM_10154	rs17222589	Ch10:101564023
ABCC2	K495E	ABCC2_c.1483A>G(K495E)	AM_10155	rs17222561	Ch10:101565157
ABCC2	F562L	ABCC2_c.1686T>G(F562L)	AM_10156	rs17216233	Ch10:101567857
ABCC2	602FS	ABCC2_c.1803insC	AM_10157	rs72558198	Ch10:101567974
ABCC2	I670T	ABCC2_c.2009T>C(I670T)	AM_10158	rs17222632	Ch10:101572816
ABCC2	N	ABCC2_c.2073C>A(V691V)	AM_10159	rs17222624	Ch10:101572880
ABCC2	N718S	ABCC2_c.2153A>G(N718S)	AM_10160	rs3740072	Ch10:101577123
ABCC2	R768W	ABCC2_c.2302C>T(R768W)	AM_10161	rs56199535	Ch10:101578577
ABCC2	S789F	ABCC2_c.2366C>T(S789F)	AM_10162	rs56220353	Ch10:101578641
ABCC2	L849R	ABCC2_c.2546T>G(L849R)	AM_10163	rs17222617	Ch10:101578952
ABCC2	E893Q	ABCC2_c.2677G>C(E893Q)	AM_10164	rs3740071	Ch10:101590120
ABCC2	Y967X	ABCC2_c.2901C>A(Y967X)	AM_10166	rs17222547	Ch10:101591385
ABCC2	N	ABCC2_c.2934G>A(S978S)	AM_10167	rs3740070	Ch10:101591418
ABCC2	I982V	ABCC2_c.2944A>G(I982V)	AM_10168	rs17222554	Ch10:101591428
ABCC2	I1036T	ABCC2_c.3107T>C(I1036T)	AM_10169	rs45441199	Ch10:101591737
ABCC2	R1066X	ABCC2_c.3196C>T(R1066X)	AM_10171	rs72558199	Ch10:101591826
ABCC2	N	ABCC2_c.3396T>C(I1132I)	AM_10172	rs17216345	Ch10:101594274
ABCC2	I1173F	ABCC2_c.3517A>T(I1173F)	AM_10174	rs72558201	Ch10:101595950
ABCC2	R1181L	ABCC2_c.3542G>T(R1181L)	AM_10175	rs8187692	Ch10:101595975
ABCC2	N	ABCC2_c.3561G>A(E1187E)	AM_10176	rs17216324	Ch10:101595994
ABCC2	V1188E	ABCC2_c.3563T>A(V1188E)	AM_10177	rs17222723	Ch10:101595996
ABCC2	T1273A	ABCC2_c.3817A>G(T1273A)	AM_10178	rs8187699	Ch10:101603631
ABCC2	P1291L	ABCC2_c.3872C>T(P1291L)	AM_10179	rs17216317	Ch10:101604107
ABCC2	K1299Q	ABCC2_c.3895A>C(K1299Q)	AM_10180	rs4148400	Ch10:101604130
ABCC2	N	ABCC2_c.3927C>T(Y1309Y)	AM_10181	rs4148401	Ch10:101604162
ABCC2	R1310X	ABCC2_c.3928C>T(R1310X)	AM_10182	rs66898362	Ch10:101604163
ABCC2	N	ABCC2_c.3972C>T(I1324I)	AM_10183	rs3740066	Ch10:101604207
ABCC2	N	ABCC2_c.4062C>T(A1354A)	AM_10184	rs17216275	Ch10:101605455
ABCC2	N	ABCC2_c.4110C>T(L1370L)	AM_10185	rs7899457	Ch10:101605503
ABCC2	N	ABCC2_c.4290G>T(V1430V)	AM_10188	rs1137968	Ch10:101606861
ABCC2	A1450S, A1450T	ABCC2_c.4348G>T>A(A1450SorT)	AM_10189	rs56296335	Ch10:101610393, Ch10:101610393
ABCC2	N	ABCC2_c.4410G>A(E1470E)	AM_10190	rs8187706	Ch10:101610455
ABCC2	N	ABCC2_c.4488C>T(H1496H)	AM_10191	rs8187707	Ch10:101610533
ABCC2	N	ABCC2_c.4527C>T(N1509N)	AM_10192	rs8187709	Ch10:101611277
ABCC2	C1515Y	ABCC2_c.4544G>A(C1515Y)	AM_10193	rs8187710	Ch10:101611294
ABCC3	A528G	ABCC3_c.1583C>G(A528G)	AM_11225	rs1003355	Ch17:48745066
ABCC3	N	ABCC3_c.3039C>T(G1013G)	AM_11231	rs4148416	Ch17:48753423
ABCC3	R1297H	ABCC3_c.3890G>A(R1297H)	AM_11235	rs11568591	Ch17:48761053

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ABCC3	N	ABCC3_c.3942C>T(H1314H)	AM_11236	rs2277624	Ch17:48761105
ABCC3	L1362V	ABCC3_c.4084C>G(L1362V)	AM_11239	rs1051625	Ch17:48761439
ABCC3	N	ABCC3_c.4509A>G(E1503E)	AM_11243	rs1051640	Ch17:48768486
ABCC4	C171G	ABCC4_c.511T>G(C171G)	AM_10640	rs4148460	Ch13:95886884
ABCC4	K304N	ABCC4_c.912G>T(K304N)	AM_10634	rs2274407	Ch13:95859035
ABCC4	N	ABCC4_c.951A>G(R317R)	AM_10633	rs2274406	Ch13:95858996
ABCC4	N	ABCC4_c.969G>A(S323S)	AM_10632	rs2274405	Ch13:95858978
ABCC4	631FS	ABCC4_c.1892delT(L631X)	AM_10624	rs72559753	Ch13:95818554
ABCC4	E757K	ABCC4_c.2269G>A(E757K)	AM_10622	rs3765534	Ch13:95815415
ABCC4	N	ABCC4_c.2712G>A(L904L)	AM_10618	rs1678339	Ch13:95727780
ABCC4	N	ABCC4_c.2844C>T(F948F)	AM_10617	rs1189466	Ch13:95726541
ABCC4	N,1116FS	ABCC4_c.3348A>GorDel (K1116KorX)	AM_10611	rs1751034	Ch13:95714976, Ch13:95714976
ABCC4	N	ABCC4_c.*38T>G	AM_10604	rs3742106	Ch13:95673791
ABCC4	N	ABCC4_c.*311G>A	AM_10603	rs4148551	Ch13:95673518
ABCC4	N	ABCC4_c.*694G>A	AM_10602	rs4148553	Ch13:95673135
ABCC4	N	ABCC4_c.*879T>C	AM_10601	rs1059751	Ch13:95672950
ABCC4	N	ABCC4_c.*1282T>C	AM_10600	rs4148554	Ch13:95672547
ABCC4	N	ABCC4_c.*1351T>C	AM_10599	rs1059754	Ch13:95672478
ABCC4	N	ABCC4_c.*1564A>T	AM_10598	rs4148555	Ch13:95672265
ABCC5	N	ABCC5_c.1146A>G(Q382Q)	AM_13400	rs7636910	Ch3:183699516
ABCC5	N	ABCC5_c.1200C>T(S400S)	AM_13397	rs1053386	Ch3:183696387
ABCC5	N	ABCC5_c.1782C>T(C594C)	AM_13393	rs939336	Ch3:183685534
ABCC5	1147FS	ABCC5_c.3441_3442insC(V1147X)	AM_13381	rs72551384	Ch3:183663700
ABCC5	Y1202X	ABCC5_c.3606C>A(Y1202X)	AM_13378	rs1053351	Ch3:183660603
ABCC5	N	ABCC5_c.3624C>T(L1208L)	AM_13377	rs3749442	Ch3:183660585
ABCC5	T1383N	ABCC5_c.4148C>A(T1383N)	AM_13370	rs1053387	Ch3:183643407
ABCC5	N	ABCC5_c.*1243G>A	AM_13365	rs562	Ch3:183637845
ABCC5	N	ABCC5_c.*1366A>C	AM_13364	rs3805114	Ch3:183637722
ABCC6	N	ABCC6_c.1890C>G(T630T)	AM_10978	rs8058696	Ch16:16278869
ABCC6	H632Q	ABCC6_c.1896C>A(H632Q)	AM_10977	rs8058694	Ch16:16278863
ABCC6	V665A	ABCC6_c.1994T>C(V665A)	AM_10976	rs4341770	Ch16:16276737
ABCC6	N	ABCC6_c.2400G>A(G800G)	AM_10968	rs7500834	Ch16:16272670
ABCC6	V848M	ABCC6_c.2542G>A(V848M)	AM_10966	rs6416668	Ch16:16271357
ABCC6	N	ABCC6_c.2835C>T(P945P)	AM_10964	rs2856585	Ch16:16263663
ABCC6	R1268Q	ABCC6_c.3803G>A(R1268Q)	AM_10959	rs2238472	Ch16:16251599
ABCC8	R248X	ABCC8_c.742C>T(R248X)	AM_10301	rs72559730	Ch11:17483210
ABCC8	631FS	ABCC8_c.1891delC(P631X)	AM_10287	rs72559725	Ch11:17450144
ABCC8	701FS	ABCC8_c.2101_2102insT(I701X)	AM_10285	rs72559724	Ch11:17449428
ABCC8	R836X	ABCC8_c.2506C>T(R836X)	AM_10281	rs72559722	Ch11:17434263
ABCC8	L1014X	ABCC8_c.3041T>A(L1014X)	AM_10277	rs67706538	Ch11:17428556
ABCC8	S1369A	ABCC8_c.4105T>G(S1369A)	AM_10270	rs757110	Ch11:17418477
ABCC8	V1572I	ABCC8_c.4714G>A(V1572I)	AM_10260	rs8192690	Ch11:17414570
ABCC9	N	ABCC9_c.669G>T(L223L)	AM_10558	rs17846788	Ch12:22068749
ABCC9	N	ABCC9_c.789C>T(C263C)	AM_10557	rs58386780	Ch12:22068629

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ABCC9	N	ABCC9_c.1035G>A(K345K)	AM_10556	rs72559752	Ch12:22063889
ABCC9	N	ABCC9_c.1296C>T(P432P)	AM_10555	rs10770865	Ch12:22063115
ABCC9	N	ABCC9_c.1848C>T(D616D)	AM_10554	rs61001398	Ch12:22040823
ABCC9	A1513T	ABCC9_c.4537G>A(A1513T)	AM_10546	rs72559751	Ch12:21958221
ABCG1	N	ABCG1_c.589-407A>G	AM_12204	rs492338	Ch21:43701977
ABCG1	N	ABCG1_c.973+672G>A	AM_12205	rs3788007	Ch21:43706776
ABCG1	N	ABCG1_c.974-898C>G	AM_12206	rs425215	Ch21:43707101
ABCG1	N	ABCG1_c.1430-293C>G	AM_12207	rs914189	Ch21:43710909
ABCG1	N	ABCG1_c.1809-252G>A	AM_12208	rs3788010	Ch21:43716022
ABCG1	N	ABCG1_c.*399G>A	AM_12209	rs1044317	Ch21:43716901
ABCG1	N	ABCG1_c.*1981G>A	AM_12210	rs1541290	Ch21:43718483
ABCG2	N	ABCG2_c.369C>T(Y123Y)	AM_13690	rs2231139	Ch4:89052964
ABCG2	Q126X	ABCG2_c.376C>T(Q126X)	AM_13689	rs72552713	Ch4:89052957
ABCG2	Q141K	ABCG2_c.421C>A(Q141K)	AM_13688	rs2231142	Ch4:89052323
ABCG2	Q166E	ABCG2_c.496C>G(Q166E)	AM_13687	rs1061017	Ch4:89052248
ABCG2	N	ABCG2_c.564A>G(G188G)	AM_13686	rs3116439	Ch4:89042912
ABCG2	F208S	ABCG2_c.623T>C(F208S)	AM_13683	rs1061018	Ch4:89042853
ABCG2	S248P	ABCG2_c.742T>C(S248P)	AM_13682	rs3116448	Ch4:89039360
ABCG2	E334X	ABCG2_c.1000G>T(E334X)	AM_13681	rs3201997	Ch4:89034649
ATP7A	L142V	ATP7A_c.424C>G(L142V)	AM_15437	rs61743418	ChX:77244041
ATP7A	Q167X	ATP7A_c.499C>T(Q167X)	AM_15438	rs72554635	ChX:77244116
ATP7A	I189V	ATP7A_c.565A>G(I189V)	AM_15439	rs2228447	ChX:77244182
ATP7A	R409X	ATP7A_c.1225C>T(R409X)	AM_15440	rs72554636	ChX:77245343
ATP7A	N	ATP7A_c.1390T>C(L464L)	AM_15441	rs2234934	ChX:77254028
ATP7A	V767L	ATP7A_c.2299G>C(V767L)	AM_15454	rs2227291	ChX:77268502
ATP7A	V1401L	ATP7A_c.4201G>C(V1401L)	AM_15472	rs5959130	ChX:77301044
CDA	*2	CDA*2_c.79A>C(K27Q)	AM_11499	rs2072671	Ch1:20915701
CDA	*3	CDA*3_c.208G>A(A70T)	AM_11506	rs60369023	Ch1:20931474
CDA	N	CDA_c.-88G>A	AM_11498	rs602946	Ch1:20915535
CDA	N	CDA_c.154+1015A>G	AM_11500	rs818202	Ch1:20916791
CDA	N	CDA_c.154+3136T>C	AM_11501	rs10916824	Ch1:20918912
CDA	N	CDA_c.435C>T(T145T)	AM_11520	rs1048977	Ch1:20945055
CES2	N	CES2_c.268+947A>T	AM_11065	rs11568314	Ch16:66970561
CES2	N	CES2_c.269-965A>G	AM_11066	rs4783745	Ch16:66970975
CES2	N	CES2_c.269-683G>A	AM_11067	rs11568311	Ch16:66971257
CES2	R98W	CES2_c.292C>T(R98W)	AM_11068	rs72547531	Ch16:66971963
CES2	V206M	CES2_c.616G>A(V206M)	AM_11070	rs72547532	Ch16:66974125
CES2	IVS8	CES2_c.1330-2A>G(SpliceDefect)	AM_11075	rs72547533	Ch16:66976006
CES2	IVS10	CES2_c.1613-88C>T(SpliceDefect)	AM_11076	rs3893757	Ch16:66977114
CHST7	N	CHST7_c.*31+10802G>A	AM_15403	rs6521128	ChX:46445660
CHST7	N	CHST7_c.*32-7531A>C	AM_15404	rs11796837	ChX:46449664
CHST7	N	CHST7_c.*32-2930C>A	AM_15405	rs12012841	ChX:46454265
CHST7	N	CHST7_c.*58G>A(3'UTR)	AM_15406	rs7056956	ChX:46457221
CHST7	N	CHST7_c.*623A>G(3'UTR)	AM_15407	rs735716	ChX:46457786
CHST7	N	CHST7_c.*675+785T>G(3'UTR)	AM_15408	rs732316	ChX:46458623

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CHST7	N	CHST7_c.*675+2777C>T(3'UTR)	AM_15409	rs12014644	ChX:46460615
CYP1A1	*6	CYP1A1*6_1635G>T(M331I)	AM_10774	rs56313657	Ch15:75013804
CYP1A1	*7	CYP1A1*7_2345insT	AM_10771	rs72547510	Ch15:75013093
CYP1A1	*8	CYP1A1*8_2413T>A(I448N)	AM_10770	rs72547509	Ch15:75013026
CYP1A1	*4	CYP1A1*4_2452C>A(T461N)	AM_10769	rs1799814	Ch15:75012987
CYP1A1	*2C	CYP1A1*2C_2454A>G(I462V)	AM_10768	rs1048943	Ch15:75012985
CYP1A1	*5,*9	CYP1A1*5or*9_2460C>A>T |(R464SorC)	AM_10766	rs41279188	Ch15:75012979, Ch15:75012979
CYP1A1	*10	CYP1A1*10_2499C>T(R477W)	AM_10765	rs56240201	Ch15:75012940
CYP1A1	*3	CYP1A1*3_3204T>C(3'UTR)	AM_10762	rs1800031	Ch15:75012235
CYP1A1	G45D	CYP1A1_134G>A(G45D)	AM_10778	rs4646422	Ch15:75015305
CYP1A1	R279W	CYP1A1_1390C>T(R279W)	AM_10776	rs34260157	Ch15:75014049
CYP1A1	I286T	CYP1A1_1412T>C(I286T)	AM_10775	rs4987133	Ch15:75014027
CYP1A1	F381L	CYP1A1_1876C>A(F381L)	AM_10772	rs2856833	Ch15:75013563
CYP1A1	A463G	CYP1A1_2458C>G(A463G)	AM_10767	rs2278970	Ch15:75012981
CYP1A2	PR	CYP1A2*1C_-3860G>A(Promoter)	AM_10780	rs2069514	Ch15:75038220
CYP1A2	*1K	CYP1A2*1K_-729C>T(Promoter)	AM_10784	rs12720461	Ch15:75041351
CYP1A2	PR	CYP1A2*1F_-163C>A(Promoter)	AM_10785	rs762551	Ch15:75041917
CYP1A2	*2	CYP1A2*2_63C>G(F21L)	AM_10787	rs56160784	Ch15:75042142
CYP1A2	*15	CYP1A2*15_125C>G(P42R)	AM_10788	rs72547511	Ch15:75042204
CYP1A2	*11	CYP1A2*11_558C>A(F186L)	AM_10793	rs72547513	Ch15:75042637
CYP1A2	*3	CYP1A2*3_2116G>A(D348N)	AM_10796	rs56276455	Ch15:75044195
CYP1A2	*16	CYP1A2*16_2473G>A(R377Q)	AM_10798	rs72547515	Ch15:75044552
CYP1A2	*4	CYP1A2*4_2499A>T(I386F)	AM_10799	rs72547516	Ch15:75044578
CYP1A2	*5	CYP1A2*5_3497G>A(C406Y)	AM_10801	rs55889066	Ch15:75045575
CYP1A2	*7	CYP1A2*7_3533G>A(SpliceDefect)	AM_10802	rs56107638	Ch15:75045612
CYP1A2	*6	CYP1A2*6_5090C>T(R431W)	AM_10803	rs28399424	Ch15:75047169
CYP1A2	*8	CYP1A2*8_5166G>A(R456H)	AM_10805	rs72547517	Ch15:75047245
CYP1A2	*1D	CYP1A2*1D_-2467delT(Promoter)	AM_10782	rs35694136	Ch15:75039613
CYP1A2	PR	CYP1A2*1K_-739T>G(Promoter)	AM_10783	rs2069526	Ch15:75041341
CYP1A2	N	CYP1A2_5347T>C(N516N)	AM_10807	rs2470890	Ch15:75047426
CYP1B1	*12	CYP1B1*12_182G>A(G61E)	AM_12493	rs28936700	Ch2:38302350
CYP1B1	*17	CYP1B1*17_4096del13 (RVQAE355X)	AM_12473	rs72549380	Ch2:38298421
CYP1B1	*18	CYP1B1*18_4125G>T(G365W)	AM_12471	rs55771538	Ch2:38298404
CYP1B1	*19	CYP1B1*19_4168C>T(P379L)	AM_12467	rs56305281	Ch2:38298361
CYP1B1	*20	CYP1B1*20_4191G>A(E387K)	AM_12466	rs55989760	Ch2:38298338
CYP1B1	*21	CYP1B1*21_4201G>A(R390H)	AM_12465	rs56010818	Ch2:38298328
CYP1B1	*3	CYP1B1*3_4326C>G(L432V)	AM_12461	rs1056836	Ch2:38298203
CYP1B1	*23	CYP1B1*23_4342C>T(P437L)	AM_12460	rs56175199	Ch2:38298187
CYP1B1	*7	CYP1B1*7_4360C>G(A443G)	AM_12458	rs4986888	Ch2:38298169
CYP1B1	*24	CYP1B1*24_4377delG(D449X)	AM_12456	rs72549375	Ch2:38298152
CYP1B1	*4	CYP1B1*4_4390A>G(N453S)	AM_12454	rs1800440	Ch2:38298139
CYP1B1	*25	CYP1B1*25_4437C>T(R469W)	AM_12452	rs28936701	Ch2:38298092
CYP1B1	N	CYP1B1_81G>C(L27L)	AM_12498	rs4987135	Ch2:38302451

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CYP1B1	R355X	CYP1B1_4095C>T(R355X)	AM_12474	rs72549381	Ch2:38298434
CYP1B1	M372V	CYP1B1_4146A>G(M372V)	AM_12469	rs4398252	Ch2:38298383
CYP1B1	N, D449DorE	CYP1B1_4379C>TorA(D449DorE)	AM_12455	rs1056837	Ch2:38298150, Ch2:38298150
CYP1B1	N	CYP1B1_4424A>T(S464S)	AM_12453	rs4986889	Ch2:38298105
CYP2A6	*9	CYP2A6*9_-48T>G(Promoter)	AM_11361	rs28399433	Ch19:41356379
CYP2A6	*13	CYP2A6*13_13G>A(G5R)	AM_11360	rs28399434	Ch19:41356319
CYP2A6	*6	CYP2A6*6_1703G>A(R128Q)	AM_11353	rs4986891	Ch19:41354629
CYP2A6	*2	CYP2A6*2_1799T>A(L160H)	AM_11351	rs1801272	Ch19:41354533
CYP2A6	*20	CYP2A6*20_2141delAA(K196X)	AM_11346	rs28399444	Ch19:41354190
CYP2A6	*11	CYP2A6*11_3391T>C(S224P)	AM_11344	rs111033610	Ch19:41352941
CYP2A6	*17	CYP2A6*17_5065G>A(V365M)	AM_11337	rs28399454	Ch19:41351267
CYP2A6	*7	CYP2A6*7_6558T>C(I471T)	AM_11330	rs5031016	Ch19:41349774
CYP2A6	*28	CYP2A6*28_5750G>C(E419D)	AM_11332	rs8192730	Ch19:41350582
CYP2A6	*8	CYP2A6*8_6600G>T(R485L)	AM_11327	rs28399468	Ch19:41349732
CYP2A6	*1D	CYP2A6*1D_-1013A>G	AM_11364	rs4803381	Ch19:41357344
CYP2A6	N	CYP2A6_22C>T(L8L)	AM_11359	rs8192720	Ch19:41356310
CYP2A6	N	CYP2A6_51G>A(V17V)	AM_11358	rs1137115	Ch19:41356281
CYP2A6	N	CYP2A6_1874G>T	AM_11349	rs28399442	Ch19:41354458
CYP2A6	N	CYP2A6_3420A>G(P233P)	AM_11343	rs3891219	Ch19:41352912
CYP2A6	N	CYP2A6_3570C>G	AM_11342	rs4079369	Ch19:41352762
CYP2A6	N	CYP2A6_4365A>G(K289K)	AM_11341	rs2644905	Ch19:41351967
CYP2A6	387FS	CYP2A6_5132delA(K387X)	AM_11336	rs72547582	Ch19:41351200
CYP2A6	N	CYP2A6_5336G>A	AM_11334	rs8192729	Ch19:41350996
CYP2A13	N	CYP2A13_2366C>T	AM_11440	rs1645691	Ch19:41596742
CYP2A13	N	CYP2A13_6432C>T	AM_11446	rs1645694	Ch19:41600808
CYP2A13	N	CYP2A13_7233T>G	AM_11449	rs1709082	Ch19:41601609
CYP2A13	P321L	CYP2A13_5289C>T(P321L)	AM_11443	rs3885816	Ch19:41599665
CYP2B6	*22	CYP2B6*22_-82T>C	AM_11398	rs34223104	Ch19:41497129
CYP2B6	*2	CYP2B6*2_64C>T(R22C)	AM_11399	rs8192709	Ch19:41497274
CYP2B6	*11	CYP2B6*11_136A>G(M46V)	AM_11401	rs35303484	Ch19:41497346
CYP2B6	*12	CYP2B6*12_12820G>A(G99E)	AM_11403	rs36060847	Ch19:41510030
CYP2B6	*8	CYP2B6*8_13072A>G(K139E)	AM_11405	rs12721655	Ch19:41510282
CYP2B6	*14	CYP2B6*14_13076G>A(R140Q)	AM_11406	rs35773040	Ch19:41510286
CYP2B6	*26	CYP2B6*26_15614C>G(P167A)	AM_11409	rs3826711	Ch19:41512824
CYP2B6	*20	CYP2B6*20_15618C>T(T168I)	AM_11410	rs36056539	Ch19:41512828
CYP2B6	*6	CYP2B6*6_15631G>T(Q172H)	AM_11411	rs3745274	Ch19:41512841
CYP2B6	*27	CYP2B6*27_15708T>C(M198T)	AM_11413	rs36079186	Ch19:41512918
CYP2B6	*3	CYP2B6*3_18045C>A(S259R)	AM_11414	rs45482602	Ch19:41515255
CYP2B6	*4	CYP2B6*4_18053A>G(K262R)	AM_11415	rs2279343	Ch19:41515263
CYP2B6	*16	CYP2B6*16_21011T>C(I328T)	AM_11417	rs28399499	Ch19:41518221
CYP2B6	*19	CYP2B6*19_21034C>T(R336C)	AM_11418	rs34826503	Ch19:41518244
CYP2B6	*28	CYP2B6*28_21160C>T(R378X)	AM_11419	rs34097093	Ch19:41518370
CYP2B6	*15	CYP2B6*15_21388T>A(I391N)	AM_11420	rs35979566	Ch19:41518598
CYP2B6	*21	CYP2B6*21_21498C>A(P428T)	AM_11422	rs35010098	Ch19:41518708

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CYP2B6	*5	CYP2B6*5_25505C>T(R487C)	AM_11426	rs3211371	Ch19:41522715
CYP2B6	N	CYP2B6_12740G>C(P72P)	AM_11402	rs2279341	Ch19:41509950
CYP2B6	N	CYP2B6_14593C>G	AM_11407	rs4803418	Ch19:41511803
CYP2B6	N	CYP2B6_15582C>T	AM_11408	rs4803419	Ch19:41512792
CYP2B6	N	CYP2B6_18273G>A	AM_11416	rs2279344	Ch19:41515483
CYP2B6	N	CYP2B6_21563C>T	AM_11423	rs8192719	Ch19:41518773
CYP2C8	*3	CYP2C8*3_2130G>A(R139K)	AM_10135	rs11572080	Ch10:96827030
CYP2C8	*5	CYP2C8*5_2189delA(T159X)	AM_10133	rs72558196	Ch10:96826971
CYP2C8	*7,*8	CYP2C8*7or*8_4517C>T>G (R186XorG)	AM_10131	rs72558195	Ch10:96824643, Ch10:96824643
CYP2C8	*4	CYP2C8*4_11041C>G(I264M)	AM_10129	rs1058930	Ch10:96818119
CYP2C8	*2	CYP2C8*2_11054A>T(I269F)	AM_10128	rs11572103	Ch10:96818106
CYP2C8	*3	CYP2C8*3_30411A>G(K399R)	AM_10125	rs10509681	Ch10:96798749
CYP2C8	*12	CYP2C8*12_32184_32186delTTG (V461X)	AM_10123	rs3832694	Ch10:96796974
CYP2C8	N	CYP2C8_-86A>C	AM_10138	rs11572066	Ch10:96829245
CYP2C8	L390S	CYP2C8_30384T>C(L390S)	AM_10126	rs72558194	Ch10:96798776
CYP2C8	P404A	CYP2C8_30425C>G(P404A)	AM_10124	rs66501115	Ch10:96798735
CYP2C8	N	CYP2C8_32364C>T(3'UTR)	AM_10122	rs28399518	Ch10:96796796
CYP2C9	*13	CYP2C9*13_3276T>C(L90P)	AM_10093	rs72558187	Ch10:96701715
CYP2C9	*25	CYP2C9*25_3531_3540del10	AM_10095	rs72558188	Ch10:96701970
CYP2C9	*14	CYP2C9*14_3552G>A(R125H)	AM_10097	rs72558189	Ch10:96701991
CYP2C9	*2	CYP2C9*2_3608C>T(R144C)	AM_10100	rs1799853	Ch10:96702047
CYP2C9	*15	CYP2C9*15_9100C>A(S162x)	AM_10104	rs72558190	Ch10:96707539
CYP2C9	*9	CYP2C9*9_10535A>G(H251R)	AM_10106	rs2256871	Ch10:96708974
CYP2C9	*10	CYP2C9*10_10598A>G(E272G)	AM_10107	rs9332130	Ch10:96709037
CYP2C9	*6	CYP2C9*6_10601delA(K273X)	AM_10108	rs9332131	Ch10:96709039
CYP2C9	*16	CYP2C9*16_33497A>G(T299A)	AM_10109	rs72558192	Ch10:96731936
CYP2C9	*11	CYP2C9*11_42542C>T(R335W)	AM_10111	rs28371685	Ch10:96740981
CYP2C9	*3	CYP2C9*3_42614A>C(I359L)	AM_10113	rs1057910	Ch10:96741053
CYP2C9	*4	CYP2C9*4_42615T>C(I359T)	AM_10114	rs56165452	Ch10:96741054
CYP2C9	*5	CYP2C9*5_42619C>G(D360E)	AM_10115	rs28371686	Ch10:96741058
CYP2C9	*12	CYP2C9*12_50338C>T(P489S)	AM_10121	rs9332239	Ch10:96748777
CYP2C9	Y358C	CYP2C9_42612A>G(Y358C)	AM_10112	rs1057909	Ch10:96741051
CYP2C9	N	CYP2C9_55221C>T(A441A)	AM_10119	rs2017319	Ch10:96748635
CYP2C9	N	CYP2C9_55323A>T(G475G)	AM_10120	rs1057911	Ch10:96748737
CYP2C18	Y68X	CYP2C18_c.204T>A(Y68X)	AM_10039	rs41291550	Ch10:96447562
CYP2C18	195FS	CYP2C18_c.582insT	AM_10042	rs72558183	Ch10:96454774
CYP2C18	K232E	CYP2C18_c.694A>G(K232E)	AM_10044	rs2296681	Ch10:96466592
CYP2C18	T385M	CYP2C18_c.1154C>T(T385M)	AM_10047	rs2281891	Ch10:96493058
CYP2C18	N	CYP2C18_c.*31C>T(3'UTR)	AM_10048	rs2860840	Ch10:96495232
CYP2C18	N	CYP2C18_c.*592C>A(3'UTR)	AM_10052	rs1326830	Ch10:96495793
CYP2C19	*17	CYP2C19*17_-806C>T	AM_10053	rs12248560	Ch10:96521657
CYP2C19	*4	CYP2C19*4_1A>G(M1V)	AM_10054	rs28399504	Ch10:96522463
CYP2C19	*14	CYP2C19*14_50T>C(L17P)	AM_10055	rs55752064	Ch10:96522512

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CYP2C19	*15	CYP2C19*15_55A>C(I19L)	AM_10056	rs17882687	Ch10:96522517
CYP2C19	*2B	CYP2C19*2B_12460G>C(E92D)	AM_10059	rs17878459	Ch10:96534922
CYP2C19	*8	CYP2C19*8_12711T>C(W120R)	AM_10060	rs41291556	Ch10:96535173
CYP2C19	*6	CYP2C19*6_12748G>A(R132Q)	AM_10062	rs72558184	Ch10:96535210
CYP2C19	*9	CYP2C19*9_12784G>A(R144H)	AM_10064	rs17884712	Ch10:96535246
CYP2C19	*3	CYP2C19*3_17948G>A(W212X)	AM_10068	rs4986893	Ch10:96540410
CYP2C19	*10	CYP2C19*10_19153C>T(P227L)	AM_10069	rs6413438	Ch10:96541615
CYP2C19	*2	CYP2C19*2_19154G>A(P227P)	AM_10070	rs4244285	Ch10:96541616
CYP2C19	*7	CYP2C19*7_19294T>A (SpliceDefect)	AM_10072	rs72558186	Ch10:96541756
CYP2C19	*13	CYP2C19*13_87290C>T(R410C)	AM_10077	rs17879685	Ch10:96609752
CYP2C19	*5	CYP2C19*5_90033C>T(R433W)	AM_10079	rs56337013	Ch10:96612495
CYP2C19	*12	CYP2C19*12_90209A>C(X491C)	AM_10082	rs55640102	Ch10:96612671
CYP2C19	439FS	CYP2C19_90052delG	AM_10080	rs5787121	Ch10:96612514
CYP2C19	241FS	CYP2C19_721insG	AM_10071	rs72558185	Ch10:96541656
CYP2C19	V331I	CYP2C19_80161G>A(V331I)	AM_10075	rs3758581	Ch10:96602623
CYP2D6	P34S	CYP2D6_100C>T(P34S)	AM_12285	rs1065852	Ch22:42526694
CYP2D6	*12	CYP2D6*12_124G>A(G42R)	AM_12284	rs5030862	Ch22:42526670
CYP2D6	*15	CYP2D6*15_137insT	AM_12283	rs72549357	Ch22:42526657
CYP2D6	*11	CYP2D6*11_883G>C(SpliceDefect)	AM_12281	rs5030863	Ch22:42525912
CYP2D6	T107I	CYP2D6_1023C>T(T107I)	AM_12280	rs28371706	Ch22:42525772
CYP2D6	*29	CYP2D6*29_1659G>A(V136I)	AM_12278	rs61736512	Ch22:42525134
CYP2D6	*6	CYP2D6*6_1707delT(W152X)	AM_12276	rs5030655	Ch22:42525086
CYP2D6	*14,*8	CYP2D6*14or*8_1758G>A>T (G169RorX)	AM_12275	rs5030865	Ch22:42525035, Ch22:42525035
CYP2D6	*4	CYP2D6*4_1846G>A(SpliceDefect)	AM_12274	rs3892097	Ch22:42524947
CYP2D6	*40	CYP2D6*40_1863ins(TTTCGCCCC)2	AM_12272	rs72549356	Ch22:42524929
CYP2D6	*20	CYP2D6*20_1973insG	AM_12270	rs72549354	Ch22:42524819
CYP2D6	*19	CYP2D6*19_2539delAACT	AM_12268	rs72549353	Ch22:42524251
CYP2D6	*3	CYP2D6*3_2549delA(R259X)	AM_12267	rs35742686	Ch22:42524244
CYP2D6	*21	CYP2D6*21_2573insC	AM_12266	rs72549352	Ch22:42524213
CYP2D6	*38	CYP2D6*38_2587delGACT	AM_12265	rs72549351	Ch22:42524203
CYP2D6	*9	CYP2D6*9_2615delAAG	AM_12264	rs28371720	Ch22:42524178
CYP2D6	R296C	CYP2D6_2850C>T(R296C)	AM_12261	rs16947	Ch22:42523943
CYP2D6	*7	CYP2D6*7_2935A>C(H324P)	AM_12259	rs5030867	Ch22:42523858
CYP2D6	*44	CYP2D6*44_2950G>C(SpliceDefect)	AM_12258	rs72549349	Ch22:42523843
CYP2D6	*41	CYP2D6*41_2988G>A(SpliceDefect)	AM_12257	rs28371725	Ch22:42523805
CYP2D6	*29	CYP2D6*29_3183G>A(V338M)	AM_12255	rs59421388	Ch22:42523610
CYP2D6	*56	CYP2D6*56_3201C>T(R344X)	AM_12254	rs72549347	Ch22:42523592
CYP2D6	*42	CYP2D6*42_3259insGT	AM_12252	rs72549346	Ch22:42523533
CYP2D6	*18	CYP2D6*18_4125dupGTGCCCACT	AM_12248	rs1135836	Ch22:42522660
CYP2D6	N	CYP2D6_-2178G>A	AM_15506	rs28360521	Ch22:42528976
CYP2D6	N	CYP2D6_-1961C>G>A	AM_15503	N/A	Ch22:42528759, Ch22:42528759
CYP2D6	N	CYP2D6_-1770G>A	AM_15502	rs1080983	Ch22:42528568

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CYP2D6	N	CYP2D6_-1584C>G	AM_12291	rs1080985	Ch22:42528382
CYP2D6	N	CYP2D6_1661G>C(V136V)	AM_12277	rs1058164	Ch22:42525132
CYP2D6	S486T	CYP2D6_4180G>C(S486T)	AM_12247	rs1135840	Ch22:42522613
CYP2E1	*5	CYP2E1*5_-1293G>C(Promoter)	AM_10240	rs3813867	Ch10:135339605
CYP2E1	*5	CYP2E1*5_-1053C>T(Promoter)	AM_10241	rs2031920	Ch10:135339845
CYP2E1	*7C	CYP2E1*7C_-352A>G(Promoter)	AM_10242	rs2070672	Ch10:135340548
CYP2E1	*7	CYP2E1*7_-333T>A(Promoter)	AM_10243	rs2070673	Ch10:135340567
CYP2E1	*7B	CYP2E1*7B_-71G>T(Promoter)	AM_10244	rs6413420	Ch10:135340829
CYP2E1	*2	CYP2E1*2_1132G>A(R76H)	AM_10249	rs72559710	Ch10:135342034
CYP2E1	*4	CYP2E1*4_4768G>A(V179I)	AM_10251	rs6413419	Ch10:135345675
CYP2E1	*3	CYP2E1*3_10023G>A(V389I)	AM_10257	rs55897648	Ch10:135351264
CYP2E1	N	CYP2E1_6498C>T(I321I)	AM_10253	rs915909	Ch10:135347397
CYP2E1	N	CYP2E1_10463T>C(F421F)	AM_10258	rs2515641	Ch10:135351362
CYP2F1	N	CYP2F1_96G>A(P32P)	AM_11456	rs305968	Ch19:41622189
CYP2F1	*4	CYP2F1*4_112T>C(S38P)	AM_11457	rs58285195	Ch19:41622205
CYP2F1	*6	CYP2F1*6_388G>C(R98P)	AM_11458	rs57670668	Ch19:41622481
CYP2F1	*3	CYP2F1*3_11887C>T(P490L)	AM_11462	rs7246981	Ch19:41633980
CYP2F1	V175L	CYP2F1_5308G>C(V175L)	AM_11459	rs2287941	Ch19:41627401
CYP2J2	*7	CYP2J2*7_-76G>T(Promoter)	AM_11637	rs890293	Ch1:60392494
CYP2J2	*2	CYP2J2*2_14488A>G(T143A)	AM_11630	rs55753213	Ch1:60377930
CYP2J2	*3	CYP2J2*3_14533C>T(R158C)	AM_11629	rs56307989	Ch1:60377885
CYP2J2	*4	CYP2J2*4_15029T>A(I192N)	AM_11627	rs66515830	Ch1:60377389
CYP2J2	*5	CYP2J2*5_21709G>A(D342N)	AM_11625	rs56053398	Ch1:60370710
CYP2J2	*6	CYP2J2*6_25662A>T(N404Y)	AM_11622	rs72547598	Ch1:60366757
CYP2J2	N	CYP2J2_10769C>G(R111R)	AM_11632	rs1056595	Ch1:60381650
CYP2J2	L378Q	CYP2J2_21818T>A(L378Q)	AM_11624	rs1056596	Ch1:60370601
CYP2J2	N	CYP2J2_33084T>A(V499V)	AM_11621	rs2228114	Ch1:60359335
CYP2S1	N	CYP2S1_1300G>A(P66P)	AM_11463	rs60694775	Ch19:41700469
CYP2S1	N	CYP2S1_1324C>G(P74P)	AM_11464	rs338599	Ch19:41700493
CYP2S1	N	CYP2S1_4624G>A(A151A)	AM_11466	rs57266494	Ch19:41703793
CYP2S1	N	CYP2S1_4594G>A(G141G)	AM_11465	rs16975056	Ch19:41703763
CYP3A4	*14	CYP3A4*14_44T>C(L15P)	AM_14835	rs12721634	Ch7:99381661
CYP3A4	*7	CYP3A4*7_6004G>A(G56D)	AM_14834	rs56324128	Ch7:99375702
CYP3A4	*4	CYP3A4*4_13871A>G(I118V)	AM_14831	rs55951658	Ch7:99367825
CYP3A4	*8	CYP3A4*8_13908G>A(R130Q)	AM_14830	rs72552799	Ch7:99367788
CYP3A4	*15	CYP3A4*15_14269G>A(R162Q)	AM_14828	rs4986907	Ch7:99367427
CYP3A4	*10	CYP3A4*10_14304G>C(D174H)	AM_14826	rs4986908	Ch7:99367392
CYP3A4	*16	CYP3A4*16_15603C>G(T185S)	AM_14825	rs12721627	Ch7:99366093
CYP3A4	*17	CYP3A4*17_15615T>C(F189S)	AM_14824	rs4987161	Ch7:99366081
CYP3A4	*5	CYP3A4*5_15702C>G(P218R)	AM_14821	rs55901263	Ch7:99365994
CYP3A4	*2	CYP3A4*2_15713T>C(S222P)	AM_14820	rs55785340	Ch7:99365983
CYP3A4	*6	CYP3A4*6_17661insA	AM_14816	rs4646438	Ch7:99364034
CYP3A4	*18	CYP3A4*18_20070T>C(L293P)	AM_14815	rs28371759	Ch7:99361626
CYP3A4	*11	CYP3A4*11_21867C>T(T363M)	AM_14812	rs67784355	Ch7:99359829
CYP3A4	*12	CYP3A4*12_21896C>T(L373F)	AM_14811	rs12721629	Ch7:99359800

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CYP3A4	*13	CYP3A4*13_22026C>T(P416L)	AM_14809	rs4986909	Ch7:99359670
CYP3A4	*3	CYP3A4*3_23171T>C(M445T)	AM_14807	rs4986910	Ch7:99358524
CYP3A4	*19	CYP3A4*19_23237C>T(P467S)	AM_14804	rs4986913	Ch7:99358459
CYP3A4	*20	CYP3A4*20_25889insA	AM_14802	rs67666821	Ch7:99355806
CYP3A4	N	CYP3A4_-392A>G	AM_14846	rs2740574	Ch7:99382096
CYP3A4	K96E	CYP3A4_11451A>G(K96E)	AM_14832	rs3091339	Ch7:99370245
CYP3A4	I193V	CYP3A4_15626A>G(I193V)	AM_14823	rs3208361	Ch7:99366070
CYP3A4	S252A	CYP3A4_16898T>G(S252A)	AM_14817	rs3208363	Ch7:99364798
CYP3A4	N	CYP3A4_20230G>A	AM_14814	rs2242480	Ch7:99361466
CYP3A4	I431T	CYP3A4_23130T>C(I431T)	AM_14808	rs1041988	Ch7:99358566
CYP3A4	465FS	CYP3A4_23231insC	AM_14805	rs72552795	Ch7:99358465
CYP3A5	*8	CYP3A5*8_3699C>T(R28C)	AM_14765	rs55817950	Ch7:99273821
CYP3A5	*3B	CYP3A5*3B_3705C>T(H30Y)	AM_14764	rs28383468	Ch7:99273815
CYP3A5	*3L	CYP3A5*3L_3775A>G(Y53C)	AM_14762	rs72552791	Ch7:99273745
CYP3A5	*3	CYP3A5*3_6986A>G(SpliceDefect)	AM_14759	rs776746	Ch7:99270539
CYP3A5	*3D	CYP3A5*3D_7249T>G(L82R)	AM_14757	rs56244447	Ch7:99270276
CYP3A5	*5	CYP3A5*5_12952T>C(SpliceDefect)	AM_14753	rs55965422	Ch7:99264573
CYP3A5	*4	CYP3A5*4_14665A>G(Q200R)	AM_14749	rs56411402	Ch7:99262860
CYP3A5	*6	CYP3A5*6_14690G>A(SpliceDefect)	AM_14748	rs10264272	Ch7:99262835
CYP3A5	*9	CYP3A5*9_19386G>A(A337T)	AM_14739	rs28383479	Ch7:99258139
CYP3A5	*7	CYP3A5*7_27131insT	AM_14737	rs41303343	Ch7:99250393
CYP3A5	*2	CYP3A5*2_27289C>A(T398N)	AM_14735	rs28365083	Ch7:99250236
CYP3A5	*3K	CYP3A5*3K_29753T>C(F446S)	AM_14730	rs41279854	Ch7:99247772
CYP3A5	*3F	CYP3A5*3F_31551T>C(I488T)	AM_14726	rs28365085	Ch7:99245974
CYP3A5	S100Y	CYP3A5_7303C>A(S100Y)	AM_14756	rs41279857	Ch7:99270222
CYP3A7	*1B	CYP3A7*1B_-314C>T(Promoter)	AM_14798	rs45465393	Ch7:99333030
CYP3A7	*1C	CYP3A7*1C_-284T>A(Promoter)	AM_14796	rs45494802	Ch7:99333000
CYP3A7	*1C	CYP3A7*1C_-281A>T(Promoter)	AM_14794	rs45467892	Ch7:99332997
CYP3A7	*1C	CYP3A7*1C_-232A>C(Promoter)	AM_14791	rs45446698	Ch7:99332948
CYP3A7	*1D	CYP3A7*1D_-91G>A(Promoter)	AM_14790	rs55798860	Ch7:99332807
CYP3A7	*1E	CYP3A7*1E_-49G>A(Promoter)	AM_14789	rs28451617	Ch7:99332765
CYP3A7	*2	CYP3A7*2_26041C>G(T409R)	AM_14781	rs2257401	Ch7:99306685
CYP3A43	*3	CYP3A43*3_31867C>G(P340A)	AM_14861	rs680055	Ch7:99457605
CYP3A43	*1B	CYP3A43*1B_33518C>T(A349A)	AM_14862	rs17342647	Ch7:99459256
CYP3A43	N	CYP3A43_14956C>T	AM_14856	rs533486	Ch7:99440694
CYP3A43	N	CYP3A43_19394C>T(L114L)	AM_14857	rs4646474	Ch7:99445132
CYP3A43	M145I	CYP3A43_20053G>T(M145I)	AM_14858	rs45450092	Ch7:99445791
CYP3A43	N	CYP3A43_21503T>C(N198N)	AM_14859	rs800667	Ch7:99447241
CYP3A43	M275I	CYP3A43_28744G>A(M275I)	AM_14860	rs45621431	Ch7:99454482
CYP4B1	R173W	CYP4B1_c.517C>T(R173W)	AM_11543	rs4646487	Ch1:47279175
CYP4B1	294FS	CYP4B1_c.881_882delAT(D294X)	AM_11546	rs3215983	Ch1:47280747
CYP4B1	*4	CYP4B1*4_c.964A>G(S322G)	AM_11547	rs45467195	Ch1:47280830
CYP4B1	M331I	CYP4B1_c.993G>A(M331I)	AM_11549	rs2297810	Ch1:47280859
CYP4B1	R340C	CYP4B1_c.1018C>T(R340C)	AM_11550	rs4646491	Ch1:47280884
CYP4B1	N	CYP4B1_c.330G>A(K110K)	AM_11539	rs7513658	Ch1:47276819

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CYP4B1	R375C	CYP4B1_c.1123C>T(R375C)	AM_11554	rs2297809	Ch1:47282772
CYP4F2	*2	CYP4F2*2_34T>G(W12G)	AM_11310	rs3093105	Ch19:16008388
CYP4F2	*3	CYP4F2*3_18000G>A(V433M)	AM_11299	rs2108622	Ch19:15990431
CYP4F2	W12C	CYP4F2_36G>C(W12C)	AM_11309	rs2906891	Ch19:16008386
CYP4F2	P13R	CYP4F2_38C>G(P13R)	AM_11308	rs2906890	Ch19:16008384
CYP4F2	N	CYP4F2_165A>G(P55P)	AM_11307	rs3093106	Ch19:16008257
CYP4F2	N	CYP4F2_2042A>C(G93G)	AM_11305	rs8100960	Ch19:16006380
CYP4F2	N	CYP4F2_2099C>T(N112N)	AM_11304	rs8110714	Ch19:16006323
CYP4F2	N	CYP4F2_5034C>G(A116A)	AM_11303	rs3093136	Ch19:16003388
CYP4F2	G185V	CYP4F2_7207G>T(G185V)	AM_11302	rs3093153	Ch19:16001215
CYP4F2	L278F	CYP4F2_8103C>T(L278F)	AM_11301	rs4605294	Ch19:16000319
CYP4F2	N	CYP4F2_11602C>T(H343H)	AM_11300	rs2074900	Ch19:15996820
CYP17A1	N	CYP17A1_-34T>C	AM_10206	rs743572	Ch10:104597152
CYP17A1	C22W	CYP17A1_66C>G(C22W)	AM_10203	rs762563	Ch10:104597053
CYP17A1	N	CYP17A1_138C>T(H46H)	AM_10202	rs6162	Ch10:104596981
CYP17A1	N	CYP17A1_195G>T(S65S)	AM_10201	rs6163	Ch10:104596924
CYP17A1	N	CYP17A1_4249C>T(D283D)	AM_10198	rs1042386	Ch10:104592870
CYP17A1	N	CYP17A1_6417G>A(P428P)	AM_10194	rs6164	Ch10:104590702
CYP19A1	*2	CYP19A1*2_115T>C(W39R)	AM_10749	rs2236722	Ch15:51534995
CYP19A1	*4	CYP19A1*4_27142C>T(R264C)	AM_10747	rs700519	Ch15:51507968
CYP19A1	N	CYP19A1_32124C>T(3'UTR)	AM_10744	rs10046	Ch15:51502986
CYP19A1	N	CYP19A1_32266G>T(3'UTR)	AM_10742	rs4646	Ch15:51502844
CYP19A1	N	CYP19A1_-12829G>C(5'UTR)	AM_10750	rs1062033	Ch15:51547938
CYP19A1	N	CYP19A1_5998A>G(V80V)	AM_10748	rs700518	Ch15:51529112
CYP19A1	R264H	CYP19A1_27143G>A(R264H)	AM_10746	rs2304462	Ch15:51507967
CYP19A1	N	CYP19A1_30554C>T(P408P)	AM_10745	rs2304461	Ch15:51504556
CYP19A1	N	CYP19A1_32217C>A(3'UTR)	AM_10743	rs1050677	Ch15:51502893
CYP19A1	N	CYP19A1_32812T>C(3'UTR)	AM_10741	rs1050760	Ch15:51502298
CYP19A1	N	CYP19A1_33028T>A(3'UTR)	AM_10740	rs1050787	Ch15:51502082
DCK	I24V	DCK_c.70A>G(I24V)	AM_13643	rs66878317	Ch4:71859622
DCK	A119G	DCK_c.356C>G(A119G)	AM_13644	rs66472932	Ch4:71888232
DCK	P122S	DCK_c.364C>T(P122S)	AM_13645	rs67437265	Ch4:71888240
DCK	N	DCK_c.*165C>T(3'UTR)	AM_13646	rs4643786	Ch4:71895260
DPYD	*9	DPYD*9_c.85T>C(C29R)	AM_11669	rs1801265	Ch1:98348885
DPYD	*7	DPYD*7_c.295delTCAT	AM_11668	rs72549309	Ch1:98205971
DPYD	*8	DPYD*8_c.703C>T(R235W)	AM_11663	rs1801266	Ch1:98157332
DPYD	*11	DPYD*11_c.1003G>T(V335L)	AM_11660	rs72549306	Ch1:98058899
DPYD	*4	DPYD*4_c.1601G>A(S534N)	AM_11653	rs1801158	Ch1:97981421
DPYD	*13	DPYD*13_c.1679T>G(I560S)	AM_11651	rs55886062	Ch1:97981343
DPYD	*3	DPYD*3_c.1897delC(P633X)	AM_11649	rs72549303	Ch1:97915622
DPYD	*2	DPYD*2_c.1905+1G>A	AM_11647	rs3918290	Ch1:97915614
DPYD	*9B	DPYD*9B_c.2657G>A(R886H)	AM_11643	rs1801267	Ch1:97564154
DPYD	*10	DPYD*10_c.2983G>T(V995F)	AM_11639	rs1801268	Ch1:97544627
DPYD	R21X	DPYD_c.61C>T(R21X)	AM_11670	rs72549310	Ch1:98348909
DPYD	M166V	DPYD_c.496A>G(M166V)	AM_11667	rs2297595	Ch1:98165091

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DPYD	N	DPYD_c.525G>A(S175S)	AM_11666	rs6670886	Ch1:98165062
DPYD	N	DPYD_c.756T>C(G252G)	AM_11662	rs6675198	Ch1:98157279
DPYD	N	DPYD_c.1035T>C(F345F)	AM_11659	rs1042478	Ch1:98058867
DPYD	N	DPYD_c.1074T>A(R358R)	AM_11658	rs1042479	Ch1:98058828
DPYD	N	DPYD_c.1896T>C(F632F)	AM_11650	rs17376848	Ch1:97915624
DPYD	N	DPYD_c.1905C>T(N635N)	AM_11648	rs3918289	Ch1:97915615
FAAH	P129T	FAAH_c.385C>A(P129T)	AM_11525	rs324420	Ch1:46870761
FAAH	H370R	FAAH_c.1109A>G(H370R)	AM_11526	rs12094805	Ch1:46874804
FMO2	113FS	FMO2_c.337delG(V113X)	AM_11933	rs28369860	Ch1:171165803
FMO2	N413K	FMO2_c.1239T>G(N413K)	AM_11948	rs2020865	Ch1:171176912
FMO2	X472Q	FMO2_23238T>C(X472Q)	AM_11958	rs6661174	Ch1:171178090
FMO2	D36G	FMO2_c.107A>G(D36G)	AM_11925	rs2020870	Ch1:171154959
FMO2	V59I	FMO2_c.175G>A(V59I)	AM_11927	rs55708639	Ch1:171162516
FMO2	70InsD	FMO2_c.210_211insGAC(70InsD)	AM_11929	rs2020868	Ch1:171162551
FMO2	F81S	FMO2_c.242T>C(F81S)	AM_11931	rs2020860	Ch1:171162583
FMO2	F182S	FMO2_c.545T>C(F182S)	AM_11936	rs2307492	Ch1:171168545
FMO2	N	FMO2_c.585A>G(S195S)	AM_11938	rs2020861	Ch1:171168585
FMO2	R238Q	FMO2_c.713G>A(R238Q)	AM_11940	rs28369895	Ch1:171173089
FMO2	R249X	FMO2_c.745C>T(R249X)	AM_11941	rs2020866	Ch1:171173121
FMO2	E314G	FMO2_c.941A>G(E314G)	AM_11942	rs2020863	Ch1:171174531
FMO2	N	FMO2_c.1101G>A(A367A)	AM_11943	rs7536646	Ch1:171174691
FMO2	R387ins8	FMO2_c.1160_1161ins8(R387X)	AM_11944	rs72549336	Ch1:171174750
FMO2	R391T	FMO2_c.1172G>C(R391T)	AM_11945	rs28369899	Ch1:171174762
FMO2	N	FMO2_c.1206G>A(E402E)	AM_11947	rs6671692	Ch1:171176879
FMO2	N	FMO2_c.*60A>G	AM_11959	rs2020869	Ch1:171178152
FMO2	N	FMO2_c.*113_*114insT	AM_11960	rs72549337	Ch1:171178205
FMO2	N	FMO2_c.*933T>C	AM_11966	rs6664553	Ch1:171179025
FMO2	N	FMO2_c.*1195C>T	AM_11968	rs7512785	Ch1:171179287
FMO2	N	FMO2_c.*1385C>T	AM_11970	rs7515157	Ch1:171179477
FMO3	E32K	FMO3_c.94G>A(E32K)	AM_11857	rs72549320	Ch1:171061893
FMO3	A52T	FMO3_c.154G>A(A52T)	AM_11866	rs72549321	Ch1:171072947
FMO3	N61S	FMO3_c.182A>G(N61S)	AM_11867	rs72549322	Ch1:171072975
FMO3	M66I	FMO3_c.198G>T(M66I)	AM_11868	rs72549323	Ch1:171072991
FMO3	M82T	FMO3_c.245T>C(M82T)	AM_11869	rs72549324	Ch1:171073038
FMO3	D132H	FMO3_c.394G>C(D132H)	AM_11871	rs12072582	Ch1:171076888
FMO3	N	FMO3_c.441C>T(S147S)	AM_11872	rs1800822	Ch1:171076935
FMO3	G148X	FMO3_c.442G>T(G148X)	AM_11873	rs72549325	Ch1:171076936
FMO3	P153L	FMO3_c.458C>T(P153L)	AM_11874	rs72549326	Ch1:171076952
FMO3	E158K	FMO3_c.472G>A(E158K)	AM_11875	rs2266782	Ch1:171076966
FMO3	I199N	FMO3_c.596T>A(I199N)	AM_11877	rs72549327	Ch1:171077331
FMO3	202FS	FMO3_c.604_605insT(E202X)	AM_11878	rs72549328	Ch1:171077339
FMO3	N	FMO3_c.627+10C>G	AM_11880	rs2066534	Ch1:171077372
FMO3	N	FMO3_c.717T>C(F239F)	AM_11884	rs1050902	Ch1:171080028
FMO3	V257M	FMO3_c.769G>A(V257M)	AM_11885	rs1736557	Ch1:171080080
FMO3	V277A	FMO3_c.830T>C(V277A)	AM_11887	rs2066530	Ch1:171083149

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FMO3	N	FMO3_c.855C>T(N285N)	AM_11888	rs909530	Ch1:171083174
FMO3	N	FMO3_c.906C>T(N302N)	AM_11889	rs2066536	Ch1:171083225
FMO3	E305X	FMO3_c.913G>T(E305X)	AM_11890	rs61753344	Ch1:171083232
FMO3	E308G	FMO3_c.923A>G(E308G)	AM_11891	rs2266780	Ch1:171083242
FMO3	E314X	FMO3_c.940G>T(E314X)	AM_11892	rs72549330	Ch1:171083259
FMO3	L360P	FMO3_c.1079T>C(L360P)	AM_11893	rs28363581	Ch1:171083398
FMO3	E362Q	FMO3_c.1084G>C(E362Q)	AM_11894	rs2066532	Ch1:171083403
FMO3	R387L	FMO3_c.1160G>T(R387L)	AM_11895	rs72549331	Ch1:171083479
FMO3	I486M	FMO3_c.1458A>G(I486M)	AM_11902	rs1050906	Ch1:171086441
FMO3	R492W	FMO3_c.1474C>T(R492W)	AM_11903	rs72549334	Ch1:171086457
FMO3	G503R	FMO3_c.1507G>A(G503R)	AM_11905	rs72549335	Ch1:171086490
GSTM1	*B	GSTM1*B_c.519G>C(K173N)	AM_11707	rs74837985	Ch1:110233138
GSTM1	N	GSTM1_c.178-78T>C	AM_11705	rs737497	Ch1:110231592
GSTP1	*B	GSTP1*B_c.313A>G(I105V)	AM_10440	rs1695	Ch11:67352689
GSTP1	*C	GSTP1*C_c.341C>T(A114V)	AM_10442	rs1138272	Ch11:67353579
GSTP1	N	GSTP1_c.-18G>A(Promoter)	AM_10430	rs8191439	Ch11:67351297
GSTP1	N	GSTP1_c.21C>G(V7V)	AM_10434	rs8191444	Ch11:67351624
GSTP1	D147Y	GSTP1_c.439G>T(D147Y)	AM_10444	rs4986949	Ch11:67353677
GSTT1	*B	GSTT1*B_c.310A>C(T104P)	AM_12242	rs11550605	Ch22:24379402
GSTT1	A21T	GSTT1_c.61G>A(A21T)	AM_12245	rs2266635	Ch22:24384171
GSTT1	F45C	GSTT1_c.134T>G(F45C)	AM_12243	rs17856199	Ch22:24381766
GSTT1	N	GSTT1_c.354G>A(V118V)	AM_12241	rs2266636	Ch22:24376996
GSTT1	V169I	GSTT1_c.505G>A(V169I)	AM_12239	rs2266637	Ch22:24376845
NAT1	*11	NAT1*11_c.-344C>T	AM_14965	rs4986988	Ch8:18079213
NAT1	*11	NAT1*11_c.-40A>T	AM_14966	rs4986989	Ch8:18079517
NAT1	*27	NAT1*27_c.21T>G(L7L)	AM_14968	rs4986992	Ch8:18079577
NAT1	*19	NAT1*19_c.97C>T(R33X)	AM_14969	rs56318881	Ch8:18079653
NAT1	R64W	NAT1_c.190C>T(R64W)	AM_14970	rs56379106	Ch8:18079746
NAT1	*5	NAT1*5_c.350GG>CC(R117T)	AM_14971	rs72554606	Ch8:18079906
NAT1	V149I	NAT1_c.445G>A(V149I)	AM_14974	rs4987076	Ch8:18080001
NAT1	*11	NAT1*11_c.459G>A(T153T)	AM_14975	rs4986990	Ch8:18080015
NAT1	*5	NAT1*5_c.497GGG>CCC(RE166TQ)	AM_14976	rs72554608	Ch8:18080053
NAT1	*15	NAT1*15_c.559C>T(R187X)	AM_14977	rs5030839	Ch8:18080115
NAT1	*14	NAT1*14_c.560G>A(R187Q)	AM_14978	rs4986782	Ch8:18080116
NAT1	*11	NAT1*11_c.640T>G(S214A)	AM_14981	rs4986783	Ch8:18080196
NAT1	*22	NAT1*22_c.752A>T(D251V)	AM_14982	rs56172717	Ch8:18080308
NAT1	N	NAT1_c.777T>C(S259S)	AM_14983	rs4986991	Ch8:18080333
NAT1	*5	NAT1*5_884A>G(3'UTR)	AM_14986	rs55793712	Ch8:18080440
NAT1	*5	NAT1*5_976delA(3'UTR)	AM_14988	rs72554612	Ch8:18080532
NAT1	N	NAT1_c.-11982A>T	AM_14963	rs72554605	Ch8:18067575
NAT1	N	NAT1_c.363C>T(V121V)	AM_14973	rs8190858	Ch8:18079919
NAT1	T207I	NAT1_c.620C>T(T207I)	AM_14980	rs4987195	Ch8:18080176
NAT1	N	NAT1_931C>T(3'UTR)	AM_14987	rs4986994	Ch8:18080487
NAT1	N	NAT1_1191G>T(3'UTR)	AM_14993	rs4986993	Ch8:18080747
NAT2	*19	NAT2*19_c.190C>T(R64W)	AM_14998	rs1805158	Ch8:18257703

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NAT2	*14	NAT2*14_c.191G>A(R64Q)	AM_14999	rs1801279	Ch8:18257704
NAT2	*5	NAT2*5_c.341T>C(I114T)	AM_15001	rs1801280	Ch8:18257854
NAT2	*12D	NAT2*12D_c.364G>A(D122N)	AM_15002	rs4986996	Ch8:18257877
NAT2	*17	NAT2*17_c.434A>C(Q145P)	AM_15005	rs72554616	Ch8:18257947
NAT2	*10	NAT2*10_c.499G>A(E167K)	AM_15007	rs72554617	Ch8:18258012
NAT2	*6	NAT2*6_c.590G>A(R197Q)	AM_15008	rs1799930	Ch8:18258103
NAT2	*18	NAT2*18_c.845A>C(K282T)	AM_15011	rs56054745	Ch8:18258358
NAT2	*7	NAT2*7_c.857G>A(G286E)	AM_15012	rs1799931	Ch8:18258370
NAT2	N	NAT2_c.111T>C(F37F)	AM_14997	rs72554615	Ch8:18257624
NAT2	N	NAT2_c.282C>T(Y94Y)	AM_15000	rs1041983	Ch8:18257795
NAT2	L137F	NAT2_c.411A>T(L137F)	AM_15004	rs4986997	Ch8:18257924
NAT2	N	NAT2_c.481C>T(L161L)	AM_15006	rs1799929	Ch8:18257994
NAT2	N	NAT2_c.759C>T(V253V)	AM_15009	rs56011192	Ch8:18258272
NAT2	K268R	NAT2_c.803A>G(K268R)	AM_15010	rs1208	Ch8:18258316
PTGIS	*3	PTGIS*3_c.354T>A(S118R)	AM_12133	rs5622	Ch20:48164401
PTGIS	N	PTGIS_c.1117C>A(R373R)	AM_12126	rs5629	Ch20:48129706
PTGIS	*4	PTGIS*4_c.1135C>A(R379S)	AM_12125	rs56195291	Ch20:48129688
PTGIS	N	PTGIS_c.258G>T(V86V)	AM_12134	rs5621	Ch20:48164497
PTGIS	Q134H	PTGIS_c.402G>T(Q134H)	AM_12132	rs6067121	Ch20:48160961
PTGIS	E154A	PTGIS_c.461A>C(E154A)	AM_12131	rs5623	Ch20:48160902
PTGIS	F171L	PTGIS_c.511T>C(F171L)	AM_12130	rs5624	Ch20:48160852
PTGIS	N	PTGIS_c.591C>T(R197R)	AM_12129	rs5625	Ch20:48156189
PTGIS	R236C	PTGIS_c.706C>T(R236C)	AM_12128	rs5626	Ch20:48140744
PTGIS	N	PTGIS_c.723A>G(L241L)	AM_12127	rs5627	Ch20:48140727
PTGIS	N	PTGIS_c.1239C>T(D413D)	AM_12124	rs5630	Ch20:48127684
PTGIS	P500S	PTGIS_c.1498C>T(P500S)	AM_12123	rs5584	Ch20:48124462
SLC15A1	N	SLC15A1_c.22-40G>C	AM_10664	rs8187819	Ch13:99378743
SLC15A1	V21I	SLC15A1_c.61G>A(V21I)	AM_10663	rs8187818	Ch13:99378664
SLC15A1	F28Y	SLC15A1_c.83T>A(F28Y)	AM_10662	rs8187817	Ch13:99378642
SLC15A1	N	SLC15A1_c.258G>A(S86S)	AM_10661	rs8187823	Ch13:99376273
SLC15A1	N	SLC15A1_c.330C>T(N110N)	AM_10660	rs8187822	Ch13:99376201
SLC15A1	S117N	SLC15A1_c.350G>A(S117N)	AM_10659	rs2297322	Ch13:99376181
SLC15A1	S117R	SLC15A1_c.351C>A(S117R)	AM_10658	rs8187821	Ch13:99376180
SLC15A1	N	SLC15A1_c.1179C>T(N393N)	AM_10653	rs8187836	Ch13:99358478
SLC15A1	G419A	SLC15A1_c.1256G>C(G419A)	AM_10652	rs4646227	Ch13:99358401
SLC15A1	441FS	SLC15A1_c.1321insT	AM_10651	rs72547504	Ch13:99356637
SLC15A1	N	SLC15A1_c.1347T>C(A449A)	AM_10650	rs1339067	Ch13:99356612
SLC15A1	V450I	SLC15A1_c.1348G>A(V450I)	AM_10649	rs2274828	Ch13:99356611
SLC15A1	T451N	SLC15A1_c.1352C>A(T451N)	AM_10648	rs8187838	Ch13:99356607
SLC15A1	R459C	SLC15A1_c.1375C>T(R459C)	AM_10647	rs2274827	Ch13:99356584
SLC15A1	N	SLC15A1_c.1446A>G(E482E)	AM_10645	rs8187828	Ch13:99354754
SLC15A1	N	SLC15A1_c.1527C>T(N509N)	AM_10644	rs8187832	Ch13:99340771
SLC15A1	P537S	SLC15A1_c.1609C>T(P537S)	AM_10643	rs8187830	Ch13:99340576
SLC15A1	P586L	SLC15A1_c.1757C>T(P586L)	AM_10642	rs56120058	Ch13:99339905
SLC15A1	N	SLC15A1_c.1848G>A(S616S)	AM_10641	rs8187840	Ch13:99338531

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SLC15A2	L350F	SLC15A2_c.1048C>T(L350F)	AM_13300	rs2257212	Ch3:121643804
SLC15A2	P409S	SLC15A2_c.1225C>T(P409S)	AM_13306	rs1143671	Ch3:121647286
SLC15A2	R509K	SLC15A2_c.1526G>A(R509K)	AM_13307	rs1143672	Ch3:121648168
SLC15A2	N	SLC15A2_c.141C>T(A47A)	AM_13282	rs1143669	Ch3:121615288
SLC15A2	R57H	SLC15A2_c.170G>A(R57H)	AM_13283	rs1920305	Ch3:121615317
SLC15A2	N	SLC15A2_c.852G>A(A284A)	AM_13298	rs2293616	Ch3:121641693
SLC15A2	N	SLC15A2_c.1161A>G(A387A)	AM_13301	rs1143670	Ch3:121646641
SLC15A2	M704L	SLC15A2_c.2110A>C(M704L)	AM_13315	rs1920314	Ch3:121659774
SLC15A2	N	SLC15A2_c.*6C>T(3'UTR)	AM_13316	rs1920313	Ch3:121659860
SLC15A2	N	SLC15A2_c.*146T>G(3'UTR)	AM_13318	rs4388019	Ch3:121660000
SLC22A1	S14F	SLC22A1_c.41C>T(S14F)	AM_14342	rs34447885	Ch6:160543008
SLC22A1	19FS	SLC22A1_c.54insC	AM_14343	rs72552761	Ch6:160543021
SLC22A1	L23V	SLC22A1_c.67C>G(L23V)	AM_14344	rs34570655	Ch6:160543034
SLC22A1	G38D	SLC22A1_c.113G>A(G38D)	AM_14345	rs35888596	Ch6:160543080
SLC22A1	N	SLC22A1_c.156T>C(S52S)	AM_14347	rs1867351	Ch6:160543123
SLC22A1	R61C	SLC22A1_c.181C>T(R61C)	AM_14348	rs12208357	Ch6:160543148
SLC22A1	L85F	SLC22A1_c.253C>T(L85F)	AM_14349	rs35546288	Ch6:160543220
SLC22A1	C88R	SLC22A1_c.262T>C(C88R)	AM_14350	rs55918055	Ch6:160543229
SLC22A1	F160L	SLC22A1_c.480C>G(F160L)	AM_14351	rs683369	Ch6:160551204
SLC22A1	S189L	SLC22A1_c.566C>T(S189L)	AM_14353	rs34104736	Ch6:160553314
SLC22A1	G220V	SLC22A1_c.659G>T(G220V)	AM_14354	rs36103319	Ch6:160553407
SLC22A1	P283L	SLC22A1_c.848C>T(P283L)	AM_14357	rs4646277	Ch6:160557260
SLC22A1	R287G	SLC22A1_c.859C>G(R287G)	AM_14358	rs4646278	Ch6:160557271
SLC22A1	P341L	SLC22A1_c.1022C>T(P341L)	AM_14359	rs2282143	Ch6:160557643
SLC22A1	G401S	SLC22A1_c.1201G>A(G401S)	AM_14361	rs34130495	Ch6:160560824
SLC22A1	N	SLC22A1_c.1209C>T(I403I)	AM_14362	rs35373824	Ch6:160560832
SLC22A1	V408M	SLC22A1_c.1222G>A(V408M)	AM_14363	rs628031	Ch6:160560845
SLC22A1	M420del	SLC22A1_c.1260_1262delGAT (M420del)	AM_14368	rs72552763	Ch6:160560883
SLC22A1	M440I	SLC22A1_c.1320G>A(M440I)	AM_14371	rs35956182	Ch6:160564616
SLC22A1	V461I	SLC22A1_c.1381G>A(V461I)	AM_14372	rs34295611	Ch6:160564677
SLC22A1	G465R	SLC22A1_c.1393G>A(G465R)	AM_14373	rs34059508	Ch6:160575837
SLC22A1	R488M	SLC22A1_c.1463G>T(R488M)	AM_14374	rs35270274	Ch6:160575907
SLC22A1	N	SLC22A1_c.1647A>C(S549S)	AM_14376	rs16891138	Ch6:160579596
SLC22A1	N	SLC22A1_c.*15G>A(3'UTR)	AM_14377	rs9457846	Ch6:160579629
SLC22A1	N	SLC22A1_c.*23G>A(3'UTR)	AM_14379	rs34108432	Ch6:160579637
SLC22A2	45FS	SLC22A2_c.134insA	AM_14403	rs72552765	Ch6:160679656
SLC22A2	P54S	SLC22A2_c.160C>T(P54S)	AM_14402	rs8177504	Ch6:160679630
SLC22A2	N	SLC22A2_c.390G>T(T130T)	AM_14401	rs624249	Ch6:160679400
SLC22A2	*5	SLC22A2*5_c.481T>C(F161L)	AM_14400	rs8177509	Ch6:160677683
SLC22A2	M165V	SLC22A2_c.493A>G(M165V)	AM_14399	rs8177508	Ch6:160677671
SLC22A2	M165I	SLC22A2_c.495G>A(M165I)	AM_14398	rs8177507	Ch6:160677669
SLC22A2	*8	SLC22A2*8_c.669C>T(I223I)	AM_14396	rs8177510	Ch6:160671584
SLC22A2	A270S	SLC22A2_c.808G>T(A270S)	AM_14395	rs316019	Ch6:160670282
SLC22A2	A297G	SLC22A2_c.890C>G(A297G)	AM_14394	rs8177513	Ch6:160668283

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SLC22A2	*7	SLC22A2*7_c.1198C>T(R400C)	AM_14392	rs8177516	Ch6:160664685
SLC22A2	N	SLC22A2_c.1203C>T(I401I)	AM_14391	rs8177515	Ch6:160664680
SLC22A2	K432Q	SLC22A2_c.1294A>C(K432Q)	AM_14390	rs8177517	Ch6:160663420
SLC22A2	N	SLC22A2_c.1398C>T(G466G)	AM_14388	rs8177520	Ch6:160662609
SLC22A2	N	SLC22A2_c.1506A>G(V502V)	AM_14386	rs316003	Ch6:160645832
SLC22A2	N	SLC22A2_c.1587C>T(A529A)	AM_14385	rs8177521	Ch6:160645751
SLC22A2	R463K	SLC22A2_c.1388G>A(R463K)	AM_14389	rs3907239	Ch6:160663326
SLC22A6	G14S	SLC22A6_c.40G>A(G14S)	AM_10348	rs72559735	Ch11:62752123
SLC22A6	R50H	SLC22A6_c.149G>A(R50H)	AM_10347	rs11568626	Ch11:62752014
SLC22A6	57FS	SLC22A6_c.169delC(L57X)	AM_10346	rs67264610	Ch11:62751994
SLC22A6	I226T	SLC22A6_c.677T>C(I226T)	AM_10340	rs11568623	Ch11:62749434
SLC22A6	A256V	SLC22A6_c.767C>T(A256V)	AM_10339	rs11568624	Ch11:62749344
SLC22A6	R293W	SLC22A6_c.877C>T(R293W)	AM_10337	rs45607933	Ch11:62748776
SLC22A6	R454Q	SLC22A6_c.1361G>A(R454Q)	AM_10335	rs11568634	Ch11:62746960
SLCO1A2	I13T	SLCO1A2_c.38T>C(I13T)	AM_10542	rs10841795	Ch12:21487544
SLCO1A2	N	SLCO1A2_c.61-5605T>A	AM_10540	rs7957203	Ch12:21477462
SLCO1A2	N	SLCO1A2_c.61-5126T>C	AM_10539	rs4078	Ch12:21476983
SLCO1A2	N	SLCO1A2_c.61-4734C>T	AM_10538	rs7484455	Ch12:21476591
SLCO1A2	N	SLCO1A2_c.61-3770A>G	AM_10537	rs7298982	Ch12:21475627
SLCO1A2	N	SLCO1A2_c.61-3234G>A	AM_10536	rs7312628	Ch12:21475091
SLCO1A2	N128Y	SLCO1A2_c.382A>T(N128Y)	AM_10534	rs11568567	Ch12:21459876
SLCO1A2	N135I	SLCO1A2_c.404A>T(N135I)	AM_10533	rs45502302	Ch12:21459854
SLCO1A2	R168C	SLCO1A2_c.502C>T(R168C)	AM_10532	rs11568564	Ch12:21457448
SLCO1A2	E172D	SLCO1A2_c.516A>C(E172D)	AM_10531	rs11568563	Ch12:21457434
SLCO1A2	T277N	SLCO1A2_c.830C>A(T277N)	AM_10528	rs11568553	Ch12:21453362
SLCO1A2	278FS	SLCO1A2_c.833delA(N278X)	AM_10527	rs11568555	Ch12:21453359
SLCO1A2	I281V	SLCO1A2_c.841A>G(I281V)	AM_10526	rs11568551	Ch12:21453351
SLCO1A2	L323P	SLCO1A2_c.968T>C(L323P)	AM_10525	rs11568579	Ch12:21450445
SLCO1A2	I355V	SLCO1A2_c.1063A>G(I355V)	AM_10524	rs45628437	Ch12:21450350
SLCO1A2	T668S	SLCO1A2_c.2003C>G(T668S)	AM_10518	rs11568557	Ch12:21422492
SLCO1B1	*17	SLCO1B1*17_c.11187G>A(Promoter)	AM_10493	rs4149015	Ch12:21283322
SLCO1B1	*2	SLCO1B1*2_c.217T>C(F73L)	AM_10494	rs56101265	Ch12:21325716
SLCO1B1	V82A	SLCO1B1_c.245T>C(V82A)	AM_10495	rs56061388	Ch12:21327529
SLCO1B1	N130D	SLCO1B1*1B_c.388A>G(N130D)	AM_10496	rs2306283	Ch12:21329738
SLCO1B1	*16	SLCO1B1*16_c.452A>G(N151S)	AM_10497	rs2306282	Ch12:21329802
SLCO1B1	*4	SLCO1B1*4_c.463C>A(P155T)	AM_10498	rs11045819	Ch12:21329813
SLCO1B1	*3	SLCO1B1*3_c.467A>G(E156G)	AM_10499	rs72559745	Ch12:21329817
SLCO1B1	*5	SLCO1B1*5_c.521T>C(V174A)	AM_10500	rs4149056	Ch12:21331549
SLCO1B1	*18	SLCO1B1*18_c.578T>G(L193R)	AM_10502	rs72559746	Ch12:21331606
SLCO1B1	*6	SLCO1B1*6_c.1058T>C(I353T)	AM_10509	rs55901008	Ch12:21353529
SLCO1B1	*7	SLCO1B1*7_c.1294A>G(N432D)	AM_10512	rs56387224	Ch12:21355583
SLCO1B1	*8	SLCO1B1*8_c.1385A>G(D462G)	AM_10513	rs72559748	Ch12:21358855
SLCO1B1	*9	SLCO1B1*9_c.1463G>C(G488A)	AM_10514	rs59502379	Ch12:21358933
SLCO1B1	*10	SLCO1B1*10_c.1964A>G(D655G)	AM_10516	rs56199088	Ch12:21392011
SLCO1B1	*11	SLCO1B1*11_c.2000A>G(E667G)	AM_10517	rs55737008	Ch12:21392047

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SLCO1B1	N	SLCO1B1_c.571T>C(L191L)	AM_10501	rs4149057	Ch12:21331599
SLCO1B1	N	SLCO1B1_c.597C>T(F199F)	AM_10503	rs2291075	Ch12:21331625
SLCO1B1	P336R	SLCO1B1_c.1007C>G(P336R)	AM_10508	rs72559747	Ch12:21353478
SLCO1B3	A112S	SLCO1B3_c.334G>T(A112S)	AM_10481	rs4149117	Ch12:21011480
SLCO1B3	I233M	SLCO1B3_c.699A>G(I233M)	AM_10482	rs7311358	Ch12:21015760
SLCO1B3	281FS	SLCO1B3_c.841delA(K281X)	AM_10484	N/A	Ch12:21028282
SLCO1B3	N	SLCO1B3_c.1272A>G(L424L)	AM_10485	rs4149143	Ch12:21032506
SLCO1B3	N	SLCO1B3_c.1557G>A(A519A)	AM_10487	rs2053098	Ch12:21036411
SLCO1B3	N	SLCO1B3_c.1833A>G(G611G)	AM_10490	rs3764006	Ch12:21054369
SLCO1B3	695FS	SLCO1B3_c.2083insC	AM_10492	rs72559744	Ch12:21069155
SLCO2B1	*2	SLCO2B1*2_c.1175C>T(T392I)	AM_10458	rs1621378	Ch11:74904362
SLCO2B1	N	SLCO2B1_c.405G>A(P135P)	AM_10454	rs1109407	Ch11:74876951
SLCO2B1	D215V	SLCO2B1_c.644A>T(D215V)	AM_10456	rs72559740	Ch11:74880413
SLCO2B1	N	SLCO2B1_c.1434-138G>T	AM_10460	rs2306167	Ch11:74907421
SLCO2B1	N	SLCO2B1_c.1512C>T(C504C)	AM_10462	rs59305495	Ch11:74907637
SULT1A1	*2	SULT1A1*2_c.638G>A(R213H)	AM_11005	rs9282861	Ch16:28617514
SULT1A1	*3	SULT1A1*3_c.667A>G(M223V)	AM_11004	rs1801030	Ch16:28617485
SULT1A2	T7I	SULT1A2_c.20C>T(T7I)	AM_10997	rs1136703	Ch16:28607232
SULT1A2	P19L	SULT1A2_c.56C>T(P19L)	AM_10996	rs10797300	Ch16:28607196
SULT1A2	N235T	SULT1A2_c.704A>C(N235T)	AM_10989	rs1059491	Ch16:28603655
SULT1A2	N239S	SULT1A2_c.716A>G(N239S)	AM_10988	rs45472392	Ch16:28603643
SULT1A3	P101L, P101H	SULT1A3_C>T>A(P101LorH)_alternate	AM_11016	rs67944833	Ch16:29473201, Ch16:29473201
SULT1A3	R144C	SULT1A3_C>T(R144C)_alternate	AM_11017	rs67878449	Ch16:29474723
SULT1A3	K234N	SULT1A3_G>T(K234N)_alternate	AM_11018	rs35044222	Ch16:29475588
SULT1A3	P101L, P101H	SULT1A3_C>T>A(P101LorH)	AM_11027	rs67944833	Ch16:30212544, Ch16:30212544
SULT1A3	R144C	SULT1A3_C>T(R144C)	AM_11028	rs67878449	Ch16:30214068
SULT1A3	K234N	SULT1A3_G>T(K234N)	AM_11029	rs35044222	Ch16:30214933
TBXAS1	*2	TBXAS1*2_c.182G>A(R61H)	AM_14917	rs6138	Ch7:139572123
TBXAS1	*1B	TBXAS1*1B_c.360G>A(S120S)	AM_14918	rs41275018	Ch7:139636013
TBXAS1	*3	TBXAS1*3_c.483C>A(D161E)	AM_14920	rs5768	Ch7:139653196
TBXAS1	*4	TBXAS1*4_c.737A>G(N246S)	AM_14923	rs55856189	Ch7:139657478
TBXAS1	*5	TBXAS1*5_c.1069C>G(L357V)	AM_14928	rs4529	Ch7:139661964
TBXAS1	*6	TBXAS1*6_c.1249C>G(Q417E)	AM_14932	rs4528	Ch7:139715542
TBXAS1	*7	TBXAS1*7_c.1348G>A(E450K)	AM_14936	rs8192868	Ch7:139715641
TBXAS1	*8	TBXAS1*8_c.1352C>A(T451N)	AM_14937	rs5763	Ch7:139715645
TBXAS1	*9	TBXAS1*9_c.1397G>A(R466Q)	AM_14939	rs41311778	Ch7:139717500
TBXAS1	*1D	TBXAS1*1D_c.*121G>A(3'UTR)	AM_14941	rs56091454	Ch7:139720020
TBXAS1	V125I	TBXAS1_c.373G>A(V125I)	AM_14919	rs8192833	Ch7:139636026
TBXAS1	L163I	TBXAS1_c.487C>A(L163I)	AM_14921	rs6137	Ch7:139653200
TBXAS1	N	TBXAS1_c.585C>G(A195A)	AM_14922	rs1042561	Ch7:139655300
TBXAS1	K258E	TBXAS1_c.772A>G(K258E)	AM_14924	rs5769	Ch7:139657513
TBXAS1	R261G	TBXAS1_c.781A>G(R261G)	AM_14925	rs5770	Ch7:139657522
TBXAS1	Q317K	TBXAS1_c.949C>A(Q317K)	AM_14926	rs5771	Ch7:139661844

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TBXAS1	I332T	TBXAS1_c.995T>C(I332T)	AM_14927	rs6140	Ch7:139661890
TBXAS1	E388K	TBXAS1_c.1162G>A(E388K)	AM_14929	rs3735354	Ch7:139706915
TBXAS1	G390V	TBXAS1_c.1169G>T(G390V)	AM_14930	rs5760	Ch7:139706922
TBXAS1	N	TBXAS1_c.1206G>A(L402L)	AM_14931	rs5761	Ch7:139706959
TBXAS1	R425C	TBXAS1_c.1273C>T(R425C)	AM_14933	rs5762	Ch7:139715566
TBXAS1	A430T	TBXAS1_c.1288G>A(A430T)	AM_14934	rs4526	Ch7:139715581
TBXAS1	N	TBXAS1_c.1305C>T(A435A)	AM_14935	rs4527	Ch7:139715598
TBXAS1	N	TBXAS1_c.1384C>A(R462R)	AM_14938	rs5764	Ch7:139717487
TBXAS1	R502Q	TBXAS1_c.1505G>A(R502Q)	AM_14940	rs8192864	Ch7:139717608
TPMT	*2	TPMT*2_c.238G>C(A80P)	AM_13986	rs1800462	Ch6:18143955
TPMT	E98X	TPMT_c.292G>T(E98X)	AM_13985	rs72552739	Ch6:18143901
TPMT	*3B	TPMT*3B_c.460G>A(A154T)	AM_13980	rs1800460	Ch6:18139228
TPMT	*24	TPMT*24_c.537G>T(Q179H)	AM_13978	rs6921269	Ch6:18134078
TPMT	*4	TPMT*4_c.626-1G>A(SpliceDefect)	AM_13977	rs1800584	Ch6:18131012
TPMT	*8	TPMT*8_c.644G>A(R215H)	AM_13976	rs56161402	Ch6:18130993
TPMT	*3C	TPMT*3C_c.719A>G(Y240C)	AM_13973	rs1142345	Ch6:18130918
TPMT	N	TPMT_c.474C>T(I158I)	AM_13979	rs2842934	Ch6:18139214
UGT1A COMMON	*76	UGT1A1*76_c.*211C>T(3'UTR)	AM_13067	rs10929303	Ch2:234681416
UGT1A COMMON	*78	UGT1A1*78_c.*339C>G(3'UTR)	AM_13068	rs1042640	Ch2:234681544
UGT1A COMMON	*79	UGT1A1*79_c.*440C>G(3'UTR)	AM_13070	rs8330	Ch2:234681645
UGT1A COMMON	*11	UGT1A1*11_c.923G>A(G308E)	AM_13043	rs62625011	Ch2:234675738
UGT1A COMMON	*9	UGT1A1*9_c.992A>G(Q331R)	AM_13044	rs72551348	Ch2:234675807
UGT1A COMMON	*10	UGT1A1*10_c.1021C>T(R341X)	AM_13045	rs72551349	Ch2:234676519
UGT1A COMMON	*4	UGT1A1*4_c.1069C>T(Q357X)	AM_13046	rs72551350	Ch2:234676567
UGT1A COMMON	*16	UGT1A1*16_c.1070A>G(Q357R)	AM_13047	rs72551351	Ch2:234676568
UGT1A COMMON	*29	UGT1A1*29_c.1099C>G(R367G)	AM_13049	rs55750087	Ch2:234676880
UGT1A COMMON	*20	UGT1A1*20_c.1102G>A(A368T)	AM_13050	rs72551352	Ch2:234676883
UGT1A COMMON	*3	UGT1A1*3_c.1124C>T(S375F)	AM_13051	rs72551353	Ch2:234676905
UGT1A COMMON	*17	UGT1A1*17_c.1143C>G(S381R)	AM_13052	rs72551354	Ch2:234676924
UGT1A COMMON	*18	UGT1A1*18_c.1201G>C(A401P)	AM_13053	rs72551355	Ch2:234676982
UGT1A COMMON	*24	UGT1A1*24_c.1309A>T(K437X)	AM_13057	rs72551357	Ch2:234680912
UGT1A COMMON	N	UGT1A1_c.1428C>T(P476P)	AM_13060	rs28900406	Ch2:234681031

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UGT1A COMMON	*55	UGT1A1*55_c.1487T>A(L496X)	AM_13063	rs72551361	Ch2:234681090
UGT1A COMMON	A511P	UGT1A1_c.1531G>C(A511P)	AM_13064	rs1042709	Ch2:234681134
UGT1A1	*60	UGT1A1*60_c.-3279T>G(Promoter)	AM_13018	rs4124874	Ch2:234665659
UGT1A1	*93	UGT1A1*93_c.-3156G>A(Promoter)	AM_13019	rs10929302	Ch2:234665782
UGT1A1	*28	UGT1A1*28_c.TATA-box(Promoter)	AM_13024	rs8175347	Ch2:234668881
UGT1A1	*6	UGT1A1*6_c.211G>A(G71R)	AM_13030	rs4148323	Ch2:234669144
UGT1A1	*27	UGT1A1*27_c.686C>A(P229Q)	AM_13037	rs35350960	Ch2:234669619
UGT1A1	N	UGT1A1_c.-2950A>G	AM_13020	rs111741722	Ch2:234665983
UGT1A1	*112	UGT1A1*112_c.-1353A>C	AM_13021	rs3755319	Ch2:234667582
UGT1A1	*80	UGT1A1*80_c.-364C>T	AM_13022	rs887829	Ch2:234668570
UGT1A1	*45	UGT1A1*45_c.222C>A(Y74X)	AM_13031	rs72551340	Ch2:234669155
UGT1A1	*62	UGT1A1*62_c.247T>C(F83L)	AM_13032	rs56059937	Ch2:234669180
UGT1A1	*12	UGT1A1*12_c.524T>A(L175Q)	AM_13033	rs72551341	Ch2:234669457
UGT1A1	*15	UGT1A1*15_c.529T>C(C177R)	AM_13034	rs72551342	Ch2:234669462
UGT1A1	*8	UGT1A1*8_c.625C>T(R209W)	AM_13035	rs72551343	Ch2:234669558
UGT1A1	*43	UGT1A1*43_c.698T>G(L233R)	AM_13038	rs72551344	Ch2:234669631
UGT1A1	*14	UGT1A1*14_c.826G>C(G276R)	AM_13040	rs72551345	Ch2:234669759
UGT1A3	*5	UGT1A3*5_c.17A>G(Q6R)	AM_13004	rs28898617	Ch2:234637789
UGT1A3	W11R	UGT1A3_c.31T>C(W11R)	AM_13005	rs3821242	Ch2:234637803
UGT1A3	*4	UGT1A3*4_c.133C>T(R45W)	AM_13008	rs45625338	Ch2:234637905
UGT1A3	N	UGT1A3_c.81G>A(E27E)	AM_13007	rs6706232	Ch2:234637853
UGT1A3	N	UGT1A3_c.477A>G(A159A)	AM_13011	rs7574296	Ch2:234638249
UGT1A3	177FS	UGT1A3_c.529delC(P177X)	AM_13012	rs72551338	Ch2:234638301
UGT1A4	*2	UGT1A4*2_c.70C>A(P24T)	AM_12991	rs6755571	Ch2:234627536
UGT1A4	207FS	UGT1A4_c.621delC(F207X)	AM_12999	rs68014726	Ch2:234628087
UGT1A5	T78A	UGT1A5_c.232A>G(T78A)	AM_12980	rs72551334	Ch2:234621869
UGT1A5	N	UGT1A5_c.285T>C(G95G)	AM_12981	rs17874942	Ch2:234621922
UGT1A5	H142N	UGT1A5_c.424C>A(H142N)	AM_12982	rs3755320	Ch2:234622061
UGT1A5	207FS	UGT1A5_c.621delC(F207X)	AM_12986	rs72551335	Ch2:234622258
UGT1A6	S7A	UGT1A6_c.19T>G(S7A)	AM_12969	rs6759892	Ch2:234601669
UGT1A6	N	UGT1A6_c.315A>G(L105L)	AM_12973	rs1105880	Ch2:234601965
UGT1A6	T181A	UGT1A6_c.541A>G(T181A)	AM_12974	rs2070959	Ch2:234602191
UGT1A6	N	UGT1A6_c.627G>T(V209V)	AM_12976	rs17863783	Ch2:234602277
UGT1A6	N	UGT1A6_c.57A>T(A19A)	AM_12971	rs1042707	Ch2:234601707
UGT1A6	S70Y	UGT1A6_c.209C>A(S70Y)	AM_12972	rs1042708	Ch2:234601859
UGT1A7	*12	UGT1A7*12_c.-57T>G(5'UTR)	AM_12956	rs7586110	Ch2:234590527
UGT1A7	*5	UGT1A7*5_c.343G>A(G115S)	AM_12961	rs61261057	Ch2:234590926
UGT1A8	*3	UGT1A8*3_c.830G>A(C277Y)	AM_12932	rs17863762	Ch2:234527183
UGT1A8	L105M	UGT1A8_c.313T>A(L105M)	AM_12911	rs1126788	Ch2:234526666
UGT1A8	N	UGT1A8_c.315G>A(L105L)	AM_12912	rs1126790	Ch2:234526668
UGT1A8	F109L	UGT1A8_c.327T>A(F109L)	AM_12913	rs1126792	Ch2:234526680
UGT1A8	L110M	UGT1A8_c.328C>A(L110M)	AM_12914	rs1126793	Ch2:234526681
UGT1A8	N118D	UGT1A8_c.352A>G(N118D)	AM_12915	rs1126798	Ch2:234526705

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UGT1A8	M212V	UGT1A8_c.634A>G(M212V)	AM_12924	rs1126803	Ch2:234526987
UGT1A8	E216D	UGT1A8_c.648A>C(E216D)	AM_12925	rs1126804	Ch2:234527001
UGT1A9	*1b	UGT1A9*1b_c.-118insT	AM_12947	rs3832043	Ch2:234580463
UGT1A9	*3	UGT1A9*3_c.98T>C(M33T)	AM_12949	rs72551330	Ch2:234580678
UGT1A9	*4	UGT1A9*4_c.726T>G(Y242X)	AM_12951	rs66915469	Ch2:234581306
UGT1A9	*5	UGT1A9*5_c.766G>A(D256N)	AM_12953	rs58597806	Ch2:234581346
UGT1A9	C3W	UGT1A9_c.9C>G(C3W)	AM_12948	rs72551329	Ch2:234580589
UGT1A10	*5	UGT1A10*5_c.177G>A(M59I)	AM_12938	rs56935833	Ch2:234545345
UGT1A10	N	UGT1A10_c.597T>C(D199D)	AM_12941	rs45523834	Ch2:234545765
UGT1A10	*6	UGT1A10*6_c.605C>T(T202I)	AM_12942	rs58704432	Ch2:234545773
UGT2B7	N	UGT2B7*2_c.-327G>A(Promoter)	AM_13458	rs7662029	Ch4:69961912
UGT2B7	N	UGT2B7*2_c.-161C>T(Promoter)	AM_13459	rs7668258	Ch4:69962078
UGT2B7	*3	UGT2B7*3_c.211G>T(A71S)	AM_13460	rs12233719	Ch4:69962449
UGT2B7	N	UGT2B7_c.735A>G(T245T)	AM_13463	rs28365062	Ch4:69964271
UGT2B7	N	UGT2B7*2_c.801T>A(P267P)	AM_13464	rs7438284	Ch4:69964337
UGT2B7	*2	UGT2B7*2_c.802C>T(H268Y)	AM_13465	rs7439366	Ch4:69964338
UGT2B7	N	UGT2B7_c.1062C>T(Y354Y)	AM_13468	rs4348159	Ch4:69972952
UGT2B15	D85Y	UGT2B15_c.253G>T(D85Y)	AM_13439	rs1902023	Ch4:69536084
UGT2B15	K523T	UGT2B15_c.1568A>C(K523T)	AM_13437	rs4148269	Ch4:69512847
UGT2B15	K523T	UGT2B15_c.1568A>C(K523T)	AM_13444	rs4148269	Ch4:69512847
UGT2B15	A500T	UGT2B15_c.1498G>A(A500T)	AM_13445	rs72551390	Ch4:69512917
UGT2B15	N	UGT2B15_c.*131C>G(3'UTR)	AM_13443	rs72551389	Ch4:69512692
UGT2B15	N	UGT2B15_c.*168C>T(3'UTR)	AM_13442	rs3100	Ch4:69512655
UGT2B15	N	UGT2B15_c.*185A>T(3'UTR)	AM_13441	rs4148271	Ch4:69512637
UGT2B17	N	UGT2B17_c.1006-2603T>C	AM_13428	rs4860305	Ch4:69420232
UGT2B17	N	UGT2B17_c.1313+840A>G	AM_13425	rs7436962	Ch4:69415555
UGT2B17	H450Y	UGT2B17_c.1348C>T(H450Y)	AM_13423	rs72551385	Ch4:69403588
VKORC1	N	VKORC1_c.-1639G>A(Promoter)	AM_11054	rs9923231	Ch16:31107689
VKORC1	N	VKORC1_c.173+324T>G	AM_11049	rs2884737	Ch16:31105554
VKORC1	N	VKORC1_c.173+525C>T	AM_11047	rs17708472	Ch16:31105353
VKORC1	N	VKORC1_c.174-136C>T	AM_11045	rs9934438	Ch16:31104878
VKORC1	N	VKORC1_c.283+124G>C	AM_11043	rs8050894	Ch16:31104509
VKORC1	N	VKORC1_c.283+837C>T	AM_11040	rs2359612	Ch16:31103796
VKORC1	N	VKORC1_c.*134G>A(3'UTR)	AM_11034	rs7294	Ch16:31102321
VKORC1	N	VKORC1_c.-5014T>C(Promoter)	AM_11061	rs17884388	Ch16:31111064
VKORC1	N	VKORC1_c.-1877A>G(Promoter)	AM_11055	rs17878544	Ch16:31107927
VKORC1	V29L	VKORC1_c.85G>T(V29L)	AM_11052	rs104894539	Ch16:31105966
VKORC1	V45A	VKORC1_c.134T>C(V45A)	AM_11051	rs104894540	Ch16:31105917
VKORC1	R58G	VKORC1_c.172A>G(R58G)	AM_11050	rs104894541	Ch16:31105879
VKORC1	N	VKORC1_c.173+486C>A	AM_11048	rs13337470	Ch16:31105392
VKORC1	N	VKORC1_c.174-429C>T	AM_11046	rs13336384	Ch16:31105171
VKORC1	V66M	VKORC1_c.196G>A(V66M)	AM_11044	rs72547529	Ch16:31104720
VKORC1	N	VKORC1_c.283+186T>C	AM_11042	rs17886199	Ch16:31104447
VKORC1	N	VKORC1_c.283+231G>A	AM_11041	rs17884850	Ch16:31104402
VKORC1	N	VKORC1_c.284-882A>T	AM_11039	rs17884982	Ch16:31103545

Gene	Summary Flag	Common Name	Probe Set ID	dbSNP RS ID	Chrom
VKORC1	R98W	VKORC1_c.292C>T(R98W)	AM_11038	rs72547528	Ch16:31102655
VKORC1	N	VKORC1_c.358C>T(L120L)	AM_11037	rs7200749	Ch16:31102589
VKORC1	L128R	VKORC1_c.383T>G(L128R)	AM_11036	rs104894542	Ch16:31102564
VKORC1	N	VKORC1_c.*131C>A(3'UTR)	AM_11035	rs11540137	Ch16:31102324

4

Chapter 05

Developing treatment for adrenocortical carcinoma

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Abstract

Cancer of the adrenal cortex (ACC) is a rare endocrine malignancy with limited treatment options. Patients typically present with autonomous hormonal overproduction and/or a large abdominal mass. Hormonal assays and medical imaging can be diagnostic, but urinary steroid profiling might be a more sensitive technique to assess malignancy in adrenal tumours. Stage of disease at diagnosis is the most important prognostic factor. The current staging system needs refinement, especially to separate aggressive from indolent disease in stage IV patients and to select patients who need adjuvant treatment after complete surgical resection. Regarding the latter, assessing the proliferation index Ki-67 seems the best tool currently available. Genomic profiling is expected to become of clinical relevance in the future. Medical therapy is centred on the adrenolytic drug mitotane, which carries considerable toxicity and is not easy to manage. Its tolerability and long plasma level build-up phase may be improved by therapeutic drug monitoring based on pharmacokinetic modelling and intensive counselling of patients. Current chemotherapy regimens can offer disease stabilization in about 50% of patients, but an objective response should be expected in less than 25%. Research on targeted therapy and immunotherapy is difficult in this rare disease with often heavily pre-treated patients and has not yet been successful. Quality of care should be ensured by treating patients in centres with established experience in multidisciplinary oncologic care, who adhere to prevailing guidelines and state-of-art in diagnostic and treatment concepts. International collaboration in fundamental research and clinical trials is the key to further elucidate the pathogenesis and to improve patient care.

Introduction

Cancer of the adrenocortical cortex (ACC) occurs in about 1-2 persons per million population, which renders it a rare endocrine malignancy (Kebebew *et al*. 2006, Fassnacht and Allolio 2009). Complete surgical resection is the sole curative option. In about half of all patients this is impossible because of presentation with metastasized disease (Kerkhofs *et al*. 2013c). Moreover, recurrent disease occurs in approximately 60% of operated patients according to national database studies (Hermsen *et al*. 2012, Erdogan *et al*. 2013). Upon presentation, prognosis is mainly determined by stage of disease. Patients with localized disease have a 5-year survival of 60 to 80%, in patients with locally advanced disease this rate drops to 35 to 50%. In metastasized disease, prognosis varies from 13 to 28%, but heterogeneity in this group precludes an adequate estimation (Fassnacht *et al*. 2009, Tran *et al*. 2013, Kerkhofs *et al*. 2013c). The cornerstone of medical treatment is mitotane, a 55-year-old drug that is derived from an agricultural insecticide (Lubitz *et al*. 1973). The aim of this review is to discuss changes we foresee in diagnostic and prognostic instruments, as well as suggestions for clinical management and possibilities for future treatment of ACC.

Diagnostics

ACC patients may present with symptoms of an abdominal mass, symptoms related to autonomous hormonal production by the tumour, or both. In rare cases, ACC is discovered incidentally during diagnostic tests or medical imaging for reasons unrelated to the adrenal glands. Figure 1 contains a flow-chart of the diagnostic work-up of patients with suspect ACC. Data on the incidence of hormonal overproduction in ACC differ as some studies report clinical symptoms in symptomatic patients and others report biochemical results. Clinical symptoms caused by autonomous and excessive production of glucocorticoids alone occur in about 30% of patients according to an international multicenter study (Berruti *et al*. 2014). Interestingly, studies based on biochemical results report a lower rate of isolated glucocorticoid overproduction: 14 to 20% (Berruti *et al*. 2005, Abiven *et al*. 2006). The discrepancy may be explained by underestimation of combined glucocorticoid/androgen excess in the multicenter clinical study given the reported rates of 8.3% versus 23 to 36% in the biochemical studies mentioned before. After all, biochemical analysis is expected to have higher sensitivity for hormonal abnormalities compared to clinical examination only. Overproduction of androgens alone was reported in 5 to 20% (Berruti *et al*. 2005, Abiven *et al*. 2006, Berruti *et al*. 2014). Overproduction of oestrogens, mineralocorticoids or other steroid combinations are rare, being diagnosed in <5% of patients. In 23 to 31% (after endocrine work-up) to 48% (based on clinical evaluation) of all ACC patients, hormonal overproduction is not apparent upon presentation. These patients typically seek medical attention because of abdominal pain, fullness

or a palpable mass. Non-specific symptoms related to malignancy such as weight loss, night sweats or fever may also be present (Berruti *et al*. 2014). Research suggests that despite absence of clinically apparent hormonal overproduction, these tumours do produce excess steroid precursors such as tetrahydro-11-deoxycortisol (THS), pregnanediol (P2), pregnanetriol (P3) and etiocholanolone (E) (Arlt *et al*. 2011). Examination of urine samples using highly sensitive methods such as gas chromatography/mass spectrometry (urinary steroid profiling, USP) is able to detect small amounts of these substances. Retrospective studies suggest that THS excretion >2.35 μmol/24h could be diagnostic for the presence of ACC (Kerkhofs *et al*. 2015b). Also, USP can be used to distinguish ACC from adenomas with degenerative or regressive characteristics on imaging (Perna *et al*. 2014). We expect the diagnostic value of urinary steroid profiling will be confirmed in prospective trials and this technique will gain a place in the diagnostic armamentarium used in analysing patients with adrenal tumours. A prospective study aimed at assessing cost-effectiveness of urinary steroid profiling in the work-up of adrenal tumours is currently recruiting (Structured Evaluation of adRENal Tumors Discovered Incidentally - Prospectively Investigating the Testing Yield [SERENDIPITY-trial], NCT02324647). A secondary objective is to assess the frequency of ACC among patients with adrenal incidentaloma at baseline or during follow-up. It is tempting to speculate that urinary excretion of steroid precursors could be a marker for disease recurrence and/or occurrence of metastases. Following this reasoning, it is not inconceivable that urinary samples could eliminate the need of frequent CT-scans and the associated exposure to ionizing radiation. Further research will have to determine whether USP has adequate sensitivity for recurrent disease in post-operative follow-up of patients with ACC. Another promising technique is measurement of circulating tumour cells in the bloodstream. Although difficult to detect in peripheral blood, these cells are considered to be an early marker of metastatic disease in several solid tumours and possibly a marker of therapeutic response. Thanks to technical advances, methods of detection are improving which results in increased sensitivity and specificity (Sun *et al*. 2011). In a recent preliminary study among 24 patients with adrenal tumours, circulating tumour cells were detected in all ACC patients, but not in patients with an adrenal adenoma (Pinzani *et al*. 2013). Additional larger studies are needed, since this technique seems potentially valuable as marker for detection of malignancy and perhaps even for disease progression or treatment monitoring.

Clinical prognostic factors

Currently, the most important prognostic factor in ACC is stage of disease at presentation (Fassnacht *et al*. 2009). The ENSAT staging system was introduced in 2009 and proved to be superior to the International Union Against Cancer (UICC)

staging system in a 2010 validation study (Lughezzani *et al*. 2010). Additional prognostic factors are needed, since the ENSAT system does not result in a differentiation firm enough to base important treatment decisions on.

Localized disease

Overall survival was not significantly different between patients with ENSAT stage I and stage II disease in validation studies (Lughezzani *et al*. 2010). In this group, the main question is which patients should receive adjuvant treatment after surgery. The immunohistochemical proliferation marker Ki-67 (Mib1) is regarded as the most promising prognostic factor to be included in standard work-up of ACC patients (McNicol *et al*. 1997, Terzolo *et al*. 2001, Sasano *et al*. 2006, Morimoto *et al*. 2008). A retrospective study among 569 patients with disease stages I-III who underwent a complete resection including an independent validation cohort demonstrated that Ki-67 is associated with recurrent disease (hazard ratio [HR] 1.042 per 1% increase) and overall survival (HR for death, 1.051) (Beuschlein *et al*. 2015). The authors suggest clinical application of the Ki-67 index based on expression in tumour tissue of <10%, 10-19%, and ≥20%. There are limitations to clinical application of the Ki-67 index that deserve further attention. Research shows that inter-observer variability in determining the index is high, even when performed by expert pathologists. Preliminary results suggest that automated software assessment of the Ki-67 index may result in more consistent results and better correlation with clinical outcome (Pucci *et al*. 2014). In addition, Ki-67 proliferation within a single tumour is characterized by a highly heterogeneous pattern. Fields with highly proliferative cells are often surrounded by areas of non-staining cells or even necrosis, which holds a significant risk of sampling error. Therefore, a uniform methodology for assessing the Ki-67 index in ACC needs to be established.

Advanced disease

A second shortcoming of the ENSAT staging system is considerable heterogeneity in the stage IV group. In some patients disease progresses aggressively and hardly responds to treatment. As a result, survival is only a few weeks or months (Kerkhofs *et al*. 2013c). In other patients, the disease follows a more indolent course and survival of up to several years has been observed (Hermsen *et al*. 2008). The search for clinical predictors of survival in these patients is still ongoing. Several parameters have been suggested, such as age, mitotic rate or presence of autonomous hormone production (Abiven *et al*. 2006, Assie *et al*. 2007). A recent retrospective study from the ENSAT ACC working group proposes a modification of the ENSAT staging system regarding patients with stage III-IV disease (Libe *et al*. 2015). In this proposal, stage IV is divided

in three subgroups (A, B and C, Table 1). Patients with $T_{3-4}N_1M_0$ disease shift from stage III to stage IV-A. Also in IV-A are patients with $T_{1-4}N_{0-1}M_1$, as long as no more than two different organs are affected (including lymph node metastases). Patients with $T_{1-4}N_{0-1}M_1$ and three affected organs form group IV-B, all other patients (i.e. $T_{1-4}N_{0-1}M_1$, >3 organs) form group IV-C. In addition, the prognostic significance of four parameters was validated in a large cohort of stage III-IV patients (n=349). Tumour grade (G) was proposed as a binomial parameter defined as Weiss ≤ 6 and Ki-67 <20% or Weiss >6 and/or Ki-67 >20%). Resection status (R), also binomial, was defined as complete resection (R_0) or other. Age (A) below 50 had a favourable effect on overall survival. Functional symptoms (F) was defined as the presence of tumour- or hormone related symptoms at diagnosis. The exact role of these parameters in further stratification of patients with advanced ACC will have to be established in future studies. It is expected that the differentiation between stage IV-A, B and C will have consequences for treatment initiation, for example to start with mitotane monotherapy in metastatic disease with relatively favourable characteristics, but start immediate combination chemotherapy in metastasized disease with unfavourable features.

Genetic prognostic factors

In addition to establishing clinical prognostic factors, research is focused on the emerging possibilities of genomic characterization of tumours. Gene expression profiling on large cohorts of adrenal tumours identified among ACCs two distinct subgroups that differed in long-term overall survival, while distinguishing histological features could not be found (De Reynies *et al*. 2009, Giordano *et al*. 2009). In an effort to further characterize genomic alterations in ACC, a study combined exome sequencing, SNP arrays, DNA methylation analysis, mRNA expression arrays and miRNA sequencing (Assie *et al*. 2014). Two clusters were identified based on mRNA expression that were associated with clinical outcome, i.e. overall survival. Tumours from the cluster with poor outcome (named C1A) could subsequently be divided in three groups based on DNA methylation profile. In this particular example, DNA methylation refers to hypermethylation of Cytosine-Guanine dinucleotides (CpG). A region of DNA with a high frequency of CpG sites is called a CpG island. If hypermethylation of CpG islands occurs in the promotor region of a specific gene (for example a tumour suppressor gene), this may cause their dysfunction. In accordance with observations in colon cancer, subsets of hypermethylated ACCs have been identified and labelled CpG island methylator phenotype (CIMP) (Barreau *et al*. 2013). Hence, the three subgroups in the C1A cluster are labelled CIMP-high, CIMP-low and non-CIMP. Tumours from the cluster with indolent course of disease (named C1B) could be further divided in two groups based on miRNA expression (Mi1 and Mi2). Earlier research also suggested circulating miRNA was associated with clinical outcome: high levels of miR-483-5p

and low levels of miR-195 were correlated with shorter recurrence-free and overall survival (Chabre *et al*. 2013). Analysis of suspect driver genes and pathways revealed most alterations in the subgroup with poor clinical outcome, as well as a higher mutation rate. It appears that inter-individual differences are large, given that the most frequently altered gene (ZNRF3, related to the β-catenin pathway) was altered in 21% of cases (Assie *et al*. 2014). It is expected that further genomic characterization will lead to increased understanding of ACC pathogenesis. In the future, genomic characterization of ACC is expected to be of prognostic value and improve clinical management. For example, an unfavourable genomic profile might warrant aggressive adjuvant (chemo)therapy and close surveillance, whereas a favourable profile could justify more lenient follow-up. In addition we expect improved understanding of the pathogenesis will also yield therapeutic targets and thus therapeutic possibilities.

Organization and quality of care

Concentration of care in a limited number of specialized hospitals aimed at improving outcomes is an important concept in general healthcare, but particularly in oncological care, high risk surgical care and rare diseases. Regarding ACC, increasing evidence suggests that establishment of (inter)national collaborative networks of expert centres has a favourable effect on survival (Fassnacht *et al*. 2010, Lombardi *et al*. 2012, Kerkhofs *et al*. 2013b). The concept that rare diseases should be treated in a limited number of specialized hospitals seems intuitively logical. Interestingly, a better outcome of oncological treatment in specialized centres is not necessarily correlated with volume requirements (Ho *et al*. 2012). Similar observations were reported in high-risk surgical treatment (Finks *et al*. 2011). Moreover, common minimum volume requirements as instituted for other cancers are hardly feasible for ACC due to the low incidence. For example, the number of new patients with ACC per year in the Netherlands is about 20 (median 21, range 13-26 between 1993 and 2010). Although the number of surgical procedures for suspect adrenal malignancy will be higher, the grand total is expected to be too low to institute meaningful minimum volume requirements. Of course, numbers are higher in other (bigger) countries, but it is questionable whether geographical spread would allow for concentration of care on this scale. We expect quality criteria other than volume are of greater importance in rare diseases. It seems more important that centres adhere to current state-of-art treatment concepts, which in turn seems best feasible in a specialized centres with dedicated physicians. Experience with multidisciplinary oncologic surgery and preferably adrenal/endocrine surgery is a strong recommendation and maybe even a prerequisite. In addition, participation in international networks and clinical trials should be encouraged. By doing so, local specialists remain up-to-date and rapid adjustments can be made while respecting national and local regulations. Figure 2

contains a flowchart depicting current treatment strategy in which multidisciplinary decision-making has a key role. The following paragraphs discuss the steps of this treatment algorithm and highlight several possibilities for future improvement. Table 2 should be regarded as supplement to Figure 1 and 2 and summarizes the considerations that in our view should be part of current state-of-art diagnostic and treatment concepts in ACC.

Surgery: the primary treatment modality

In patients who present with local or locally advanced disease, curative treatment is possible by performing complete surgical resection (Schulick and Brennan 1999, Grubbs *et al*. 2010). In practice, this is not as straightforward as it may seem. In the first place, completeness of resection can be difficult to determine due to the often close anatomical relationship between tumour capsule and adjacent organs such as liver or kidney. As a consequence, in some cases the largest possible margin consists of tumour capsule only, which may be only few cell layers thick. Secondly, the necessity of lymph node dissection (LND) and subsequent resection has not yet been established. It has been suggested in (few) retrospective studies that locoregional LND is associated with improved survival (Reibetanz *et al*. 2012, Tran *et al*. 2013, Erdogan *et al*. 2013). In contrast, a study from the SEER database could not confirm a survival benefit after LND (Saade *et al*. 2015). Results are difficult to interpret since it remains unknown whether presumed LNDs were performed intentionally and, the other way around, whether pathologists recognized and/or described lymph nodes that in fact were intentionally dissected. Also, the anatomical extent of LND for adrenal tumours is not standardized. Reibetanz et al. presented a proposal for the field of LND on a sound theoretical basis, but this has not yet been tested in clinical practice (Reibetanz *et al*. 2012). The only way to settle this debate is to perform a prospective randomized trial with standardized surgical technique, but this seems difficult in such a rare disease. A third subject of debate is the surgical approach, i.e. laparoscopy or laparotomy. A minimally invasive technique is supposed to offer short-term benefits such as lower morbidity and shorter hospital stay (Brix *et al*. 2010, Porpiglia *et al*. 2010, Lombardi *et al*. 2012, Donatini *et al*. 2014). Those in favour of laparotomy advocate this approach holds a lower risk of tumour rupture (Miller *et al*. 2010, Miller *et al*. 2012, Cooper *et al*. 2013, Mir *et al*. 2013, Toniato. 2013). It is expected that individual preferences and skills are highly influential on the outcome. A literature review of 23 retrospective studies addressing this question concluded that open surgery should remain the default option in ACC (Jurowich *et al*. 2013). However, in case of 'limited size tumours' (<10cm) radical resection through a laparoscopic approach should be technically feasible if performed by an experienced surgeon. If there is evidence of invasive disease, laparotomy should be performed as extensive resection might be necessary.

In selected patients presenting with metastasized disease, debulking surgery might yield a survival benefit (Kerkhofs *et al*. 2013b, Dy *et al*. 2015). Prospective studies on this topic are not yet available, and retrospective studies inevitably hold selection bias: chances are that patients who underwent debulking surgery were in better clinical condition compared to patients who were not operated on. Patients with metachronous oligometastases might benefit from local surgical resection or other local ablative measures. Evidence is limited and these interventions are probably best applicable to patients with otherwise favourable prognostic characteristics (Wood *et al*. 2003, Cazejust *et al*. 2010, Baudin *et al*. 2011). In recurrent (local) disease, surgery is the treatment of choice. Main predictors of outcome in this situation are time to first recurrence and resectability of the recurred tumour (Erdogan *et al*. 2013). Patients with a time to recurrence longer than one year and a completely resected recurrence have the best prognosis. In patients with early and/or not completely resectable recurrence, surgical treatment may be considered.

Mitotane

Drug therapy with mitotane has since 1959 been in use for treatment of patients with irresectable or metastasized ACC (Bergenstal *et al*. 1960, Lubitz *et al*. 1973, Baudin *et al*. 2011, Fassnacht *et al*. 2011). Mitotane is derived from the insecticide dichlorodiphenyltrichloroethane (DDT) and is administered orally in tablets in two to three daily doses. The drug exerts an anti-neoplastic effect on ACC tissue and in addition inhibits cortisol synthesis, which is beneficial in patients with Cushing's syndrome (Southren *et al*. 1966, Fukushima *et al*. 1971, Touitou *et al*. 1978, Ghataore *et al*. 2012). The antineoplastic effect is correlated with the plasma level of mitotane. A therapeutic response was observed in patients with plasma levels >14mg/L, which is therefore considered as the lower limit of the therapeutic window (Haak *et al*. 1994, Baudin *et al*. 2001). Mitotane is well known for its toxicity, in particular for the gastro-intestinal tract (nausea, diarrhoea, vomiting) and the nervous system (ataxia, amnesia, confusion) (Daffara *et al*. 2008). Toxicity of mitotane appears to be correlated with its plasma concentration, which is why a plasma level of 20mg/L is considered the upper limit of the therapeutic window (Baudin *et al*. 2001). Patients (and their physicians) intuitively want to achieve therapeutic mitotane concentrations as early as possible in order to rapidly establish antiproliferative efficacy. A problematic aspect is that the drug's half-life is quite variable between patients but is in general extremely long (13-159 days) (Moolenaar *et al*. 1981). Therefore, it takes a very long time (on average 3 months) to reach the therapeutic level between 14 and 20 mg/L (Terzolo *et al*. 2000, Faggiano *et al*. 2006, Kerkhofs *et al*. 2013a). Especially in patients presenting with stage IV disease (+/- 50% of patients), this long build-up phase delays effective treatment. Also, co-administration of chemotherapy might

affect mitotane pharmacokinetics by inducing or inhibiting enzymes involved in drug metabolism (Kerkhofs *et al*. 2013a). Pharmacokinetic studies demonstrated that mitotane concentrations in fatty tissue are approximately 200-fold higher than in plasma and drug distribution appears to be far more important than drug elimination (von Slooten *et al*. 1982). Currently, research is focused at optimizing dosing schedules with help of therapeutic drug monitoring and pharmacogenetics (D'Avolio *et al*. 2013, Kerkhofs *et al*. 2015a). The aim is to shorten the plasma level build-up time and to minimize drug toxicity. For example, in patients presenting with stage IV disease not amenable to surgical resection, short-term achievement of adequate mitotane monotherapy may postpone the necessity of the often toxic and invalidating etoposide/doxorubicin/cisplatin (EDP) chemotherapy. On a side note, EDP chemotherapy may be more effective in combination with adequate mitotane levels due to an interaction at the drug resistance protein, which is an additional argument to optimize mitotane treatment first (Bates *et al*. 1991, Berruti *et al*. 1998). In addition to the established application of mitotane in patients with advanced disease, mitotane is increasingly being used as adjuvant treatment after complete resection. This is based on convincing retrospective evidence suggesting prolonged recurrence-free and overall survival (Terzolo *et al*. 2007). According to the 2012 ESMO guidelines, adjuvant mitotane is recommended in patients with a high risk of disease recurrence defined by stage III disease, Ki-67 >10%, or incomplete resection (Berruti *et al*. 2012). Adjuvant treatment with mitotane is prospectively compared in a randomized trial that is currently recruiting patients with stage I-III disease who underwent a complete resection and had a Ki-67 <10% (Efficacy of Adjuvant Mitotane Treatment in Prolonging Recurrence-free Survival in Patients With Adrenocortical Carcinoma at Low-intermediate Risk of Recurrence [ADIUVO-trial], NCT00777244). In this era of targeted therapy and drug engineering, it may seem unwise to rely on an old and toxic drug. However, no 'modern' drug has yet proven to be effective and possible new agents will have to be compared to mitotane first. Since mitotane is currently the only effective drug available, it will remain the backbone of medical therapy in ACC during the following years. Figure 3 contains a comprehensive flowchart of mitotane clinical management based on available literature and personal experience. It is important to realize that despite optimization of dosing schedules, the key factor influencing build-up of mitotane plasma levels is patient tolerability. From this perspective, the importance of adequate supportive treatment cannot be stressed enough, i.e. administration of hydrocortisone, anti-emetic and anti-diarrheal drugs if necessary. The strong CYP3A4 induction by mitotane necessitates careful selection of supportive drugs (van Erp *et al*. 2011, Kroiss *et al*. 2011). A possible strategy is to start concomitant treatment on day 1 including hydrocortisone 20mg, metoclopramide 10mg two times daily (as needed) and loperamide 2mg two to four times daily (as needed). Hydrocortisone should be administered in supraphysiological

doses because of CYP3A4 induction, which increases glucocorticoid metabolism (Chortis *et al*. 2013). Frequent assessment (every 3 months) of thyroid hormone status is necessary as mitotane may induce a clinical picture similar to central hypothyroidism, possibly through a direct effect on the pituitary gland or induction of thyroid hormone metabolism (Zatelli *et al*. 2010). Based on clinical experience, treatment with thyroxine supplementation restores euthyroidism. In men with signs of hypogonadism, assessment of testosterone and sex hormone binding globulin levels is warranted, as (yet unexplained) disturbances in mitotane treated patients are common (Berruti *et al*. 2012). Since mitotane inhibits 5-alpha-reductase, the enzyme that converts testosterone in the more potent metabolite 5α-dihydrotestosterone, treatment with testosterone supplementation may be ineffective. Supplementation of synthetic dihydrotestosterone should have the desired effect. Another (presumed) consequence of 5-alpha inhibition is increased conversion of testosterone to 17β-estradiol, which could explain the occurrence of gynecomastia in mitotane-treated men (Daffara *et al*. 2008, Chortis *et al*. 2013). Psychological and social aspects of treatment should not be neglected, i.e. professional counselling may be warranted. Follow-up on patient's well-being may be performed by questionnaire-based assessment of toxicity upon start of treatment and by repeating this assessment every 3 months. This facilitates early recognition and optimization of supportive treatment, and if necessary mitotane dose adjustments. In our experience educating patients about side-effects, taking time to register side-effects and providing instruments to alleviate them improves compliance and patient motivation to stay on treatment.

Chemotherapy

One of the greatest achievements in ACC research was the completion of the FIRM-ACT trial, which established the regimen EDP combined with mitotane as first-line chemotherapy in advanced ACC. An objective response was achieved in 23% of patients (Fassnacht *et al*. 2012). The secondary endpoint was defined as progression free survival at 8 weeks, which was achieved in 58% of patients. The question could be asked whether a three-drug regimen is necessary to achieve these results. Therefore, a non-inferiority trial seems justified. On the other hand, given the overall poor prognosis in this group, it may be wiser to deploy available resources to a trial comparing new drugs to the existing regimen of EDP. Problematic is that no other regimens of cytotoxic drugs demonstrated results that even approximate those of EDP. Based on results of a phase II trial, a combined regimen of gemcitabine/capecitabine is now offered as second-line therapy in Dutch and German expert centres. This regimen resulted in disease control during 6 months in 29% of 28 patients (Sperone *et al*. 2010). Other studies including cytotoxic chemotherapy only produced disappointing results. Among the tested agents were capecitabine

(combined with bevacizumab) (Wortmann *et al*. 2010), paclitaxel (Berruti *et al*. 2011), irinotecan (Baudin *et al*. 2002) and docetaxel (combined with cisplatin) (Urup *et al*. 2013). Again, mitotane is a problem in investigating new drugs since virtually all patients in advanced stages are pre-treated.

Radiotherapy

The application of adjuvant radiotherapy in ACC has so far only been studied in retrospective setting (Fassnacht *et al*. 2006, Sabolch *et al*. 2011, Habra *et al*. 2013). None of these studies demonstrated a benefit in disease-free or overall survival after adjuvant radiotherapy, whereas two studies did report a beneficial effect on the local recurrence rate (Fassnacht *et al*. 2006, Sabolch *et al*. 2011). Since local recurrence does not seem to be the most important predictor of survival, it is expected that systemic adjuvant therapy is more important than local control. In a different setting, i.e. after incomplete surgical resection, radiotherapy in combination with mitotane is recommended (Berruti *et al*. 2012). The rationale behind this recommendation is that patients with residual disease after surgery perform worse compared to patients who had a complete resection and aggressive treatment is warranted (Libe *et al*. 2015). Polat et al. suggested a dosing regimen based on clinical experience consisting of a total dose >40 Gy with single fractions of 1.8 Gy to 2 Gy. This includes a boost volume to reach 50 Gy to 60 Gy in individual patients (Polat *et al*. 2009). As discussed above, there is a subgroup of ACC patients with indolent disease, for example with slowly progressive oligometastases in ≤2 organs. Based on personal clinical experience (unpublished), we believe these patients might benefit from stereotactic body radiation therapy. This technique is continuously being refined and deserves further study as it holds the potential of prolonging local control in patients with often limited tumour load, in whom toxic systemic therapy would otherwise be the only option (Salama and Milano 2014). Another new technique in patients with metastasized disease is treatment with radioactive isotopes. A treatment protocol based on the radionuclide [^{131}I]iodometomidate (IMTO) has been tested on 11 patients and resulted in a partial response in one patient and stable disease in five patients (Hahner *et al*. 2011). Further research will have to prove the potential of this promising treatment modality. Radiotherapy with palliative intent, for example irradiation of painful bone metastases or a large irresectable primary tumour, is widely accepted as a considerably effective measure. Evidence is again retrospective and mostly based on single-institution experience (Polat *et al*. 2009, Hermsen *et al*. 2010, Ho *et al*. 2013).

Targeted therapy

Several targeted therapies have been tested in mostly small-scale trials; none of these has yet earned a place in the treatment algorithm. Among the targets examined in case series were the platelet derived growth factor receptor (PDGFR-R) and stem cell ligand receptor (Gross *et al*. 2006); epidermal growth factor receptor (EGFR)(Quinkler *et al*. 2008); vascular endothelial growth factor (VEGF)(Wortmann *et al*. 2010) and the mammalian target of rapamycin (mTOR) pathway (Fraenkel *et al*. 2013). An expanded phase I study combined the IGF-1R inhibitor cixutumumab and the mTOR inhibitor temsirolimus in 26 patients. Two phase II trials examined the efficacy of multi-tyrosine kinase inhibitors sorafenib and sunitinib in 10 and 38 patients respectively (Berruti *et al*. 2011, Kroiss *et al*. 2012). In general, these studies demonstrated disappointing results without any objective response and with only few patients experiencing a short-lived stabilization of their disease. So far, the only phase III trial involving a targeted treatment is a study on the IGF-1R inhibitor linsitinib (Fassnacht *et al*. 2015). A confirmed partial response was observed in three patients, but in the total population there was no increase in overall or progression-free survival. Interpretation of results from trials with targeted therapies is difficult for several reasons. In the first place, most patients who participated in these studies were pre-treated with mitotane and chemotherapy and were in advanced stage of their disease. It is likely that these tumours became drug-resistant and were hardly responsive to any therapy, or not responsive at all. Secondly, pre-treatment with mitotane and consequently CYP3A4 induction might have impaired the effectiveness of the therapies studied. This is a problematic aspect of mitotane treatment, especially since the half-life is so long and mitotane remains detectable in plasma months after cessation of treatment. It is unknown how long it takes for the inductive effect to wear off. As a result, timing of targeted therapies in clinical management (or trial participation for that matter) is difficult to determine. It seems logical to test potential new drugs in a setting where they can be compared to mitotane. Patients with advanced ACC (modified ENSAT stage IV-A) who had no prior systemic therapy and who would qualify for treatment with mitotane monotherapy (possibly after debulking surgery) could form the most suitable subgroup for such a trial. Thirdly, recent research made it increasingly clear that there is a subgroup of patients with metastatic disease and an indolent course of disease (Assie *et al*. 2014). It is not inconceivable that their natural course of disease resembles stable disease for several months while on a study drug. In future studies patients should be meticulously stratified, not only based on tumour characteristics but also based on disease progression in months or even years before participation. Research on immunotherapy in adrenocortical carcinoma is still at a preliminary stage. Blocking the Programmed Death-1 (PD-1) or Programmed Death-Ligand 1 (PD-L1) receptor in an attempt to direct a T-cell response to tumour tissue has been successful in renal cell carcinoma, melanoma and non-small cell lung carcinoma

(Topalian *et al*. 2012). Exploratory research on a small scale (n=28) demonstrated ACC may express PD-L1 on the cell surface (Fay *et al*. 2015). A phase I study with the PD-L1 inhibitor Avelumab is currently recruiting and is open to ACC patients (NCT01772004). Other data demonstrated high expression of the survivin protein in a cohort of 29 ACCs, associated with a trend towards poor prognosis (Sbiera *et al*. 2013). These findings suggest survivin might be a target for immunotherapy, but this has not yet lead to a clinical trial.

Research

As discussed, there are many clinical questions yet unanswered and many trials to be performed. The European Network for the Study of Adrenal Tumours (ENSAT) is an expanding collaboration formed by researchers and clinicians. It holds a database with clinical data of >2200 ACC patients (as of June 2015), which is of great potential for future studies. Collection of biomaterial such as blood, urine and tumour samples is important in order to facilitate research. Due to high costs associated with especially genomic research projects, upscaling to European or even global platforms seems necessary to be able to fund these undertakings. National laws on the use of archival tumour tissue may differ. It is recommended to consult the local ethics committee on prevailing regulations regarding the establishment of a local biobank. A starting point should be to obtain informed consent from the patient for future (genomic) research on tumour tissue before surgery, even if the exact content of said research is not specified. International networks could facilitate this by supplying standard consent forms, issue standard operating procedures for tissue handling or even sponsor a central storage facility. In addition, international networks should promote development of international standards for reporting surgery and pathology results, determination of the Ki-67 index and urinary steroid profiling cut-off values.

Conclusion

The present review discusses current and future ACC diagnostics, prognostics and treatment concepts. In addition, it provides a view on organization of care and international collaboration. In the near future, we expect urinary steroid profiling to gain a place in daily practice diagnostics of adrenal tumours. Application in follow-up of ACC patients is promising but needs additional research. A recent proposal for refinement of the most important clinical prognostic factor, stage of disease, is expected to become standard practice. Currently, the best indicator of recurrence-free and overall survival is determination of the proliferation marker Ki-67. This technique needs standardization, since it will take several years before its successor,

genomic profiling, will be ready for clinical practice. Risk stratification is needed to select patients for adjuvant treatment, since complete surgical resection alone, with or without lymph node dissection, cannot guarantee recurrence-free survival. Mitotane will remain the backbone of drug therapy in years to come. Its tolerability and long plasma level build-up phase may be improved by therapeutic drug monitoring based on pharmacokinetic modelling and intensive counselling of patients. Due to the rarity of disease, it is questionable whether expert centres should be instituted based on volume requirements. We advocate treatment of patients in centres with established experience in multidisciplinary oncologic care, who adhere to prevailing guidelines and state-of-art in diagnostic and treatment concepts. International collaboration is necessary to facilitate fundamental research and clinical trials to test new treatment possibilities that are so badly needed.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Table 1: Proposal for modified ENSAT staging system (Libé et al. 2014).

Stage	Definition
III	$T_{3-4}N_0M_0$
IV-A	$T_{1-4}N_1M_0$ or $T_{1-4}N_{0-1}M_1$ (max. 2 organs including N)
IV-B	$T_{1-4}N_{0-1}M_1$ (3 organs)
IV-C	$T_{1-4}N_{0-1}M_1$ (>3 organs)

Table 2: Summary of considerations that should be part of current state-of-art diagnostic and treatment concepts in adrenocortical carcinoma.

Step in algorithm	Consideration
Diagnostics in patients with suspect ACC (Figure 1)	
Hormonal work-up	Urinary steroid profiling can be of added value in patients with inconclusive imaging results.
Pre-operative multidisciplinary meeting	Takes place within 2 weeks after completion of diagnostic tests. Attended by endocrinologist, oncologist, surgeon and radiologist.
Treatment algorithm (Figure 2)	
Surgery	Adrenalectomy within 3 weeks after multidisciplinary meeting.
Post-operative multidisciplinary meeting	Resection status is determined by discussing surgeon's and pathologist's observations and conclusions. Pathology report contains itemized Weiss-score and Ki-67 index. Lymph node involvement is determined. Definitive ENSAT-stage is determined.
Surveillance	FDG-PET scan performed 3-6 months after (complete) resection of primary tumour.
Consider ADIUVO	Possibility to enrol patients in (international) trials
Adjuvant mitotane	Treatment instituted according to protocol (Figure 3) and maintained for at least 2 years.
Radiotherapy	Consider total dose >40 Gy with single fractions of 1.8 Gy to 2 Gy (including boost volume to reach 50 Gy to 60 Gy in individual patients) (Polat et al. 2009).
Patient-tailored decision in metastatic disease	Consider cut-off values for number of affected organs from least to worst risk: 2, 3 or >3 (Libé et al. 2015). Weiss-score >6 and/or Ki-67 >20% correlates to most aggressive behaviour (Libé et al. 2015). Consider performance status ECOG 2 or better as baseline before EDP-M treatment. Treatment goal: take into account patient's wishes and expectations.
Mitotane monotherapy	Treatment instituted according to protocol (Figure 3).
Local ablative measures	Consider radio-frequency ablation, stereotactic body radiation therapy or surgical resection of oligometastases.
EDP+mitotane	Treatment instituted according to FIRM-ACT protocol (Fassnacht et al. 2012)
Optional: research facilitation	
Obtain informed consent to store and share clinical data for future research.	
Obtain informed consent to store and share tumour tissue, blood sample, urine sample for future research.	
Store freshly frozen sample of tumour tissue.	

ECOG: Eastern Cooperative Oncology Group.

Figure legends:

Figure 1: Diagnostic work up of patients with suspect adrenocortical carcinoma.

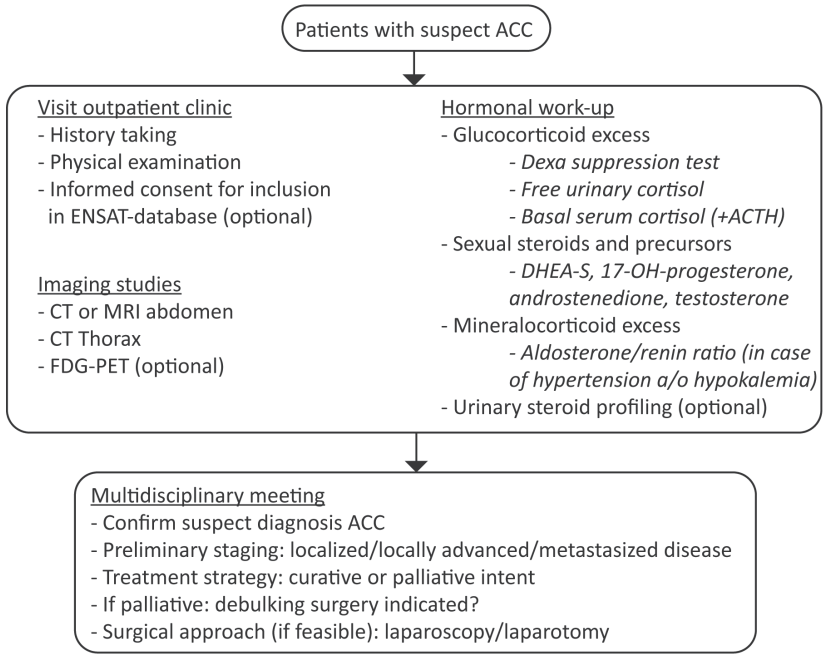


Figure 2: Treatment algorithm for patients with adrenocortical carcinoma.

R0: complete resection, R1: microscopic residual tumour, R2: macroscopic residual tumour, Rx: resection status undetermined, EDP: Etoposide/Doxorubicin/Cisplatin, ADIUVO: Clinical trial testing efficacy of Adjuvant Mitotane Treatment in Prolonging Recurrence-free Survival in Patients With Adrenocortical Carcinoma at Low-intermediate Risk of Recurrence.

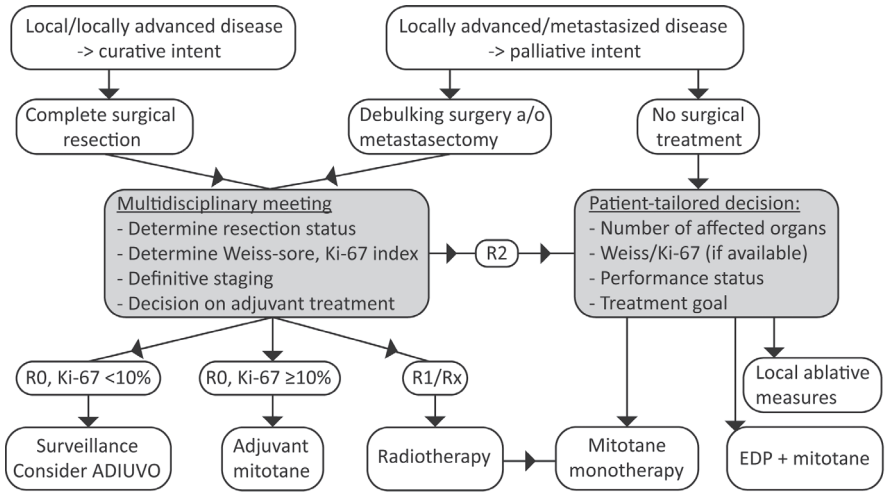
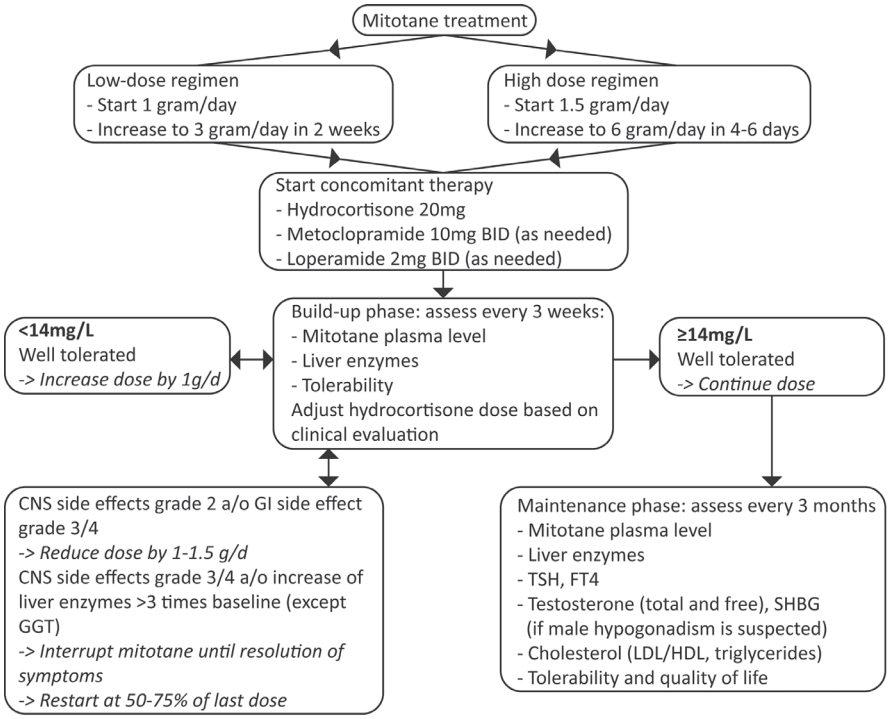


Figure 3: Flowchart of clinical management in starting and maintaining mitotane treatment.

CNS: central nervous system, GI: gastro-intestinal, grading of side effects according to National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 4, GGT: gamma-glutamyltransferase.



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5

Chapter 06

Past, Present and Future of Epigenetics in Adrenocortical Carcinoma

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Abstract

DNA methylation profiling has been suggested a reliable technique to distinguish between benign and malignant adrenocortical tumors, a process which with current diagnostic methods remains challenging and lacks diagnostic accuracy of borderline tumors. Accurate distinction between benign and malignant adrenal tumors is of the essence, since ACC is a rare but aggressive endocrine disease with an annual incidence of about 2.0 cases per million people per year. The estimated five-year overall survival rate for ACC patients is <50%. However, available treatment regimens are limited, in which a radical surgical resection is the only curable option. Nevertheless, up to 85% of patients with radical resection show recurrence of the local disease often with concurrent metastases. Adrenolytic therapy with mitotane, administered alone or in combination with cytotoxic agents, is currently the primary (palliative) treatment for patients with advanced ACC and is increasingly used in adjuvant setting to prevent recurrence. Prognostic stratification is important in order to individualize adjuvant therapies. On April 1, 2020, there were 7404 publications on adrenocortical carcinoma ((((((adrenocortical carcinoma) OR adrenocortical carcinoma [MeSH Terms]) OR adrenal cortex cancer[MeSH Terms]) OR adrenal cortical carcinoma [MeSH Terms]) OR adrenal cortex neoplasm [MeSH Terms]) OR adrenocortical cancer [MeSH Terms]), yet the underlying pathophysiology and characteristics of ACC is not fully understood. Knowledge on epigenetic alterations in the process of adrenal tumorigenesis is rapidly increasing and will add to a better understanding of the pathogenesis of ACC. DNA methylation profiling has been heralded as a promising method in the prognostication of ACC. This review summarizes recent findings on epigenetics of ACC and its role in diagnosis, prognosis and therapeutic strategies.

Keywords: adrenocortical carcinoma; epigenetics; DNA methylation

1. Introduction

Adrenocortical tumors (ACTs) are frequently discovered as incidentaloma due to increased use of imaging in a variety of medical settings. The first computed tomography (CT) series published in the early 1980s showed a prevalence of adrenal incidentaloma between 0.7-1.3% [1,2]. In a series published between 1982 to 1994 the mean prevalence of adrenal incidentaloma was 0.64% (ranging from 0.35–1.9%) [3], whereas, in 1991 Herrera et al. showed that only 0.4% of all the CT scans showed serendipitously discovered adrenal masses [4]). Bovio et al. showed a prevalence of 4.4% in their series of high resolution CT scans [5].

Most of these incidentalomas are benign adrenocortical adenomas (ACA), non-functional and clinically irrelevant. Their malignant counterpart, adrenocortical carcinoma (ACC), is a rare and aggressive type of cancer. Although estimates varied widely, the frequency of primary adrenal carcinoma in patients with adrenal incidentaloma ranges from 1.2–11% [6]. It should be kept in mind that due to the nature of these studies, selection bias is very probable (the populations studied not reflecting a random sample of all patients with an adrenal incidentaloma) and most likely leads to an overestimation of the frequency of ACC. Differentiating between these two types of tumors can be challenging, considering that clinical, laboratory, radiological and even histological features may overlap. Although ACC occurs in children (only 0.2% of pediatric cancers [7], annual incidence of 0.2 to 0.3 cases per 1 million individuals [8]), most cases appear between ages 30 and 50 (0.02% to 0.2% of adult cancers [9], annual incidence of 0.5–2.0 patient per million people per year [10]. An exception to these epidemiologic data is described in southern Brazil, where the annual incidence of adrenal cancer in children is unusually high, ranging from 3.4–4.2 per million children [11]. The distribution of tumors seems to follow a regional rather than familial pattern, therefore environmental factors have been considered, but so far none have been identified.

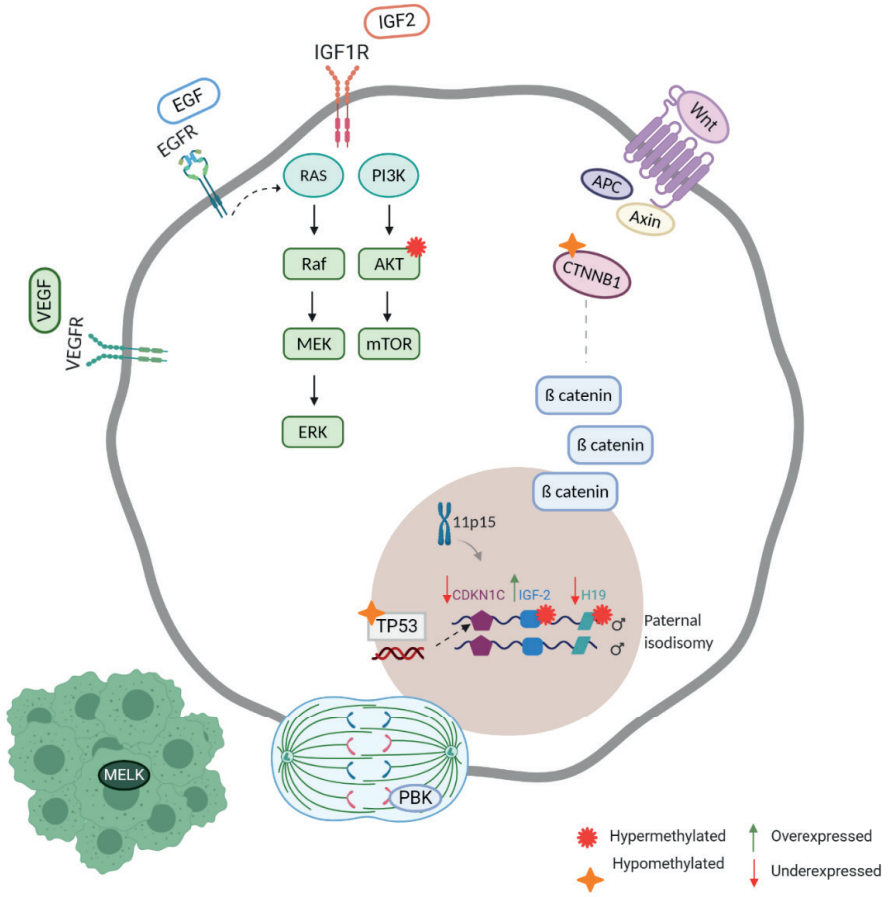

Figure 1. Major dysfunctional molecular pathways in adrenocortical carcinoma, resulting in abnormal survival, proliferation, apoptosis resistance, metastasis and angiogenesis [Created with BioRender.com].

1.1. Molecular Alterations

The understanding of adrenocortical tumorigenesis has been challenging since molecular studies on adrenocortical carcinoma have been based on a small number of samples. Until recently they have been directed mainly to candidate genes. It was with this approach that the first genetic studies on ACC started with the elucidation of rare genetic syndromes (e.g. Li Fraumeni syndrome, Beckwith Wiedemann syndrome) in which ACTs are a manifestation [12]. They have led to the discovery of major dysfunctional molecular pathways in adrenocortical tumors, such as the IGF pathway, the Wnt pathway and TP53 (Figure 1) [12]. TP53 germline mutations have been described with a mutation prevalence of 3.9% [13].

Gene expression analysis showed that *IGF2*, a fetal growth factor imprinted at chromosome 11p15 locus, is upregulated and overexpressed in ACC. Another gene at the 11p15 locus, *H19* (a non-protein coding RNA) associated with the inhibition of *IGF2* expression, is under expressed in ACC [14].

In ACC, the Wnt/β-catenin (*CTNNB1*) is frequently activated trough *CTNNB1* mutations and even associated with a poor outcome [15]. In approximately 25% of both benign and malignant sporadic adrenocortical neoplasms β-catenin gain-of-function mutations are evident [16]. Zheng et al (2016) found 41% of ACC cases to have alterations of ZNRF3, CTNNB1, APC and MEN1 resulting in modification of the Wnt/β-catenin pathway [17].

Tyrosine-kinase coupled receptors have been confirmed to be abnormally active in the IGF pathway, the epidermal growth factor (EGF), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) pathway. These pathways are associated with cell survival, proliferation, angiogenesis, apoptosis resistance and metastasis [12].

Recently, efforts have been made to overcome the problem of small number of ACC samples by developing new preclinical models (CU-ACC1 and CU-ACC2) to advance ACC research [18]. For a long time H295R, initially established in 1980 from a 48-year-old female patient diagnosed with ACC, was the only cell line available for research [19]. With these new preclinical models Kiseljak-Vassiliades et al. have attempted to find new therapies by focusing on identifying the cell cycle kinases in ACC and pinpointing defects in the DNA damage response pathway. They analyzed publicly available expression data sets and observed that maternal embryonic leucine zipper kinase (MELK) was one of the most upregulated kinases in adrenal cancer compared to normal tissue [20].

This research group also observed the mitotic PDZ-binding kinase (PBK; also known as T-lymphokine-activated killer cell-originated protein kinase (TOPK)), a master mitotic kinase known for its role in mitotic division and regulation, to be highly overexpressed in ACC tissues compared to normal adrenal samples [21].

1.2. Prognostication

Clinical behavior among ACCs is heterogeneous and stage dependent. The extent of the disease at the time of diagnosis is best assessed by the European Network for the Study of Adrenal Tumor (ENSAT) staging score (Table 1) [22].

Table 1. ENSAT score.

Stage	TNM
I	T1,N0,M0 Tumor ≤5 cm
II	T2,N0,M0 >5 cm
III	T1–2,N1,M0 or T3–4,N0–1,M0
IV	T1–4,N0–1,M1

T, tumor. N, lymph node. M, metastasis. T1, tumor size ≤5 centimeter (cm); T2, tumor size >5 cm, T3, tumor infiltration in surrounding tissue; T4, tumor invasion in adjacent organs or venous tumor thrombus in vena cava or renal vein. N0, no positive lymph nodes; N1, positive lymph node(s). M0, no distant metastases; M1, presence of distant metastasis.

Prognosis in stage IV disease can vary from a few months to many years [23]. The immunohistochemical Ki67 index < 10% has been correlated with a relatively good prognosis where an index over 20% correlates with a more grim prognosis [24]. Despite the high inter- and intra-observer variability [25], Ki67 is currently the most accepted and used prognostic parameter [26] as confirmed by five studies [27-31]. It is recommended in the guidelines to use the Ki67 immunohistochemistry for every resection specimen of an adrenocortical tumor, and therapeutic strategies are suggested based on low-risk (Ki67 ≤10%) or high-risk (Ki67 >10%) stratification [32]. However, the ACC guideline does not provide directions for a standardized pre-analytical process or scoring approach for Ki67. There is a need for new reliable and reproductive diagnostic tests that can add to current classification scores or even outperform them and correlate better with biological behavior. It is currently not possible to predict whether a patient is cured after complete surgery commonly resulting in the prescription of adjuvant mitotane although some patients may not benefit from it. Over 20% of patients with tumor stages I to III die within the first 3 years, opting for a more aggressive post-operative systemic treatment. Furthermore, long-term survival of patients with metastatic disease at diagnosis has been described [23]. It is relevant to understand which patient with stage IV disease at primary diagnosis should be offered a treatment strategy facilitating such a long-term survival, or for which patients therapy should be aimed at quality of life and comfort [22].

Genomic profiling approaches have proven to be able to differentiate between adenomas and carcinomas and also to differentiate between carcinomas with different clinical outcome [33,34]. De Reynies et al. specified a malignant molecular cluster C1A and C1B versus a benign molecular cluster C2. Tumors of the C1A group had a very poor outcome and were enriched in transcription and mitotic cell cycle genes, whereas the good prognosis malignant C1B group was enriched in cell metabolism, intracellular transport, apoptosis and cell differentiation genes [34].

Giordano et al. showed a cluster analysis of the ACCs which revealed two subtypes that reflected tumor proliferation, as measured by mitotic counts and cell cycle genes [33]. Such accurate distinction is essential since treatment is radically different between adenoma and carcinomas. We were unable to find a study that compared a genomic profile versus regular immunohistochemical classification to differentiate between adrenal adenoma and carcinoma, therefore it is not clear whether genomics is superior to pathology.

Giordano showed that pathologists accurately classify the large majority of ACTs using traditional clinicopathologic techniques, yet concluded that occasionally ACTs pose diagnostic challenges and would benefit from additional approaches and tools [35]. Lippert et al. found an improved prognostic stratification when implementing a modified Grading (G; ENSAT and Ki67), resection status (R), Age (A) and as tumor- or hormone-related symptoms (S) (mGRAS) system, by recognizing four ACC subgroups with a different clinical outcome by merging mGRAS and a molecular score (number of somatic mutations, alterations in the Wnt/b-catenin and p53/Rb pathways and promoter region methylation pattern) into a combined (COMBI) score. This molecular profile further improves the progression risk stratification identifying a group of patients with a favorable prognosis [36]. When superiority of COMBI with respect to mGRAS score was tested by discriminating patients with the best clinical outcome (at least 24 months free of disease progression).

COMBI score showed a better prognostic performance, proven by superior specificity (58.6% vs. 31.0%) and accuracy (83.3% vs. 74.5%). Also, when evaluating the disease-free survival (DFS) in a selected group of patients with ACC who were successfully operated (R0). In this subgroup, only COMBI score was able to identify a category of patients with an extremely longer DFS [36]. However, molecular profiling is not part of the European Guideline on ACC [32]. In addition, genomic studies lead to better understanding of tumor biology and hopefully could yield new insights to develop new therapies where current therapeutic options are limited, and available (chemo) therapies of limited effectiveness [37].

Over the last decade the understanding of adrenocortical tumorigenesis has improved and recently studies started focusing on epigenetic changes associated with adrenocortical tumors. Global DNA hypomethylation is a thoroughly studied example of an epigenetic alteration which is a hallmark of both benign and malignant tumors with unique methylation patterns [38]. Epigenetic alterations occur frequently in cancer cells and have the ability to mimic the effects of the latter [14,39,40]. Such epigenetic alterations are becoming increasingly accessible to analyze for an individual patient, and would be an interesting layer of additional molecular information to existing clinicopathological methods. Additionally, Feinberg (2018) suggested that differences in tumor types are related to the tissue of origin and often to the

spectrum of mutations associated with that organ, whereas properties of the tumor heterogeneity and therapeutic resistance are epigenetic and are shared among tumor types. Therefore, understanding epigenetic regulation in cancer in general could provide valuable information needed to improve therapeutic strategies [41].

2. Genome Wide DNA Methylation

Rechache et al. [42] were the first to report a genome-wide DNA methylation profiling study in adrenocortical tumors: 19 normal, 48 benign, eight primary malignant and 12 metastatic malignant ones. They found, using the Infinium HumanMethylation450 BeadChips (Illumina, San Diego, CA, USA), that methylation patterns were distinctly different between normal, benign, primary malignant and metastatic tissue samples. Differentially methylated sites were found in both coding and noncoding regions of DNA.

Interestingly, analysis of methylation patterns of benign adrenocortical tumor samples by functional status (cortisol secreting, aldosterone secreting, and nonfunctioning) showed different methylation patterns. Aldosterone-secreting tumor samples compared with nonfunctioning samples showed mostly hypomethylated CpG sites (75,3). There were only a small amount of differentially hypermethylated CpG sites between cortisol-secreting tumor samples compared with aldosterone-secreting tumor samples. No significant differences in the methylation pattern between cortisol secreting tumors and nonfunctioning tumor samples were found [42].This raises the question whether it is the methylation analysis that is unable to show difference between cortisol secreting tumors and nonfunctioning tumor samples or it is the clinical definition of 'cortisol secreting' and 'nonfunctioning'.

In cancer, hypomethylation usually occurs at repeated DNA sequences whereas hypermethylation predominantly involves CpG Islands [43]. This was also observed in ACC. Primary and metastatic ACC samples were globally hypomethylated compared to normal and benign samples. Hypermethylation in primary and metastatic ACC samples was predominantly seen in islands [42,44]. DNA methylation of the *H19* promoter has been shown to be involved in the abnormal expression of both *H19* and *IGF2* genes in the single gene study by Gao et al. (2002) [45]. Rechache et al. (2012) found 52 genes to be hypermethylated and downregulated in ACC (Table 2). Furthermore, of the differentially methylated genes in primary ACC, compared with benign tissue samples, several CpG sites were differentially methylated including those associated with *KCTD12*, *KRREL*, *SYNGR1*, and *NTNG2* and those in chromosome 11p15 imprinted region including *IGF2* and *H19*. Other sites were also in the *IGF2* pathway, including *IGF1R* that *IGF2* binds to and *AKT1*, a downstream signaling molecule in the cell survival pathway of *IGF1R*. *TP53* and *CTNBB1* both had hypomethylated sites, *RARRES2* and *SC16A9* had several hypermethylated sites in ACC tissue samples.

Table 2. Whole genome methylation studies on adrenocortical carcinoma.

Study	Country	Year	N	Population	19 NA; 47 Benign; 8 Primary malignant; 12 Metastatic malignant adrenals.
[42]	USA	2012	87	Method	Infinium HumanMethylation 450 BeadChips (Illumina, San Diego, CA, USA)
				Results	ACC show unique methylation patterns in which gene methylation status may be an important regulator of gene expression.
				Hypomethylated	TP53, β catenin (CTNNB1)
				Hypermethylated	↓ABCA1, CD55, CD74, COL4A3, GOS2, GATA6, **HSD3B2**, KCNQ1, MAP3K5, NCOA, RAPGEF4, RARRES2, S100A6, SPTBN1, TNFSF13, TNS1, ADCK3, ALDH3B1, CSDC2, CYP7B1, **GIPC2**, HOOK1, MEIS1, MLH3, MRPL33, NME5, RGNEF, TCIRG1, AMPD3, B4GALT6, CAB39L, GYPC, NDRG4, **RAB34**, RBPMS, SEMA6A, TNFS1F2-TNFSF13, SLC16A9, PHF11
				Diagnostic	'Determination of the methylation difference in certain probe sites in ACT may be a useful diagnostic adjunct to histopathology for localized primary ACC.'
				Prognostic	-
				Therapeutic	-
[46]	USA	2012	48	Population	6 NA; 27 ACA (9 Nonfunctional,9 Cortisol producing, 9 Aldosterone producing); 15 ACC (9 Nonfunctional,6 Cortisol producing)
				Method	Infinium HumanMethylation27 Beadchip (Illumina, San Diego, CA)
				Results	CpG islands in the promoter regions are significantly hypermethylated in ACC.
				Hypomethylated	
				Hypermethylated	ZNF154, ALX4, ↓CDKN2A, GATA4, SCGB3A1/HIN1, PYCARRD, HDAC10 and DLEC1
				Diagnostic	-
				Prognostic	-
				Therapeutic	Treatment of ACC cell line H295R with 5-aza-2'-deoxytide showed significant restoration of gene expression of CDKN2A, GATA4, DLEC1, HDAC10, PYCARD and SCGB3A1/HIN1.

Study	Country	Year	N	Population	19 NA; 47 Benign; 8 Primary malignant; 12 Metastatic malignant adrenals.
[47]	France	2013	135	Population	84 ACA; 51 ACC
				Method	Infinium HumanMethylation27 Beadchip (Illumina, San Diego, CA) MS-MLPA
				Results	ACC samples can be categorized according to CpG island methylator phenotype.
				Hypomethylated	
				Hypermethylated	↓H19, GSTM1, GSTP1, G0S2, GSTT1, **RAB34**, GYPC, **GIPC2**, PLAGL1, LY6D, PCOLCE, NDN, AMT, LGALS3BP, APOC1, TM7SF2, PPAPDC3, PTPN7, SCNN1A, **HSD3B2**, ACAA2, CTSZ, PYGM, KRT8, NDRG2
				Diagnostic	-
				Prognostic	The global level of methylation in CpG islands was associated with survival. CIMP carcinomas were associated with poorer prognosis.
				Therapeutic	-
[48]	France/ Europe (ENSAT)	2014	81**	Population	51 ACC; 30 ACA
				Method	Infinium HumanMethylation27 Beadchip (Illumina, San Diego, CA)
				Results	Confirmed CIMP in ACC. Tumor clusters based on different genomic approaches correlate.
				Hypomethylated	Nfs
				Hypermethylated	Nfs
				Diagnostic	-
				Prognostic	Transcriptome clusters were strongly correlated with DNA methylation clusters. The C1A subgroup with poor prognosis included almost all CIMP and Mi3 tumors. C1B tumors with good prognosis were generally non-CIMP and belonged to the Mi1 or Mi2 miRNA cluster.
				Therapeutic	-

Study	Country	Year	N	Population	19 NA; 47 Benign; 8 Primary malignant; 12 Metastatic malignant adrenals.
[49]	USA	2015	116	Population	20 ACC; 75 Benign, 21NA
				Method	Infinium HumanMethylation 450 BeadChips (Illumina, San Diego, CA)
				Results	A cumulative comparison among gene methylation, copy number and miRNA profiling found that oncostatin M signaling, retinoic acid receptor activation (RXR) and PI3K/AKT and CDC42 signaling pathways were among the top pathways altered in ACC.
				Hypomethylated	
				Hypermethylated	TIPARP, RAPGEF4, RAB34, PPTC7, PDZRN3, OBSL1, NCEH1, MTMR6, METTL7A, LONRF2, LIMCH1, KLF9, KIAA1024, JAK1, ITGAV, ITGA2, **HSD3B2**, HLA-DPB1, DDC2, FOSL2, FGF12, FAMI198B, CYP1B1, CLU, CD59, CD55, C1QB, B4GALT6, IL13RA2, CDK1, ZMIZ1, TNS1, TBC1D4, SPTBN1, SLC16A9, SKAP2, SEMA6A, S100A6, RBPMS, RARRES2, RAB8B, PTPRG, PPP1R14A, NCOA7, MEIS1, MAP3K5, KCTD12, IL6ST, HTR2B, HOXA5, **GIPC2**, GATA6, G0S2, FSTL1, FMNL2, DDAH1, CD9, CD74, CD14, C3, C1RL, BNIP3L, AS3MT, APOC1, ABCA1, LPPR1, C9orf84
				Diagnostic	-
				Prognostic	-
				Therapeutic	Treatment of the ACC cell line, H295R, with decitabine (a global methylation inhibitor) increased the gene expression of CYP1B1 dramatically. It was found that oncostatin M inhibits ACC cell proliferation. Oncostatin M could inhibit ACC cell growth

Study	Country	Year	N	Population	19 NA; 47 Benign; 8 Primary malignant; 12 Metastatic malignant adrenals.
[44]	USA	2016	24	**Population**	18 ACC (17 adrenal carcinomas, 1liver metastasis); 6 NA
				Method	Infinium HumanMethylation 450 BeadChips (Illumina, San Diego, CA)
				Results	It was demonstrated that ACC are globally hypomethylated compared to normal adrenal tissue. Hypomethylation was most frequent in 'open seas' and hypermethylation mostly in CpG islands. Epigenetic modulation of genes involved in TP53 stability and function, WNT signaling, and tumor suppressor genes were found.
				Hypomethylated	TMEM132D, ADCY2
				Hypermethylated	i.a. EPHX3, MEIS, CCDC8, TBX3, PAX8, DUSP7, DYRK2, RBM5, SETD7, NDRG1, UBE2D1
				Diagnostic	-
				Prognostic	-
				Therapeutic	-
[17]	USA	2016	79***	**Population**	91 adrenal tumors: 84 usual type, 4 oncocytic, 2 sarcomatoid and 1 myxoid variant.
				Method	Infinium HumanMethylation 450 BeadChips (Illumina, San Diego, CA)
				Results	Identified three Coc subtypes
				Hypomethylated	Nfs
				Hypermethylated	CDKN2A; Nfs
				Diagnostic	A methylation signature consisting of 68 probes robustly classified their cohort into three ACC survival groups with 92,4% accuracy.
				Prognostic	Coc analysis showed that molecular data can determine outcome with high significance.
				Therapeutic	-

Underlined results show overlap between two studies/Genes in **bold** were found to be hypermethylated in multiple studies. ↓ Under expressed genes. Adrenocortical Adenoma (ACA); Adrenocortical Carcinoma (ACC); Adrenocortical Tumors (ACT); CpG island promoter methylation (CIMP); Clusters of Cluster (Coc); European Network for the study of Adrenal Tumors (ENSAT); Normal adrenal (NA); Not further specified (nfs) ** [48] studied a total of 130 ACCs: 53 ACCs in their discovery cohort and 77 ACCs in their validation cohort. Only 51 samples from the discovery cohort were analyzed for DNA methylation profiling. *** [17] only analyzed 79 samples for DNA methylation profiling.

Genetic studies have found IGF2 overexpression and *CDKN1C* and *H19* downregulation in 90% of ACC cases [33,50]. In pediatric ACC, *IGF1R* overexpression is associated with a worse prognosis. Genetic mutations of the β-catenin gene are common in preferentially non-functioning adenomas and in ACC [15,51].

Fonseca et al. [46] also analyzed the genome-wide methylation pattern of normal, benign and malignant adrenocortical tumors (Table 2). When comparing benign versus malignant ACT's, they found that CpG islands were identified as significantly hypermethylated in ACC.

Primarily, genes involved in regulation of apoptosis, transcriptional, and cell cycle control showed significant and frequent hypermethylation. Only six genes known to be involved in the pathogenesis of other malignancies were further analyzed at mRNA level.

Expression of the significantly hypermethylated genes *CDKN2A*, *GATA4*, *DLEC1*, *HDAC10*, *PYCARD*, and *SCB3A1/HIN1* was reduced in both ACAs and ACCs compared to normal adrenal tissue. When treating the H295R ACC cell line in vitro with a demethylating agent (5-aza-2'-deoxycytidine), expression of all hypermethylated genes increased.

This was observed earlier in the study by Gao et al. in which *H19*, hypermethylated expression increased after treating the H295R ACC cell line with a demethylating agent [45]. These results suggest that epigenetic alterations could be reversible and a potential therapeutic target [49].

3. Candidate Gene Approach (Table 3)

Since *IGF2* is the most frequently overexpressed gene in ACC it is not surprising that single gene studies have focused on *IGF2*. Both Nielsen and Creemers studied differentially methylated regions patterns of *IGF2*. Three differentially methylated regions (DMRs) are involved in the regulation of *IGF2* expression. DMR0, DMR2 and the imprinting control region (ICR). *IGF2* DMR0 and DMR2 are located between exons 2 and 3 and exons 8 and 9 respectively.

Table 3. Single gene methylation studies on adrenocortical carcinoma.

Study	Country	Year	N	Population	16 NA; 10 ACC (2 Virilizing, 2 Nonfunctional, 6 Cushing's); 16 ACA (2 Virilizing, 5 Cushing's, 5 Conn's)
[45]	Finland	2002	46	Gene	H19
				Method	Bisulfite-PCR
				Results	CpG sites in the H19 promoter are hypermethylated in ACC. IGFII is over expressed (methylation of IGFII not analyzed)
				Hypermethylated	H19
				Diagnostic	-
				Prognostic	-
				Therapeutic	ACC cell line NCIH295R was treated with Azad, a demethylating agent. It induced an increase in the H19 RNA content.
				Population	7 ACC; 8 ACA; 6 NA
[52]	USA	2013	21	Gene	RASSF1
				Method	Epitect methyl II PCR
				Results	There is a potential oncosuppressor role for RASFF1 in adrenocortical carcinogenesis.
				Hypermethylated	RASSF1
				Diagnostic	-
				Prognostic	All ACC showed reduced expression of RASSf1A, irrespective of their clinical characteristics or malignant stages.
				Therapeutic	-
				Population	39 ACA (16 Nonfunctional, 16 Aldosterone producing, Cortisol producing); 3 ACC; 23 NA
[53]	Japan	2014	65	Gene	Wif-1
				Method	MSP, USP & Bisulfite-PCR
				Results	57,1% of the adrenal tumours were found to be positive for Wif-1 methylation. No sub analysis specific for ACC.
				Hypermethylated	Wif-1
				Diagnostic	-
				Prognostic	-

Study	Country	Year	N	Population	16 NA; 10 ACC (2 Virilizing, 2 Nonfunctional, 6 Cushing's); 16 ACA (2 Virilizing, 5 Cushing's, 5 Conn's)
				Therapeutic	-
				Population	3 NA; 19 ACC
[54]	Netherlands	2014	22	Gene	INHA
				Method	Bisulfite-PCR
				Results	A subset of ACCs has an increased methylation ratio of several CpGs in the INHA promoter.
				Hypermethylated	INHA
				Diagnostic	-
				Prognostic	No association with van Slooten index or ENSAT stage.
				Therapeutic	-
				Population	12 Conn's; 10 Pheochromocytoma; 20 ACA, 20 ACC
[55]	France	2015	62	Gene	IGF2
				Method	Pyro-sequencing Bisulfite-PCR
				Results	IGF2 overexpressed in 85% of ACCs and 100% of PCC. Significant decreased expression of H19 in ACCs. 15/19 ACCs had somatic copy number alterations at the IGF2/H19 locus, with 6/15 having an extra copy of the allele.
				Hypomethylated	IGF2-DMR2
				Hypermethylated	H19-ICR (CTCF2, CTCF3, CTCF6); 3 CPGs of DMR0
				Diagnostic	3 CPGs of DRM0 correlated positively with the Weiss score.
				Prognostic	Expression levels of IGF2 did not correlate with clinical parameters such as presence of metastases or TNM stage. The presence of more paternal alleles than maternal alleles was significantly associated with the presence of metastases.

Study	Country	Year	N	Population	16 NA; 10 ACC (2 Virilizing, 2 Nonfunctional, 6 Cushing's); 16 ACA (2 Virilizing, 5 Cushing's, 5 Conn's)
				Therapeutic	-
[56]	Italy	2015	26	**Population**	3 NA; 15 ACA (3 Nonfunctional, 10 Aldosterone producing, 2 Cortisol producing); 8 ACC
				Gene	VDR
				Method	Bisulfite-PCR
				Results	Methylation in the VDR promoter was observed in 3/8 ACCs. Methylation sites were identical in all 3 ACCs. No VDR promoter methylation was found in the other 5 ACCs, 3 NAs and 15 ACAs.
				Hypomethylated	Nfs
				Hypermethylated	Nfs
				Diagnostic	-
				Prognostic	-
				Therapeutic	VDR promoter methylation is mentioned as potential drug target in ACC.
[57]	Netherlands	2016	49 + 22	**Population**	Cohort (*n* = 49): 24 ACC; 14 ACA; 11 NA Validation cohort (*n* = 22): 9 ACC; 13 ACA
				Gene	IGF2
				Method	Pyro-sequencing Bisulfite-PCR
				Results	DMR0, DMR2 no significant differences between ACC and ACA. CTCF3, CTCF6 and H19 hypermethylated.
				Hypermethylated	CTCF3, CTCF6, H19

Study	Country	Year	N	Population	16 NA; 10 ACC (2 Virilizing, 2 Nonfunctional, 6 Cushing's); 16 ACA (2 Virilizing, 5 Cushing's, 5 Conn's)
				Diagnostic	IGF2 expression, DMR2, CTCF3 and H19 showed a significant predictive value for the diagnosis of ACC.
				Prognostic	-
				Therapeutic	Treatment of three human ACC cell lines (H295R, HAC15 and SW13) with the demethylating drug AZA significantly decreased IGF2 expression and increased H19 expression.

Adrenocortical carcinoma (ACC); Differentially methylated regions (DMR); Imprinting Control Region (ICR); Methylation-specific PCR (MSP); Normal adrenals (NA) Pheochromocytoma (PCC); Not further specified (Nfs); Unmethylation-specific PCR (USP).

The *H19* and *IGF2* genes are separated from each other by the ICR. Nielsen found that 85% of the ACCs showed *IGF2* overexpression and *H19* down regulation, but did not found a correlation with clinical parameters such as the presence of metastases or TNM stage. The *H19* DMR is located upstream of the *H19* transcription start site. It harbors seven binding sites for the methylation-sensitive insulator CCCTC-binding factor (CTCF).

Methylation status of the ICR is of direct influence on CTCF binding. CTCF only binds unmethylated ICR on the maternal chromosome. Upon binding, CTCF prevents communication between the proximal *H19* enhancer and the *IGF2* promoter, therefore keeping *IGF2* inactivated. CTCF cannot bind the paternal chromosome because the ICR is methylated. The enhancer is able to active *IGF2* transcription from the paternal chromosome. CTCF serves as a position-dependent insulator element to block inappropriate enhancer signals and protect against forged gene activation [58]. Creemers et al. (2016) found a significant change in methylation in CTCF3 and CTCF6 between ACC and ACA, where methylation in ACCs was higher [57]. Also, *H19* and *IGF2* showed significant hypermethylation in ACC [57].

Recently an increased interest has been raised for the relation between vitamin D and the adrenal gland [59]. Pilon et al. (2015) found methylation of the promoter of the vitamin D receptor and a reduced expression of the vitamin D receptor in ACC [56].

Earlier, inhibin alpha-subunit (INHA) was found to have a tumor suppressive role in adrenocortical tumorigenesis. In INHA knockout mice, 99% developed steroid-secreting ACCs after gonadectomy [60,61]. Hofland et al. (2014) investigated the methylation and expression of Inhibin α-subunit (encoded by INHA) in adrenal tumors. They found a significant difference in methylation of the INHA promotor between normal adrenals and ACCs. However, the promoter methylation in the ACC samples was not associated with tumor characteristics or ENSAT stage.

4. CIMP

It was in a study for colorectal cancer that the CpG island promoter methylation, or CIMP, was first discovered [62]. The term CIMP is controversial [63] since there is actually no universal standard or consensus with respect to defining CIMP. It is used to describe the increased prevalence of CpG island promoter methylation. So far this phenomenon has been described in multiple types of cancer: bladder, breast, endometrial, gastric, glioblastoma, hepatocellular, lung, ovarian, pancreatic, prostate, and renal cell cancers as well as in leukemia, melanoma and neuroblastoma. In 2012 Barreau et al. [47] analyzed CIMP in adrenocortical cancer. Tumor material of 135 patients with adrenal tumors was collected and clinical outcome was registered. ACCs were globally more hypermethylated than ACAs at the CpG islands in the promoter regions.

ACCs were clustered in two groups with different methylation levels. The first group carcinomas was slightly hypermethylated compared with adenomas, the second group was hypermethylated compared with both the adenomas and the carcinomas from the first group. The second group was again subdivided according to methylation level: a CIMP-high subgroup and a CIMP-low subgroup. The level of methylation was associated with survival and CIMP carcinomas show a worse prognosis compared to non-CIMP tumors.

Gene expression levels were increasingly down-regulated when comparing non-CIMP, CIMP-low and CIMP high carcinomas. These data suggest that differential methylation in the CpG promoter regions could be of clinical importance since they provide a classification based on methylation as a marker for prognosis in patients with adrenal tumors.

Assié et al. (2014) [48] analyzed a cohort of 47 ACCs and an independent validation cohort of 77 ACCs, recruited from the European Network for the Study of Adrenal Tumors centers. Four DNA methylation-based tumor clusters were found. Two clusters corresponded to the CIMP-high and CIMP-low as described by Barreau et al. [47] associated with poor prognosis. Two other groups were categorized non-CIMP of which one showed widespread hypomethylation of CpG sites located outside CpG islands.

miRNA expression was assayed and showed MIR483 to be overexpressed in ACC. MIR483 is located on the IGF2 locus, known to be involved in ACC. Based on miRNA expression levels Assié et al. (2014) identified three stable tumor clusters, Mi1-Mi3, with Mi1 having a significantly better overall survival rate then Mi2 and Mi3. Clusters were also established for mRNA expression in which two profiles were confirmed [34] to correlate strongly with survival, the aggressive C1A and indolent C1B. More importantly, a substantial overlap was found between the different omics of classifications. C1A (gene expression) with poor prognosis include almost all CIMP (DNA methylation) and Mi3 (miRNA expression) tumors. C1B tumors with a good prognosis were generally non-CIMP and belonged to the Mi1 or Mi2 mRNA clusters.

The Cancer Genome Atlas (TCGA), an unique landmark cancer genomics program, began in 2006 and since then molecularly characterized over 20,000 primary cancer and matched normal samples spanning 33 cancer types (PubMed searched on 1 April 2020: 8422 citations of TCGA {search: "tcga"[Text Word]}. In 2016 Zheng et al. [17] analyzed 91 histologically confirmed adrenal tumors and matched it with tissue from a global cohort including 84 usual type, four oncoytic, two sarcomatoid, and one myxoid variant. Their pan-genomic approach yielded four mRNA-expression groups, six microRNA-expression groups, three copy-number groups, three protein-expression groups and three DNA-methylation groups. They integrated these ACC subsets through a cluster of clusters (CoC) analyses, resulting in three CoC subtypes, recognizing the fact that implementing four parallel profiling platforms poses a clinical challenge. The three methylation subtypes found (CIMP- low, intermediate and high) rendered discriminative representations of each CoC group, and classified the cohort into three ACC survival groups with 92.4% accuracy and were validated with an independent cohort.

Furthermore, it was shown that collectively the genes altered most frequently by somatic mutations, DNA copy-number alterations and epigenetic silencing were TP53 (21%), ZNRF3 (19%), CDKN2A (15%), CTNNB1 (16%), *TERT* (14%) and *PRKAR1A* (11%) [17].

5. Histone Modification

Histone modification is involved in the regulation of chromatin and gene expression. The best studied modifications are the acetylation and methylation of histones. Acetylation and deacetylation of histones is performed by histone modifying proteins such as histone acetyltransferases (HATs) and histone deacetylases (HDACs). At the transcriptional level, histone methylation is defined as the transfer of one, two, or three methyl groups from S-adenosyl-L-methionine to lysine or arginine residues of histone proteins by histone methyltransferases (HMTs). HMTs regulate DNA methylation through chromatin-dependent transcriptional repression or activation [64,65].

Drelon et al. performed a screen of histone methyltransferases, demethylases and associated factors in publicly available transcriptome data from ACC patients [66]. They observed the histone methyltransferase EZH2 to be overexpressed in ACC. High EZH2 expression levels, a result of deregulated P53/RB/E2f pathway, were associated increased cell proliferation/aggressive tumor behavior and poor prognosis in their study.

Zheng et al. reported that 22% of their analyzed samples had dysregulated mRNA expression levels of histone modification genes *MLL*, *MLL2*, and *MLL4* and chromatin remodeling genes *ATRX* and *DAXX* [17]. Interestingly, seven percent of ACC cases have mutation in gene *MEN1*. *MEN1* encodes the tumour suppressor, menin, which has been reported to interact with HMTs *MLL*, *MLL2* [17,48].

6. Epigenetics and ACC Treatment

Genetic studies aimed at targeting biological pathways have not yet resulted in a significant breakthrough regarding therapeutic options. Inhibitors of both *IG2/IGF1R* and the mTOR pathways cause inhibition of cell proliferation of human ACC cell lines in vitro, and of growth of tumor xenografts in vivo [67,68]. A dual inhibitor of both *IGF-1R* and *IR*, linsitinib (OSI-906), was studied for the first time in humans in an open-label phase I study of 79 patients with advanced solid tumors, of which 15 patients had ACC. Although efficacy was not the primary end point of the study, two patients with ACC had partial responses [69]. Therefore, linsitinib versus placebo was studied in a double-blind placebo-controlled phase III trial. Unfortunately, linsitinib failed to show a difference in median overall survival (OS) or progression free survival (PFS) [70].

Also, everolimus, an mTOR inhibitor, showed no clinically meaningful response in patients with stage IV ACC [71]. Other attempts targeting VEGF and EGFR have also met with modest success [72,73]. Studies suggest that DNA methylation, in addition to genetic modifications causes altered patterns of gene expression resulting in tumorigenesis and harvest potential therapeutic markers [12,74,75].

A role for temozolomide (TMZ), a cytotoxic and antiproliferative agent, has been proposed in the treatment for ACC, which is thought to act primarily by alkylation of specific sites on especially the O^6 position of guanine, which mispairs with thymine during the next DNA replication cycle [76]. The methyl group in O6-methylguanine can be removed by the O6-methylguanine-DNA methyltransferase (MGMT) gene, which leads to and impaired efficacy of TMZ. Epigenetic marks regulating MGMT expression are used as a predictive marker for response to TMZ in glioblastoma patients, since epigenetic silencing of MGMT sensitizes glioblastoma cells to TMZ [77]. Creemers et al. showed that ACC cell lines appear to have a low MGMT promoter methylation and observed a trend toward a slightly higher MGMT methylation in the responsive primary ACC

cultures [76]. Curiously, overexpression of PBK/TOPK promotes the chemotherapeutic resistance to TMZ in glioma [78]. Both low MGMT promoter methylations as well as PBK overexpression could contribute to the observed short-lived control rate and poor prognosis in a clinical study with TMZ in 28 ACC patients [79].

Epigenetic targeted drug reports are still limited to in vitro ACC cell line studies. The study by Gao et al. was mentioned earlier: they treated H295R with Azad (also known as 5-aza/decitabine), a demethylating agent (5-aza-2'-deoxycytidine), which led to a significant increase in the H19 RNA content [45]. Decitabine is a drug which is currently approved by the Food and Drug Administration (FDA) for the treatment of myelodysplastic syndromes. It reverses the DNA promoter methylation.

Suh et al. also tested decitabine NCI-H295R cells. They observed a significant decrease in ACC cell proliferation by 39% to 47% at 5 days after treatment compared with control specimens ($p < .001$) [74]. Interestingly, decitabine has been shown to potentiate the cytotoxic effects of current chemotherapies, such as doxorubicin, cisplatin and etoposide, in neuroblastoma, suggesting that a combination of 5-aza with standard therapies could lead to more effective treatment [80].

Vorinostat was one of the first drugs to be approved that influence post-translational modification of histone proteins. Demeure et al. tested vorinostat in an ACC cell line which resulted growth inhibition. No studies on HMT inhibition were found. EHZ2 being overexpressed in ACC, HMT inhibition could be a potential treatment strategy in ACC.

Further research is required to determine the role of epigenetic targeted drugs in the treatment of ACC and overcoming drug resistance, where in other types of cancer epigenetic therapies are an emerging option for overcoming drug resistance [81].

Pan-genomic studies will initially contribute to the process of matching clinical/molecular profiles of patients with ACC with specific therapeutic programs and the understanding of therapeutic failures in the past. As Grisanti et al. noted, the COC1 cluster identified by Zheng et al. is characterized by a low grade of aneuploidy, better survival outcome, and high expression of the IGF2 pathway [17,82]. It could be considered that patients falling in this category would likely be more responsive to antieIGF2/IGF-1R compounds. Whereas, patients in the COC3 group characterized by mutations involving the cell cycle and DNA damage repair machinery would probably better respond to chemotherapy. Eventually the focus will shift to the understanding and identification of cancer dependencies based on functional genomic data and selection of priority drug candidates/drug repositioning in ACC.

Actually, Zheng et al. found 51 potentially actionable alterations in 22 ACCs, considering existing clinical trials and FDA-approved drugs for cancers [17]. Recently two excellent reviews have been published on this issue [75,83] which we encourage interested readers to consult.

7. Discussion

In cancer in general, there has been growing evidence to suggest that DNA methylation in addition to direct genetic modification causes altered gene expression resulting in tumorigenesis. Epigenetic analysis in adrenocortical tumors so far has been a significant addition to the understanding of molecular events involved in adrenocortical carcinogenesis. DNA methylation- based tumor clusters show overlap with other omics classifications. Clustering on epigenetic level allows differentiation between benign and malignant tumors and could be a significant addition to current histological parameters. Moreover, it might serve as an addition to current ENSAT staging in order to estimate prognosis and tumor aggressiveness. Currently no biomarker is included in the European Society of Endocrinology Clinical Practice Guidelines on ACC, but it is stated in the guideline that patients' participation in registries and the collection of biological material as part of structured research programs aimed at defining biomarkers of diagnosis, prognosis and treatment response is encouraged [32].

An interesting observation in the studies discussed above is the comparison between genetic and transcriptome-based studies (Figure 2). *IGF2* overexpression and structural abnormalities of 11p15 are present in up to 90% of cases of human ACC. *IGF2* expression is mediated by the insulin-like growth factor 1 receptor (IGF1R) which is also overexpressed in ACC. These genes have altered DNA methylation expression patterns in ACC. Zheng et al. found that 69% of tumours had at least one alteration of potential driver genes when combining somatic mutations, copy-number alterations, and epigenetic modification [17].

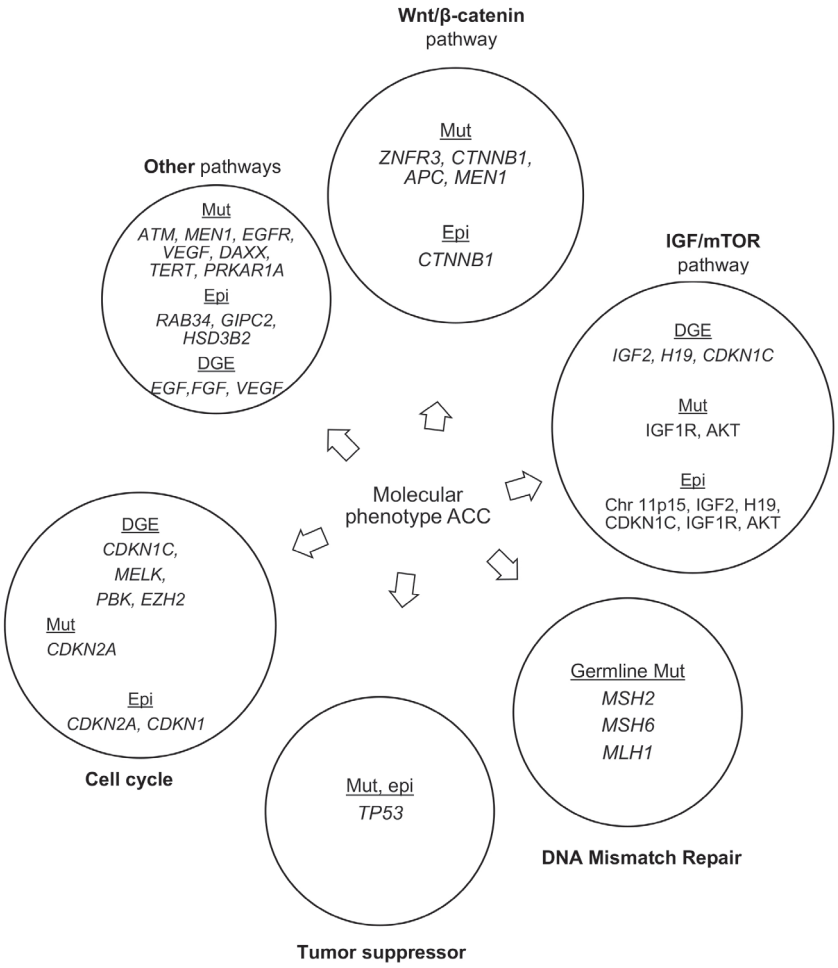

Figure 2. Frequently altered pathways in adrenocortical carcinoma as discussed in this review. Mut: mutations; DGE: differential gene expression; Epi: epigenetic modifications.

Further research is needed to understand the implications of epigenetic changes in adrenal tumorigenesis. Comprehensive data on well powered series are needed. For an orphan disease like ACC, united multinational consortia studies have the best chance of providing well powered data.

Results of methylation associated gene expression levels in the articles discussed show heterogeneity (Table 2). For example, the 52 genes identified by Rechache et al. do not completely overlap with the top genes identified by Barreau et al. This discrepancy may be caused by methodological differences. Rechache et al. [42] used

the 450 BeadChip, which provides a more comprehensive coverage of the genome with 17 times more CpG sites than the 27BeadChip that was used in the other studies. Nevertheless, when using Infinium 450k microarrays, data still is restricted to the particular genomic locations of the probes used in the array, which might not even necessarily capture the most relevant methylation sites. Koch et al. already advocated that a better understanding and more detailed analysis of the clinical relevance of the genomic location of DNA methylation is required to increase the number biomarkers that can be successfully implemented in patient care [84].

Also, it should be decided which platform of genomic analysis will be used in daily practice. Because, effectively, what is probably needed is a multi-omic molecular panel with the best selected biomarker predictors.

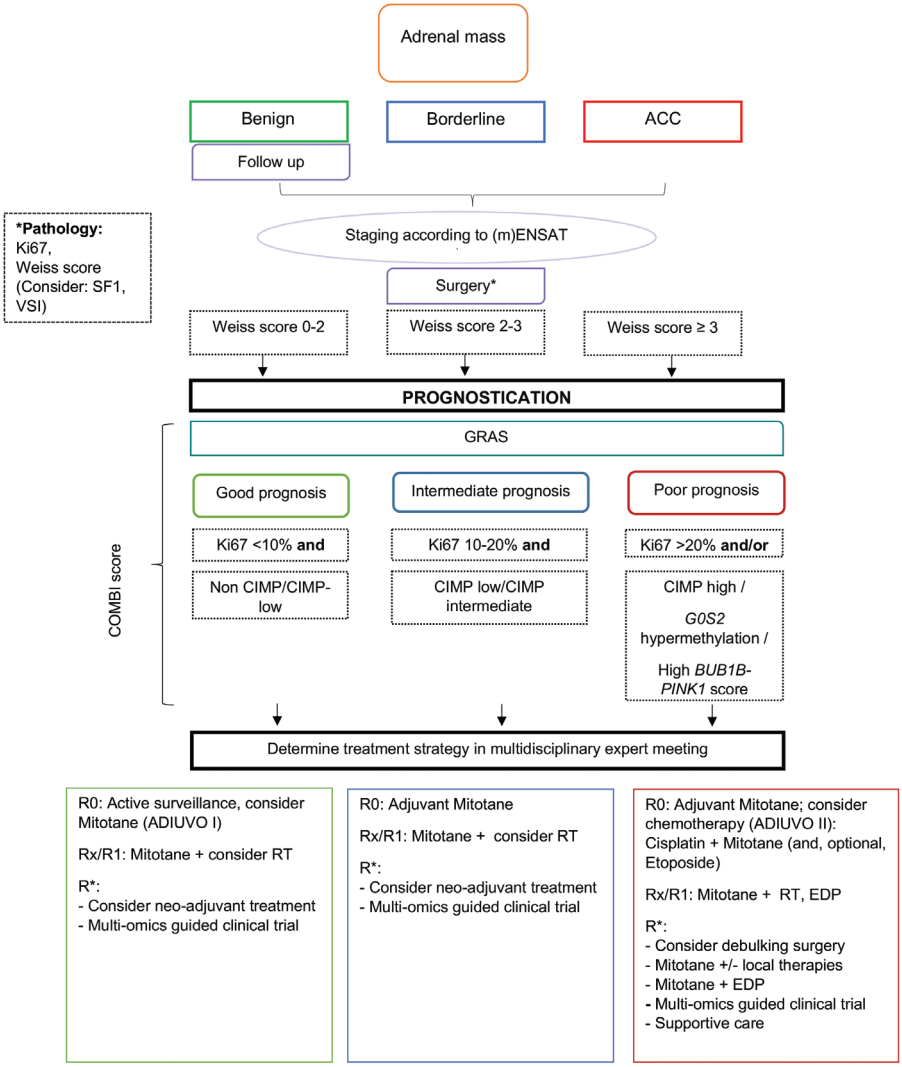

Figure 3. Flowchart on the potential management of the adrenal mass with the implementation of genomic analysis. Adrenocortical Carcinoma (ACC); Etoposide doxorubicin cisplatin (EDP); (modified) European Network for the Study of Adrenal Tumors ((m)ENSAT); Grade, Resection status, Age, Symptoms (GRAS); Complete resection (R0); Unknown radicality (Rx); Microscopically irradical (R1); ACC not amendable to radical resection (R*); Radiotherapy (RT); Steroidogenic factor-1 (SF1); Van Slooten Index (VSI).

DNA-methylation has proven to be replicable and able to provide accurate data on formalin-fixed or paraffin-embedded tumor samples [85,86] but no data has been published comparing the available genomic cluster entities in their ability to correctly diagnose adrenal malignancy and to predict recurrence, progression free survival and overall survival. Currently have been opted: C1A/C1B cluster [33,34,48] based on gene expressions, CIMP low/intermediate/high [17] cluster or CIMP low/high and non-CIMP [48] based on methylation profiles, CoC I-III as an integrated subset based on DNA copy number, DNA-methylation, mRNA-expression and miRNA-expression and Mi1-2 based on MiRNA expression. It is of importance to see how these clusters will perform when tested prospectively in a large cohort of adrenal tumors to validate these data and also establish the required cutoff values for the diagnosis of malignancy. Within these discriminating clusters, studies already are making an effort to identify markers representing these cluster sub-types. *G0S2* hypermethylation was shown to be a hallmark of the CIMP-high cluster [87]. When validated *G0S2* hypermethylation and the *BUB1B-PINK1* score could be potential markers on a molecular panel for ACC [34,87,88].

Next steps will include the prospective comparison of the pathologic classification (Ki67 and Weiss score) of adrenal tumors versus a genomic assay versus the combination of both in the process of accurately diagnosing adrenal tumors. Evidence is needed that molecular data can improve the current diagnostic tools and that it does not matter whether it is genetic or epigenetic data. Finally, the bold step needs to be made to test the predictive value of these classifications in clinical practice by choosing a treatment regimen (Figure 3) based on the ACC prognostic cluster. Creemers et al. already showed that including *IGF2* methylation status to the pathology review could be supportive for the decision of adjuvant mitotane treatment [57]. Only after these steps, biomarkers could be officially implemented in the guidelines and acquire FDA approval.

Epigenetic changes may contribute to adrenocortical tumorigenesis by modulating size of the stem/progenitor population, altering phenotypic plasticity and enhancing sensitivity to subsequent mutations. ACC may develop in a multistep process. Therefore, it could be suggested that the level of DNA methylation is correlated with the risk of subsequent mutations, in which quantifying the influence of DNA methylation on gene expression remains difficult. Mutations in the *Wnt/β catenin* pathway have been shown to occur during progression [16,89]. Epigenetic and genetic mutations reflect alternate ways of inactivation during tumor progressions, i.e. a synergy between epigenetic and genetic alterations causing tumorigenesis, suggesting that combined inhibition of multiple affected pathways may hold the key to successful targeted therapy for ACC.

8. Conclusions

Research on adrenocortical tumors has been dominated by gene expression profiling and by analysis of genetic disorders associated with the predisposition of these tumors. With epigenetic studies, we are entering a new and complex phase in the understanding of ACC tumorigenesis. Analyzing the relationship between alterations in different layers of gene regulation could yield interesting insights.

Finally, it will be challenging to not only use epigenetic analysis for diagnostic and prognostic purposes but also to keep investing in the development of new pharmacologic therapies and explore the potential of demethylating agents, because currently no significant therapeutic breakthrough is emerging. In the near future it will become interesting to see how the vast development of artificial intelligence, radiomics etc. will be of impact on diagnosis, prognosis and treatment of ACC.

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Part III

Elaboration

Chapter 07

General discussion and future perspectives

General Discussion

This thesis focuses on prediction. The prediction of the pathologic behavior and clinical outcome of ACC, the treatment strategy of mitotane as cornerstone in the management of ACC and the future of treating ACC. In this chapter the main findings of this thesis are discussed in the light of current literature. Furthermore, strategies and topics for future research are explored (Table Future directions in ACC).

Predicting Clinical Course in Adrenocortical Carcinoma

Adrenocortical carcinoma prognosis is grim and varies widely. It ranges from tumors curable by complete surgery to those that are resectable but with high recurrence rate, or unresectable, fast growing and with an aggressive potential for metastatic spread. Reliable prognostication in ACC is critical to patients and treating physicians for making decisions regarding (adjuvant) treatment, type, and frequency of follow-up, as well as for providing patients with helpful information about short-term and long-term outcomes and to make a shared decision concerning treatment modalities.

Current prognostic factors include tumor stage, best assessed by the European Network for the Study of Adrenal Tumors (ENSAT) and tumor proliferation index measured by Ki67 immunostaining. Other commonly reported prognostic factors include cortisol secretion and age.

In **chapter 2** it is shown that synchronous metastases of ACC are associated with a worse prognosis compared to metachronous metastases of ACC and patients with late synchronous metastases have a better prognosis than those with metastases at initial diagnosis. Furthermore, number of affected organs and number of metastases are associated with poor prognosis. Interestingly, it is shown that adrenalectomy had a strong positive influence on survival, even in the presence of metastases. This result should be interpreted cautiously, because it could be hypothesized that patients with stage IV amendable to surgery are in a condition that allows an operative intervention and therefore have better overall survival compared to other patients with advanced disease not amendable for surgery. However, positive results on surgical management in advanced disease has been observed in other studies (1-4). Current guideline suggest against the routine use of adrenal surgery in case of widespread metastatic disease. Still, the guideline states that in selected cases (e.g. patients with severe hormone excess) debulking surgery might be an option, taking anti-hormonal drugs into account, reasoning that surgery might be appropriate if >80% of the tumor burden can be removed safely (5).

In **chapter 3** a prediction model is presented capable of predicting ACC-specific mortality which included age, modified European Network for the Study of Adrenal Tumors (mENSAT) stage, and radical resection.

Over the years many attempts have been made to identify clinical parameters and pathological index/scores, for the prediction of clinical outcome in ACC, but accurate prognostication remains a challenge. It seems that for stage I-II risk of recurrence after resection is the most important question whereas for stage III and IV survival is more relevant.

ENSAT stage unmistakably holds up in many studies as predictor of survival (see appendix 3 supplementary data to Fassnacht et al. 2018©, European Society of Endocrinology Clinical Practice Guidelines on the Management of Adrenocortical Carcinoma in Adults) (5-7). In **chapter 2** we found that spread to lymph nodes affects survival to the same extent as hematogenous metastasis. Other studies have found similar negative effects of nodal metastasis on survival (8,9). Libé et al. refined the ENSAT stage and suggested mENSAT. In this new classification, the N1 status shifted from ENSAT stage III to the mENSAT stage IV category, with Stage III defined as T3-4N0M0 and stage IVa, IVb, IVc according to the number of tumor-involved organs (including the primary tumor and the 'N' as 'organ'): two, three or more than three, respectively(10). Increasing evidence suggested beneficial prognostic impact of a prophylactic loco regional lymphadenectomy during primary tumor resection, where it has been found to improve tumor staging and survival outcome in patients with localized ACC(11,12). It was even added to the European guidelines on the management of ACC (2018): routine loco-regional lymphadenectomy should be performed with adrenalectomy for highly suspected or proven ACC, including (as a minimum) the periadrenal and renal hilum nodes. Furthermore, it is advised that all suspicious or enlarged lymph nodes identified on preoperative imaging or intraoperatively should be resected (5,9,11,13). The importance of surgery in the management of ACC is also illustrated by its impact on oligometastatic disease. In **chapter 2** we noticed a positive effect of a metastasectomy in patients with limited spread of disease to the lungs, on survival. Other studies have found similar results (2,14,15), endorsing prospective studies on the role of oligo-metastasectomy in ACC in well-defined study and control groups.

In **chapter 3** it is shown that the mENSAT performed slightly better than ENSAT in a clinical prediction model. Other modifications of the ENSAT have been proposed. Asare et al. suggested adding age to the ENSAT staging for better prediction of overall survival (OS) in stage I-II ACC (16). Miller et al. suggested the Ann Arbor modification of the ENSAT staging system incorporating tumor grade (17). Still none of these staging systems has been implemented in the guideline. Insufficient prospective validation maybe the reason. Also, some studies did not find age/tumor grade to be of impact on prognosis. Studies that included multivariate models did not find age as a predictor of recurrence (6,18,19) or cancer-specific mortality (20). Age could be associated with an increase in all-cause mortality due to an increase in treatment-related mortality or in non-specific mortality. It just could be a confounding factor of frailty and

comorbidities and subsequent treatment decisions. But it should be acknowledged that recurrence is a different outcome measure than overall survival. Presumably, to predict recurrence and overall survival in ACC accurately, separate prognostic models are needed. Implementing multiple models could be an inconvenience and confusing when used in clinical practice.

The problem with tumor grade, defined as Ki67, as prognostic factor is high inter- and intra-observer variability (21) and limited reproducibility. The heterogeneity in the preanalytical process (fixation) and immunostaining is not completely standardized and might affect Ki67 measurement (22,23). It has been suggested that digital microscopy-enabled methods could provide critical aid in reducing variation, increasing reproducibility. In 2014 an open source automated detection quantitative ranking of hotspots was published to support histopathologists in selecting the 'hottest' hotspot areas, but this has not been implemented in daily practice(21,24). Even when standardized, interpretation of Ki67 staining and scoring may be subjected to predicaments: Does the sample analyzed represent the whole tumor? How many cells or high-power fields have to be included for evaluation? Do we need to score Ki67 into hot spot areas, or average score across the section? And do we have to score multiple samples from one tumor? And if so, do we take the average of all the samples or the highest score measured? These difficulties may explain why in **chapter 3** we are missing so many Ki67 data. This calls for a more objective and reproducible prognostic marker, in which there is a potential role for genomics (discussed later). Also, as we plead for in **chapter 5**, a standardized pathology record would guarantee a complete report, containing itemized Weiss-score and standardized assessment of Ki67-index. On the other hand, it could be argued that the best prediction model is one that is easy to use in clinical practice and therefore consist of 'simple' prognosticators as we showed in **chapter 3.**

The impact of cortisol secretion of the tumor remains uncertain in ACC. It appears that it mostly negatively impacts patients undergoing surgery (19,25), whereas it showed no prognostic value in other series involving localized (6) or metastatic ACC patients (26). It could be suggested that hypercortisolism, as implied for age, is associated with a general increase in mortality. Hypercortisolism is associated with an increased risk of thromboembolism, and it suppresses the immune system increasing the risk of infection and post-operative complications.

In addition, it should be noted that there is statistical difference in showing an association of a variable in relation to outcome using a regression analysis or creating a predictive formula or nomogram that can calculate a predefined outcome for an individual patient. Most important, a general concern for studies on prognosis is the number of variables included in models, relative to the number of events, because when outbalanced this may have the potential to lead to false-positive results.

Improvement of current strategies and developing new treatments

Although prognostication allows for a more personalized treatment strategy, it has not generated new therapeutic options. Fortunately, the survival rate of ACC has slightly improved over the last years. Does this mean we are treating patients better? The reasons are not totally clear, studies have shown that a higher percentage of patients are diagnosed in earlier stages (27) which may be explained by improved imaging techniques.

Also, the improvement of surgical techniques with a complete tumor removal even for advanced disease stages, or the widespread use of adjuvant therapy with mitotane may explain, at least in part, this positive evolution. Possibly, the repeat use of local therapy in selected patients might have led to prolonged stabilization and control of the disease (28,29). Moreover, it could be hypothesized that the appeal of treating ACC patients in dedicated centers might have positively contributed to the referral, and the improvement of, multidisciplinary expert team meetings, consequently leading to better individualized (multidisciplinary) treatment strategies and hence better outcome.

In **chapter 5** future ACC diagnostics, prognostics and treatment concepts are discussed. It is disappointing that since this publication, despite effort, no new therapies have been established for ACC. Understanding why we are failing in treatment in ACC is key. Drug resistance is a very important part of this challenge. ACC has a medium-to-low sensitivity to alkylating drugs (cisplatin, streptozotocin), drugs interfering with DNA-topoisomerase (etoposide, doxorubicin, irinotecan) or drugs interfering with DNA bases (gemcitabine, capecitabine) (30). Also attempted targeted therapy, aimed at three most altered pathways in ACC (IGF2, Wnt/beta catenin and TP53) failed to show significant improvement of survival. Overexpression of a molecular pathway as a result of one single genetic alteration has been proven insufficient for making a good therapeutic target for ACC (e.g. linsitinib trial aimed at the IGF1R (31), everolimus aimed at mTOR (32)) or gefitinib and erlotinib aimed at the EGFR pathway (33,34). Tumor heterogeneity between patients, but also heterogeneity among different metastatic sites within the same patient, pose a problem with this single pathway approach. Unfortunately, the identification of targets linked to the multiple driver molecular alterations are lacking.

In the meantime, mitotane remains the main therapeutic backbone of ACC and actually still is the only Food and Drug Administration (FDA)/European Medicines Agency (EMA)-approved drug. It is used treating ACC in either adjuvant or metastatic setting. Therefore, improving mitotane treatment is a logical consequence. Our current clinical knowledge on mitotane is mostly based on retrospective studies. The exact mechanism of action of mitotane is not yet fully understood and remains an object of investigation as well as how it influences other therapeutic agents when given concomitantly.

Mitotane is a complicated drug. Its effectiveness is correlated with a therapeutic level of 14 mg/l (35-37). Unfortunately most patients require a long treatment period to reach those levels because of unfavorable pharmacokinetics of mitotane: low oral bioavailability of 30–40% (38), high volume of distribution due to its high lipophilicity, and its elimination half-life varying between 18–159 days (39-41). Almost all patients experience side effects. In the study by Daffara et al. 76% of patients even experienced multiple side effects.

Earlier it was shown by our group that weight, body surface area (BSA) and lean body weight (LBA) were significantly correlated with volume of distribution in the central compartment (V1) in a pharmacokinetic model for mitotane (42). However, it was acknowledged that a residual variance in the population pharmacokinetic (PK) parameters was not explained by clinical parameters and it was hypothesized that genetic factors are involved in mitotane pharmacokinetics.

Earlier, two studies suggested possible roles for CYP2B6 and CYP2C9 (43). One study demonstrated that the genotype of CYP2B6*6 (rs3745274) was significantly correlated with mitotane plasma concentrations at 3 and 6 months after initiation of treatment (44). However, after nine months, treatment difference was no more significant. Also, they only investigated CYP2B6 and ABCB1. The other study showed that one patient with high mitotane concentration was a CYP2C9 intermediate metabolizer (43).

In **chapter 4** we showed that lean body weight (LBW), genotypes of CYP2C19*2 (rs4244285), SLCO1B3 699A>G (rs7311358), and SLCO1B1 571T>C (rs4149057) were identified to affect mitotane clearance (CL/F) significantly, which decreased the coefficient of variation (CV%) of random inter-individual variability of CL/F from 67.0% to 43.0%. Fat amount was identified to affect the central distribution volume significantly. A major limitation of this study is that the effect of co-medication was not included in the analysis because of missing data. Mitotane is a known strong inducer of CYP3A4 (45). Van Erp et al. found an increased metabolism of midazolam, when given concomitantly with mitotane, indicative of CYP3A4 upregulation (45). In addition, it has been demonstrated that mitotane induced liver microsomal enzymes and, more specifically, CYP3A4 through binding to the pregnane X receptor (PXR) in liver cells (46,47). Considering that CYP3A4 is one of the most important drug-metabolizing microsomal monooxygenases, which is considered to be implicated in the metabolism of about 50% of the drugs on the market, many drugs potentially are affected by mitotane-related CYP3A4 induction(48). It could also be hypothesized that this (partially) explains the therapeutic failure of tested targeted pharmaceuticals in ACC, e.g. everolimus (32), an mTOR inhibitor, imatinib (49), a tyrosine kinase inhibitor and gefitinib (34), an inhibitor of epidermal growth factor (EGR). All these drugs are substrates of CYP3A4, and hence, insufficient drug exposures may have been reached in these clinical trials, since patients were treated with mitotane in the recent past, and therefore with measurable levels in blood because of the long elimination half-life, or even concomitantly.

In the case of PK modelling it is more interesting to know whether co-medication influences mitotane. Statins are known to promote the effect of mitotane. In a retrospective study by Hescot et al. 26 patients with ACC the combination of mitotane and statins (Rosuvastatin not metabolized by CYP3A4 but, presumably, CYP2C9 and CYP2C19) was shown to be significantly associated with a better tumor control according to Response Evaluation Criteria in Solid Tumors criteria (RECIST) (50). Boulate et al recently showed in NCI-H295R human ACC cells that rosuvastatin potentiated the effects of mitotane by reducing cell viability, inducing apoptosis, increasing intracellular free cholesterol levels, and by decreasing the expression of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and ATP binding cassette subfamily a member 1 (ABCA1), genes involved in cholesterol metabolism, and inhibiting steroidogenesis(51). As mentioned earlier, CYP2C9 has been correlated with mitotane levels (43) and we show in **chapter 4** a role for CYP2C19 in the clearance of mitotane. Both enzymes are involved in the metabolism of rosuvastatin, perhaps contributing to their synergy.

Noteworthy, mitotane induces dyslipidemia with increased LDL, HDL and triglycerides concentrations (52,53). Remarkably, this dyslipidemia reduces mitotane efficacy in vitro as suggested by the higher anti-proliferative and pro-apoptotic effects of mitotane when NCI-H295R cells are cultured in a lipoprotein-free medium (50). Also, hyperlipidemia leads to an overestimation of plasma mitotane levels in patients (54). Further research should focus on the understanding of how mitotane is associated with lipid changing on the one hand and the mechanism through which statins may potentiate the effects of mitotane on the other hand.

In contrast to what we already know about drug synergism and effect of mitotane on other medication, little is known about medication that reduce mitotane efficacy. It has been suggested that spironolactone, commonly used in the management of the hypokalemia of Cushing's syndrome, blocks the action of mitotane and should be avoided if possible (55).

Mitotane is advised to be taken with food, preferably a fatty meal, where this could enhance absorption (39). When taking this into account it could be hypothesized that also the acidity of gastric fluid might affect mitotane absorption. However, the role of proton pump inhibitors (PPI) and their influence on mitotane absorption has not been investigated. While for many other medications, e.g. ketoconazole, it is known that absorption is rendered when given in combination with a PPI (56).

For some drugs synergism with mitotane have been proposed. Kroiss et al. showed a synergism of bortezomib and carfilzomib, proteasome inhibitors who indirectly activate endoplasmic reticulum (ER) stress, with mitotane in the NCI-H295 cell culture model for ACC (57). Such enhancement of the effect of mitotane could be clinically very relevant, where only half of patients treated with mitotane reach therapeutic levels (58).

It can be concluded that therapeutic drug monitoring (TDM) is very important not only for mitotane but also for certain concomitant given drugs. In our experience, the major problem with TDM is the delay in test results for mitotane. This is caused by two factors: firstly, the concentration of mitotane in blood can only be determined in one medical center in the Netherlands, and it generally takes one week from submitting a blood sample to receiving the result. The alternative, Lysosafe® (Lysosafe is a is a free of charge mitotane plasma level testing service in France offered by HRA Pharma – the pharmaceutical producing mitotane {Lysodren®} – to all hospitals in Europe using Lysdoren® in the treatment of ACC), is not quicker. Secondly, in our treatment protocol the first blood sample is taken two to four weeks after starting mitotane treatment in the building up phase. So, when the result is in, we might find that the patient may have received too little or too much mitotane for at least three weeks. A good example is the case report of Di Paolo et al. they showed that thanks to TDM their patient did not have Central Nervous System (CNS) or gastrointestinal toxicities, but this took a long time of dose adjusting (six months) (59).

The Population PK (PopPK) model proposed in **chapter 4** could fill this hiatus, where it could allow for the predicting of mitotane levels, considering previous individual plasma level measurements as well.

Predicting mitotane levels in advance allows for an increase in dosage when the predicted level is not at a therapeutic level of 14mg/l within expected time frame, or to lower the dosage when it is expected that toxic levels above 20mg/l will be reached. Off course in the latter situation, side effects experienced by the patient should be weighed, because when levels of 20 or above are well tolerated by the patient, continuing the dosage can be considered. Further, it could allow for a personalized dosage regimen to build up to a therapeutic level and inform about the dosage needed to maintain a therapeutic range. Currently there is no consensus regarding the optimal dosage strategy for mitotane which depends on a physician's expertise and is therefore prone to error (58,60). A median of three to five months is reported in studies (36,61) to reach therapeutic levels. This can be an unacceptable long time for patients with advanced disease or at high risk of recurrence. Besides supportive medication to temper experienced side effects it could be hypothesized that a predictive PK model could motivate the patient to continue therapy knowing they will reach a therapeutic level with the advised dosage regimen. In the study by Mauclère-Denost et al. was transiently discontinued 50% of patients, due to toxicity (60) and also Kerkhofs et al. report about the discontinuation of mitotane treatment (58). Discontinuation should be minimized by regular counseling about the management of adverse effects, careful adjustment of hormone replacement therapies, and tailoring of mitotane dosage. The latter could be based on the PK model in which the measured levels can be entered, and, of course considering the experienced side effects. For this prospective validation of our presented PK model is warranted.

Molecular profiling/biomarkers

In the last decades, multiple molecular alterations have been found to promote ACC carcinogenesis. Understanding ACC tumorigenesis is of importance where it might yield the identification of new markers that improve diagnostic accuracy, predict treatment response or even provide novel therapeutic targets.

In **chapter 6** an overview of new insights in the evolving field of epigenetic studies on ACC is provided. Epigenetics involves genetic modification without altering the DNA sequence. Most epigenetic changes concern DNA methylation, attaching methyl groups to segments of DNA, and histone modification. Hypermethylation is a common phenomenon seen in cancer. It has been found that there is significantly more hypermethylation at CpG sites in ACC. Different studies were able to find such CpG clusters to correlate with ACC survival, so called CIMP-high (poor prognosis) and CIMP-low clusters (62-64). Also, studies have identified that expression levels of, on mRNA levels of mitotic regulator, BUB1 Mitotic Checkpoint Serine/Threonine Kinase B (BUB1B) and, mitochondrial kinase PTEN Induced Putative Kinase 1 (PINK1), help in identifying different subgroups of ACC with different survival rates (65,66).

The term CIMP, often referring to the CpG island promoter methylation, is controversial since there is actually no universal standard or consensus with respect to defining CIMP. It is used to describe the increased prevalence of CpG island promoter methylation. CpG islands are genomic regions larger than 500 base pairs (bp). More than 55% of these nucleotides are composed of CpG dinucleotides (67).

As Koch et al. already advocated at the beginning of 2018, international effort needs to be made to make molecular profiling of clinical relevance (68). They reported that in January 2018 there were more than 14,000 scientific publications describing DNA methylation- based biomarkers and their clinical associations in cancer, but only 14 of these biomarkers had been translated into a commercially available clinical test. They recommend that the location of a potential DNA methylation biomarker and the number of CpGs to cover should be critically evaluated. Most genetic studies on ACC clearly describe the type of (Infinium) assay (see **chapter 6**). There is heterogeneity in the microarrays used and therefore in the results. Also, the microarray restricts data to the particular genomic locations of the probes used in the array, which might not necessarily capture the most relevant methylation sites. Microarrays that cover over 850,000 CpG methylation sites (850K) have shown to also find CpG clusters not only in islands but also CpG shores (sequences 2 kilobytes (kb) upstream and downstream from CpG island), in CpG shelves (sequences 2 kb upstream and downstream from shore regions) and outside these regions ('open sea').

What is missing in most studies on ACC is an accurate and thoroughly report on the exact genomic location of the biomarker, which is depending on the microarray and

cut-off values used.

However, it seems that we are on the verge of the development of a biomarker for prognosis in ACC. Despite the methodologic differences the CIMP phenotype has been shown in three different studies.

The development of a biomarker that can clearly distinguishes ACA and ACC (potentially BUB1-PINK1 (69)), predicts recurrence or treatment response in ACC appears more challenging. Sterol-O-acyl- transferase 1 (SOAT1) has been suggested a potential biomarker for treatment response of mitotane (70).

Most important, multi-omics studies are establishing insight into the heterogeneous and complex molecular biology of ACC and identifying drug targets (see table below). Unfortunately, for many of these targets no treatment is available yet (see table 3 Crona & Beuschlein 2019©, Adrenocortical carcinoma - towards genomics guided clinical care, Nature Endocrinology (71)). Increasingly, it is stated that ACC research should be focused on biomarker driven clinical trials. This means international effort should be made to implement biomarkers in the guideline and, as a result, daily practice.

Drug target (mutation frequency in ACC)	**Therapy**	**Studies**
NF1 (17%)(71,72) *ATM (3-10%)*(71,72)	Everolimus, Temsirolimus	Fraenkel et al. 2013 – Everolimus: clinically no meaningful response was observed in four patients with advanced ACC*(32).
CDKN2A/B (11-15%)(63,64) *CDK4(2-7%)*(63,72) *ATM (10%)*(71,72)	*CDK 4/6 inhibitors:* Abemaciclib Palbociclib Ribociclib	Fiorentini et al. 2018 – Palbociclib: significantly affected cell viability in NCI-H295R cell line for ACC (73).
PTCH1 (3%)(71,72)	Vismodegib	LoRusso et al. 2011 – Phase I trial Vismodegib: included 2 ACC patients. Progressive disease/ missing, no details specified (74).
* Patients used mitotane, potentially diminishing therapeutic effect of Everolimus.		

Future perspectives

Adrenocortical carcinoma is an orphan disease. Collaborative efforts, like the European Network for the Study of Adrenal Tumors (ENSAT), and the more recent established A5: American–Australian–Asian Adrenal Alliance, are making important contributions in the research field of ACC. Such strong international collaborations and large consortia are

necessary to improve diagnostic and therapeutic strategies for patients with ACC.

The Dutch Adrenal Network (DAN) should make an effort to initiate a national biobank. The prognostic model presented in this thesis had a disadvantage because of lacking Ki67 data as a result of pathology records missing or Ki67 not evaluated and therefore not mentioned in the pathology report. An attempt to retrieve these tumor samples for revision proved to be very difficult with material being unavailable. A centralized biobank could overcome this problem, making the storage and accessibility of patient samples easier for research purposes. Additionally, a standardized pathology report for ACC could ensure a thorough report including Weiss and Ki67.A potential improvement would be a national collaboration between the Netherlands Cancer Registry, which is managed by the Netherlands Comprehensive Cancer Organization (IKNL), and the DAN.

Prognostication of ACC has improved with the introduction of the ENSAT-staging and the proliferation marker, Ki67. Recently there have been relevant publications that ought to further improve prognostication. This is necessary, because predicting recurrence still is problematic, as is the heterogeneous prognosis within the different ENSAT stages. It appears that both selected clinical factors (e.g. Resection, Age, Symptoms) and molecular biomarkers (COMBI score) have the potential to improve prognostication. Prospective trials, with large patient cohorts are needed to further investigate the discriminative value of these prognostic factors.

New therapeutic strategies in ACC should be stratified according to patients' molecular subtype by using thorough validated biomarkers that permit prospective assessment. It must be stressed that individual therapeutic approaches are unlikely to be efficacious in all ACC subtypes. An interesting approach has been suggested by Crona & Beuschlein. They argue that a, so called, umbrella clinical trial design. This design, in which a distinct disease is selected for study and patients are stratified on the basis of molecular data towards different treatment baskets, would allow researchers to study the feasibility and efficacy of the entire concept of genetics-guided therapy rather than evaluating different agents separately, each with very low numbers of patients (71).

Furthermore, it could be thoroughly considered whether new agents should be tested concomitant with mitotane. Because of the long elimination half-life of mitotane, after termination, an interaction could be expected for a long period.

For many years it has been accepted that one type of medication is given to various types of patients. With the upcoming field of pharmacogenetics, we are entering a new era of personalized medicine.

For mitotane it could allow for a personalized dosage strategy in order to attain a therapeutic level within three months preferably. We showed that *CYP2C19*2*,

SLCO1B3 699A>G and *SLCO1B1 571T>C* influence pharmacokinetics of mitotane. Further validation is needed to confirm these findings in an independent cohort.

Considering that mitotane remains important in the therapy of ACC, effort should be made to better understand its mechanism of action. Markers that predict therapeutic efficacy would help deciding on adjuvant therapy. Investment should be made to improve mitotane treatment form. Its current form as a tablet of 500 milligram is not very patient friendly, considering taking up to 24 tablets a day is necessary, and uptake is reliant on a patients' diet. Intravenous treatment with mitotane in a Lipomul suspension is described in only one study in 1961 (38) and failed to be superior to oral treatment. Lipid-carriers or special tablet coatings could enhance the uptake and pharmacokinetics of mitotane.

Finally, and maybe most important, we need to gain insight into the health-related quality of life (HRQoL) impact of adrenocortical carcinoma (ACC). For patients with a rare disease, limited treatment options with high treatment burden and limited survival HRQoL research is of utmost importance. Shared decision making, as opposed to clinicians making decisions, requires that a healthcare professional should know how the course of the disease and the treatments of ACC impact a patients HRQoL. When discussing diagnosis and treatment options, respecting ones wishes, treatment strategy should be aimed at improving quality of life and enable well-being where possible. This again emphasizes that ACC care should be managed by a multidisciplinary expert team.

Those medical experts, together in teams, may have improved ACC survival over the last couple of years, and it's their collaboration that brings hope for the future.

Diagnostics	Prognostics	Therapy
	Implementation necessary	
• Implementation of a standardized (pre)analytic protocol for Ki67 in ACC. ~Line number 7 ~ • Implementation of a standardized pathology record for ACC. ~Line number 88, 319~	• Implementation of a multi-omic molecular panel with the best selected biomarker predictors. ~Line number 280~	• Implementation of a combine pharmacokinetic and genetic model for personalized dosage of mitotane. ~Line number 156, Chapter 4~
	Validation required	
	• Validate mENSAT in a prospective cohort. ~Line number 57~ • Validate Grade, Resection status, Age, Symptoms (GRAS) criteria in a prospective cohort. ~Chapter 3~ • Comprehensive international prospective validation of multi-omic predictive markers. ~Chapter 6~	*Mitotane* • Prospective validation of the single nucleotide polymorphisms of the CYP enzymes identified in **chapter 4** ~Line number 241, chapter 4~ • Prospective validation of the two compartment pharmacokinetic model of mitotane presented in **chapter 4** ~Line number 241~
	Need for future studies	
• Prospective comparison of current pathologic classification (Ki67 and Weiss score) of adrenal tumors versus a genomic assay versus the combination of both in the process of accurately diagnosing adrenal tumors. ~Line number 86~	• Consider a predictive model combining clinical, pathologic predictors and biomarkers. ~Chapter 6~	Prospective study on the role of adrenalectomy in stage IV patients. ~Line number 19~ Prospective study on the role of a metastasectomy in well-defined study and control groups of ACC patients. ~Line number 55~
	Necessity for future initiatives	
• Centralized biobank in the Netherlands for adrenal tumors. ~Line number 314~		

Diagnostics	Prognostics	Therapy
	Innovative ideas	
• Role of artificial intelligence in diagnosis of ACC. ~chapter 6~ • Role of radiomics in diagnosis of ACC. ~chapter 6~		• Focus on the understanding of how mitotane is associated with lipids and the mechanism through which statins may potentiate the effects of mitotane on the other hand. ~Line number 191~ • Identify markers that predict therapeutic efficacy of mitotane. ~Line number 351~ • Attempt to improve mitotane administration form. ~Line number 356~ *Genomic driven treatment* • Initiate studies on distinct genetic, transcriptional, and epigenetic mutations in ACC that are pharmacologically targetable. ~Line number 291~
Quality of life ~ Line number 360 ~		

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Chapter 08

Summary

Summary

The present thesis was designed to investigate adrenocortical carcinoma (ACC) from a clinical perspective with focus on survival.

ACC is a rare but aggressive malignancy. Unfortunately, most patients present with advanced disease at presentation. **Chapter 2** presents a retrospective population-based study with the focus on the time and extent of metastasis. Patients who presented with ACC between January 1, 2004 to October 31, 2013 in one of the nine centers of the Dutch Adrenal Network (DAN) were included. The total number of ACC patients was 160. One hundred seventeen patients (73%) were diagnosed with metastases during the follow-up period of this study: eighty-four patients had synchronous metastases and thirty-three developed metachronous metastases. Overall survival (OS) rate was calculated from the date of diagnosis of metastasis until death from any cause.

Median OS after diagnosis of first metastases for patients with synchronous or metachronous metastases was 12 months and 29 months, respectively, (p=0.046).

Forty patients (34%) had one to three metastases. The other 77 patients (66%) had four or more metastases. Overall survival for these two groups was significantly different with 50% and 12% respectively (p<0.001).

Furthermore, the number of affected organs was of influence on median survival. Seventy-five patients had only one affected organ at presentation, in 42 patients two or more organs were involved: OS percentages were 33% and 10% respectively (p<0.001).

ACC prognosis is heterogeneous. European Network for the Study of Adrenal Tumors (ENSAT) stage is the most used prognosticator, advised in the guidelines. In **chapter 3** a prediction model for ACC-specific mortality is developed. One hundred fifty-nine patients from the DAN database were selected for this prospective cohort study.

Internal validation of the model was performed using standard bootstrapping techniques.

The multivariable modeling yielded a prediction model which included age, modified European Network for the Study of Adrenal Tumors (mENSAT) stage, and radical resection. The c-statistic was 0.77 (95% Confidence Interval: 0.72, 0.81), indicating good predictive performance of the model.

Internal validation proofed the model not be over fitted. Applicability of the model is illustrated by calculating the two-year probability of ACC-specific mortality of a 64-year old patient with an mENSAT stage of 4a, and no radical resection of the tumor: 1 – 0.56exp(LP). LP = 0.02 * (64 - 54.5) + 0.63 * (4 - 3.2) – 0.44 * (0 - 0.8) = 1.046; hence, the probability = 1 – 0.56exp (1.046) = 0.81, being 81%.

Mitotane is the adrenolytic drug recommended for treatment of primary and recurrent adrenocortical carcinoma. Mitotane exerts an anti-neoplastic effect on ACC tissue and inhibits cortisol synthesis. Ultimately, mitotane efficacy is limited by its high lipophilicity, poor pharmacokinetic properties, and dose-limiting toxicities. A plasma level between 14-20mg/l is considered as therapeutic range. Reaching and maintaining these therapeutic levels is complicated and takes months. In **chapter 4** a population pharmacokinetic (PK) and pharmacogenetic analysis was performed for mitotane in patients with ACC utilizing the retrospectively collected PK data on 48 patients. The effect of genes encoding drug absorption, distribution, metabolism, and elimination (ADME), patients' demographic information, and clinical characteristics on mitotane PK were investigated as covariates.

A two-compartment model with first-order absorption and elimination best described the PK data of mitotane. Lean body weight (LBW), genotypes of CYP2C19*2 (rs4244285), SLCO1B3 699A>G (rs7311358), and SLCO1B1 571T>C (rs4149057) were identified to affect mitotane clearance (CL/F) significantly, which decreased the coefficient of variation (CV %) of random inter-individual variability of CL/F from 67.0% to 43.0%. Fat amount (i.e. body weight – LBW) was identified to affect the central distribution volume significantly. The developed model is beneficial to optimize mitotane treatment schedules and to guide the initial dose selection for patients.

Chapter 5 contains a review with perspectives on the future of ACC treatment. When optimizing the diagnostic and therapeutic process in ACC care, minimum volume requirements as instituted for other cancers are hardly feasible due to the low annual incidence of 0.5 -2.0 patient per million people per year. However, we advocate the establishment of an international 'standard of care' that defines mandatory elements in clinical management of ACC patients. Uniformity can be achieved by defining in detail the entire process a patient undergoes from the first diagnostic test until follow-up visits. A summary of key points that should be present in an ACC-specialized center are presented. This comprises for example timing of diagnostic evaluations, standardized formats of pathology and surgery reports, guidelines for (supportive) therapy, etcetera. In addition, participation in international networks and clinical trials should be encouraged.

The understanding of adrenocortical tumorigenesis has improved over the years and the last decade studies have been focusing on epigenetic changes associated with adrenocortical tumors. In **chapter 6** a descriptive review provides an overview on the evolving field of epigenetic studies on adrenal tumors. The level of methylation in adrenal carcinoma has been found to be associated with survival. The level of CpG island promoter methylation, or CIMP, has been shown in various studies to provide a classification based on methylation as a marker for prognosis in patients with ACC. Actually, we are on the verge of the development of a biomarker for prognosis in

ACC. *G0S2* hypermethylation was shown to be a hallmark of the CIMP-high cluster, associated with poor outcome. The establishment of a reproducible biomarker, will have implications for the diagnostic and prognostic process of ACC in the near future.

Nederlandse samenvatting

Dit proefschrift richt zich op onderzoek van kanker van de buitenste laag, het schors, van de bijnier: het bijnierschorscarcinoom. De bijnieren zijn hormoon producerende organen die bij de nieren liggen. Bijnierschorscarcinoom is een zeldzame vorm van kanker met een slechte prognose.

In Nederland is er een samenwerkingsverband van medisch specialisten, het Bijnier Netwerk Nederland (BNN), dat zich inspant om patiënten met bijnierkanker een zo goed mogelijke behandeling te geven. Dit is een samenwerking van alle Universitair Academisch Medische Centra en het Máxima Medisch Centrum.

Tot 30 procent van de patiënten met bijnierschorskanker, presenteren zich met reeds uitgezaaide (niet meer te genezen) ziekte, stadium IV kanker. In **hoofdstuk 2** wordt een studie beschreven waar wordt gekeken of het moment van ontwikkelen van uitzaaiingen (metastasen), van invloed is op de overleving. Patiënten van 18 jaar of ouder, die zich tussen 1 januari 2004 en 31 oktober 2013 presenteerden in een van de centra van het BNN, werden geïncludeerd in de studie. Patiënten die tot 6 maanden na primaire diagnose metastasen kregen de definitie synchroon gemetastaseerd. Patiënten die pas metastasen kregen vanaf 6 maanden na de primaire diagnose kregen de definitie metachroon gediagnostiseerd. Er werden 160 patiënten in het onderzoek geïncludeerd. Honderd-zeventien patiënten werden geconfronteerd met metastasen. Vierentachtig patiënten hadden synchroon gemetastaseerd bijnierschorskanker en 33 metachroon. Patiënten met metachroon gemetastaseerde ziekte hadden een betere overleving dan patiënten met synchroon gemetastaseerde ziekte.

Tevens werd er gekeken naar de invloed van het aantal metastasen en aantal organen waarin uitzaaiingen aanwezig waren, op de overleving. Er werd geobserveerd dat patiënten met drie of meer metastasen en twee of meer organen met metastasen een slechtere prognose hadden.

De overlevingsduur voor bijnierschorskanker patiënten is globaal genomen somber. Een stadiëring systeem wordt gebruikt om op een generaliseerbare manier de grootte en de graad van verspreiding van de kanker te beschrijven. Bij bijnierschorskanker is dit het Europese netwerk voor studies naar bijniertumoren (European Network for the Study of Adrenal Tumors; ENSAT) systeem. Aan de hand van dit systeem kan een voorspelling worden gedaan over de kans op overleven.

Als er wordt gekeken naar de overleving van bijnierschorskanker patiënten binnen de verschillende ENSAT stadia is er heterogeniteit en er is dan ook behoefte aan een methode, dan wel stadiering, waarmee een meer nauwkeurige inschatting over de prognose kan worden gemaakt. In **hoofdstuk 3** wordt een voorspellend model beschreven waarmee het mogelijk is de kans op overlijden aan (de gevolgen van)

bijnierschorskanker voor een individuele patiënt te berekenen. Het model bestaat uit een aantal voorspellende factoren (predictoren).

In 2015 is door een onderzoeksgroep in Frankrijk een suggestie voor een aanpassing op de ENSAT stadiering gepubliceerd: mENSAT. Patiënten met betrokkenheid van lymfeklieren worden in dit stadiëring systeem als stadium IV, in plaats van stadium III, beschouwd. Tevens zijn de stadium IV patiënten verdeeld in sub stadia a-c afhankelijk van het aantal organen met uitzaaiingen (2, 3 of >3) waarbij lymfeklieren als orgaan worden gerekend en ook de primaire tumor als aangetast orgaan wordt meegerekend.

Het in **hoofdstuk 3** gepresenteerde model is in staat om met goede accuraatheid de kans op mortaliteit te berekenen voor een individuele patiënt aan de hand van leeftijd (≥ 18 jaar), mENSAT stadium en in hoeverre de kanker radicaal is verwijderd door de chirurg.

De resultaten uit **hoofdstuk 2** en **hoofdstuk 3** van dit proefschrift bemoedigen de implementatie van mENSAT. Het aantal aangedane organen speelt een rol in de prognose. Dit zien we ook terug in mENSAT. Een grootschalig prospectief onderzoek wat ENSAT en mENSAT tegen elkaar uitzet in hun vermogen prognose van de patiënt te voorspellen, kan extra bewijs leveren mENSAT op te nemen in de richtlijn.

Tevens wordt gesuggereerd dat patiënten met lymfeklier en orgaan metastasen wellicht extra aandacht verdienen van de behandelaren, waar er heterogeniteit is in overleving. Dit vereist maatwerk in de behandeling van een patiënt met uitzaaiingen. Aanvullend onderzoek is nodig om patiënten met metastasen doch een relatief gunstige prognose beter te kunnen detecteren zodat de primaire behandeling zich daar op aanpast en dat bijvoorbeeld de chirurg wordt gevraagd zo veel mogelijk afwijkingen te verwijderen.

De enige curatieve optie bij niet-uitgezaaid bijnierschorscarcinoom is chirurgische resectie van de primaire tumor. Echter, zelfs nadat de tumor volledig is weggesneden is er een kans op terugkeren van de kanker. Medicamenteuze opties zijn beperkt. Hierin speelt het medicijn mitotaan een hoofdrol. Dit medicijn kent echter ernstige bijwerkingen en wordt de therapie door patiënten als invaliderend ervaren.

Mitotaan vernietigt vooral bijnierschorsweefsel en wordt al langer gebruikt bij patiënten met gemetastaseerde ziekte. Onderzoek heeft aangetoond dat behandeling met mitotaan na complete resectie van de tumor de ziektevrije overleving verlengt. Over het exacte werkingsmechanisme van mitotaan is onvoldoende bekend. Wel zijn er sterke aanwijzingen dat het therapeutisch effect afhankelijk is van de plasmaconcentratie in het bloed: een plasmaspiegel van 14 milligram per liter wordt algemeen aangehouden als ondergrens voor werkzaamheid. Plasmaspiegels boven de 20mg/L zijn geassocieerd met toxiciteit die belastend kan zijn voor de patiënt. Dit smalle therapeutisch venster maakt nauwkeurige dosering en monitoring

noodzakelijk. Echter, de optimale opbouw- en onderhoudsdosering blijkt onder patiënten sterk te verschillen, wat het doseren van mitotaan danig bemoeilijkt. In de praktijk heeft dit tot gevolg dat er bij bepaalde patiënten sprake is van onder-dosering, wat ongunstig is omdat er dan geen effectieve behandeling van de tumor plaatsvindt, of juist overdosering waarbij er neurotoxische of gastro-intestinale bijwerkingen kunnen optreden.

In **hoofdstuk 4** is er specifiek gekeken naar DNA van genen betrokken bij de absorptie, distributie, activering en excretie van geneesmiddelen van patiënten met bijnierschorskanker die behandeld zijn met mitotaan. Aan de hand van de patiënt eigenschappen en hun DNA, is een model ontwikkeld waarmee de optimale mitotaan dosering kan worden berekend om tot een therapeutische spiegel te komen. De klaring van mitotaan wordt significant beïnvloedt door de vetvrije massa en de genen (erfelijk materiaal) met het volgende genotype: CYP2C19*2 (rs4244285), SLCO1B3 699A>G (rs7311358) en SLCO1B1 571T>C (rs4149057).

In **Hoofdstuk 5** van dit proefschrift wordt een oproep gedaan om de zorg voor bijnierschorskankerpatiënten te verbeteren. Het hoofdstuk biedt een overzicht van de diagnostiek, voorspellende factoren en behandeling van bijnierschorskanker. Er wordt een suggestie gedaan voor een uniforme benadering die moet waarborgen dat de zorg volgens een bepaalde standaard wordt geleverd. Belangrijke onderdelen van deze uniforme zorg is onder andere een standaard richtlijn voor de frequentie van de controles, een standaard format voor het operatieverslag en pathologieverslag. Daarnaast is het ontzettend belangrijk om nationaal en internationaal te blijven samenwerken om met een groter aantal patiënten participatie onderzoek te kunnen doen.

Er is dringend behoefte aan aanvullende behandelingen om de overleving van bijnierschorskanker te verbeteren.

Om dit te kunnen bewerkstelligen is met name begrip van de pathofysiologie van de ziekte van belang. Waarom en hoe ontstaat het bijnierschorscarcinoom?

Kanker kunnen we slechts gedeeltelijk begrijpen als we alleen in het DNA naar de genen kijken. Bij het epigenetisch onderzoek kijken we naar niet coderende genen die onder druk van omgevingsfactoren mee bepalen of een coderend gen ‘aan’ of ‘uit’ wordt gezet en of een gen dus wel of niet wordt afgelezen. Op je DNA zitten moleculaire ‘dimmers’ die regelen of genen ‘aan’ of ‘uit’ kunnen staan. Een voorbeeld van moleculaire ‘dimmers’ waarover we al veel weten, zijn de zogeheten methylgroepen. Zijn er veel methylmoleculen aan genen gekoppeld, dan zijn deze genen uitgeschakeld; zijn er weinig methylmoleculen, dan kunnen de genen actief worden. Zo een cluster van ‘dimmers’ kom je veel tegen rond een speciaal gebied in het DNA, het zogenaamde promotor gebied. De promotor is de startknop voor het lezen van je DNA.

Indien we zulke gebieden op de genen bij bijnierkanker die overmatig ‘aan’ of ‘uit’ staan kunnen identificeren dan verschaft dit niet alleen meer inzicht in het ontstaan van de ziekte maar het kan ook leiden tot de ontwikkeling van nieuwe therapieën. **Hoofdstuk 6** is een review artikel bestaand uit een groot literatuuronderzoek. Het geeft een samenvatting van de stand van zaken op het gebied van epigenetica van bijnierschorskanker. De verwachting is dat methyl patronen op korte termijn een belangrijke rol gaan spelen in de voorspelling van overleving van bijnierschorskanker waar deze patronen een associatie tonen met mate van agressiviteit van de kanker.

Hoofdstuk 7 bevat een algemene discussie waarin de bevindingen uit dit proefschrift worden bediscussieerd in een breder perspectief en waarin suggesties voor toekomstig onderzoek worden gedaan.

8

Chapter 09

Impact paragraph

Adrenocortical carcinoma (ACC) is a rare type of cancer. This thesis focuses on the identification of markers that could improve the accuracy of diagnosing, treating, and estimating survival of ACC.

Survival

In **chapter 2** of this thesis it is concluded that patients who develop metastases within six months after initial diagnosis of ACC have a more poor overall survival (OS) than patients with metachronous metastasis. Also, patients with a high disease burden at diagnosis, four or more metastases or two or more affected organs, have a more grim overall survival than patients with limited disease. Furthermore, it is suggested that surgery might have a positive impact on OS for patient with synchronous metastases. In **chapter 3** a prediction model for ACC specific mortality is proposed. The model is based on age, modified European Network for the Study of Adrenal Tumors (mENSAT) stage, and radical resection.

ACC has a heterogeneous prognosis. Studies have been published describing aggressive stage III ACC, biologically behaving like stage IV, but also stage IV patients with exceptional long survival. This emphasizes that the biological heterogeneous behavior of ACC is not fully captioned with ENSAT stage. Therefore, there is a need for better prognostication in ACC to inform the individual patient on survival and assist in deciding on treatment strategy. The impact of **chapter 2** and **3** is the addition of information on ACC prognosis, to better capture the biologically heterogeneous behavior of ACC, not fully endorsed by the ENSAT stage. We hope that this new information will support the implementation of mENSAT in the ACC guideline. Better staging may encourage earlier, for example, surgical intervention through defining of (severe) prognosis, and may support further research into surgery in advanced ACC.

The information on prognostication is relevant for both ACC patients as well as their caregivers. Our research has been published and has been presented at national and international congresses.

If, in the future, our study outcomes will be implemented in daily practice it would be relevant to inform patients, for example using online platforms (international Facebook patient groups, accsupport.org.uk etc.).

Treatment

Mitotane is a keystone in the treatment of ACC. It is used both in adjuvant setting as well as in patients with metastatic disease. Mitotane has a small therapeutic window of 14-20 milligram per liter. Survival benefit has been proven in patients with blood

levels higher than 14mg/L whereas blood levels higher than 20mg/L are associated with increased toxicity. Toxicity is primarily gastro-intestinal and neurological and even leads to temporary or final discontinuation of mitotane therapy in some cases.

There is a large inter-patient variability in reaching and maintaining therapeutic concentrations, without a clear relationship between the mitotane dose and the serum concentration. The build-up of the therapeutic plasma concentration is in general slow. In most patients this level is reached after three months of treatment with a daily dose of about 6.0 gram (12 tablets of 500 milligrams). This means a delay of the optimal therapeutic effect. This complicates timing of therapy evaluation and consequently, the decision to add cytotoxic chemotherapy to the treatment.

Dosing regimens are based on clinical experience and adjusted according to plasma concentration and tolerability. The inability to predict mitotane levels leads to relative under dosing and a prolonged build-up phase in some patients, while others unexpectedly demonstrate high plasma levels early in therapy, causing increased toxicity. Earlier it was shown that there are only weak correlations between weight, age, gender, height and the pharmacokinetics of mitotane. The main goal in **chapter 4** was identifying pharmacogenetic differences among patients treated with mitotane by analyzing genes related involved in drug absorption, distribution, metabolism and elimination (ADME). In **chapter 4** it is shown that pharmacokinetics of mitotane can partially be explained by pharmacogenetics: a two compartment pharmacologic model is presented best describing the population pharmacokinetic (PK) data of mitotane. Lean body weight (LBW), genotypes of CYP2C19*2 (rs4244285), SLCO1B3 699A>G (rs7311358), and SLCO1B1 571T>C (rs4149057) were identified to affect mitotane clearance (CL/F) significantly.

The developed model is beneficial to optimize mitotane treatment schedules and to guide the initial dose selection for patients. Therefore it is relevant for ACC patients starting with mitotane therapy.

Currently only half of the patients treated with mitotane reach therapeutic levels and up to 50% of patients discontinue treatment because of toxicity. Our pharmacokinetic model could assist optimization of mitotane dosage to guide the build up to a therapeutic level, but prevent overshooting to toxic levels.

The model needs to be externally validated: it needs to be tested with a different set of patients to see if the results of our study can be generalized to and across other patients in different contexts.

If validated we would encourage the use of an online resource to make our model available worldwide for experts who treat ACC patients. The shiny app could for example be implemented in the ENSAT website.

Orphan disease

Adrenocortical carcinoma is an orphan disease. There are multiple definitions of orphan disease: it has been used to describe diseases that are neglected by doctors, or used to designate diseases that affect only small numbers of individuals and as disease that has not been adopted by the pharmaceutical industry because it provides little financial incentive for the private sector to make and market new medications to treat or prevent it. It defines a large group of diseases which are characterized by a low prevalence in the population. They frequently are associated with problems in diagnosis and treatment.

In **chapter 5** foreseen changes in diagnostic and prognostic instruments are discussed and suggestions are made for clinical management and possibilities for future treatment of ACC.

It has been proven repeatedly that the establishment of (inter)national collaborative networks of expert centers has a favorable effect on survival of ACC patients. Because of ACC being an orphan disease, we suggest that quality criteria other than volume criteria might be of greater importance: it seems more important that centers adhere to treatment as suggested in up to date guidelines, which in turn seems best feasible in a specialized centers with dedicated physicians. Furthermore, these international collaborative networks, such as ENSAT, encourage clinical trials. By working together sufficient patient numbers can be provided in order to achieve adequate reductions in uncertainties about e.g. treatment effects and run those clinical trials to test new treatment possibilities that are so badly needed. The impact of **chapter 5** is that it goes against current ideology of treating cancer patients in high-volume facilities. It endorses the treatment of ACC with mitotane by experts, even if this means treatment in low volume centers. It encourages initiatives as the European Network for the Study of Adrenal Tumors (ENSAT) and the A5: American–Australian–Asian Adrenal Alliance. It is a plea for collaboration even in the current competitive scientific culture where the credo seems to be 'to publish or perish'.

Many of the suggestions made in **chapter 5** (additional prognostic factors are needed; a uniform methodology for assessing the Ki-67 index in ACC needs to be established; centers should adhere to current state-of-art treatment concepts which seems best feasible in a specialized centers with dedicated physicians, adequate supportive treatment during mitotane therapy cannot be stressed enough; international collaboration is necessary to facilitate fundamental research and clinical trials to test new treatment possibilities) are included in the European guideline published in 2018, for example the recommendation that the pathology report should at least contain Weiss score (including the exact mitotic count), exact Ki67 index, resection status and pathological tumor stage (indicating invasion or not of the capsule and/or surrounding tissue and organs) and nodal status.

Also, **chapter 5** has been cited for at least 14 times by other scientific authors.

Epigenetics

Adrenocortical carcinoma occurs both as an inherited form of cancer as well as a sporadic neoplasm, being prominent in the Brazilian population.

Inherited ACC, associated with familiar syndromes, as well as the increased incidence in the Brazilian population is associated with a mutation in TP53, a gene that codes for a protein that regulates the cell cycle and hence functions as a tumor suppression. In sporadic ACC a TP53 mutation is often lacking.

Overall, the understanding of the pathophysiology, especially genetic cause, of ACC is limited. Mutations in insulin-like growth factor 2 (IGF2), β-catenin (CTNNB1 or ZNRF3), and TP53 have been associated with ACC. So, if the usual mutation analysis doesn't explain the familial cause, how can we solve the conundrum of how ACC develops? The answer might be epigenetics.

Epigenetic alterations occur in cancer cells as commonly as genetic mutations and have the ability to mimic the effects of the latter. Therefore, epigenetics are currently an increasing interest in the subject of cancer research. Epigenetics refers to the non-sequence-based modifications of DNA or its associated factors (e.g., histones) that are maintained during cell division. Most epigenetic changes concern DNA methylation, attaching methyl groups to segments of DNA, and histone modification. Hypermethylation is a common phenomenon seen in cancer. Studies suggests that epigenetic alterations precede tumor formation and increase the probability of cancer when genetic changes arise instead of being the consequence of tumor progression. Understanding the methylation process in adrenal tumours could expand our knowledge of tumorigenesis and improve current therapy strategies providing potential drug targets.

Chapter 6 summarizes recent findings on epigenetics of ACC and its role in diagnosis, prognosis and therapeutic strategies.

Different studies are presented who have been able to show clustering on epigenetic level, allowing differentiation based on these specific epigenetic findings not only between benign and malignant adrenals tumors, but also correlate with ACC aggressiveness. Therefore, epigenetics is suggested a significant addition to current histological parameters. Moreover, it might serve as an addition to current ENSAT staging in order to estimate prognosis and tumor aggressiveness. In **chapter 6** suggestions are made for follow up research, stimulating the implementation of a reproducible epigenetic (cluster) biomarker in the guideline. Also, a new flowchart on the potential management of the adrenal mass with the implementation of genomic analysis is presented.

The addition of a well reproducible biomarker would be of significance, where current diagnostic and prognostic markers occasionally are unable to differentiate (borderline) malignant from benign and insufficiently correlate with biological behavior of ACC.

Chapter 6 could form the base of which in the future a study protocol could be proposed to prospectively compare the pathologic classification (Ki67 and Weiss score) of adrenal tumors versus a genomic assay versus the combination of both (PA vs genomics vs PA + genomics) in the process of accurately diagnosing adrenal tumors.

Personalization

The impact of this thesis in general, is that it attempts to individualize diagnosis, prognostication and treatment of ACC. Health professionals are often uncomfortable with prognostic uncertainty but the process of addressing "what to expect" for an individual's disease course is essential for meaningful decision-making and end-of-life planning. Additional information on prognostication as provided in this thesis could support such conversation. The same goes for tailoring of treatment. A professional should explore: does the patient strive for quantity of life or quality of life? In the process of shared decision making, patients are required to make trade-offs between the two. Some patients are willing to endure toxicities associated with treatment in order to increase their length of life, while others value quality more and are reluctant to spend their remaining years in a compromised state. This involves weighing the risks and benefits of treatment and managing the patients' expectations and concerns. In order to do so international effort needs to be made to investigate ACC patients experience with the diagnostic process and treatment of ACC and study patient reported outcomes, which are currently scarce for ACC. Furthermore, international collaborative research should continue to invest in the optimization of current- and development of new ACC treatment.

9

Appendix

Curriculum vitae

List of publications

Acknowledgments

Curriculum vitae

Hester Ettaieb was born in 1988 in Naarden, the Netherlands.

After completing high school in 2006, she studied Biomedical Sciences at the University of Amsterdam. To complete this undergraduate degree, Hester undertook an internship at the NKI (Nederlands Kanker Instituut, Netherlands Cancer Institute) where she was introduced to several molecular-biological techniques used to study cancer. She obtained her Bachelor of Science in 2009 and then left Amsterdam for Maastricht where she attended the selective four-year Master's degree programme called AKO (Arts-Klinisch Onderzoeker, Doctor–Clinical Researcher) from which she graduated in 2013. In that same year she started her PhD research studying adrenocortical carcinoma under supervision of professor Harm Haak.

During her PhD she worked for over four years at the outpatient care treating patients with adrenal tumours, as ANIOS (resident not in training) on call at the Internal Medicine department and part-time as the only doctor on shift during the evenings and nights at the Maxima Medical Center Eindhoven for emergency care.

In 2018 she worked as ANIOS at the different oncology departments of the Antoni van Leeuwenhoek hospital (NKI) and in 2019 started working at Tergooi hospital at the department of Internal Medicine.

Currently she is attending residency training in Internal Medicine at the Amsterdam University Medical Center and Tergooi hospital.

List Of Publications

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