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A new method to determine the optimal exposure to mitotane treatment in patients with adrenocortical carcinoma

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1. INTRODUCTION

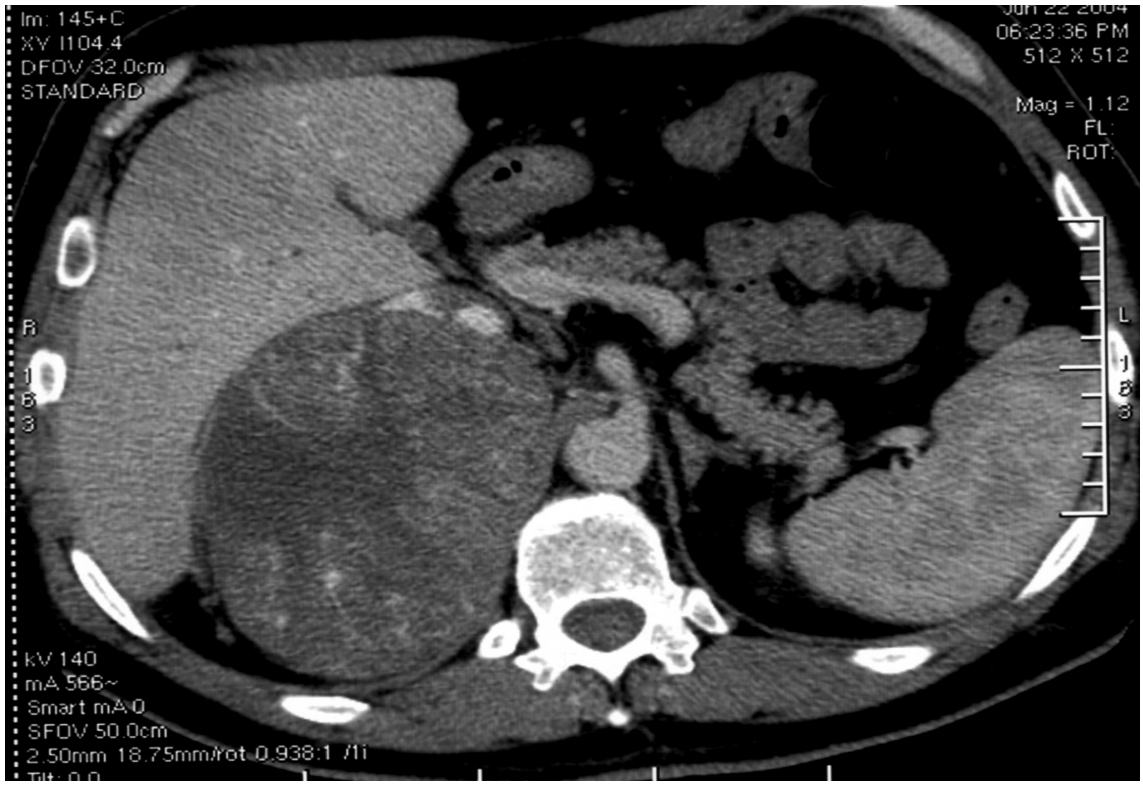
1.1 Adrenocortical carcinoma

1.1.1 Diagnosis

Adrenocortical carcinoma (ACC) is a rare tumor (0.5–2 cases per million per year) with a peak incidence between 40–60 years, and with women being more affected (55– 60%) (1, 2). ACC is characterized by a poor prognosis in most cases (3). However, prognosis is heterogeneous being mainly influenced by tumor stage at diagnosis (5-year survival rate is 81, 61, 50, and 13%, respectively, from stage 1 to stage 4) and completeness of surgical extirpation (4). ACC usually present as a sporadic tumor, but can be encountered in the setting of hereditary tumor syndromes, such as Li Fraumeni (TP53 germline and somatic mutations), familial adenomatous polyposis coli (β-catenin somatic mutations) and Beckwith–Wiedeman (IGF-2 overexpression) (5).

The diagnostic approach to any adrenal mass should include use of high-resolution imaging techniques to ascertain the risk of malignancy. In current practice, Computed Tomography (CT) is the most frequently used test for this aim and also for staging purpose. Magnetic Resonance Imaging (MRI) and Fluorodeoxyglucose-Positron Emission Tomography (FDG-PET). FDG-PET are often used as second-line tests, when CT findings are inconclusive (3, 6-8). There is still insufficient evidence to judge which modality is superior (9); however, unenhanced CT is generally considered the primary imaging test to exclude an adrenal malignancy, in presence of a homogeneous adrenal mass with density ≤10 Hounsfield Units and size ≤4 cm (7). Conversely, ACC should be suspected in case of an inhomogeneous mass with elevated density (due to low fat content). In addition, a large size enhances the probability of malignancy, especially when the mass is ≥ 4 cm, as other characteristics like intra-tumoral necrosis and irregular mass shape and borders (**Figure 1**).

Figure 1. Typical CT appearance of ACC (large, necrotic, inhomogeneous adrenal mass).



When an adrenal mass is suspected to be an ACC, it is key to conduct a full staging by extending imaging evaluation to the thorax and pelvis, since ACC may present with distant metastases in about 25% of cases (3, 4, 6-8).

Fine needle aspiration biopsy (FNAB) has no established role in securing the diagnosis, if not in case of unresectable ACC, when it can serve to inform further management (7). One of the reasons that do not support a routine use of FNAB is that making a pathologic diagnosis of ACC may be a real challenge that becomes even more difficult if only limited material is available, as with FNAB.

The Weiss score is the cornerstone of pathological diagnosis. It includes nine criteria of proliferation, nuclear abnormality and tumor extension and may have also a prognostic stratification power (10, 11) (**Table 1**).

Table 1. The Weiss score (Weiss et al., 1989).

Nuclear atypia
Atypical mitoses,
Mitotic rate >5 in 50 HPF
Character of cytoplasma
Architecture of tumor cells
Necrosis
Invasion of venous structure
Invasion of sinusoidal structure
Invasion of the capsule of tumor
Invasion of the capsule of tumor

A Weiss score of 0–2 defines benign adrenal tumors, while tumors with a Weiss score of 3, or more, are considered malignant. Tumors with a Weiss score of 2 or 3 may eventually display an undetermined behavior. A correct assessment of this morphological score is strictly dependent on individual expertise and an easier standardization is urgently needed.

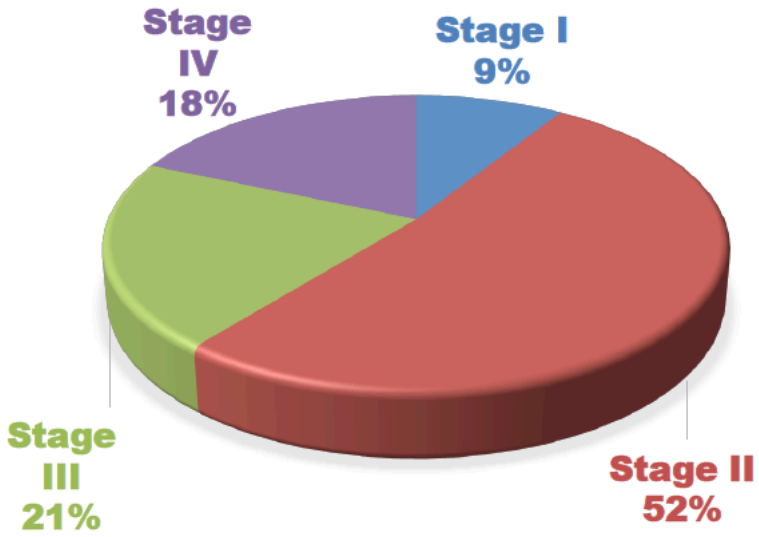
Several staging systems have been used in the past, but in the last years the system developed by the European Network for the Study of Adrenal Tumors (ENSAT) has emerged as the most useful one (**Table 2**). The ENSAT staging system allows a more precise prognostic differentiation among stages, and in this system, tumor infiltration in surrounding tissues, tumor thrombus in caval or renal vein, and/or positive lymph nodes define stage III, whereas the presence of distant metastasis is the only criteria for stage IV (4). It is worth of note that the stage stratification depends heavily on the accuracy of staging procedures and heterogeneity of populations (i.e. it is generally lower in surgical series). At San Luigi Hospital (Orbassano), less than one third of cases have advanced disease **(Figure 2),** and this may depend on the particular referral pattern of our center, since many of the patients are seeking advice for possible post-operative adjuvant therapy.

Table 2. Staging System for ACC proposed by the European Network for the Study of Adrenal Tumors (ENSAT) (4).

Stage	
I	T1, N0, M0
II	T2, N0, M0
III	T1-T2, N1, M0; T3-T4, N0-N1, M0
IV	any T, any N, M1

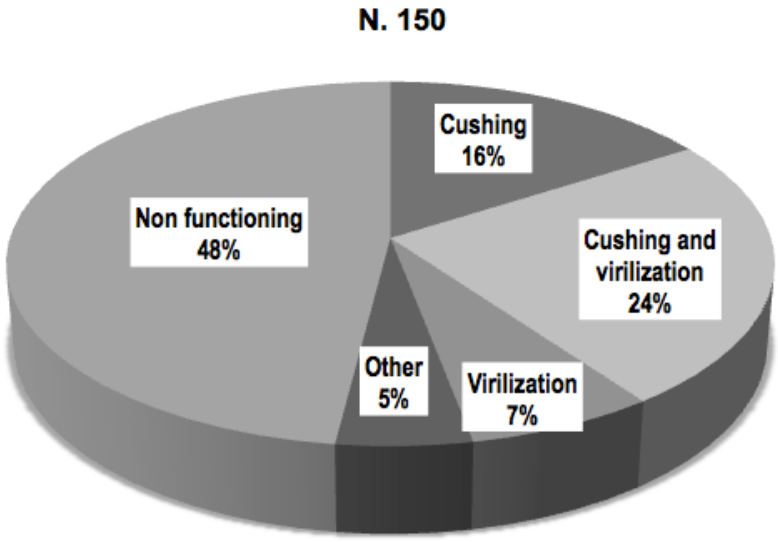
T1, tumor ≤5 cm; T2, tumor >5 cm; T3, tumor infiltration into surrounding tissue; T4, tumor invasion into adjacent organs or venous tumor thrombus in vena cava or renal vein; N0, no positive lymph nodes; N1, positive lymph node(s); M0, no distant metastases; M1, presence of distant metastasis.

Figure 2. Stage at diagnosis in 198 patients with ACC (San Luigi Hospital series).



ACC has the propensity to produce and secrete steroids; thus, in all patients with suspected ACC, signs and symptoms of cortisol, aldosterone, and sex steroids should be actively investigated (6). Manifestations of adrenal steroid hormone excess represent the most common presentation of ACC in up to 60% of cases (**Figure 3**) (3, 12).

Figure 3. Hormonal secretion in patients with ACC (S. Luigi series).



Concomitant secretion of different steroids is a hallmark of ACC. The most frequent condition is a cortisol secreting ACC causing a Cushingoid phenotype including facial plethora, easy bruising, weight gain, proximal myopathy, severe hypertension, and uncontrolled diabetes mellitus. Hypokalemia is common with severe hypercortisolism because mineralocorticoid receptors are triggered by the large amount of cortisol that overwhelms the inactivating capacity of corticosteroid 11β-dehydrogenase isoenzyme 2 (HSD11B2). Women frequently complain of acne, hirsutism, and oligomenorrhea (13). The differential diagnosis in these situations is PCOS, especially with mild or subclinical hypercortisolism. Clinical clues that are helpful to the diagnosis of ACC are the concomitant existence of a Cushingoid phenotype with signs of marked androgen excess, with cancer-related symptoms (anorexia, cachexia, mass effect). With rapidly growing tumors, cancer-related features dominate the clinical presentation. ACC can also cause deep venous thrombosis or pulmonary embolism due to either cortisol excess or malignancy (3). Moreover, the presence of cortisol excess may consistently increase the toxicity of chemotherapy since it is associated with immune depression that may favor

infections particularly in the neutropenia phase. Cortisol excess should be excluded in all patients with suspected ACC, even if they do not present with typical Cushing features (7).

A detailed hormonal workup (**Table 3**) should be performed preoperatively in all patients with suspected ACC for the following reasons:

- Demonstration of steroid excess establishes the adrenocortical origin of the tumor, while other differential diagnoses are being ruled out (i.e. lymphoma, sarcoma);
- The steroid profile may be helpful to evaluate the malignant potential (i.e. estradiol excess in males, high concentration of dehydroepiandrosterone sulfate (DHEAS) or steroid precursors);
- Presence of autonomous cortisol secretion in a patient with ACC indicates a risk of postoperative adrenal insufficiency, which can be potentially life-threatening;
- Demonstration of steroid excess at baseline establishes tumor markers that can be useful to detect persistence or recurrence of disease postoperatively (3, 8).

Table 3. Endocrine assessment in patients with suspected or proven ACC

CONDITION	**TESTS**
CORTISOL EXCESS	[a]Serum cortisol following 1-mg DST (v.n. < 1.8 mcg/dl) [a]Morning plasma ACTH Urinary free cortisol (24 h collection) Morning plasma ACTH Night-time salivary cortisol
ALDOSTERONE EXCESS	Plasma aldosterone and plasma renin activity (PRA) or direct renin *(if hypokalemia and/or arterial hypertension)*

SEX STEROID EXCESS	Androstenedione Testosterone (in women) 17β-estradiol (in men and postmenopausal women)
STEROID PRECURSORS EXCESS	[a]DHEAS [a]17OH-progesterone
CATHECOLAMINE EXCESS	Urinary fractionated metanephrines (24 h collection) or free plasma metanephrines

[a] *Mandatory tests*

A standard 1 mg overnight dexamethasone suppression test (1-mg DST) is recommended to exclude autonomous cortisol secretion in accordance with low or suppress levels of ACTH, similar with adrenal incidentaloma (7) This test has higher sensitivity (95% at a cortisol threshold of 1.8 μg/dL), compared with 24-h urinary-free cortisol (UFC) which is not helpful in cases of mild hypercortisolism (14). If cortisol levels following the 1-mg DST are not suppressed despite lack of overt Cushing syndrome, the condition of autonomous cortisol secretion may be present. The recent guidelines of the European Society of Endocrinology and the European Network for the Study of Adrenal Tumors (ENSAT) promoted this definition to the classic 'subclinical Cushing's syndrome' (7). Autonomous cortisol secretion is certain for a cortisol levels above 5 μg/dL after 1-mg DST, while values between 1.8 μg/dL and 5 μg/dL require additional investigation to confirm the diagnosis (7). Recognizing asymptomatic cortisol excess preoperatively identifies the patients who benefit from glucocorticoid replacement in anesthesia induction and after adrenalectomy and during follow-up (15).

Aldosterone-producing ACC is rare and is generally associated with severe hypertension and marked hypokalemia (16). Screening by measuring plasma aldosterone and plasma

renin activity (PRA) (or direct renin concentration) is recommended in all hypertensive and/or hypokalemic patients with adrenal masses (17). In some cases, pseudo-aldosteronism is present, due to increased production of deoxycorticosterone. Pure estrogen excess is rare and may cause gynecomastia, loss of libido and testicular atrophy in men, while in women menstrual irregularities (8). Hypersecretion of sexual steroids is frequently associated to cortisol excess in ACC patients. Baseline 17-OH progesterone levels are frequently increased, as well as androstenedione and DHEAS, which leads to increased plasma testosterone in females with signs of androgen excess (hirsutism, acne, alopecia) (3). Measurement of steroid precursors in blood or urine may be exploited for diagnostic purposes. However, the value of increased DHEAS levels to predict malignancy of an adrenal mass is rather low (18). More recently, it was demonstrated that serum steroid paneling by LC-MS/MS is a useful tool to discriminate ACC from other adrenal tumor lesions. In this study, both the number of steroids secreted in high amounts and the marked elevation of several steroid intermediates without biological activity was characteristic of ACC and useful for the differential diagnosis. The cortisol precursor 11-deoxycortisol was found the most discriminating between ACC and non-ACC adrenal lesions (19). Assessment of plasma or urine fractionated metanephrines is recommend in patients with suspected ACC to exclude a pheochromocytoma, and avoid misdiagnosis and unexpected intraoperative complications (3, 7, 13). Pertinently, the radiological imaging of pheochromocytoma may appear as a large, heterogeneous and hypervascularized mass mimicking ACC and rarely pheocromocytoma may present with hypercortisolism, being due to ectopic ACTH production (20). Finally, the hormonal assessment is fundamental because treatment should be directed toward both cancer and hormones, and the therapeutic approach varies according to the stage at diagnosis and clinical conditions of patients. However, it is important to recognize nonspecific

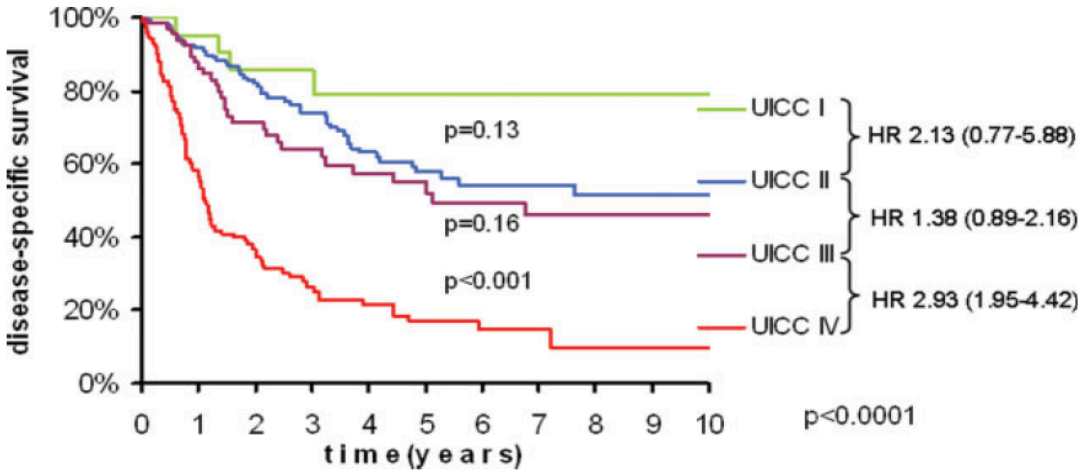
symptoms due to the mass effect, including abdominal discomfort (nausea, vomiting, abdominal fullness) and back pain, while classical malignancy-associated symptoms such as weight loss, night sweats, fatigue, or fever are less frequently observed (8).

All patients with suspected or proven ACC should be discussed in a multidisciplinary expert team meeting (including the following specialists: endocrinologist, oncologist, surgeon, radiologist, pathologist) at least at the time of initial diagnosis and at critical points during the disease course (e.g. tumor recurrence, progression). The team should have access to adrenal-specific expertise in interventional radiology, radiation therapy, nuclear medicine, and genetics as well as to palliative care facilities.

1.1.2 Prognostic factors

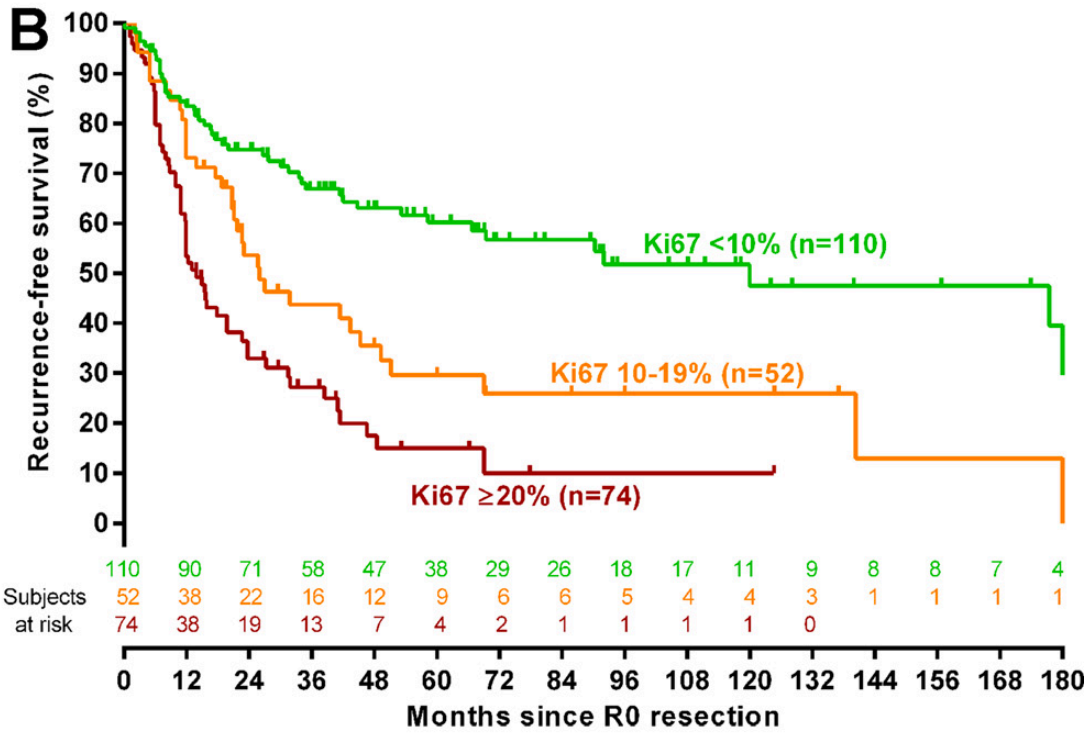
ACC stage and a margin-free resection are important and validated prognostic factors (3, 4, 6). Currently, the ENSAT staging system is the most frequently used and allows a clear stratification of prognosis by stage **(Figure 4)** (4).

Figure 4. Disease-specific survival by ENSAT stage (4).



Resection status Rx (unknown), R1 (microscopically positive margins) and R2 (macroscopically positive margins) are associated with progressively reduced survival irrespectively of other risk factors (3, 4, 6). The proliferation activity of the tumor influences the risk of recurrence following R0 surgery and proliferation is currently assessed by the immunohistochemical evaluation of the Ki-67 index, despite some problems to harmonize readings among different pathologists. Higher values of Ki-67 index are consistently associated with a worse prognosis and, in a multicenter study, a Ki-67 value at 10% was found to separate patients at good or worse prognosis, in terms of risk of recurrence following complete resection (21) **(Figure 5)**.

Figure 5. Prognostic role of Ki67 in localized ACC after complete resection (21).



Assessment of the mitotic index carries the same information, and a cutoff at >20 mitoses per 50 high-power field has been established to define high-grade tumors (22). However, studies correlating the two proliferation indexes are lacking.

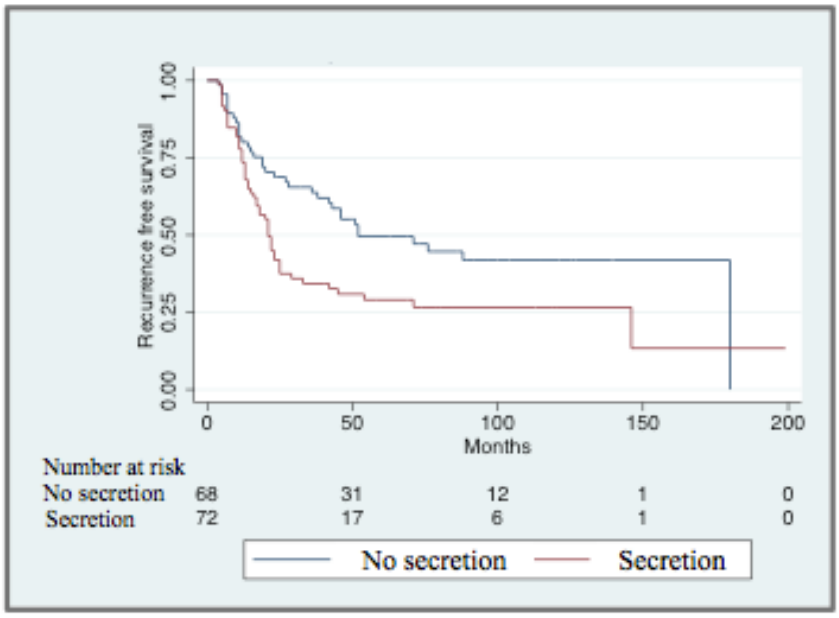
The role of overt cortisol excess as a negative prognostic factor has been first suggested in an Italian study (23), including 72 patients with metastatic or locally advanced ACC, submitted to chemotherapy with etoposide, doxorubicin, and cisplatin plus mitotane. Patients with cortisol hypersecretion had shorter overall survival, both in univariate and multivariate analysis (HR: 0.64, 0.42–0.97; P< 0.04). This finding was confirmed by a French study (24), including 202 patients with ACC at different stages. In this large series from a single endocrine center, 135 patients presented with hypercortisolism, and multivariate analysis identified cortisol overproduction as an independent prognostic factor associated with shorter survival (HR 3.90; P< 0.0001). In this subgroup of patients with cortisol-secreting tumors, adjuvant mitotane treatment had a positive effect on the risk of death (HR 0.40; P< 0.04). However, another study in 124 patients with metastatic ACC, did not find an association between cortisol secretion and prognosis (25).

The interaction between cortisol excess and adjuvant mitotane therapy has been investigated by Berruti and colleagues (26) in a multicenter, retrospective series of 524 patients with completely resected ACC, of whom 197 patients (37.6%) with overt Cushing's syndrome. After adjustment for sex, age, tumor stage and adjuvant mitotane therapy, hypercortisolism remained a strong independent predictor for both recurrence (HR: 1.30, 1.04–2.62; P= 0.02) and death (HR: 1.55, 1.15–2.09; P= 0.004). Efficacy of adjuvant mitotane treatment was not affected by the secretory status. More recently, a study carried out in US surgical institutions (27) demonstrated an association between cortisol secretion and risk of postoperative complications (HR: 2.25, 1.04-4.88; p=0.04). Moreover, the study confirmed that hypercortisolism was an independent prognostic factors associated with shorter recurrence-free survival (HR: 2.05, 1.16-3.60; p<0.01). There are several possible underlying mechanisms by which excess cortisol influences the prognosis in ACC patients. First, hypercortisolism is associated with increased

morbidity and mortality (28) that complicates the management of ACC patients. Second, although an association between cortisol secretion and tumor grading has not been demonstrated so far, tumors with cortisol production may be more aggressive. This was supported by a French study evaluating the role of SKG1 protein expression in ACC (29). SKG1 is a glucocorticoid-inducible kinase involved in cell cycle progression, acting as an anti-apoptotic factor. The study demonstrated an inverse association between SGK1 expression and cortisol overproduction (29). Low SGK1 protein level was identified as a negative prognostic factor in ACC patients, being associated with reduced OS (HR: 2.0, 1.24–3.24; P <0.005). Third, the immunosuppressive effects of overt cortisol excess before surgery may favor the development of ACC micrometastases and recurrences.

Of note, studies implicating hypercortisolism as a negative predictive factor were retrospective and used variable methods used to confirm cortisol excess. However, they suggest that cortisol excess in ACC may identify a cluster of patients who need more active surveillance and treatment **(Figure 6)** (12).

Figure 6. RFS in patients with cortisol-secreting ACC *versus* patients with non functioning ACC (S. Luigi series).



1.1.3 Surgical treatment

Surgery is the first option in ACC without evidence of metastatic disease (stages I–III) and the only possibility of cure. The 5-year survival rate is approximately 55% when radical resection is possible (30). In patients with infiltrating tumor or suspected lymph nodes open adrenalectomy (OA) is recommended; on the contrary, a localized ACC (I–II stages) can be removed by laparoscopic adrenalectomy (LA) or by OA (6), although the use of laparoscopic approach in this setting is still debated. Literature is indeed discordant: Huynh and colleagues (31) showed that use of LA may decrease survival in patients with stage II ACC, while most of the other studies failed to demonstrated significant different outcomes between LA and OA (32-35). In these studies, the recurrence rate was 54%, 50%, 53%, and 49%, respectively, after LA compared to 61%, 64%, 65%, and 64%, respectively, after OA. However, a study raised concern about the most frequent occurrence of peritoneal carcinomatosis with the use of LA (36). However, all these studies are retrospective and likely prone to selection bias, and no prospective trials are available at this moment. Whatever the surgical approach, surgery must be performed by an extremely skilled surgical team, in centers with high volume of adrenalectomies per year (37), with the goal of a R0 resection (microscopically free margins). Despite state-of-the-art surgery recurrence after intervention is frequent. Since an early detection of local recurrence or limited metastatic disease can open the possibility of a complete resection associated with a long Recurrence-free survival (RFS) (38), a tight follow-up is mandatory. Hormonal assessment and imaging (total-body CT) should be done every 3 months for at least 2 years after surgery. After this period, intervals could be gradually increased, but also in patients without evidence of disease follow-up is recommended in the long period (6).

1.1.4 Adjuvant treatment

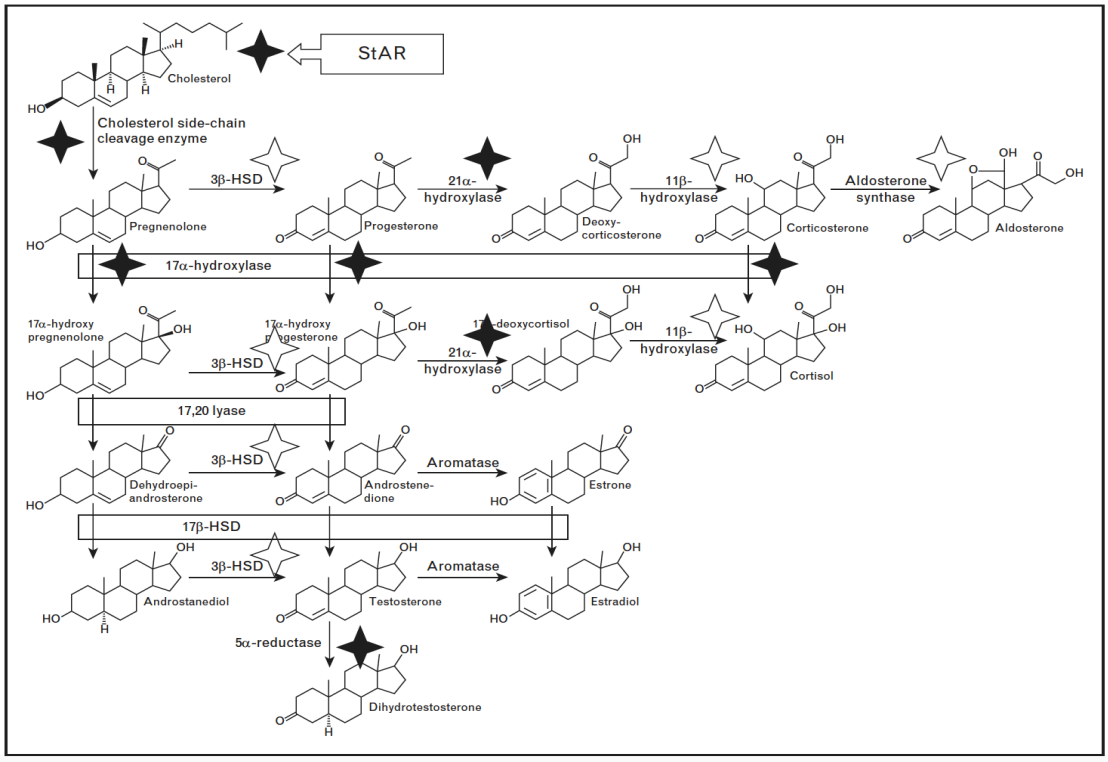
The risk for recurrence is lower for patients who undergo surgery by expert surgeons (39) but cannot be completely prevented. More than 50% of the tumors that have been completely extirpated are doomed to relapse (40) and most patients with ACC recurrence experience further tumor progression and eventually die of the disease. Therefore, the significant propensity of ACC to recur provides a rationale for adjuvant therapies.

1.1.4.1 Mitotane

To reduce the high rate of recurrence, most centers recommend adjuvant treatment with mitotane (o,p'-DDD), available in 500- mg tablets (Lysodren®) for oral administration. Mitotane is an adrenolytic drug, a parent compound of the insecticide dichlorodiphenyltrichloroethane–DDT, able to inhibit gene expression of various cytochrome P450-dependent mitochondrial enzymes of the steroidogenetic pathway (41-45). Steroidogenic acute regulatory protein (StAR) and CYP11A1, which are involved in the rate-limiting step of steroidogenesis, are most sensitive to mitotane (46) (**Figure 7**). The intracellular targets of mitotane remains to be identified; however, studies showed that mitotane effects seem to be mainly mediated by the mitochondria damage that activates an apoptotic process involving caspase 3 and caspase 7 activities (45). Moreover, mitotane was found to be a strong inducer of CYP3A4 activity leading to glucocorticoid inactivation and a consequent sharp rise in 6 β hydroxycortisol urinary excretion (47). It was calculated that mitotane is able to inactivate 50% of administered hydrocortisone and this explains why patients on mitotane have an increased dose requirement of steroid replacement. More recently, Sbiera *et al.* (48) demonstrated that mitotane is an inhibitor of sterol-O-acyl-transferase 1 (SOAT1) leading to accumulation of free cholesterol at toxic levels for the cell. The fact that SOAT1 is predominantly

expressed by the adrenals confers the specificity of action to mitotane. By inhibiting SOAT1, mitotane down-regulates steroidogenesis and exerts its cytotoxic effect due to lipid-induced endoplasmic reticulum stress.

Figure 7. Effects of mitotane on adrenal steroidogenenesis (black diamonds represent enzymatic steps that are inhibited by mitotane, white diamonds represent less affected steps).



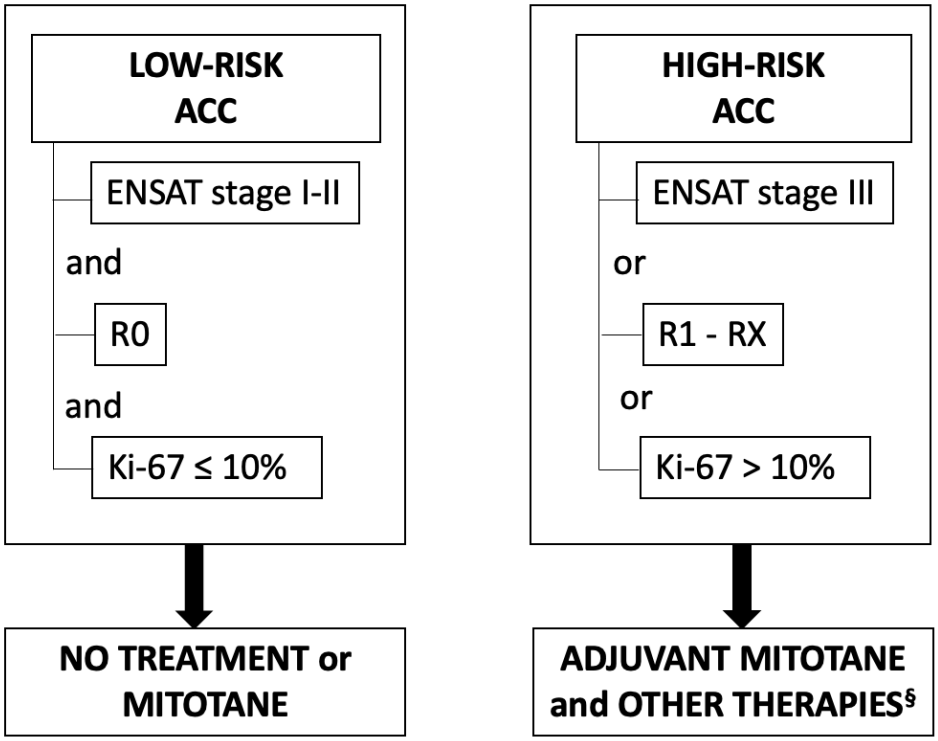
Use of adjuvant mitotane in ACC was first proposed by Schteingart and colleagues in 1982 (49). No data from randomized trials are available; however, convincing results in support of adjuvant therapy with mitotane were provided by a large retrospective study of ours, including 177 patients from different Italian and German centers. A group of patients underwent adjuvant therapy with mitotane after surgery while patients of two contemporary independent control groups were followed without any therapy. RFS was significantly longer ($p < 0.0001$) in the 47 patients treated with adjuvant therapy (42

months) compared to the groups of 55 and 75 patients not treated after surgery (10 and 25 months, respectively). Also overall survival (OS) was significantly prolonged in the mitotane group (110 months) compared to the two control groups (52 and 67 months, respectively) (50). Recently, the group has updated the follow-up of these cohorts of patients with almost 10 years of additional observation, confirming that adjuvant mitotane treatment is associated with a significant benefit in terms of RFS regardless of the hormone secretory status (51). Advantage in OS was less evident, but this may be explained by the fact that mitotane was introduced as treatment of ACC recurrence in most patients. Despite its retrospective nature, this study remains the most informative piece of evidence on the topic and represents a reference for decision-making in ACC patients. Strengths of the study are the inclusion of contemporary groups of matched patients, who were allocated to treatment or follow-up based on the treatment policy the center. Conversely, in many studies patients with unfavorable characteristics were more likely selected for adjuvant mitotane, thus introducing a bias. An example of this may be found in a recent study reporting a multicenter, retrospective analysis on 207 ACC patients, showing that adjuvant mitotane was associated with decreased RFS. However, 42% of the patients treated with mitotane had stage IV ACC and, indeed, chemotherapy was frequently associated to mitotane therapy (52). A retrospective study from the University of Michigan confirmed the finding that adjuvant mitotane treatment is associated with a significantly improved RFS although it failed to prolong significantly OS (53). The lack of effect on OS may be explained with the short follow-up (25.6 months). Despite controversy on this issue, there is general agreement on the adjuvant use of mitotane following surgical removal of ACC in high-risk patients. The condition of high risk of recurrence has been defined as stage III, or Ki-67 > 10%, or Rx-R1 resection by a panel of international experts (54). For low risk patients, who are

characterized by stage I or II, R0 resection and Ki-67 ≤ 10%, adjuvant mitotane therapy is not mandatory (**Figure 8**).

An international, multicentric, prospective, randomized trial (ADIUVO trial) is currently enrolling low-risk ACC patients, who are randomized to mitotane or observation, in order to definitely establish the effectiveness of adjuvant mitotane in this set of patients.

Figure 8. Management strategy following surgical extirpation of localized ACC.

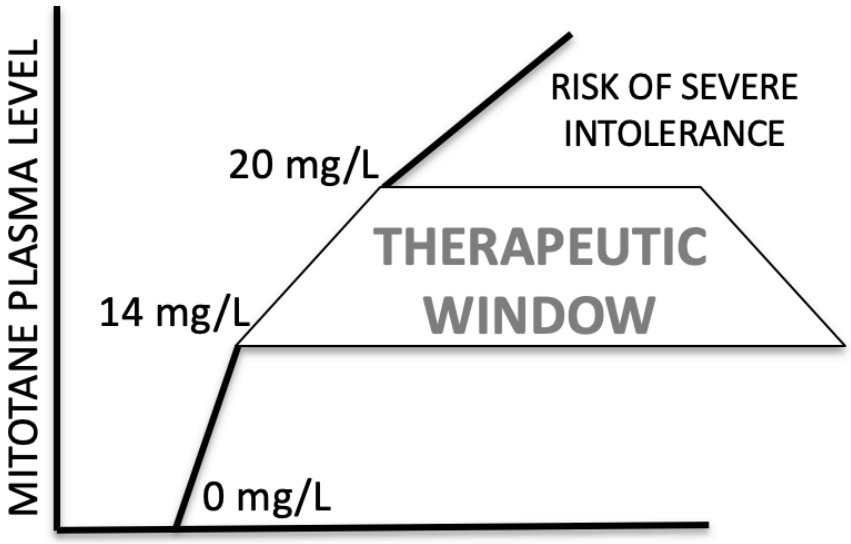


§ In patients with poor prognostic factors, local bed radiation therapy or chemotherapy can be associated

It is recommended to regularly monitor plasma mitotane levels during treatment with the aim of maintaining levels >14 mg/L (7), based on studies suggesting a link between high mitotane levels and drug efficacy (55-59). On the other hand, elevated mitotane concentrations (> 20 mg/L) have been associated with severe toxicity. Therefore, the aim

of the monitoring is also to avoid excessively high concentrations, maintaining plasma mitotane levels in the narrow therapeutic window (**Figure 9**).

Figure 9. The therapeutic window of mitotane



It is worth of note that the evidence supporting a target range when mitotane is used as an adjuvant measure is limited and conflicting (58, 60), and this may be due to the challenge in assessing the optimal exposure to mitotane in chronic treatments. Moreover, the validation of this range was done using the peak mitotane level (56, 57, 59), which cannot give an adequate representation of a chronic exposure to mitotane, being a measurement at a single point in time, or the percentage of mitotane measurements in a range (55, 58), which is strongly dependent on the number of available measurements. Therefore, new studies and new approaches are need to better clarify this issue.

There is no consensus on how to start treatment: the ESMO guidelines (54) recommend that mitotane therapy should be administered following a high-dose regimen with the aim of reaching a daily dose of 6 g/daily rather soon and then adjust the dose according to

tolerability and mitotane levels. However, a treatment at lower doses (**Table 4**) seems to be better tolerated, with less patients discontinuing treatment (61).

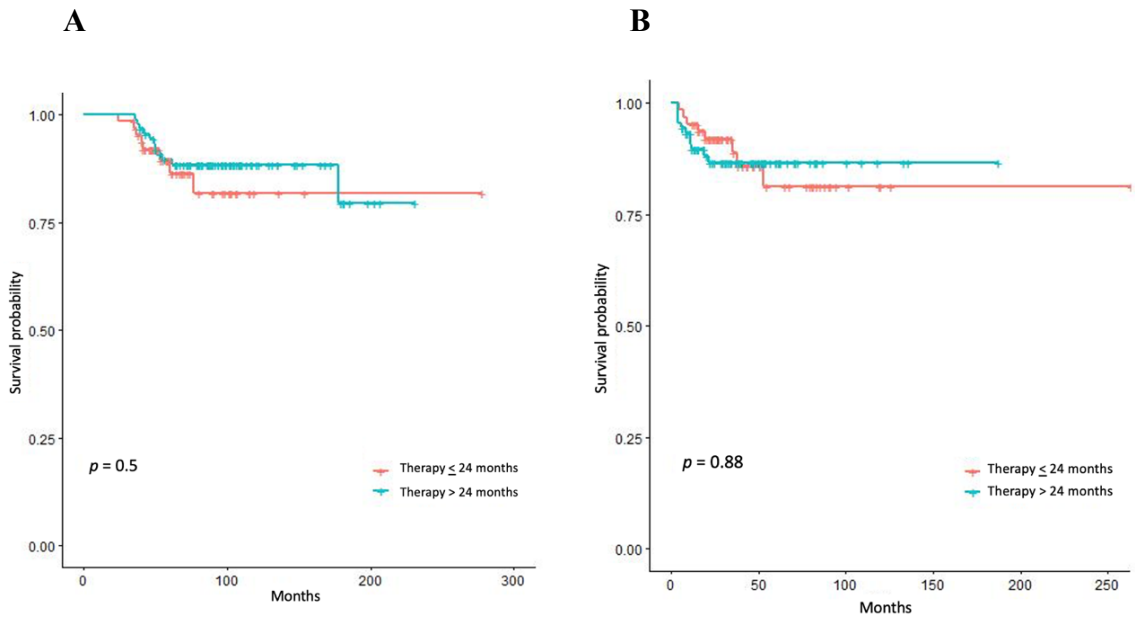
Table 4. Practical guidelines for low-dose adjuvant mitotane treatment in patients with ACC

● Start with 1 g daily and increase mitotane dose every 4-7 days up to 6-8 g daily, or the maximum tolerated dose. Give mitotane in split doses with meals or snacks.
● Check mitotane levels after 4-8 weeks to adjust dosage until reaching target levels.
● Accommodate mitotane schedule to patient's tolerance aiming at serum mitotane concentrations of 14-20 mg/L (therapeutic levels).
● At target, clinical assessment, biochemical and hormonal evaluation, and monitoring of mitotane levels every 3 months, or in case of significant side-effects. Adjust mitotane dose according to circulating levels and tolerability.
● In case of slight unwanted effects, continue mitotane and use symptomatic therapy.
● In case of moderate side effects, step down to the previously tolerated dose and use symptomatic therapy.
● In case of severe side effects, discontinue mitotane and institute specific treatment. Duration of treatment stop depends on clinics and mitotane levels. After interruption, restart with a lower dose.
● Imaging assessment with thorax, abdomen and pelvis CT every 3 months for 2 years, then the timing of re-assessment may be increased.

Adjuvant mitotane treatment must be started as soon as possible and usually no longer than 12 weeks following surgery, even if there are no data showing what is the best timing. Duration of adjuvant mitotane therapy has not been definitively established, but it is reasonable to continue therapy for at least 2 years, because this is the period when most of ACC recurrences are detected. A recent multicenter study tried to address this question, assessing whether a correlation exists between the duration of adjuvant mitotane treatment and RFS of patients (62). This retrospective analysis on 154 ACC patients do not support the concept that extending adjuvant mitotane treatment over two years is

beneficial for ACC patients with low to moderate risk of recurrence. In fact, both RFS and RFS after mitotane (RFSAM), which was calculated from the landmark time-point of mitotane discontinuation to overcome immortal time bias did not show any survival advantage in patients treated for longer than 24 months (**Figure 10**).

Figure 10. Kaplan–Meier estimates of RFS (**A**) and RFSAM (**B**) in patients treated ≤24 months versus patients treated >24 months.



The most common unwanted effects are gastrointestinal manifestations that appear early in the course of treatment, independently on mitotane levels (63). Diarrhea and nausea are particularly frequent and can be managed with temporary dose reduction and supportive therapy. Elevated gamma-glutamyltransferase levels are also frequently observed but are not actually troublesome unless values are exceedingly elevated. Clinically significant liver toxicity is characterized by a marked increase in transaminases and bilirubin, but is infrequently observed in the absence of predisposing conditions (61). Central neurologic toxicity (cerebellar symptoms, disturbed cognitive performance) is

more closely associated with elevated mitotane concentrations (20 mg/L) but subtler symptoms, such as memory impairment or attention deficit, may be observed in some patients even at lower drug concentrations (64). In this context, monitoring of circulating mitotane levels may be useful to tailor individually the therapy and limit side effects thus attaining better compliance to treatment. The implementation of blood mitotane monitoring, through a service provided in Europe by the company distributing Lysodren® (Lysosafe®, www.lysodren-europe.com), has rendered the use of this drug more feasible because it is possible to some extent to anticipate and prevent toxicity. Measurement of circulating mitotane concentration has become mandatory for a proper management of patients with ACC and it should be done every month in the starting phase of treatment, and then every 3 months once mitotane levels are at plateau; moreover, additional monitoring is adjusted according to clinical needs. Biochemical monitoring includes also blood count, liver function tests, creatinine, electrolytes, glucose, lipids, ACTH, cortisol, PRA, testosterone, DHEAS, 17-hydroxyprogesterone, androstenedione, LH, FSH, TSH, FT4. A general measure to deal with mitotane toxicity is a step down to the previously tolerated dose, or temporary drug withdrawal in case of severe manifestations (**Table 4**). However, well-informed and motivated patients are able to cope with side effects and maintain compliance to treatment. To accomplish this task, it is important to establish a close patient–physician relationship to induce and maintain adherence to treatment. Patients seek advice frequently, also because their local physicians are unfamiliar with mitotane use and its attendant complications, and it is necessary to give a timely counseling to keep patients on treatment.

Mitotane has a wide range of effects on the endocrine system and may potentially cause several endocrine disturbances that should be carefully managed (**Table 5**).

Table 5. Endocrine effects of mitotane and related treatment.

Condition	**Therapy**
Adrenal insufficiency	Glucocorticoid replacement is mandatory in all patients on mitotane therapy. High doses are needed (i.e. hydrocortisone 50–75 mg daily or cortisone acetate 75–100 mg daily). Adequacy of replacement is mostly assessed on clinical bases. High ACTH levels may herald under-replacement. A subgroup of patients requires fludrocortisone replacement (0.1–0.2 mg daily) driven by clinics and high PRA levels.
Hypothyroidism	Thyroxin supplementation in most patients driven by low FT4 levels.
Hypogonadism	Testosterone replacement in a subgroup of men driven by clinics and testosterone levels.

Because of the adrenolytic effect of mitotane, all patients should receive glucocorticoid replacement to prevent adrenal insufficiency. Steroid doses are typically higher than in Addison's disease, due to an enhanced metabolic clearance rate of glucocorticoids induced by mitotane (3, 6, 65). An inadequate treatment of adrenal insufficiency increases mitotane-related toxicity, particularly gastrointestinal side effects, and reduces tolerance (37). Mineralocorticoid supplementation is not mandatory in all patients because the zona glomerulosa is partly spared by the toxic effect of mitotane (66). Moreover, mitotane affects thyroid and gonadal function by mechanisms that are still to be completely elucidated. Mitotane administration is associated with low FT4 levels without a compensatory rise in TSH, an effect that becomes apparent early in the course of treatment. This prompts thyroxin replacement, even if the benefit of this measure may be

difficult to appreciate (64, 66). In women, gonadal function is usually preserved and most female patients have regular cycles unless PRL levels are significantly increased (6, 64, 66) due to a weak estrogen-like action of mitotane (67). Conversely, in men mitotane treatment causes sexual dysfunction as a late but common unwanted effect, due to inhibition of testosterone secretion. Sex steroid replacement may become necessary to treat hypogonadism in some patients but may worsen gynecomastia (6, 64, 66).

Mitotane use is associated with increasing levels of LDL and HDL cholesterol, and triglycerides (68). However, the value of introducing statins remains uncertain although patients may be worried about their lipid levels. The decision to use anti-lipid drugs, which may further complicate supportive therapy and is not exempt from potential toxicity, should be carefully though at considering patient life expectancy. Side effects of mitotane treatment are showed in **Table 6**.

Table 6. Side effects of mitotane treatment.

Side effect	**Frequency**
Gastrointestinal (nausea, vomiting, diarrhea, anorexia)	≥1/10
Adrenal insufficiency	≥1/10
Neurological (lethargy, somnolence, vertigo, ataxia, confusion, depression, dizziness, decreased memory)	≥1/10
Increase of hepatic enzymes (in particular gamma-GT)	≥1/10
Increase in hormone binding globulins (CBG, SHBG, TBG)	≥1/10
Alterations of thyroid hormonal values (total T4↓, free T4↓, TSH↓)	≥1/10
Hypercholesterolemia, hypertriglyceridemia	≥1/10
Primary hypogonadism in men, gynecomastia	≥1/100 to <1/10
Skin rash	≥1/100 to <1/10
Prolonged bleeding time	≥1/100 to <1/10
Leucopenia	≥1/100 to <1/10
Thrombocytopenia, anemia	≥1/10,000 to <1/1,000
Liver failure	≥1/10,000 to <1/1,000
Autoimmune hepatitis	≥1/10,000 to <1/1,000
Blood hypertension	≤1/10,000

Ocular (blurred or double vision, toxic retinopathy, cataract, macular edema)	≤1/10,000
Hemorrhagic cystitis	≤1/10,000

modified based on information published by the European Medicine Agency (EMEA) http://www.emea.eu and clinical experience

1.1.4.2 Other adjuvant therapies

Another option is adjuvant radiotherapy, that in a retrospective analysis from the United States was reported to decrease of 4.7 times the risk of local failure compared with surgery alone (69). In a retrospective analysis from the German ACC Registry, radiotherapy in an adjuvant setting resulted in a significant better 5-year RFS, but did not affect OS and disease-free survival (70). However, no difference between surgery plus radiotherapy and surgery alone was found in another retrospective study done in the United States (71). A review of the literature concluded that adjuvant radiotherapy should be considered in patients with incomplete, or R1 resection, or Rx resection, who are at high risk for local recurrence (71). A total dose of > 40 Gy with single fractions of 1.8 Gy to 2 Gy should be administered. However, prospective investigations are required, and no definitive conclusions are available at the moment.

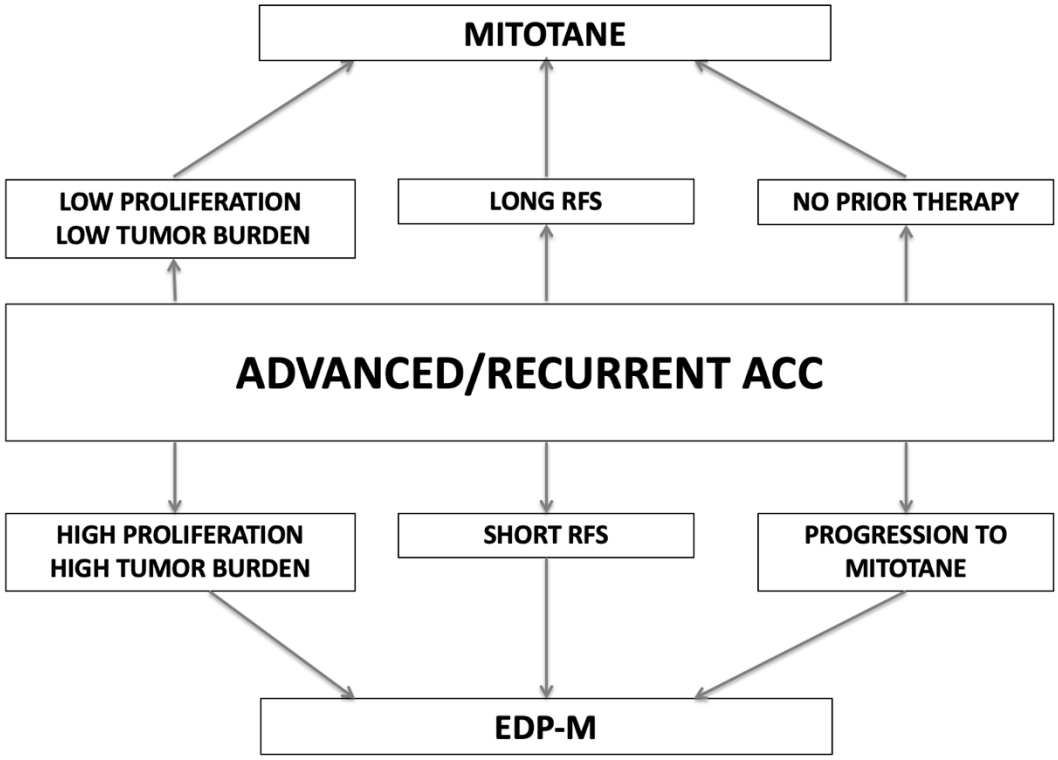
As far as chemotherapy is concerned, limited data are available. A recent paper published data on 3982 ACC patients from the National US Cancer Data Base (NCDB), revealing that adjuvant chemotherapy was performed in 10% of cases. However, the study was not able to capture in how many cases cytotoxic agents or mitotane have been used as adjuvant chemotherapy. By comparing these subjects with those treated with surgery only, OS was not different, while no RFS analysis was reported (72). Anecdotal cases reported a more favorable outcome after an adjuvant etoposide–cisplatin based chemotherapy (73). A phase II clinical trial reported that the combination of mitotane plus streptozotocin was effective in an adjuvant setting. However, the study design does not allow discriminating the relative merits of the two drugs (74).

1.1.5 Treatment of advanced disease

About 50% of newly diagnosed ACC patients present with metastatic or unresectable disease (54) and, as previously said, most ACC that underwent initial complete resection are doomed to develop recurrent or metastatic disease (54, 61). The prognosis of patients with advanced/metastatic ACC is generally poor but it is heterogeneous and long-term survivors have been described (61, 75). The management of these patients is mainly centered on systemic therapy including mitotane alone or mitotane in combination with chemotherapy. The standard chemotherapy regimen for advanced ACC is EDP (etoposide, doxorubicin and cisplatin) plus mitotane (EDPM). This scheme was introduced in a multicenter prospective phase II study conducted in Italy (23). More recently, its efficacy was compared against the combination of streptozotocin and mitotane (Sz-M) in a prospective randomized phase III clinical trial conducted worldwide (76). Three hundred and four patients were prospectively enrolled in about 6 years. Patients with disease progression to the first-line treatment received the alternate regimen. EDP-M was superior to Sz-M both in terms of disease response rate and progression-free survival (PFS). Analysis of OS also favored patients initially randomized to receive EDP-M but due to the attenuating effect of the cross over to EDP-M of patients who progressed to Sz-M, the difference failed to attain statistical significance. In addition to systemic therapy also locoregional therapies, i.e. surgery (40, 77), radiofrequency ablation (RFA) (77, 78), and chemoembolization (79) can be taken into consideration in a selected patient population. Moreover, in patients, who have contraindications to EDP, or poor performance status, either cisplatin or carboplatin administered as single agents could be reasonable options. It is worth of note that there is a small subgroup of patients with advanced/metastatic ACC presenting an oligo-metastatic disease with favorable prognostic factor and/or a relatively long disease-free interval from previous surgery (i.e.

12 months or more). These patients have a relative long survival perspective and may not benefit from an aggressive systemic treatment such as the EDP-M regimen. Therefore, single agent mitotane could be a reasonable option (**Figure 11**).

Figure 11. Management of ACC patients with advanced or recurrent disease not amenable of surgery with radical intent.



Mitotane is often associated with locoregional approaches in the treatment of these patients. Surgery of primary and or metastases can be recommended if a complete resection (R0) is achievable. Surgery of multiple metastases is considered on a case-by-case basis and should be performed mainly in patients with favorable prognostic factors, sustained disease response to systemic therapy, and long-term R0 resection expectations. In patients who are not candidates for surgery, percutaneous image-guided RFA is a locally effective treatment and chemoembolization is another possibility to treat liver

metastases. RFA in combination with surgical resection may allow better disease control in the setting of limited disease (77-79). Tumor debulking generally offers little benefit, however surgery of primary disease in newly diagnosed patients with oligo-metastatic disease and limited extra-adrenal tumor volume can be performed in case of good response to systemic therapy. It should be noted that the efficacy of local regional therapies in the management of such patients has never been assessed in a randomized prospective clinical trial, so we cannot exclude that the long-term benefit obtained in some cases can be ascribed to a patient selection. In the author opinion, the long-term benefit is due at least in part to the efficacy of systemic therapy; therefore, it is recommended that all local regional approaches should be used in combination with systemic therapy. On the contrary, the majority of metastatic ACC patients have poor prognostic features (i.e. 2 or more organs involved). For these patients, chemotherapy with EDP-M regimen represents the treatment of choice (**Figure 11**). In case of painful metastasis, palliative radiotherapy is an option, especially in bone lesions. Due to the latency of mitotane to attain the therapeutic range, the drug administered alone is not indicated in the management of patients with clinical evidence of fast growing tumors. Metastatic ACC submitted to EDP-M regimen have a survival perspective of 18 months as demonstrated by the results of the FIRM-ACT trial (76). However, 15% of patients are alive after 5 years. In terms of PFS, 50% of patients submitted to EDP-M showed disease progression after 5 months, and 25% of patients were free from progression after 12 months, and 15% after 2 years. In addition, few patients were still alive and free from progression after 5 years (76). These data show that the efficacy of chemotherapy plus mitotane is overall modest, but a small subset of patients is destined to obtain a long-term disease control. The identification of factors that may predict chemotherapy efficacy is very important to select patients destined to benefit from this aggressive strategy and to

address nonresponding patients to experimental therapies. In a recently published paper, our group has demonstrated that the expression of topoisomerase II was associated with EDP-M efficacy (80). These data need confirmation. It should be noted, however, that EDP is usually administered for a maximum of 6–8 cycles while mitotane is usually maintained till progression. It is possible that cytotoxic chemotherapy is useful to attain rapid tumor shrinkage, but the long-term efficacy observed in some cases could be attributed to the mitotane maintenance. If this is true, predictive factors of mitotane efficacy are needed. Human cytochrome P450 2B6 (CYP2B6) (81) and CYP2W1 (82) that are involved in mitotane metabolism and may activate mitotane in the adrenocortical tissue, respectively, or ribonucleotide reductase large subunit 1 (RRM1) gene expression (83) are promising predictive factors of mitotane efficacy. The value of these potential predictive factors should be assessed in prospective studies. Finally, regarding second-line therapy, the results of patients with disease progression to platinum-containing regimens plus mitotane were as a whole modest. The association of gemcitabine to metronomic capecitabine showed a limited activity in a prospective multicenter phase II trial conducted in Italy (84). Results have been confirmed a series of patients treated in a real world practice both in Germany and in Italy (85). This regimen still remains the most used option as second line therapy. Several small phase II trials have tested the efficacy of molecular agents targeting EGFR, angiogenesis, IGFR, and mTOR pathways. These treatments administered in pre-treated patients either alone or in combination with chemotherapy, or with other molecular target agents obtained poor results (86-88). In a multicenter randomized phase III trial involving most referenced centers in Europe and United States, the drug Linsitinib (OSI-906), an orally available IGFR inhibitor failed to demonstrate a superiority over placebo in terms of both progression free and OS in advanced pretreated ACC patients (89). Also, modern immunotherapy failed to show

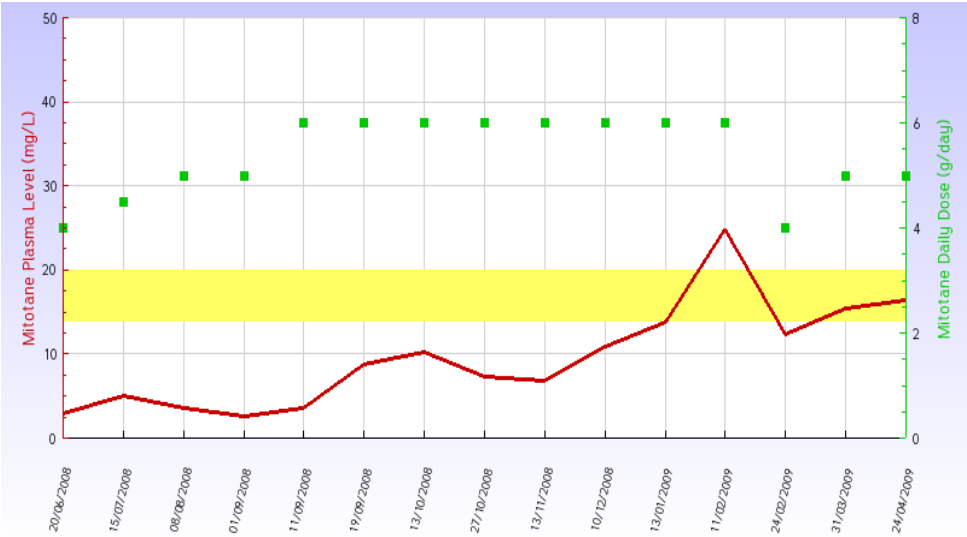
efficacy in advanced ACC. In a phase 1b cohort (NCT01772004), 50 patients with metastatic ACC and prior platinum-based therapy received avelumab at 10 mg/kg IV every 2 weeks, until progression. Only two patients (5%) attained a disease response while PFS was 5.5 and 1.5 months in patients with PDL-1 positive and negative ACC patients, respectively (90).

1.2 Lysosafe Online®

Lysosafe Online® is a login-protected website that stores mitotane plasma concentrations of patients treated by physicians who have registered with the Lysosafe service®, a free-of-charge service of measurement of plasma mitotane concentrations in ACC patients offered by HRA Pharma to European prescribers since 2005 and associated with the use of Lysodren®. Samples are collected at the centers, sent to a centralized laboratory, extracted by precipitation with ethanol, and tested by a standardized gas chromatography/mass spectrometry method. Plasma mitotane values of any patient are available for the treating physician on www.lysosafe.com, in a historical and graphic plot that matches mitotane levels with the relative Lysodren® dose (**Figure 12**).

Patient data are anonymous during the whole process since patients are recorded using an acronym and their date of birth.

Figure 12. An example of the graphic plot that matches mitotane levels with the relative Lysodren® dose.

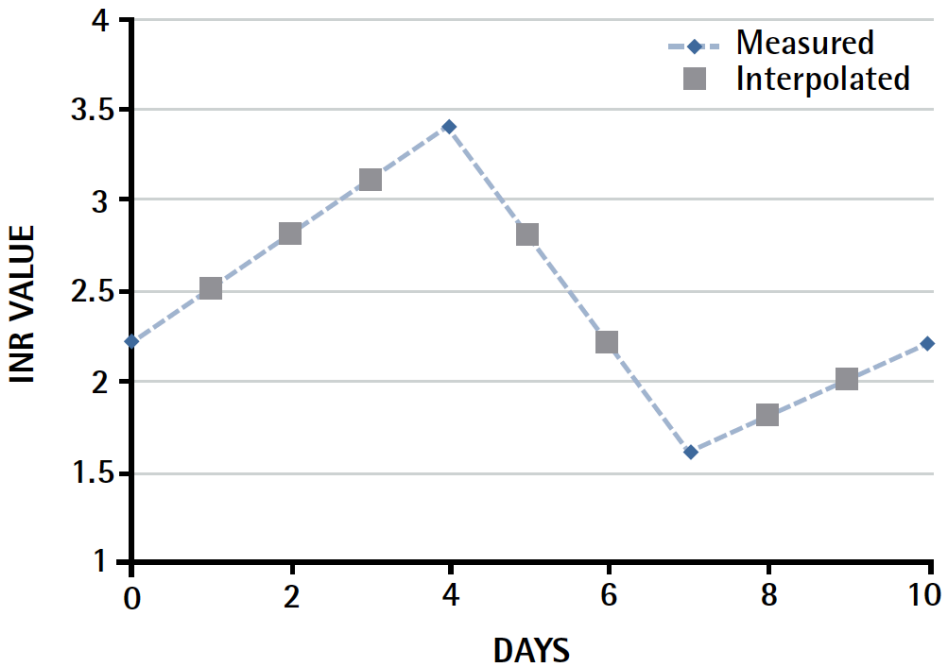


1.3 The concept of "Time in Therapeutic Range"

The concept of "Time in Therapeutic Range" (TTR) is closely related to warfarin therapy. Warfarin is a vitamin K antagonist and, although new oral anticoagulants are now available, it is one of the most used oral anticoagulants because of its availability and cost (91). Vitamin k antagonists have been shown to be effective in the treatment and prevention of thromboembolic events. However, they possess a narrow therapeutic window (92). The therapeutic range for warfarin therapy is defined in terms of the International Normalized Ratio (INR). The INR is calculated as the prothrombin time ratio, with the International Sensitivity Index (ISI) as exponent to standardize PT variations due to the use of different reagents and instruments in the measurement (patient prothrombin time/mean of normal prothrombin time for laboratory)^{ISI}. Obtaining exact and consistent INR levels maximizes the desired benefits and safety of warfarin (93). The Time in Therapeutic Range (TTR) estimates the percentage of time a patient's INR is within the desired treatment range or goal and is widely-used as an indicator of anticoagulation control. TTR is commonly used to evaluate the quality of warfarin therapy and is an important tool for assessing the risks versus benefits of warfarin therapy (94). The efficacy and safety of oral vitamin K antagonists such as warfarin depend strongly on the percentage of TTR, with the maximum benefits being evident when the TTR is >70% (95, 96). It is well-known that poor control of anticoagulant intensity increases the risks of thrombotic and hemorrhagic events (97). The consistency of an effective INR is reflected by the TTR, which is a measure of the period in which the patient was in an optimal INR range. One of the methods for assessing TTR in patients taking warfarin is the Rosendaal method (98, 99), which assumed that a linear relationship existed between consecutive values when a measurement was not available. Therefore, a linear interpolation can be used to calculate the TTR for each patient (**Figure 13**) (98).

The unknown INR values between dates of observation were interpolated using a linear function so as to apply an estimated INR value to every day within the observation period. The TTR was calculated as the number of days within target range divided by the total number of days in the observation period. Additionally, this method allowed for the combining of ranges of data that had been split by warfarin interruption. Calculations can be performed with the assistance of a template produced and made freely available by INR Pro (www.inrpro.com/rosendaal.asp).

Figure 13. Linear interpolation example



2. AIM OF THE STUDY

The aim of this study consisted in the validation of a new method for monitoring circulating mitotane concentration that it is based on the concept of the "time in therapeutic range" used for monitoring warfarin therapy, and that we named "time in target range" (TtR).

TtR was defined as the number of months in which mitotane concentrations were greater than 14 mg/L, a value considered as the lower limit of the therapeutic range that is associated with anti-neoplastic activity of mitotane in patients with adrenocortical carcinoma (56-59).

We tried to validate this new method in two different clinical settings: adjuvant treatment following complete ACC removal and palliative treatment of patients with advanced ACC by performing two different studies (TtR Study 1 and TtR Study 2).

In the TtR Study 1, we evaluated whether the TtR of plasma mitotane concentrations may influence the risk of recurrence in patients with ACC on post-operative adjuvant mitotane treatment.

In the TtR Study 2, we evaluated whether the TtR of plasma mitotane concentrations may influence tumor progression and overall survival in patients with advanced ACC.

3. MATERIALS AND METHODS

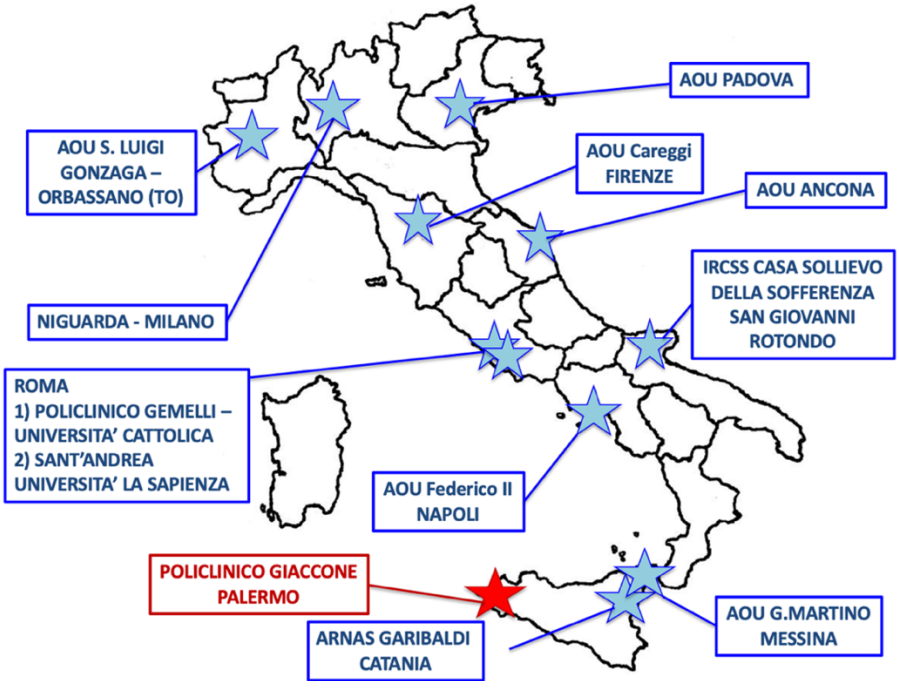
3.1 Implementation of TtR Study 1 and TtR Study 2

We invited 13 tertiary centers for the care of ACC patients in Italy to participate to the TtR Study 1 and the TtR Study 2, and to provide clinical, pathological, and biochemical data of all ACC patients who had been proactively followed at the center and treated with mitotane, according with the inclusion and exclusion criteria of the two studies. Eleven centers accepted to participate to the TtR Study 1 and twelve centers to the TtR Study 2 (**Figure 14**).

The institutional ethics committee of all centers approved the study, and all patients signed written informed consent.

All communication concerning the study between centers was by email, and a meeting was organized to harmonize the study procedures.

Figure 14. Tertiary centers for the care of ACC patients in Italy participating both to the TtR Study 1 and the TtR Study 2 (in blue) or only to the TtR Study 2 (in red).



3.2 TtR Study 1 – ADJUVANT SETTING

Inclusion criteria of the study were as follows:

- age ≥ 18 years
- pathologically confirmed diagnosis of ACC
- complete macroscopic resection
- availability of pre-operative and post-operative CT or MRI scans
- complete follow-up information
- treatment with mitotane (all patients received the same mitotane formulation, Lysodren® 500 mg tablets) for at least 6 months and with 3, or more, measurements of plasma mitotane concentrations reported on the Lysosafe Online® database.

Exclusion criteria were as follows:

- incomplete tumor staging
- history of other previous or concomitant malignancies
- R2 (macroscopic invasion of resected margins) resection
- incomplete follow-up information
- concomitant adjuvant chemotherapy and radiotherapy or both
- concomitant treatment with any drug specifically directed against ACC.

We retrieved the data of patients who were treated from July 2005 to July 2015.

Follow up for this study was closed in December 2017.

Patients' charts were reviewed and the following information was retrieved for the study: gender, age, body mass index (BMI), date of diagnosis, hormone secretion, ACC stage, pathology report, date of recurrence, last follow-up or death. Date of diagnosis was defined as the date of surgery. Biochemical confirmation of hormone excess was

requested to categorize an ACC as hormone secreting. Tumor stage was established according to the ENSAT classification (I and II, confined tumor; III, positive lymph nodes or infiltrating neighboring organs/veins without distant metastases; IV, distant metastases (4).

Date of recurrence was defined as the date of radiological evidence of a new lesion. A questionnaire was sent to the participating centers to retrieve the information requested for the study; moreover, centers were asked about indications, timing of initiation and discontinuation, reasons for discontinuation and dose regimen of adjuvant mitotane treatment, and follow-up modality. The schedule of follow-up visits was similar between centers, with an early assessment between 3–6 weeks after treatment initiation including physical exam, plasma mitotane monitoring, and biochemical work-up. Evaluations were afterwards scheduled every 3–4 months with physical exam, full body imaging, plasma mitotane monitoring, and biochemical work-up for at least 3 years. After this time limit, timing of the follow-up visits was individualized according to the preferences of the patients and physicians. Duration of treatment was calculated from the date of initiation of mitotane therapy until ACC recurrence, or discontinuation of treatment, or the end of follow-up, whichever occurred first.

Mitotane concentrations were retrieved from the Lysosafe Online® database, available at www. lysosafe.com. For the analysis of plasma mitotane concentrations, we separately considered the first six months of therapy (M0–M6) because this is the period when mitotane dose is progressively increased to attain target levels and, as a consequence, mitotane concentrations are highly variable. During the first six months, we analyzed the correlation between mitotane concentrations and the month of therapy and drug dose. We considered the period from month 7 to month 36 (M7–M36) as the maintenance phase, because mitotane dose is usually stable in chronic treatment. We set the time point at the

36th month, since the timing of follow-up visits was more consistent between centers during this period. In a multivariate regression analysis, we assessed the correlation between plasma mitotane concentrations recorded during M7–M36 and patient sex, age, and BMI. We also calculated TtR during M7–M36.

3.3 TtR Study 2 – PALLIATIVE SETTING

Inclusion criteria of the study were as follows:

- Age ≥ 18 years
- pathologically confirmed diagnosis of ACC
- availability of CT or MRI scans
- complete follow-up information
- treatment with mitotane (all patients received the same mitotane formulation, Lysodren® 500 mg tablets) for ≥ 3 months, and with > 3 measurements of plasma mitotane concentrations reported on the Lysosafe Online® database.

Exclusion criteria were as follows:

- Incomplete tumor staging
- history of other previous or concomitant malignancies
- incomplete follow-up information.

We retrieved the data of patients who were treated from July 2005 to March 2017.

Follow up for this study was closed on 1 November 2019.

Patients' charts were reviewed and the following information was retrieved for the study: gender, date of birth, date of diagnosis, hormone secretion and tumor stage at diagnosis, pathology report, date of recurrence (in case of previous adjuvant treatment), date of start of palliative treatment, hormone secretion and ACC stage at start of palliative treatment, number and type of organs/systems with metastasis at start of palliative treatment, and

last follow-up or death. Date of diagnosis was defined as the date of surgery or the date of biopsy for tumors not operated on. Biochemical confirmation of hormone excess was requested to categorize an ACC as hormone secreting. Tumor stage was established according to the ENSAT classification (I and II, confined tumor; III, positive lymph nodes or infiltrating neighboring organs/veins without distant metastases; IV, distant metastases) (4). Date of recurrence was defined as the date of radiological evidence of a new lesion. A questionnaire was sent to the participating centers to retrieve the information requested for the study; moreover, centers were asked about indications, timing of initiation and discontinuation, reasons for discontinuation of mitotane treatment, and follow-up modality. Duration of treatment was calculated from the date of the initiation of mitotane therapy until the discontinuation of treatment, or the end of follow-up, whichever occurred first.

Treatment response was evaluated according to routine radiologic assessment and qualified patients were classified on the basis of their best response to the first line of treatment using Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 (100): Complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). We stratified patients in two groups: The first group included patients with clinical benefit (CR, PR, SD) vs. patients with progression (PD).

3.4 Statistical analysis

Categorical data are presented as counts and percentages. Continuous data are presented as medians and interquartile ranges (IQR). Differences in categorical variables were analyzed by means of the chi-squared test or Fisher test as appropriate, while differences in continuous variables were analyzed by the Mann–Whitney U test. Correlation analyses were determined by calculating the Spearman's R coefficient. Multiple regression

analysis was done as appropriate. The survival curves were estimated with the Kaplan–Meyer product limit method. RFS was calculated from the time of initial surgery to the first radiological evidence of recurrence. OS was calculated from the date of initial surgery to the date of death in the TtR Study 1 (adjuvant setting) and from the start of palliative treatment to the date of death in the TtR Study 2 (palliative setting). Patients who did not experience either of those events (recurrence or death) were censored at the date of the last follow-up visit for the specific survival analysis. Cox proportional hazards regression models were fitted to determine prognostic factors on RFS and OS.

In the TtR Study 1, the following potential predictive factors for either RFS or OS were investigated: patient sex and age, tumor stage, hormone secretion, Weiss score, Ki67 index, and the time elapsed to get the first plasma mitotane level at target.

In the TtR Study 2, the following potential predictive factors for OS were investigated: patient sex, age, and hormone secretion at the starting of palliative treatment, length of RFS, the number of organs with metastasis at the start of palliative treatment, the time elapsed to get the first plasma mitotane level at target during palliative treatment, the peak of mitotane concentrations during palliative treatment, and response to treatment.

Stratification of patients into risk groups was achieved through the maximally-selected log-rank statistics approach, which provides the value of a cut-off point corresponding to the most significant relation with outcome (101). Since variable selection based on univariate analysis cannot properly control for a potential spurious relationship (102), the best subset regression approach was chosen for building a multivariate model (103). According to this approach, all the possible combinations of the candidate variables were considered, then model selection was based on the Akaike information criteria method (104).

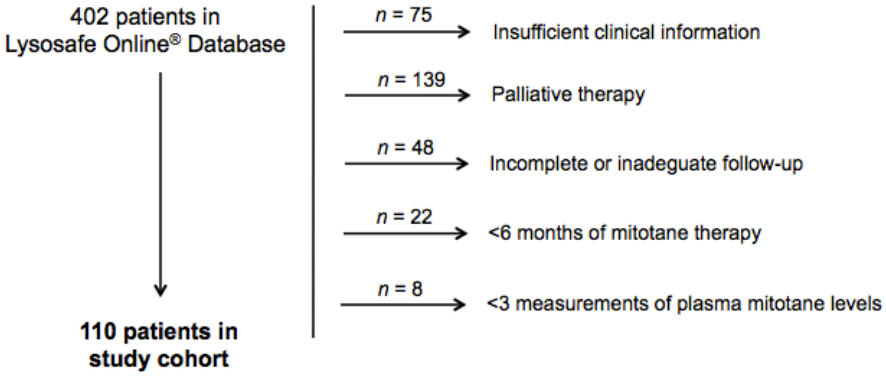
All reported p values are two-sided. The p values less than 0.05 were considered as statistically significant. The statistical analyses were performed with Statistica (StatSoft) (Dell Software, Round Rock, TX, USA) and R version 3.5.1 (R Core Team, USA).

4. RESULTS

4.1 TtR Study 1 – ADJUVANT SETTING

From a total of 402 ACC patients on the Lysosafe Online® database, 110 patients fulfilled inclusion/exclusion criteria and were retrospectively included in the study (**Figure 15**)

Figure 15. TtR Study 1 cohort.



Baseline characteristics of patients are reported in **Table 7**.

Table 7. Baseline features of patients.

Characteristics	Valid Cases (*N*)	Values
Gender, *N* (%)	110	
Male		43 (39.1%)
Female		67 (60.9%)
Age at diagnosis, year	110	
Median (IQR)		47 (35–58)
BMI, kg/m^2	95	
Median (IQR)		24.7 (21.9–29.4)
Tumor stage, *N* (%)	110	
Stage I		11 (10%)
Stage II		80 (72.7%)
Stage III		17 (15.5%)
Stage IV		2 (1.8%)
Hormone secretion, *N* (%)	106	
Yes		58 (54.7%)
No		48 (45.3%)
Weiss score	90	
Median (IQR)		6 (5–7)
Ki67	95	
Median (IQR)		17 (6.5–30)
≤10%		32 (33.7%)
>10%		63 (66.3%)

IQR = interquartile range. BMI = Body Mass Index.

The median follow-up was 63 (39–94) months. Adjuvant mitotane treatment was initiated after a median time of 1 (1–2) month from the first surgery in 102 patients (92.7%) and after surgical treatment of a recurrence in eight patients (7.3%). Median duration of treatment was 46 (28–62) months. The adjuvant therapy was discontinued permanently in 59 cases (53.6%), of which 36 (61.0%) were for end of treatment after a median time of 58 (45–62) months. Other causes of treatment discontinuation were toxicity (n = 5), patient's decision (n = 4), concomitant diseases (n = 3), or other/not available data (n = 11). Characteristics of mitotane treatment at the time of permanent discontinuation for toxicity are reported in **Table 8**.

Table 8. Characteristics of mitotane treatment at the time of permanent discontinuation for toxicity.

Patients	Mitotane Levels (mg/L)	Mitotane Dose (g/day)	Duration of Treatment (months)	Type of Toxicity
1	12.7	1.5	23	GI
2	13.2	2.0	45	GI
3	9.2	1.5	31	NEU
4	10.6	2.5	19	NEU
5	12.4	2.5	18	GI/NEU
Median (IQR)	12.4 (10.6–12.7)	2.0 (1.5–2.5)	23 (19–31)	

GI = gastrointestinal, NEU = neurological.

Gastrointestinal symptoms included nausea and diarrhea, whereas neurological manifestations were dizziness and confusion.

All centers reported recommending adjuvant mitotane treatment in all ACC patients following operation, with the exception of three centers, where treatment was o offered to high-risk patients only.

All centers but one reported using a low-dose regimen with minimal variations in the starting dose (1–2 g/day), while the velocity of further dose increments varied among

centers. Maintenance dose was guided by results of mitotane monitoring and patient tolerability.

During the period M0–M7, plasma mitotane levels increased progressively, being significantly correlated with month of therapy ($r = 0.6$, $p < 0.0001$) and mitotane doses ($r = 0.13$, $p < 0.034$). Achievement of target mitotane levels required a median time of 8 (5–19) months from start of therapy, while 11 patients (10%) never achieved levels 14 mg/L. The median mitotane dose in the maintenance phase was 2.0 (1.5–2.5) g/day. In a multiple regression analysis, sex ($\beta = -0.23$, $p = 0.02$) and BMI ($\beta = 0.22$, $p = 0.02$) were correlated with median doses of mitotane, implying that female sex was associated inversely with the dose, while BMI was correlated positively. In the group of 102 patients who started adjuvant mitotane therapy after the first surgery, recurrence occurred in 39 (38.2%) of cases. Median RFS was not reached, and the median follow-up time for RFS was 54 months (27–78). Multivariate analysis showed that the Ki67 index and time to the first mitotane level at target were independent predictors of RFS (**Table 9**).

Table 9. Univariate and multivariate analysis of predictive factors for RFS.

	Univariate Analysis			Multivariate Analysis		
Factor	**HR**	**95% CI**	***p* Value**	**HR**	**95% CI**	***p* Value**
Gender [1]	1.35	0.71–2.54	0.358	-	-	-
Age at diagnosis	1.10	0.67–1.80	0.709	-	-	-
Tumor stage [2]	2.17	1.03–4.59	**0.042**	-	-	-
Hormone secretion [3]	0.79	0.42–1.52	0.486	-	-	-
Weiss score	1.60	1.03–2.48	**0.038**	-	-	-
Ki67 index	1.49	1.06–2.10	**0.023**	4.49	1.57–12.84	**0.005**
Time to first level at target	1.22	1.00–1.50	0.053	1.48	1.06–2.07	**0.020**

Reference categories: [1] Male gender, [2] Stage III–IV, [3] Non-secreting tumors.
HR = Hazard Ratio. The bold indicates the statistically significant values.

We identified a cut-off value for the Ki67 index at 10% and for time to reach target mitotane concentrations at 17 months, which were able to differentiate significantly patients for their risk of recurrence (**Figures 16 and 17**)

Figure 16. RFS of patients stratified in “low” and “high” risk groups according to Ki67 indices of ≤10% and >10%, respectively.

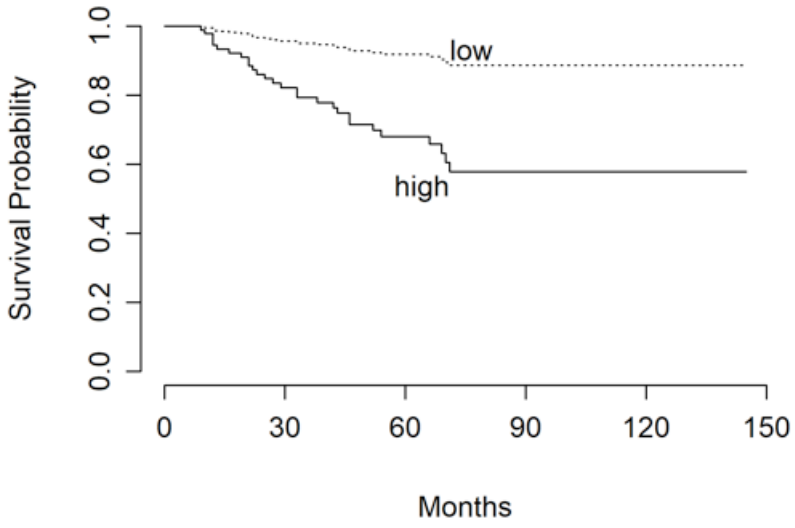
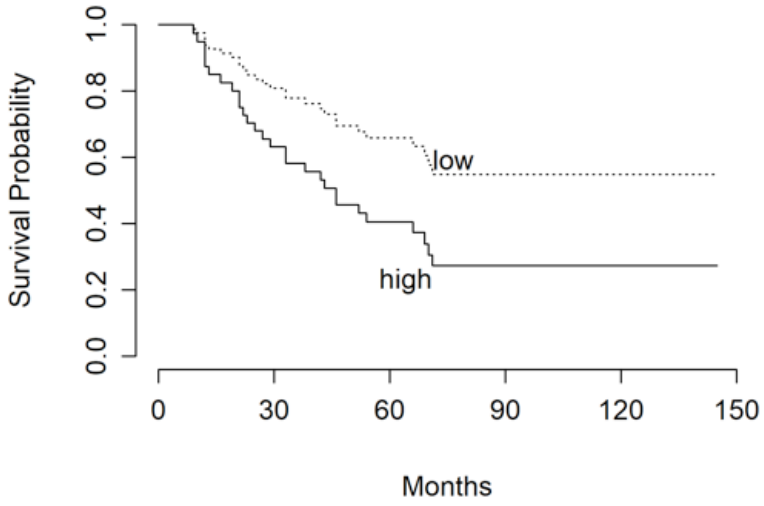


Figure 17. RFS of patients stratified in “low” and “high” risk groups according to the time needed to reach target mitotane concentrations of ≤17 and >17 months, respectively.

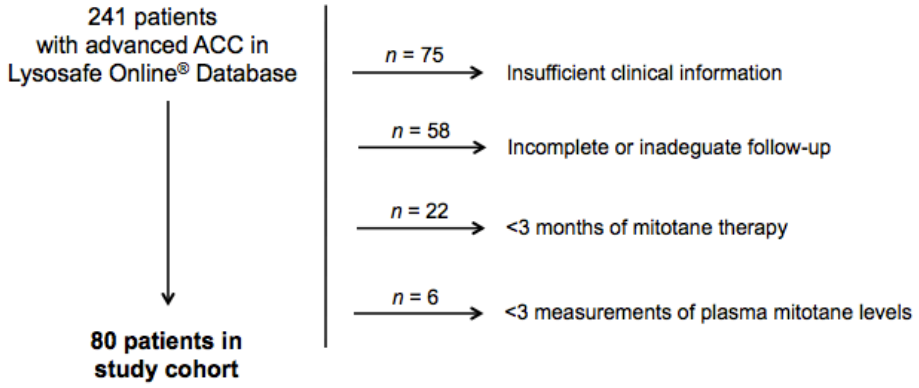


In a separate multivariate model considering only the maintenance phase (M7–M36) of treatment, TtR was associated with a significantly lower risk of recurrence (Hazard Ratio, HR = 0.93; 95% CI, 0.88–0.98; $p < 0.01$). Death occurred in 22 cases (20%). Median OS was not reached, and the median follow-up time for OS was 70 months (49–100). We did not find any predictor of the risk of death due to the low number of events.

4.2 TtR Study 2 – PALLIATIVE SETTING

From a total of 241 patients with advanced ACC on the Lysosafe Online® database, 80 patients fulfilled inclusion/exclusion criteria and were retrospectively included in the study (**Figure 18**).

Figure 18. TtR Study 2 cohort.



Baseline characteristics of the patients are reported in **Table 10**.

Table 10. Baseline features of patients.

Characteristics	Valid Cases (*N*)	Values
Gender, *N* (%)	80	
Male		25 (31.2%)
Female		55 (68.8%)
Age at diagnosis, year	80	
Median (IQR)		50 (36–59)
Tumor stage at diagnosis, *N* (%)	80	
Stage I		3 (3.7%)
Stage II		28 (35%)
Stage III		18 (22.5%)
Stage IV		31 (38.8%)
Hormone secretion at diagnosis, *N* (%)	77	
Yes		51 (66.2%)
No		26 (33.8%)
Weiss score	51	
Median (IQR)		6 (5–7)
Ki67	55	
Median (IQR)		25 (13–38)
≤10%		14 (25.5%)
>10%		41 (74.5%)
Hormone secretion at start of palliative treatment, *N* (%)	77	

Yes		25 (32.5%)
No		52 (67.5%)
ENSAT tumor stage at start of palliative treatment, *N* (%)	80	
Stage I		0 (0%)
Stage II		0 (0%)
Stage III		7 (8.7%)
Stage IV		73 (91.3%)
Number of metastatic organs	78	
Median (IQR)		2 (1–2)
1 organ, *N* (%)		29 *(37.2%)*
2 organs, *N* (%)		34 *(43.6%)*
3 organs, *N* (%)		10 *(12.8%)*
≧4 organs, *N* (%)		5 *(6.4%)*
Organ/system involved	78	
Lungs		47 *(60.3%)*
Liver		37 *(47.4%)*
Lymphatic system		15 *(49.2%)*
Local site (vena cava, adrenal loggia)		13 *(16.7%)*
Peritoneum and retroperitoneum		8 *(10.3%)*
Kidney		8 *(10.3%)*
Skeletal system		7 (9.0%)
Spleen		3 *(3.8%)*
Abdominal muscles (psoas, diaphragm)		3 *(3.8%)*
Colon		2 *(2.6%)*

IQR = interquartile range. N = number of patients.

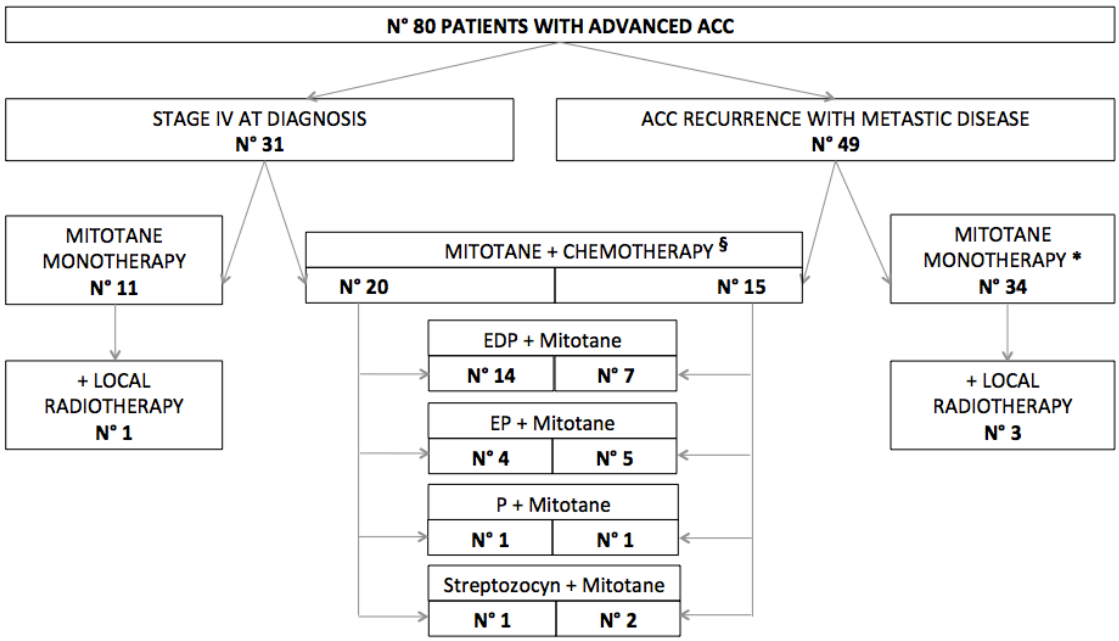
The median follow-up was 33 (22–51.2) months. Twenty-four patients were previously treated with adjuvant mitotane before ACC recurrence while 56 patients started mitotane (± chemotherapy) as first-line medical treatment of ACC recurrence or advanced disease at diagnosis. Median duration of palliative treatment was 33 (22–49) months, with a median of 8 (5–12) measurements of plasma mitotane concentration and a median time interval between two consecutive measurements of 2 (1– 3) months. At the end of the follow-up, 14 patients (17.5%) were still on mitotane therapy, after a median of 67 (43–102) months of palliative mitotane treatment, and 48 (72.7%) were treated until death, with a median duration of 31 (21–44) months. Other causes of treatment discontinuation were ACC progression (n = 10), unknown (n = 5), or patient's decision (n = 3).

In the overall group, the achievement of target mitotane levels required a median time of 6 (3–9) months from the start of therapy while 14 patients (17.5%) never achieved levels

≥ 14 mg/L. The peak of plasma mitotane concentrations was 20.8 (14.8–25.0) mg/L, which was reached after a median of 11 (6–20) months.

Forty-five patients (56.2%) were initially treated only with mitotane (4 of which received concomitant local radiotherapy) while 35 (43.8%) were treated with a combination of chemotherapy and mitotane, in most cases with the EDP-M regimen (**Figure 19**).

Figure 19. Management of advanced adrenocortical cancer in our series.



* 17 patients were previously on adjuvant mitotane.
§ 7 patients were previously on adjuvant mitotane.
EDP = etoposide, doxorubicin and cisplatin.
EP = etoposide and cisplatin.
P = cisplatin.

The comparison between the baseline characteristics of these two groups showed that patients treated with the combination of chemotherapy and mitotane were younger (43, 33–58 years, vs. 54, 45–62 years; p = 0.036) and with worse presentation at diagnosis (de novo ENSAT stage IV, 57.1% vs. 24.4%; p = 0.012) (**Table 11**).

Table 11. Comparison between patients starting palliative treatment with mitotane monotherapy vs. patients starting with the association mitotane + chemotherapy.

Characteristics	Mitotane Monotherapy N° 45	Mitotane + Chemotherapy N° 35	*p* Value
Gender, ***(valid cases)***	***(45)***	***(35)***	0.43
Male, *N* (%)	12 *(26.7)*	13 *(37.1)*	
Female, *N* (%)	33 *(73.3)*	22 *(62.9)*	
Age at time of palliative treatment, year			
(valid cases)	***(45)***	***(35)***	**0.036**
Median (IQR)	54 (45–62)	43 (33–58)	
Hormone secretion at start of palliative treatment			
(valid cases)	***(42)***	***(31)***	0.085
Yes, *N* (%)	9 *(21.4)*	14 *(45.2)*	
No, *N* (%)	33 *(78.6)*	17 *(54.8)*	
Number of metastatic organs ***(valid cases)***	***(43)***	***(35)***	0.92
≤ 2 organs, *N* (%)	35 *(81.4)*	28 *(80)*	
> 2 organs, *N* (%)	8 *(18.6)*	7 *(20)*	
De novo **stage IV***			
(valid cases)	***(45)***	***(35)***	**0.012**
N (%)	11 (24.4)	20 (57.1)	
Previous adjuvant therapy			
(valid cases)	***(45)***	***(35)***	0.17
Yes, *N* (%)	17 *(37.8)*	7 *(20)*	
No, *N* (%)	28 *(62.2)*	28 *(80)*	
Previous RFS			
(valid cases)	***(18)***	***(7)***	0.74
Median (IQR)	16 (6–26)	16 (5–54)	

***** De novo stage IV means that patients were diagnosed with stage IV ACC.

IQR = interquartile range. N = number of patients. RFS = recurrence free survival.

During the entire period of follow-up, 12 patients were treated only with mitotane while the remaining 68 received mitotane in combination with one or more chemotherapy treatments (in 61 cases, at least one regimen including platinum compound).

The comparison between the baseline characteristics of these two groups showed that patients treated with multiple lines of treatment were younger than those treated with mitotane only (48.5, 35–58.2 years, vs. 58.5, 52.7–69.2 years; p = 0.023) (**Table 12**).

Table 12. Comparison between patients treated with mitotane monotherapy during all follow-up vs. patients treated with different lines of treatment.

Characteristics	Mitotane monotherapy N° 12	Mitotane + Chemotherapy N° 68	*p* Value
Gender, ***(valid cases)***	***(12)***	***(68)***	0.90
Male, *N* (%)	4 *(33.3%)*	21 *(30.9%)*	
Female, *N* (%)	8 *(66.6%)*	47 *(69.1%)*	
Age at time of palliative treatment, year			
(valid cases)	***(12)***	***(68)***	**0.023**
Median (IQR)	58.5 (52.7–69.2)	48.5 (35–58.2)	
Hormone secretion at start of palliative treatment			
(valid cases)	***(11)***	***(62)***	0.80
Yes, *N* (%)	3 *(27.3)*	20 *(32.3)*	
No, *N* (%)	8 *(72.7)*	42 *(67.7)*	
Number of metastatic organs ***(valid cases)***	***(11)***	***(67)***	0.95
≤ 2 organs, *N* (%)	9 *(81.8)*	54 *(80.6)*	
> 2 organs, *N* (%)	2 *(18.2)*	13 *(19.4)*	
De novo stage IV			
(valid cases)	***(12)***	***(68)***	0.85
N (%)	5 (41.7)	26 (38.2)	
Previous adjuvant therapy			
(valid cases)	***(12)***	***(68)***	0.75
Yes, *N* (%)	3 *(25)*	21 *(30.9)*	
No, *N* (%)	9 *(75)*	47 *(69.1)*	
Previous RFS			
(valid cases)	***(3)***	***(22)***	0.18
Median (IQR)	27 (23–39)	13 (5–26)	

***** De novo stage IV means that patients were diagnosed with stage IV ACC.

IQR = interquartile range. N = number of patients. RFS = recurrence free survival.

We found that patients treated only with mitotane achieved a higher peak of mitotane concentrations (26.4, 21.7–29.2 mg/L, vs. 19.2, 14.7 24.6 mg/L, p = 0.028) and had a more favorable outcome (6/12 (50%) vs. 13/55 (23.6%) patients alive at last follow-up, p = 0.022).

Considering the first line of treatment, 19 patients (23.7%) experienced clinical benefit (10 out of 35 (28.6%) patients treated with mitotane + chemotherapy and 9 out of 45 (20%) patients in mitotane monotherapy), of whom 11 (57.9%) had an objective response (8 partial, 3 complete) and 8 (42.1%) had stabilization of disease, while the remaining 57

(71.2%) had progression (24 out of 35 (68.6%) patients treated with mitotane + chemotherapy and 33 out of 45 (73.3%) patients in mitotane monotherapy), according to the RECIST 1.1 criteria (100) (data not available for 4 patients).

The comparison between the baseline characteristics of these two groups is given in **Table 13**.

Table 13. Comparison between patients who had clinical benefit vs. patients who progressed at the first line of treatment.

Characteristics	Clinical benefit N° 19	Progression N° 57	*p* value
Gender, ***(valid cases)***	***(19)***	***(57)***	1.00
Male, *N* (%)	6 *(31.6%)*	18 *(31.6%)*	
Female, *N* (%)	13 *(68.4%)*	39 *(68.4%)*	
Age at time of palliative treatment, year ***(valid cases)***	***(19)***	***(57)***	0.17
Median (IQR)	50 (35.5–56)	51 (38–64)	
Hormone secretion at start of palliative treatment ***(valid cases)***	***(19)***	***(51)***	0.36
Yes, *N* (%)	4 *(21.1)*	18 *(35.3)*	
No, *N* (%)	15 *(78.9)*	33 *(64.7)*	
Number of metastatic organs ***(valid cases)***	***(19)***	***(56)***	0.72
≤ 2 organs, *N* (%)	16 *(84.2)*	44 *(78.6)*	
> 2 organs, *N* (%)	3 *(15.8)*	12 *(21.4)*	
De novo **stage IV** ***(valid cases)***	***(19)***	***(57)***	0.17
N (%)	10 (52.6)	18 (31.6)	
Previous adjuvant therapy ***(valid cases)***	***(19)***	***(57)***	0.74
Yes, *N* (%)	4 *(26.3)*	18 *(31.6)*	
No, *N* (%)	14 *(73.7)*	39 *(68.4)*	

***** De novo stage IV means that patients were diagnosed with stage IV ACC.

IQR = interquartile range. N = number of patients.

We found that patients with ACC progression had a lower TtR (8.6, 0.8–14.1 months, vs. 15.8, 2.7–32.7 months; p = 0.033) and an unfavorable outcome (7/57 patients (12.3%) vs. 12/19 (63.2%) patients alive at last follow-up, p = 0.022).

Considering the entire cohort of patients, death occurred in 61 cases (76.2%). Median overall survival (OS) was 35 months (CI95%, 31–49). Multivariate analysis showed that clinical benefit after the first-line treatment and the TtR were independent predictors of OS (**Table 14**).

Table 14. Multivariate analysis with significant predictors of OS.

	HR	**CI 95%**		***p***
Tumor response [§]	0.387	0.173	0.869	0.021
TtR *	0.484	0.308	0.759	0.002

[§] Reference category: clinical benefit *vs* progression.
***** HR is computed on a difference of 15 months.
TtR = time in target range. HR = hazard ratio. CI = confidence interval.

5. DISCUSSION

In these two studies, TtR Study 1 and TtR Study 2, we explored the relationship between target mitotane concentrations and patient outcome using the TtR, a concept analogous to that used in warfarin treatment (98). Some previous studies used the peak mitotane level, to define the attainment of target concentrations; however, the peak level cannot give an adequate representation of mitotane concentrations over time since it is a measurement at a single discrete point (56, 57). To overcome this limit, the percentage of mitotane measurements in the target range was used (58), but this method has the caveat of being strongly dependent on the number of available measurements. This may introduce a bias when comparing patients with different duration of follow-up, which may be quite prolonged in the case of adjuvant therapy. Moreover, there is no evidence to define what percentage identifies a good exposure to mitotane. These methodological issues may have contributed to the discrepancy in the literature concerning adjuvant treatment (58, 60). In our studies, we calculated the TtR, which in our opinion gives a more adequate representation of chronic exposure to mitotane, and analyzed the results in a multivariate analysis without predefining arbitrary cut-off values.

In TtR Study 1, we found an inverse relationship between the TtR and risk of ACC recurrence, implying that the greater the TtR, the lower the risk. This finding supports the clinical value of mitotane monitoring and the concept of target doses in the adjuvant setting (7). Although it is plausible that lower mitotane concentrations may be effective in adjuvant treatment, we analyzed only the level of 14 mg/L, because this was the level targeted in practice. We should acknowledge, however, that the association between the TtR and risk of recurrence was demonstrated only in a separate multivariate analysis.

The time needed to achieve target mitotane concentrations had also an impact on the risk of ACC recurrence: a longer time was associated with higher risk. This is consistent with

the concept that mitotane is a slow-acting drug in relation to the achievement of significant plasma levels. Due to the very cautious dose titration in the starting phase of treatment employed in many centers, the time needed to get into the target range was exceedingly long. We identified a time point at 17 months to achieve target concentrations, which significantly differentiates patients for their risk of recurrence. This time span is unacceptably long and the present findings call for a change in practice, aiming for a faster rise in mitotane levels and strengthening the value of mitotane monitoring. However, the potential danger of a rapid increment in mitotane dosing, which may result in important toxicity with consequent loss of compliance to treatment, should be considered (64). In addition to that, in TtR Study 1, we found only a weak relationship between mitotane dose and its plasma concentrations during the first phase of treatment, and this finding is in agreement with the concept that individual differences in mitotane metabolism and other still unknown factors influence plasma concentrations (81, 105). Interestingly, higher doses were employed in men and in patients with greater BMI and these novel findings matter for clinical practice.

In the cohort of TtR Study 1, toxicity associated with adjuvant mitotane was acceptable, though we acknowledge the fact that due to the study inclusion criteria we did not capture the patients who eventually discontinued mitotane in the first six months. Severe toxicity leading to permanent treatment discontinuation was recorded in only five patients on chronic therapy, and this is likely due to the low doses (median dose of 2 g/day) used to continue treatment in the maintenance phase. Thus, the present study shows that a few patients cannot tolerate adjuvant mitotane following the first months of treatment, proving than a careful follow-up is necessary. Despite mitotane having a reputation for being a challenging drug to manage (30), adjuvant mitotane treatment is feasible when patients are managed in expert centers. However, some patients were unable to tolerate

the drug, exhibiting neurological toxicity even when exposed to “normal” mitotane concentrations, confirming the relevance of individual factors in mitotane metabolism.

Strengths of the TtR Study 1 are the thorough characterization of adjuvant mitotane treatment of ACC patients following surgical removal of the tumor and the large data set, considering the rarity of the disease. This allowed for the capture of details of mitotane treatment that were not available in previous studies and led to observations that may be useful to informing future practice. However, we should acknowledge the limits of a retrospective analysis, and that our results are not generalizable to patients who discontinue treatment within six months for intolerability or patients with early ACC recurrence. The inclusion criteria of the study produced an immortal time of six months that may have enriched our series of a higher number of low-risk ACC compared to recently published series (53, 60).

In the TtR Study 2, the TtR of plasma mitotane concentrations is able to predict survival in patients with advanced ACC, in which higher values of TtR were associated with longer survival. Moreover, we found that patients with ACC progression had a lower time in the target range than patients experiencing clinical benefit from first-line treatment. The finding that in multivariable analysis TtR was a predictor of OS confirms that it could represent a valuable measure of mitotane efficacy also in advanced ACC. In our study, the overall activity of first-line treatment was limited, with a low number of objective responses (13.7%) and of patients experiencing clinical benefit (23.7%), with no observed difference between patients treated with mitotane + chemotherapy or mitotane monotherapy. We observed that a lower number of patients with advanced ACC benefitted from first-line treatment, either mitotane + chemotherapy or mitotane monotherapy, compared to the FIRM-ACT study and two recent retrospective studies (59, 76, 106). However, the present study was not specifically designed to analyze the

efficacy of treatment. It is worth of note that management of patients with advanced ACC remains a challenge also in referral centers. ACC may present with metastatic disease at diagnosis in about one quarter of cases, or progress to advanced disease after an initial apparently complete resection (54, 61). The prognosis of advanced ACC is generally poor when surgery is unfeasible, with a reported 5-year survival less than 15% (54, 107). Our study shows that less than a quarter of patients were alive after 3 years of follow-up, with a median survival of 35 months. These data confirm the high mortality of advanced ACC (61, 75); however, they also follow the trend of a better prognosis observed in the most recent studies (107). The TtR Study 2 shows that the lung is the organ most commonly involved by metastatic spread, with a higher frequency than previously reported in clinical series (75) but with a similar rate of autoptic (108) or surgical series (109, 110). The disease burden in our cohort was remarkable, with the presence of ≥two organs involved in about two thirds of cases, and also hormone secretion was found in a similar percentage of cases at diagnosis. It is worth noting that a remarkable number of secreting tumors at diagnosis recurred as not secreting tumors (secreting tumors 66.2% at diagnosis vs. 32.5% at the start of palliative treatment). The patient cohort enrolled in the FIRMACT study had a quite higher tumor burden and less secreting tumors, and this difference likely results from the specific inclusion criteria of that study (all patients were treated with chemotherapy in addition to mitotane) (76).

Our study reports on the current practice in the management of advanced ACC, either stage IV at diagnosis or recurrent ACC following initial surgery, focusing on the first-line treatment. It is generally agreed that the two major options are mitotane monotherapy or mitotane combined with chemotherapy, with a regimen including platinum compounds (61, 75). The choice depends on patient conditions, tumor characteristics, and center preference. The standard chemotherapy regimen is EDP-M, introduced by a multicenter

prospective phase II study carried out in Italy (23, 111) and validated in a worldwide prospective randomized phase III clinical trial (76). Our series demonstrated that this finding has been implemented in current practice, since EDP was the regimen of choice as the first treatment in most of our patients. In some cases, the parent regimens EP, or cisplatin monotherapy, were used due to limited toxicity. Conversely, the combination of streptozotocyn and mitotane was employed in less than 10% of cases. In our cohort, the two options (mitotane alone or plus chemotherapy) were almost equally chosen; however, preference toward mitotane combined with chemotherapy was given to de novo stage IV ACC and younger patients. This choice likely reflects the perception that a metastatic presentation at diagnosis implies an aggressive ACC and that younger patients are fit to sustain chemotherapy-related toxicity. However, mitotane remained the backbone of therapy because the duration of treatment was prolonged till patient death in most cases while different lines of treatment (cytotoxic drugs, loco-regional treatments) were superimposed during the disease course. The practice of continuing mitotane indefinitely, despite ACC progression, has been recently criticized (112), although we lack clear rules for mitotane discontinuation (7). A small subset of our patients was treated with mitotane without any other additional systemic treatment. Interestingly, these patients had a more favorable outcome, and this likely represents a selection bias because more aggressive tumors usually undergo multiple lines of treatment. The inclusion of this patient cohort with less aggressive ACC is one of the factors that may explain the long OS observed in the present study. Not surprisingly, higher mitotane concentrations were attained in such patients since the combination with cytotoxic agents increases toxicity and makes it difficult to give high doses of mitotane. In a small prospective trial of 12 weeks, including 40 mitotane naïve patients with metastatic ACC, assigned to a low- or high-dose mitotane regimen, the high-dose regimen resulted in higher exposure to mitotane in patients not

receiving concomitant chemotherapy, despite cumulative doses not being significantly different among the subgroups (113).

A recent retrospective study including 127 patients with advanced ACC treated with mitotane monotherapy introduced the concept that either a low tumor burden (<10 tumor lesions) or longer recurrence-free survival (RFS) after primary surgery (≥360 days) are characteristics predicting treatment efficacy (59). We did not find any difference on tumor burden, expressed as the number of metastatic organs, although we did not capture data on the number of metastatic lesions. However, RFS was almost double in the cohort of patients treated with mitotane alone, despite levels of significance not being reached for the low numbers, thus confirming the validity of the concept that tumors with lower proliferation capability (heralded by prolonged RFS) are best suited for mitotane monotherapy.

Strengths of the TtR Study 2 are the large data set of patients with available clinical information and data on mitotane measurement, considering the rarity of ACC and the high mortality in the setting of advanced disease. On the other hand, we are aware that our analysis is limited by its retrospective and multicenter nature. Therefore, we did not evaluate progression-free survival, which is heavily influenced by variable schedules of restaging in retrospective studies, and we only considered overall survival, taking into account that the inclusion criteria produced an immortal time of three months, and that we included patients with at least three mitotane measurements.

6. CONCLUSION

Our two studies suggest that TtR may be a useful tool to manage mitotane therapy of patients with ACC, both in adjuvant and palliative setting. The observations that the TtR of mitotane concentrations is associated to the risk of recurrence in the TtR Study 1 and that predicts survival in patients with advanced ACC in the TtR Study 2, are novel and have practical importance.

However, the two studies are retrospective and limited to a sample of patients followed only in Italian centers. Moreover, the association between the TtR and risk of recurrence is demonstrated only in a separate multivariate analysis of the TtR Study 1.

As the findings need to be confirmed, we have recently proposed a European multicenter retrospective study, endorsed by the ENSAT, to validate the use of TtR in a large cohort of ACC patients treated with adjuvant mitotane therapy. In this project, it will be evaluated whether the TtR, with a cut-off for mitotane concentrations $\geq$ 14 mg/L, is a factor influencing RFS, and whether different cut-off values of mitotane concentrations to define the TtR influence RFS and/or OS.

In addition to that, we will perform an analysis of the outcomes, applying the concept of TtR, in the cohort of the ADIUVO study, which is the first prospective, randomized, controlled trial done in ACC patients in the adjuvant setting. The use of TtR in a study with a prospective design could definitively validate this method.

In conclusion, although our two studies have provided promising results, the TtR method need to be tested in larger and prospective cohorts to enter in real clinical practice.

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