The Efficacy of Mitotane in Human Primary Adrenocortical Carcinoma Cultures

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

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

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

Results: The median percentage cell amount inhibition in primary ACC cultures at 50 μM mitotane was 57%. Seven patients were classified as nonresponders, 14 as partial responders, and 11 as responders. The mean median effective concentration (EC_{50}) value of mitotane for inhibition of cell amount in responders was 14.2 μM (95% CI, 11.3–17.9), in partial responders 41.6 μM (95% CI, 33.5–51.8), and could not be calculated in nonresponders. The percentage cortisol-producing ACC was 14%, 43%, and 73% for nonresponders, partial responders, and responders ($P = 0.068$). Mitotane inhibited cortisol production with a mean EC_{50} of 1.4 μM (95% CI, 0.9–2.1), which was considerably lower than the EC_{50} on cell growth. RRM1, SOAT1, and CYP2W1 expression levels were not predictive for mitotane sensitivity *in vitro*.

Conclusion: Direct antitumor effects of mitotane on human primary ACC cultures are highly variable between patients, reflecting heterogeneous responses in patients. Cortisol was inhibited at lower concentrations, compared with its effect on cell amount. Cortisol secretion by ACC might be associated with enhanced mitotane sensitivity due to increased direct antitumor effects of mitotane. (***J Clin Endocrinol Metab*** **105: 1–11, 2020)**

Adrenocortical carcinoma (ACC) is a rare malignancy with 5-year survival rates of 16–44% (1–3). Although surgery is the only curative treatment modality, medical therapy can be used in metastatic disease or to prevent recurrences after radical ACC resection (4). Mitotane is the only accepted adrenolytic drug, but response rates and efficacy in both the above-mentioned settings are limited (5), and mitotane use is accompanied by severe adverse effects.

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Abbreviations: ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; DFS, disease-free survival; NA, normal adrenal; PCR, polymerase chain reaction.

Markers that predict which patients benefit from mitotane treatment are of great importance in order to prevent overtreatment and adverse effects, as well as to safe on costs. Reaching the target plasma concentration of 14 mg/L (~50 μM) is currently considered the most important predictive marker for response to mitotane, with fewer recurrences and a longer disease-free survival (DFS) in patients who reach this plasma concentration (1, 6, 7). In a recent report, it was shown that particularly patients in which mitotane was initiated at late recurrences and patients with low tumor grade responded to mitotane (8). Volante et al. showed a correlation of expression of ribonucleotide reductase large subunit (*RRM1)* with DFS and overall survival in ACC patients (9). In patients with low tumoral *RRM1* expression, a significantly longer DFS was found in patients who received adjuvant mitotane than in patients who were only monitored during follow-up. This difference was not present in patients with high *RRM1* expression (9). As a possible explanatory mechanism, Germano et al. showed that RRM1 interferes with mitotane metabolism and bioavailability of the active metabolite in ACC cell line models *in vitro* (10). Recently, Sterol-O-Acyl-Transferase 1 (SOAT1) was identified as a key molecular target for mitotane, which expression was positively correlated with a longer time to progression and DFS in patients treated with mitotane (11). In another study, CYP2W1 immunoreactivity, adjusted for European Network for the Study of Adrenal Tumors (ENSAT) stage, was positively associated with a longer overall survival and time to progression in patients treated with mitotane (12). CYP2W1 is considered an orphan human cytochrome P450 enzyme, because its physiologic substrate is still unknown. Expression of this enzyme is known to be high during fetal life and in some cancers, and has recently gained attention as a promising tool in targeted therapy (13). Other factors in ACC have particularly been associated with diagnosis of ACC, as well as prognosis and progress (5, 14–16). In this study, however, we focus on markers that might correlate with mitotane sensitivity and not prognosis.

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

Materials and Methods

Patients and tissue samples

ACCs, adrenocortical adenomas (ACAs), and normal adrenals (NAs) were collected during surgeries performed at the Department of Surgery at the Erasmus MC between June 1990 and August 2016. Diagnosis was made on the basis of the Van Slooten Index (VSI) or the Weiss score (WS) (17, 18), dependent on the year of pathologic diagnosis. Normal adrenals were collected during nephrectomy and confirmed by the pathologist as being normal. The following clinical parameters were obtained from all ACC and ACA patients: age at diagnosis, follow-up time, sex, tumor size, ENSAT stage in case of primary tumors, hormonal secretion status, systemic therapies received prior to surgery, and development of metastases. A part of the tissue specimens was embedded in Tissue-Tek directly after resection and stored at –80°C until analysis. Another part was processed to obtain primary cultures and to isolate total RNA, as described below. *In vivo* cortisol production was identified by an increased urinary free cortisol, increased midnight salivary cortisol level, a positive dexamethasone suppression test, or a combination of tests. Informed consent was obtained from all patients. The study was conducted under the guidelines that had been approved by the Medical Ethics Committee of the Erasmus Medical Center.

Primary cultures

Immediately after surgery, a part of the ACC specimen was minced into small pieces of 2–3 mm^3, washed in culture medium and centrifuged for 5 minutes at 600 *g*. The culture medium consisted of DMEM-F12 (Fisher Scientific, Landsmeer, The Netherlands) containing 5% fetal calf serum (FCS), penicillin (1×10^5 U/; Bristol-Meyers Squibb, Woerden, The Netherlands) and l-glutamine (2 mmol/L; Fisher Scientific, Landsmeer, the Netherlands). The remaining tissue pellet was suspended in culture medium and was stored overnight at 4°C, whereafter the tissue was centrifuged again and the supernatant was removed. Tissues were dissociated in 10–25 mL of medium with collagenase type-I (2 mg/mL; Sigma-Aldrich, Zwijndrecht, The Netherlands) by incubation at 37°C for up to 2 hours. If necessary, the obtained suspension was filtered through a sterile needle. Ficoll (GE Healthcare, Eindhoven, The Netherlands) density gradient separation was used once or twice in order to separate contaminating red blood cells from the tumor cells. After centrifugation for 20 minutes at 600 g, the interphase containing the tumor cells was collected. Trypan blue exclusion was used to determine cell viability, and adrenal cells were visually counted using Türk solution. Dissociated cells were plated in quadruplicate at a density of 10^5 cells per well in a 24-well plate in 1 mL of culture medium. Medium was refreshed after 3–4 days and incubations with mitotane were initiated. Mitotane (Sigma-Aldrich, Zwijndrecht, the Netherlands) was dissolved in absolute ethanol and stored at a concentrated stock solution (10^{-2} M) at –20°C, and diluted in ethanol prior to use. After 3 days of incubation, media and mitotane were refreshed. After 7 days, media were removed and plates and media were stored at –20°C until analysis. Plated cells were routinely monitored to ascertain absence of fibroblast contamination. By using this method and when considering a minimum specimen size (2 × 2 cm) with sufficient viable tissue, the success percentage for obtaining a

primary ACC culture is >95%. To determine *in vitro* cortisol production, cortisol was measured in the media of all ACC using a chemiluminescence immunoassay system (Immulite 2000XPi). Total DNA per well, reflecting the cell amount, was determined using the bisbenzimide fluorescent dye (Hoechst 33258, Sigma-Aldrich), as previously described (19).

Real-time quantitative PCR

In primary cultures where ACC cells remained after isolation and plating for the cell culture experiments, *CYP11B1* and *STAR* mRNA expression levels were measured. *RRM1*, *SOAT1*, and *CYP2W1* mRNA expression levels were measured in NAs, ACAs, and ACCs. RNA isolation, cDNA synthesis and RT-PCR were performed as previously described, but using other primers (Supplementary Table 1 (20); Sigma-Aldrich) (21). The Vandesompele method was used to normalize the mRNA expression levels according to three housekeeping genes (22): hypoxanthine-guanine phosphoribosyl transferase 1 (*HPRT1*; Sigma-Aldrich, Zwijndrecht, the Netherlands), Beta-actin (*B-actin*; Thermo Fisher Scientific, Breda, the Netherlands), and glucuronidase beta (*GUSB*; Thermo Fisher Scientific, Breda, the Netherlands).

RRM1, SOAT1, and Chromogranin A immunohistochemistry

Construction of the TMA and immunohistochemistry of RRM1 and SOAT1 was performed as previously described in detail (23). The rabbit monoclonal RRM1 antibody (dilution 1:50; ab135383; Abcam, Cambridge, UK), the mouse monoclonal SOAT1 antibody (mAb; dilution 1:1000; Sc69836; Santa Cruz Biotechnology, Heidelberg, Germany), and the rabbit polyclonal SOAT1 antibody (PoAb; dilution 1:500; Ab39327 (11); Abcam) were used. Blinded for the tissue type, the sections were independently scored by two investigators (SGC, LJH) using a semi-quantitative well-established Immunoreactivity Score (IRS), which consists of the product of the percentage positive cells (4, >80%; 3, >51–80%; 2, >10%; 1, 0) and intensity of staining (3, strong; 2, moderate; 1, mild; 0, no staining) (24). Chromogranin A immunohistochemistry was performed on slides of the normal adrenal, as previously described (25), but using the mouse Chromogranin A primary antibody (LK2H10; Ventana Medical Systems).

Statistical analysis

For statistical analysis, Graphpad Prism 6.0 (Graphpad Software, San Diego, CA) and SPSS Statistics 21 (SPSS 21.0; SPSS Inc., Chicago, IL) were used. Response to mitotane was categorized by calculating the *in vitro* effect on cell amount at 50 µM mitotane (14 mg/L), the circulating therapeutic plasma concentration of mitotane (6), using nonlinear regression curve fitting. In two cases, the lowest concentration of mitotane caused an increase in cortisol, resulting in a top of the curve above 100%. When these curves were constrained at a top of 100%, the IC_{50} only minimally changed. For uniformity, all curves were fitted without constraint. Patient cultures were arbitrarily classified as nonresponder when the inhibitory effect on cell amount was ≤33%, as partial responders when the effect was >33% and ≤66% and responders showed a cell amount inhibition of >66% at 50 µM mitotane. Differences of categorical variables between groups were analyzed using the Fisher exact test, considering the small sample size. Continuous variables were compared using the Kruskal–Wallis test or one-way ANOVA, dependent on the distribution. Overall survival was defined as the time from diagnosis until death or last follow-up. Response to mitotane treatment *in vivo* was assessed in patients who received mitotane within 3 months after primary surgery. Time to relapse or progression was determined from the moment of mitotane initiation for patients who received mitotane as adjuvant or palliative treatment, respectively. Survival curves were computed using the Kaplan–Meier method and differences between cortisol and noncortisol secreting ACC were assessed by the log-rank (Mantel–Cox) test. A value of $P < 0.05$ was considered statistically significant. Values are presented as mean ± SEM, unless specified otherwise.

Results

Patient characteristics and sensitivity to mitotane

Ten NAs, 16 ACAs, and 45 ACCs were enrolled. Patient and tumor characteristics are listed in Supplementary Table 2 (20). *RRM1*, *SOAT1*, and *CYP2W1* mRNA expression were assessed in 55 adrenal specimens (8 NAs, 10 ACAs, 37 ACCs), and RRM1 and SOAT1 immunohistochemistry was performed in 59 tissues (7 NAs, 14 ACAs, 38 ACCs). A primary culture was obtained from 32 ACCs, including 29 primary tumors and three local recurrences. Two patients received mitotane preoperatively. The first patient was treated with mitotane after the first recurrence, whereas the primary culture was obtained from a second recurrence. The second patient had stage IV disease at diagnosis and was treated with mitotane, which resulted in a decrease in the primary tumor and the lung and liver metastases. The metastasis in the contralateral adrenal gland however showed progression. The patient underwent surgery with resection of the primary tumor, the contralateral adrenal gland and liver metastases, and tumor tissue for a primary culture was obtained. All ACC patients were postoperatively treated with mitotane.

In human primary ACC cultures, mitotane suppressed cell amount and cortisol production in a dose-dependent fashion. The median percentage of cell amount inhibition by 50 µM mitotane was 57% (IQR 39 – 71). On the basis of the percentage inhibition at 50 µM mitotane (≤33%, 33–66% or >66% inhibition), seven (22 %) ACCs were classified as nonresponders, 14 (44%) as partial responders, and 11 (34%) as responders (Table 1; Fig. 1, A and C–E). The mean EC_{50} value on cell growth

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

	Total group (ACC)	Nonresponders	Partial responders	Responders	P-value
n (%)	32	7 (22)	14 (44)	11 (34)	
% cell amount inhibition at 50 µM (median, IQR)	57 (39–71)	15 (0.0–21)	53 (44–60)	75 (70–82)	**<0.001**
EC_{50} (µM, 95% CI) cell amount	–	> 100 µM	41.6 (33.5–51.8)	14.2 (11.3–17.9)	
EC_{50} (µM, 95% CI) cortisol	1.4 (0.90 – 2.1) n = 15	15 (3.2 – 75) n = 1	1.7 (1.2 – 2.5) n = 6	0.90 (0.69 – 1.2) n = 8	**<0.0001**
Age at diagnosis (median, IQR, yrs)	51 (43–57)	52 (45– 65)	43 (38 – 51)	57 (52 – 70)	**0.003**
Male, n (%)	17 (53)	2 (29)	9 (64)	6 (55)	0.317
ENSAT staging, n (%)					0.375
I	1 (4)	0 (0)	0 (0)	1 (9)	
II	13 (46)	5 (71)	5 (42)	3 (33)	
III	2 (7)	0 (0)	2 (17)	0 (0)	
IV	13 (41)	2 (29)	5 (42)	6 (60)	
Weiss score (median, IQR)	6.0 (5.0–7.0) n = 24	6.0 (4.8–7.0) n = 6	6.0 (5.0–7.0) n = 8	7.0 (6.0–7.5) n = 9	0.372
VSI (median, IQR)	22.2 (18.9–25.1) n = 30	21.0 (16.1–25.1) n = 7	22.0 (18.3–25.3) n = 14	24.7 (19.6–28.4) n = 10	0.176
Tumor diameter (median, IQR)	14.0 (8.25–19.0)	18.0 (14.0–19.0)	11.0 (6.75–18.25)	14.0 (8.00–21.0)	0.316
***In vivo* secretion, n (%)**					
Androgens	5 (16)	1 (14)	2 (14)	2 (18)	1.000
Cortisol	15 (47)	1 (14)	6 (43)	8 (73)	0.068
Mineralocorticoids	1 (3)	0 (0)	1 (7)	0 (0)	1.000
Precursors	2 (6)	1 (14)	1 (7)	0 (0)	0.690
Estradiol	2 (6)	0 (0)	1 (7)	1 (9)	1.000
Non secreting	13 (41)	4 (57)	6 (43)	3 (27)	0.475
***In vitro* cortisol secretion, n (%)**	15 (47)	1 (14)	6 (43)	8 (73)	0.068
Metastasis during follow-up, n (%)	20 (63)	3 (43)	8 (57)	9 (82)	0.185

[a]Van Slooten Index and Weiss Score were not available for all patients, dependent on the year of diagnosis. P-value represents overall differences between the three groups. Significant *P*-values are shown in bold. Values represent mean ± SD. Nonresponders, ≤33% inhibition of cell amount at 50 µM mitotane; partial responders, >33% and ≤67% inhibition of cell amount at 50 µM mitotane; responders, >67% inhibition of cell amount at 50 µM mitotane. Abbreviations: CI, Confidence Interval; ENSAT, European Network for the Study of Adrenal Tumors, only for primary tumors; IQR, interquartile range; VSI, Van Slooten Index; yrs, years.

could not be calculated for nonresponders, because the dose–response curves did not reach the bottom. Fifteen of the 32 primary ACC cultures secreted cortisol *in vitro*. The mean EC_{50} of mitotane for inhibition of cortisol production, corrected for cell amount, was 15 µM for the single cortisol producing nonresponder (Fig. 1F; n = 1, 14%), 1.7 µM (95% CI 1.2 – 2.5; $P < 0.0001$ vs nonresponders) for partial responders (Fig. 1G; n = 6, 43%), and 0.90 µM (95% CI 0.69 – 1.2; $P < 0.0001$ vs nonresponders and partial responders) for responders (Fig. 1H; n = 8, 73%). In 14 of the 15 primary cultures with *in vitro* cortisol production, cortisol production was inhibited at significantly lower concentrations than the cell amount (Fig. 1, F–H; all $P < 0.01$).

In all of the ACC samples in which *CYP11B1* and *STAR* mRNA was measured (n = 13), *STAR* mRNA was expressed, although in one nonresponder sample at a very low level (Supplementary Fig. 1 (20)). *CYP11B1* mRNA could be detected in 12 of the 13 ACC samples. This confirms adrenocortical origin of the plated cells.

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

Patient and tumor characteristics of the ACCs of which a primary culture was obtained are listed in Table 1. There were differences in age at diagnosis between the three groups (P = 0.003); however, we did not find a linear correlation. In 81% of primary cultures, *in vitro* and *in vivo* cortisol production were concordant. The percentage *in vitro* cortisol-producing ACC gradually increased with a stronger response to mitotane *in vitro*, ie, 14%, 43%, and 73% for nonresponders, partial responders, and responders, respectively (P = 0.068; Fig. 1B, Table 1). The same difference was observed for *in vivo* glucocorticoid secretion (P = 0.068). There were no differences in pathological characteristics, tumor size, and ENSAT stage, between the three groups. A decreased overall survival was found for cortisol versus noncortisol secreting ACC *in vivo* (log rank P = 0.043, n = 45). Although all patients were treated with mitotane post surgery, evidence for a correlation between *in vitro* and *in vivo* mitotane sensitivity is yet inconclusive, considering the heterogeneous

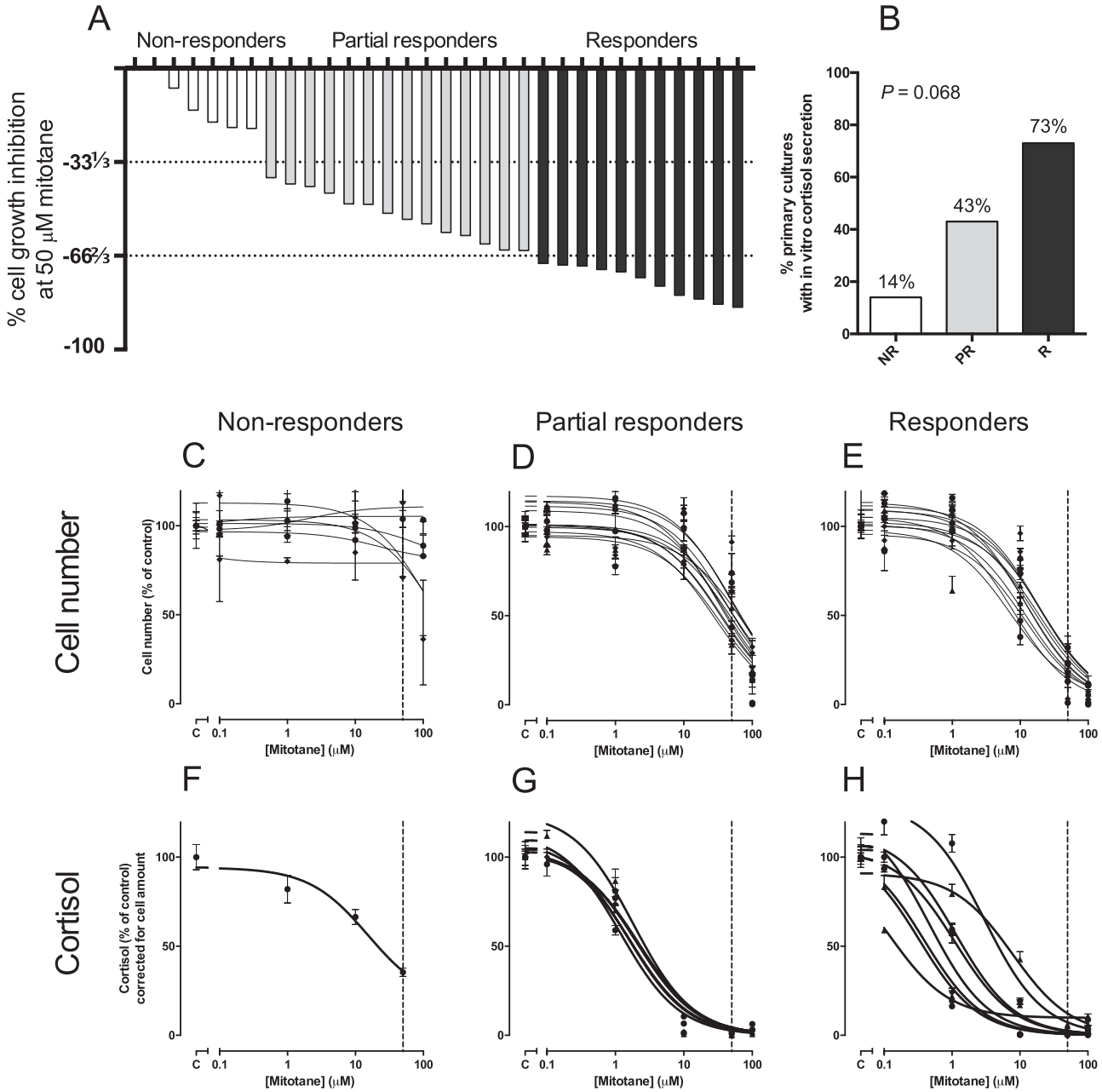

Figure 1. Effects of mitotane on cell growth and cortisol production in primary adrenocortical carcinoma (ACC) cultures ***in vitro***. (A) Percentage of inhibition of cell growth at 50 μM per primary culture and the identification of nonresponders, partial responders, and responders. (B) The percentage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

patient groups, the different indications for mitotane treatment and the small sample size. The clinical characteristics regarding *in vivo* mitotane sensitivity for the different groups can be found in Supplementary Table 3 and Supplementary Fig. 2.

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

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

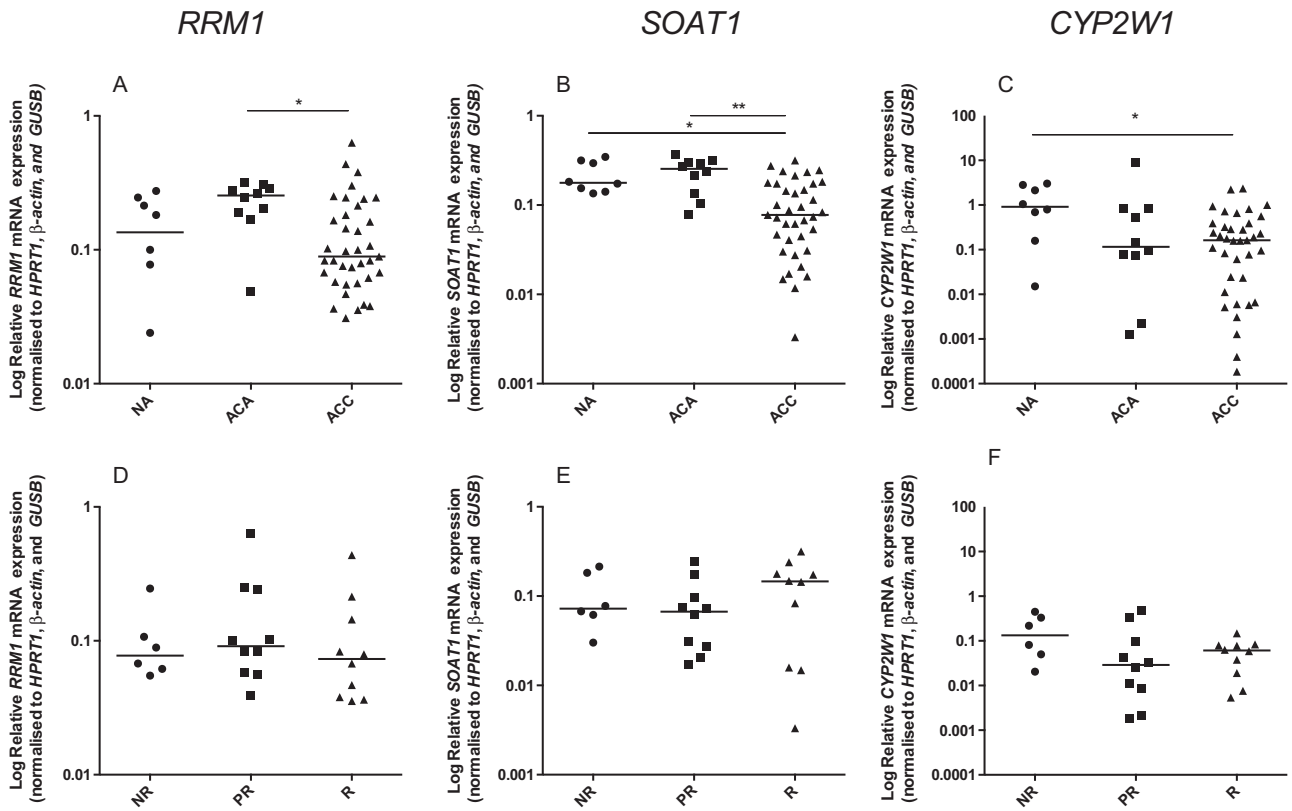

Figure 2. ***RRM1*** (A,D), ***SOAT1*** (B,E), and ***CYP2W1*** (C,F) mRNA expression in normal adrenals, adrenocortical adenomas and adrenocortical carcinomas (A–C), and in ACCs stratified for mitotane responsiveness ***in vitro*** (D–F). Lines represent medians. **P* < 0.05, ** *P* < 0.01. Abbreviations: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

stage, or histopathological criteria in ACC. *CYP2W1* mRNA expression was negatively correlated with the VSI in ACC ($P = 0.002$, $\rho = -0.530$). No correlation was found with the WS.

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

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

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

Immunoreactivity scores of SOAT1 IHC were based on expression levels relative to the normal adrenal cortex (Fig. 4A–F). Chromogranin A expression was used to differentiate between adrenal medulla and cortex (Fig. 4G–I), demonstrating that only the monoclonal SOAT1 Ab specifically stains the adrenal cortex (Fig. 4A–C and G–I). Examples of SOAT1 staining, representing different IRS scores in ACC, are stated in Fig. 4J–R. Within ACC, SOAT1 protein expression by using both antibodies was positively correlated (Supplementary Fig. 3C; $P = 0.0004$, $\rho = 0.5424$). SOAT1 protein expression as assessed by either the PoAb or the mAb was not different between NAs, ACAs, and ACCs (Supplementary Fig. 3, A and B). SOAT1 protein expression (mAb) was negatively correlated with ACC tumor size ($P < 0.0001$, $\rho = -0.550$, $n = 38$), and was significantly higher in ACC with *in vitro* cortisol production than in the ACC cultures with

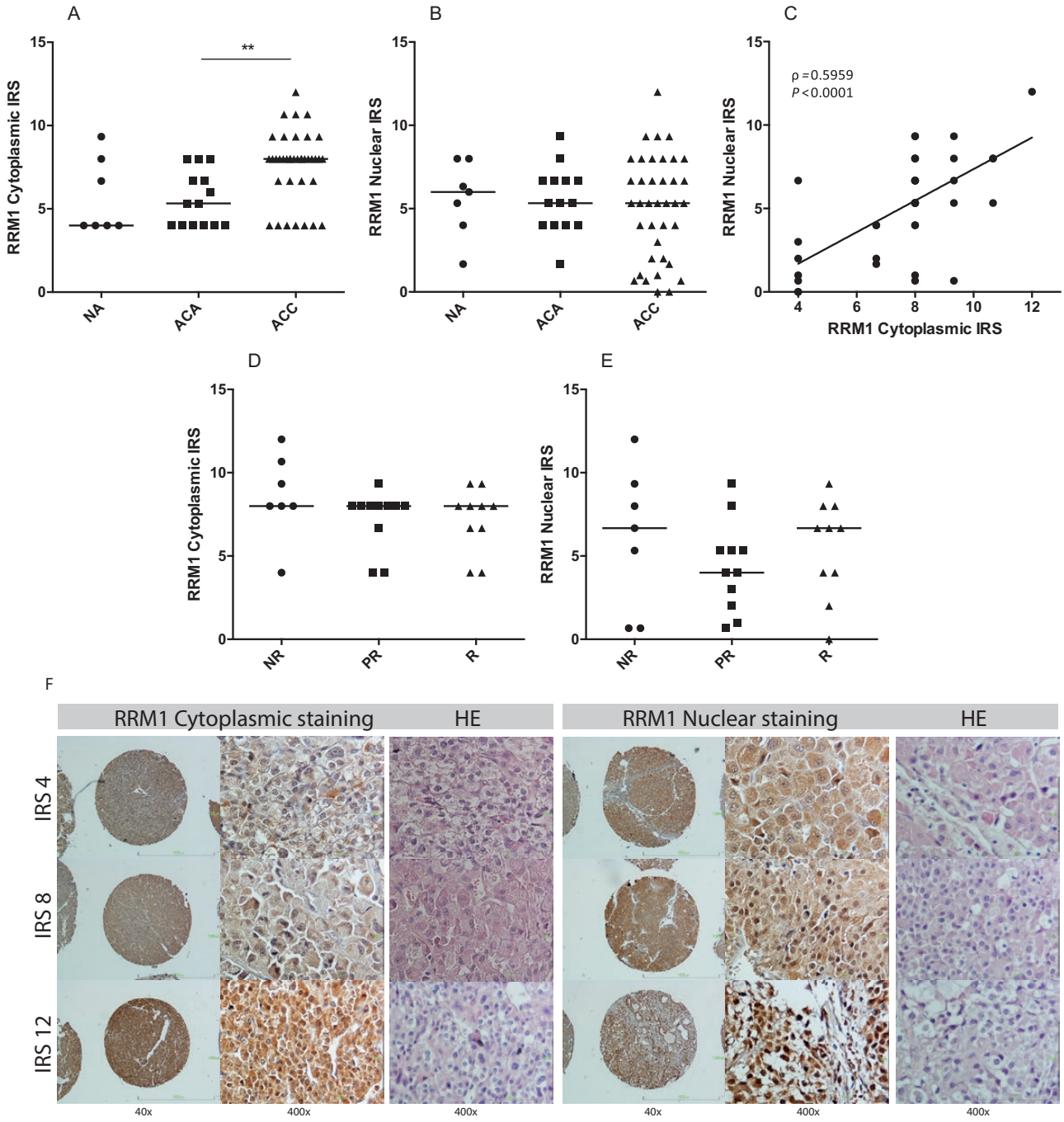

Figure 3. RRM1 protein expression in human adrenal specimens in the cytoplasm (A) and nucleus (B), and correlation of expression at both localizations in adrenocortical carcinoma (C). RRM1 protein expression in the cytoplasm (D), and nucleus (E) in adrenocortical carcinomas stratified for mitotane response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

no cortisol production (n = 28; P = 0.011). Only the SOAT1 expression as assessed by the mAb was positively correlated with *SOAT1* mRNA expression in ACC (Supplementary Fig. 3D; P = 0.0001, ρ = 0.6208), where the correlation with expression as assessed by the PoAb did not reach significance (P = 0.057, ρ = 0.334). No difference was found in SOAT1 protein expression between nonresponders, partial responders, and responders (Supplementary Fig. 3E and F). Although within a group of only 14 ACC, SOAT1 protein expression, as assessed with the mAb, was inversely correlated with the EC_{50} of mitotane on cortisol production in primary cultures (Supplementary Fig. 3G; P = 0.0025, ρ = -0.743, n = 14). This indicates that higher expression of SOAT1 results

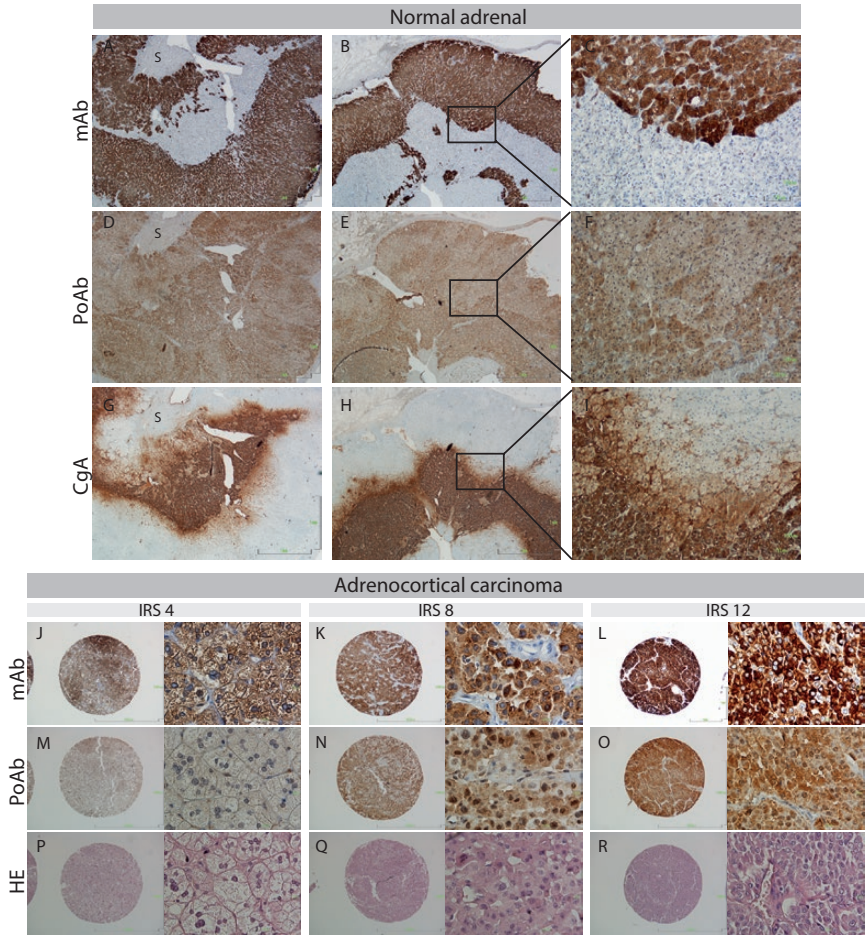

Figure 4. SOAT1 protein expression as assessed by the monoclonal and polyclonal antibody (mAb, PoAb, respectively) in human adrenal tissues. SOAT1 is primarily expressed in the cytoplasm (J–L). (A–I) SOAT1 and Chromogranin A (CgA, a marker for adrenal medulla) expression in corresponding areas in a normal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

in increased sensitivity to mitotane *in vitro*. This correlation appeared to be a trend using the PoAb (P = 0.056, ρ = -0.521, n = 14). Focusing on the correlation between higher SOAT1 protein expression (mAb) and a stronger response to mitotane as defined by the percentage cell growth inhibition at 50 µM in primary ACC cultures, there was no significant correlation (Supplementary Fig. 3H; P = 0.1201, ρ = 0.3064, n = 27).

Discussion

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

The responder classification of ACC to mitotane *in vitro* was based on the effect on cell amount at a concentration of mitotane corresponding to the therapeutic circulating plasma concentration (14 mg/L, 50 µM (1, 6, 7)). As a result, 34% of patients were classified as responder, which is consistent with clinical data, suggesting efficacy of mitotane in 25–35% of patients with advanced ACC (5). We also show that the direct antitumor effect of therapeutic concentrations of mitotane on growth of ACC cells is highly variable between ACC patients. Mitotane decreased cortisol production in all cortisol-producing ACCs, and in general at concentrations much lower than required for inhibition of cell growth. This difference suggests that a measured inhibitory effect on cortisol in patients does not necessarily resemble an antitumor effect of mitotane.

In 19% of the cases, *in vivo* and *in vitro* cortisol secretion were discordant. Discrepancy might be explained by tumor heterogeneity, considering that only a small part of the tumor is used to obtain primary cultures. Thereby, in a subset of ACC, tumor cells may

minimally secrete cortisol *in vivo*, but not sufficient to be diagnosed by clinical tests. The proportion of cortisol-producing ACC *in vitro* and *in vivo* was highest in the responder group, with a gradually decreasing percentage from the partial responder to the nonresponder group. Cortisol production has previously been identified as a negative prognostic factor in ACCs (26–28), which was confirmed in our study. Explanations for the decreased overall survival might be the presence of comorbidities associated with Cushing's syndrome and/or the immunosuppressive effects of cortisol favoring tumor progression. Regarding the relation between efficacy of mitotane and cortisol production by ACCs, data have been inconsistent (26, 29, 30). Two more recent studies have reported that mitotane treatment post surgery only increased disease-free survival in patients with cortisol-secreting tumors, whereas this was not seen in the whole patient group (26, 29). One of the possible explanations of a better response to mitotane in patients with cortisol-producing ACCs might be the decrease in cortisol secretion with concomitant improvement of Cushing's syndrome co-morbidities. However, our *in vitro* findings, ie, a trend towards more cortisol-producing ACC in the responder group, points towards a stronger direct antitumor effect of mitotane on cortisol-secreting ACC cells. It has to be acknowledged, however, that the group sizes are small. The exact mechanism of action of mitotane has yet to be convincingly established, although it is known that mitochondria-mediated intracellular stress induction plays a pivotal role in the basis for its action (31). The adrenal specificity is thought to be caused by transformation of mitotane into active metabolites specifically in mitochondria of the adrenal gland (32). A reasonable hypothesis is that cortisol-producing ACC harbor increased mitochondrial function, considering the presence of a least three mitochondrial cytochrome P450s required for steroid synthesis (CYP11A1, CYP11B1, and CYP11B2). This might result in increased sensitivity to mitotane in cortisol-producing ACC. The inhibitory effects of mitotane on steroidogenesis are besides the toxicity the result of inhibition of several enzymes necessary for cortisol and aldosterone biosynthesis, such as STAR, CYP11A, and CYP11B enzymes (33). It has also been suggested that CYP11B1 catalyzes the initial hydroxylation step of mitotane (34), which has however not been supported by a recent report by Germano et al. using CYP11B1 silencing during mitotane action (35).

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

In clinical practice, as well as in the present *in vitro* study, a great variability is observed in sensitivity to mitotane between patients. Considering the severe adverse effects of mitotane, there is an unmet need for parameters that predict treatment response. Expression levels of several genes, such as *RRM1*, *CYP2W1*, and *SOAT1*, have been proposed for this purpose (9, 11, 12). This is the first study that correlates these potential predictive factors with direct antitumor effects in primary cultures. We only found a significant correlation between mRNA expression of CYP2W1 and increased response to mitotane *in vitro*. This finding is in the opposite direction as has previously been described with *in vivo* response and CYP2W1 immunoreactivity (12). CYP2W1 protein expression was however not assessed in this study, because Nole and colleagues recently showed that, when using a more specific antibody compared with the antibody used by Ronchi et al. (12), CYP2W1 is only rarely expressed in ACC (36). Research is now focusing on the predictive value of CYP2W1 polymorphisms in ACC. For RRM1 and SOAT1, additionally immunohistochemistry was performed. We demonstrate RRM1 protein expression in both the nucleus as the cytoplasm of ACC. Localization of RRM1 protein expression is thought to be dependent on cell type, tissue of origin and cellular state (37). Further research could focus on the relevance of RRM1 expression at both localizations in ACC. No correlations were observed of RRM1 protein expression and *in vitro* mitotane sensitivity. SOAT1 IHC was performed with the polyclonal antibody that was used by Sbiera et al. (11), and furthermore by using a mouse monoclonal antibody (Sc69836; Santa Cruz Biotechnology). Protein expressions as assessed by both antibodies were correlated in ACC, but only the expression as assessed using the mAb was correlated with *SOAT1* mRNA expression in ACC. Thereby, in contrast to the SOAT1 PoAb, the SOAT1 mAb showed convincing specificity for the adrenal cortex by comparing its expression to expression of chromogranin A. Chromogranin A is a peptide produced by chromaffin cells localized in the adrenal medulla (38). Together, these data suggest that the mouse monoclonal antibody used in this study

might be a more reliable antibody for determining SOAT1 protein expression in ACC. The trend towards higher SOAT1 protein expression (with mAb) in patients with a stronger response to mitotane in primary cultures on cortisol production is in concordance with previously published *in vivo* data showing a prolonged progression-free survival in patients with high SOAT1 protein expression, although they used the PoAb (11). SOAT1 has an important role in cholesterol ester formation in the adrenal gland, which protects adrenal cells from potentially damaging effects of free cholesterol (39, 40). Sbiera and colleagues showed that mitotane inhibits SOAT1 expression, which leads to accumulation of toxic lipids that trigger endoplasmic reticulum stress (11). The increased mitotane response in patients with high SOAT1 expression might be explained by the fact that SOAT1 expression is a prerequisite for mitotane efficacy (11). Given the potential increased SOAT1 expression in cortisol-secreting ACC, this might be an additional explanation for increased direct antitumor effects of mitotane in cortisol-secreting ACC, although we did not show this in our *in vitro* study. Unfortunately, due to the lack of available mitotane serum levels in a subgroup of patients, and the diversity amongst the group of ACC patients regarding the indication for mitotane treatment (adjuvant or palliative), no correlations could be made with *in vivo* mitotane response. Further research is needed, therefore, to elucidate the predictive value of SOAT1 expression for mitotane response in patients with ACC. Furthermore, future studies could investigate whether factors that have currently been associated with prognosis in ACC, like CTNNB1 and TP53 mutations, Ki67, SF-1, Fascin-1, also correlate with mitotane sensitivity *in vivo* and *in vitro* (5, 14–16).

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

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Additional Information

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References

1. Kerkhofs TM, Verhoeven RH, Van der Zwan JM, Dieleman J, Kerstens MN, Links TP, Van de Poll-Franse LV, Haak HR. Adrenocortical carcinoma: a population-based study on incidence and survival in the Netherlands since 1993. *Eur J Cancer*. 2013;49(11):2579–2586.
2. Kebebew E, Reiff E, Duh QY, Clark OH, McMillan A. Extent of disease at presentation and outcome for adrenocortical carcinoma: have we made progress? *World J Surg*. 2006;30(5):872–878.
3. Else T, Kim AC, Sabolch A, et al. Adrenocortical carcinoma. *Endocr Rev*. 2014;35(2):282–326.
4. Fassnacht M, Dekkers O, Else T, et al. European Society of endocrinology clinical practice guidelines on the management of adrenocortical carcinoma in adults, in collaboration with the European network for the study of adrenal tumors. *Eur J Endocrinol*. 2018.
5. Creemers SG, Hofland LJ, Korpershoek E, Franssen GJ, van Kemenade FJ, de Herder WW, Feelders RA. Future directions in the diagnosis and medical treatment of adrenocortical carcinoma. *Endocr Relat Cancer*. 2016;23(1):R43–R69.
6. Terzolo M, Baudin AE, Ardito A, Kroiss M, Leboulleux S, Daffara F, Perotti P, Feelders RA, deVries JH, Zaggia B, De Francia S, Volante M, Haak HR, Allolio B, Al Ghuzlan A, Fassnacht M, Berruti A. Mitotane levels predict the outcome of patients with adrenocortical carcinoma treated adjuvantly following radical resection. *Eur J Endocrinol*. 2013;169(3):263–270.
7. Terzolo M, Berruti A. Adjunctive treatment of adrenocortical carcinoma. *Curr Opin Endocrinol Diabetes Obes*. 2008;15(3):221–226.
8. Megerle F, Herrmann W, Schloetelburg W, et al. Mitotane monotherapy in patients with advanced adrenocortical carcinoma. *J Clin Endocrinol Metab*. 2018;103(4):1686–1695.
9. Volante M, Terzolo M, Fassnacht M, Rapa I, Germano A, Sbiera S, Daffara F, Sperone P, Scagliotti G, Allolio B, Papotti M, Berruti A. Ribonucleotide reductase large subunit (RRM1) gene expression may predict efficacy of adjuvant mitotane in adrenocortical cancer. *Clin Cancer Res*. 2012;18(12):3452–3461.
10. Germano A, Rapa I, Volante M, De Francia S, Migliore C, Berruti A, Papotti M, Terzolo M. RRM1 modulates mitotane activity in adrenal cancer cells interfering with its metabolization. *Mol Cell Endocrinol*. 2015;401(C):105–110.
11. Sbiera S, Leich E, Liebisch G, Sbiera I, Schirbel A, Wiemer L, Matysik S, Eckhardt C, Gardill F, Gehl A, Kendl S, Weigand I, Bala M, Ronchi CL, Deutschbein T, Schmitz G, Rosenwald A, Allolio B, Fassnacht M, Kroiss M. Mitotane inhibits Sterol-O-Acyl Transferase 1 triggering lipid-mediated endoplasmic reticulum stress and apoptosis in adrenocortical carcinoma cells. *Endocrinology*. 2015;en20151367.
12. Ronchi CL, Sbiera S, Volante M, Steinhauer S, Scott-Wild V, Altieri B, Kroiss M, Bala M, Papotti M, Deutschbein T, Terzolo M, Fassnacht M, Allolio B. CYP2W1 is highly expressed in adrenal glands and is positively associated with the response to mitotane in adrenocortical carcinoma. *PLoS One*. 2014;9(8).
13. Karlgren M, Ingelman-Sundberg M. Tumour-specific expression of CYP2W1: its potential as a drug target in cancer therapy. *Expert Opin Ther Targets*. 2007;11(1):61–67.
14. Xiao H, Xu D, Chen P, Zeng G, Wang X, Zhang X. Identification of five genes as a potential biomarker for predicting

progress and prognosis in adrenocortical carcinoma. *J Cancer.* 2018;9(23):4484–4495.
15. Poli G, Ruggiero C, Cantini G, et al. Fascin-1 is a novel prognostic biomarker associated with tumor invasiveness in adrenocortical carcinoma. *J Clin Endocrinol Metab.* 2019;104(5):1712–1724.
16. Zheng S, Cherniack AD, Dewal N, et al. Comprehensive pan-genomic characterization of adrenocortical carcinoma. *Cancer Cell.* 2016;29(5):723–736.
17. van Slooten H, Schaberg A, Smeenk D, Moolenaar AJ. Morphologic characteristics of benign and malignant adrenocortical tumors. *Cancer.* 1985;55(4):766–773.
18. Lau SK, Weiss LM. The Weiss system for evaluating adrenocortical neoplasms: 25 years later. *Hum Pathol.* 2009;40(6):757–768.
19. Hofland LJ, van Koetsveld PM, Lamberts SW. Percoll density gradient centrifugation of rat pituitary tumor cells: a study of functional heterogeneity within and between tumors with respect to growth rates, prolactin production and responsiveness to the somatostatin analog SMS 201-995. *Eur J Cancer.* 1990;26(1):37–44.
20. Papathomas TG, Oudijk L, Zwarthoff EC, Post E, Duijkers FA, van Noesel MM, Hofland LJ, Pollard PJ, Maher ER, Restuccia DF, Feelders RA, Franssen GJ, Timmers HJ, Sleijfer S, de Herder WW, de Krijger RR, Dinjens WN, Korpershoek E. Telomerase reverse transcriptase promoter mutations in tumors originating from the adrenal gland and extra-adrenal paraganglia. *Endocr Relat Cancer.* 2014;21(4):653–661.
21. Creemers SG, van Koetsveld PM, van Kemenade FJ, Papathomas TG, Franssen GJ, Dogan F, Eekhoff EM, van der Valk P, de Herder WW, Janssen JA, Feelders RA, Hofland LJ, Methylation of IGF2 regulatory regions to diagnose adrenocortical carcinomas. *Endocr Relat Cancer.* 2016;23(9):727–737.
22. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 2002;3(7):RESEARCH0034.
23. Creemers SG, van Koetsveld PM, van den Dungen ES, Korpershoek E, van Kemenade FJ, Franssen GJ, de Herder WW, Feelders RA, Hofland LJ. Inhibition of human adrenocortical cancer cell growth by Temozolomide in vitro and the role of the MGMT gene. *J Clin Endocrinol Metab.* 2016;jc20162768.
24. Remmele W, Stegner HE. A proposal for the standardization of the immunoreactive score (IRS) for the immunohistochemical demonstration of estrogen-receptors (ER-ICA) in breast-cancer. *Pathologe.* 1987;8(3):138–140.
25. de Jong JJ, Stoop H, Nieboer D, Boormans JL, van Leenders G. Concordance of PD-L1 expression in matched urothelial bladder cancer specimens. *Histopathology.* 2018;73:983–989.
26. Abiven G, Coste J, Groussin L, Anract P, Tissier F, Legmann P, Dousset B, Bertagna X, Bertherat J. Clinical and biological features in the prognosis of adrenocortical cancer: poor outcome of cortisol-secreting tumors in a series of 202 consecutive patients. *J Clin Endocr Metab.* 2006;91(7):2650–2655.
27. Berruti A, Terzolo M, Sperone P, Pia A, Della Casa S, Gross DJ, Carnaghi C, Casali P, Porpiglia F, Mantero F, Reimondo G, Angeli A, Dogliotti L. Etoposide, doxorubicin and cisplatin plus mitotane in the treatment of advanced adrenocortical carcinoma: a large prospective phase II trial. *Endocr Relat Cancer.* 2005;12(3):657–666.
28. Vanbrabant T, Fassnacht M, Assle G, Dekkers OM. Influence of hormonal functional status on survival in adrenocortical carcinoma: systematic review and meta-analysis. *Eur J Endocrinol.* 2018;179(6):429–436.
29. Bertherat J, Coste J, Bertagna X. Adjuvant mitotane in adrenocortical carcinoma. *N Engl J Med.* 2007;357(12):1256–1257; author reply 1259.
30. Berruti A, Fassnacht M, Haak H, Else T, Baudin E, Sperone P, Kroiss M, Kerkhofs T, Williams AR, Ardito A, Leboulleux S, Volante M, Deutschbein T, Feelders R, Ronchi C, Grisanti S, Gelderblom H, Porpiglia F, Papotti M, Hammer GD, Allolio B, Terzolo M. Prognostic role of overt hypercortisolism in completely operated patients with adrenocortical cancer. *Eur Urol.* 2014;65(4):832–838.
31. Waszut U, Szyszka P, Dworakowska D. Understanding mitotane mode of action. *J Physiol Pharmacol.* 2017;68(1):13–26.
32. Cai W, Benitez R, Counsell RE, Djanegara T, Schteingart DE, Sinsheimer JE, Wotring LL. Bovine adrenal cortex transformations of mitotane [1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane; o,p'-DDD] and its p,p'- and m,p'-isomers. *Biochem Pharmacol.* 1995;49(10):1483–1489.
33. Hescot S, Slama A, Lombes A, Paci A, Remy H, Leboulleux S, Chadarevian R, Trabado S, Amazit L, Young J, Baudin E, Lombes M. Mitotane alters mitochondrial respiratory chain activity by inducing cytochrome c oxidase defect in human adrenocortical cells. *Endocr Relat Cancer.* 2013;20(3):371–381.
34. Lindhe O, Skogseid B, Brandt I. Cytochrome P450-catalyzed binding of 3-methylsulfonyl-DDE and o,p'-DDD in human adrenal zona fasciculata/reticularis. *J Clin Endocrinol Metab.* 2002;87(3):1319–1326.
35. Germano A, Saba L, De Francia S, Rapa I, Perotti P, Berruti A, Volante M, Terzolo M. CYP11B1 has no role in mitotane action and metabolism in adrenocortical carcinoma cells. *PLoS One.* 2018;13(5):e0196931.
36. Nole P, Duijndam B, Stenman A, Juhlin CC, Kozyra M, Larsson C, Ingelman-Sundberg M, Johansson I. Human cytochrome P450 2W1 is not expressed in adrenal cortex and is only rarely expressed in adrenocortical carcinomas. *PLoS One.* 2016;11(9):e0162379.
37. Engstrom Y, Rozell B. Immunocytochemical evidence for the cytoplasmic localization and differential expression during the cell cycle of the M1 and M2 subunits of mammalian ribonucleotide reductase. *EMBO J.* 1988;7(6):1615–1620.
38. Komminoth P, Roth J, Schroder S, Saremaslani P, Heitz PU. Overlapping expression of immunohistochemical markers and synaptophysin mRNA in pheochromocytomas and adrenocortical carcinomas. Implications for the differential diagnosis of adrenal gland tumors. *Lab Invest.* 1995;72(4):424–431.
39. Chang TY, Li BL, Chang CC, Urano Y. Acyl-coenzyme A:cholesterol acyltransferases. *Am J Physiol Endocrinol Metab.* 2009;297(1):E1–E9.
40. Kraemer FB. Adrenal cholesterol utilization. *Mol Cell Endocrinol.* 2007;265–266:42–45.