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Expression of SOAT1 in adrenocortical carcinoma and response to mitotane monotherapy: an ENSAT multicenter study

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Precis

Mitotane is a cornerstone of adrenal cancer treatment. In this international study, expression of putative mitotane target SOAT1 in tissue did not predict treatment response to mitotane monotherapy.

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Abstract

Context Objective response rate to mitotane in advanced adrenocortical carcinoma (ACC) is approximately 20% and adverse drug effects are frequent. To date there is no marker established that predicts treatment response. Mitotane has been shown to inhibit sterol-O-acyl transferase 1 (SOAT1) which leads to endoplasmic reticulum stress and cell death in ACC cells.

Objective To investigate SOAT1 protein expression as a marker of treatment response to mitotane.

Patients 231 ACC patients treated with single agent mitotane as adjuvant (n=158) or advanced disease therapy (n=73) from twelve ENSAT centers were included. SOAT1 protein expression was determined by immunohistochemistry on formalin-fixed paraffin-embedded (FFPE) specimens.

Setting Retrospective study at 12 ACC referral centers

Main outcome measure: recurrence-free survival (RFS), progression-free survival (PFS), disease-specific survival (DSS)

Results 61/135 patients (45%) with adjuvant mitotane treatment had recurrences and 45/68 patients (66%) with mitotane treatment for advanced disease had progressive disease. After multivariate adjustment for sex, age, hormone secretion, tumour stage and Ki67 index, RFS (HR=1.07, 95% CI 0.61-1.85, p=0.82) and DSS (HR=1.30, 95% CI 0.58-2.93, p=0.53) in adjuvantly treated ACC patients did not differ significantly between tumors with high and low SOAT1 expression. Similarly, in the advanced stage setting, PFS (HR=1.34, 95% CI 0.63-2.84, p=0.45) and DSS (HR=0.72, 95% CI 0.31-1.70, p=0.45) were comparable and response rates not significantly different.

Conclusions SOAT1 expression was not correlated with clinical endpoints RFS, PFS and DSS in ACC patients with mitotane monotherapy. Other factors appear to be relevant for mitotane treatment response and ACC patient survival.

Key words: adrenal cancer, chemotherapy, treatment, prognosis

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Introduction

Adrenocortical carcinoma (ACC) is a rare malignancy with a generally poor prognosis (1) and limited effective treatment options (1,2). Mitotane is the only approved drug for metastatic disease (3) but efficacy is very limited and the observed objective response rate is only approximately 20% (4-6). Controversy exists regarding adjuvant use which is supported by a large retrospective study (6,7) and advocated by current guidelines (2) in patients at moderate or high risk of recurrence after complete resection. Adverse drug effects like adrenal insufficiency, diarrhea, nausea and other gastrointestinal symptoms but also central nervous symptoms such as dizziness and speech disturbance may be severe and disabling (8-10) and must be balanced against potential treatment benefits. Mitotane efficacy is correlated with plasma concentrations above 14 mg/l (11). Therapeutic drug monitoring (TDM) is therefore recommended (2). Some patients for unknown reasons fail to achieve mitotane plasma concentrations within the therapeutic window which is associated with decreased efficacy (5,12). To date, few markers have been suggested for the prediction of response (13,14), but they have not been validated in a large series. Establishment of such a marker would be a major advancement in ACC treatment and enable tailored treatment of potential responders and avoidance of unnecessary mitotane exposure in non-responders.

We have provided evidence that mitotane inhibits sterol-O-acyl transferase 1 (SOAT1) also known as ACAT1 (15) (not to be mistaken with acetyl-CoA acetyltransferase known under the same name), an enzyme catalyzing the esterification of cholesterol in the adrenal cortex (16). This leads to the accumulation of toxic lipids and endoplasmic reticulum (ER) stress which results in apoptosis of adrenocortical cells (15). Accordingly, a SOAT1 inhibitor has been tested in a phase I clinical trial as a treatment for advanced ACC (17).

SOAT1 is strongly expressed in adrenocortical cell lines, normal adrenal glands and different adrenocortical tumor entities, with the highest variation among ACC, while it is only weakly to moderately expressed in non-adrenal tissues (15). Despite strong evidence of an inhibitory effect on SOAT1, other mechanisms such as impaired mitochondrial respiration and function (18-20) may contribute to the relatively tissue-specific toxicity of mitotane.

In a small cohort of patients with advanced ACC, it has been shown that SOAT1 expression was correlated with the response to mitotane treatment (15).

Here, we aimed to validate in a large multicenter study whether SOAT1 expression is a predictive marker for mitotane efficacy by investigating the association of SOAT1 tissue expression with recurrence free survival (RFS) in patients with adjuvant mitotane treatment, progression-free survival (PFS) after mitotane monotherapy administered to patients with advanced disease and disease-specific survival (DSS) for both cases.

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Patients and Methods

Setting and data acquisition

Formalin-fixed paraffin-embedded (FFPE) tumor specimens of 231 ACC were included from 12 centers belonging to the European Network for the Study of Adrenocortical Tumors (ENSAT; www.ensat.org). Only adult patients with histologically confirmed ACC were included (21). Patients that have been included in our previous analyses of SOAT1 expression (15) have been excluded from this analysis. All patients started mitotane treatment as first medical therapy no later than 3 months after complete resection in the adjuvant setting (n=158) or diagnosis of irresectable or recurrent or metastatic ACC in the advanced stage setting (n=73). Enrollment of patients is presented in a CONSORT diagram (Fig. S1) (22). Mitotane plasma levels were determined according to local standard of care (2). This retrospective study was conducted as part of the ENSAT registry, has been approved by the ethics committee at each participating institution and was conducted in accordance with the principles of the Declaration of Helsinki. All patients gave informed written consent.

Clinical and pathological data, including sex, age at diagnosis, date of diagnosis, tumor stage according to the ENSAT staging system (23), hormone secretion, Weiss score (21), Ki67 proliferation index, mitotane plasma concentrations after three and six months and response to treatment during follow-up were either provided by the participant center or collected through the ENSAT registry (https://registry.ensat.org/). Hormone excess was defined as any form of autonomous hormone secretion (glucocorticoids, androgens, aldosterone or mixed hormone secretion). Recurrence and progression were pre-defined to be based on routine local clinical judgement based on cross sectional imaging and death as ACC-related death.

Chromogenic immunohistochemistry

Full FFPE sections mounted on slides were deparaffinised, rehydrated and antigen retrieval was performed in 10mM citric acid monohydrate buffer (pH 6.5) under pressure for 13 min. Blocking of unspecific binding sites occurred with 20% human AB serum at room temperature (RT) for 1 h and the primary antibody (SOAT1; ab39327 Abcam) was incubated in a 1:1000 dilution for 1h at RT as previously described (15). The N-Universal negative control anti-rabbit (Dako) was used and signal amplification was achieved by the Advance HRP Link Kit for 40 min and developed for 10 min with the DAB+ Liquid Kit (Dako). Nuclei were counterstained using Mayer's hematoxylin for 3 min and blued for 5 min in running tap water. To ensure specificity of the antibody used (24), we overexpressed human SOAT1 in ACC cells which resulted in an increase of both detected SOAT1 bands and SOAT1 WB of 5 normal adrenal glands also resulted only in the two specific bands (Fig. S2) (22).

Semi-quantitative analysis of SOAT1 immunoreactivity

Chromogenic staining intensities were determined by two independent investigators blinded to clinical outcome (I.W. and B.A. or L.-S.L.) and graded as 0 (negative), 1 (low), 2 (medium) and 3 (high). The proportion of positive tumor cells was calculated for each slide and scored 0 if 0% were positive, 0.1 if 1-9% were positive, 0.5 if 10-49% were positive and 1 if ≥50% were positive (25,26). A semi quantitative H-Score was then calculated by multiplying the staining intensity grading score with the proportion score. Where discrepancies were observed, results were jointly assessed by

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both investigators and the final score was formed by consensus. The Spearman's correlation for inter-observer agreement for each staining was high (r>0.85).

Statistical analysis

RFS and PFS were considered as the time between diagnosis and documented recurrence and progression (based on cross sectional imaging), respectively. DSS was calculated from the time of diagnosis until disease-related death or censored at last follow-up. RFS, PFS and DSS were analysed using the Kaplan–Meier method and groups were compared by using the log-rank test. Assessment of prognostic factors (ENSAT stage, Ki67, age, sex, hormone secretion and for the group with advanced disease additionally: preM-TTP (pre mitotane time to progression= time between diagnosis and progress before initiation of mitotane treatment) was performed with the Cox proportional hazard regression model. The Chi-square test was used to investigate dichotomic variables, whereas non-parametric Kruskal-Wallis s test was used for comparison among groups for non-normal distributed variables. Correlations between H-Score and prognostic factors were evaluated by Spearman's correlation. P values *<0.05* were considered statistically significant. Statistical analyses were performed with IBM SPSS Version 23 and GraphPad Prism Version 6.

Results

Patient characteristics

Clinical characteristics of 231 ACC patients are summarised in Table 1. Median age at diagnosis was 54.2 years (range 17-83) in the adjuvant group and 51 years (range 16-80) in the group with advanced disease. In both groups, approximately 60% of the patients were female and 40% were male. At diagnosis, the majority of patients treated with mitotane monotherapy in the adjuvant setting had an ENSAT tumor stage of I-II (62.3%), whereas, in the advanced stage setting, most of the patients had a tumor stage of IV (55.6%). The remaining patients with advanced disease had a localized tumor at diagnosis and started mitotane therapy only after developing local recurrence or metastases. Data regarding Ki67 index were available in 91.2% and 83.5% of patients in the adjuvant and advanced stage setting, respectively. 31 patients (21%) of the adjuvant group and 18 patients (27.3%) of the advanced stage group had Ki67 index staining below 10% (*p=0.35*, chi-square=0.88). Median Weiss score was 6 (range 1-9) in both groups. In both arms, about 70% of the tumors were hormonally active. Median time to start mitotane were one month in the adjuvant group and less than one month in the group with advanced disease. Median mitotane plasma levels at three months of therapy were 9.3 mg/l and 10 mg/l, after six months 13.5 mg/l and 12.8 mg/l in the adjuvant and advanced stage cohort, respectively. In the advanced stage group, preM-TTP was <365 days in 51/63 patients (81%) for DSS and <365 days in 52/67 patients (78%) for PFS.

No recurrence was observed in 74/135 patients within a median follow-up of 18.5 months (range 1-216 months) in patients treated in adjuvant setting. Best response to advanced stage mitotane was complete (n=1) or partial response (n=9), stable disease in 13 and progressive disease in 45 patients. Median follow up of patients still alive (n=18) was 19.5 months (range 2-180 months) in this setting.

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SOAT1 expression and correlation with known prognostic factors of ACC

Tissue SOAT1 expression differed widely in tumors of both the adjuvant and the group with advanced disease and exhibited different intra-tumoral patterns between homogeneous and heterogeneous staining intensity (Fig. 1). Semiquantitative H-score accounts for this heterogeneity as it takes into account both the staining intensity and percentage of cells being stained and ranged from 0 to 3. Scores from 0 to <2 were designated low expression (Fig. 1J-L) while scores ≥2 were indicative of high expression (Fig. 1A-I). No difference in SOAT1 expression was found between hormone producing and endocrine inactive ACC with mean staining intensities of 1.53 ±0.9 in inactive *vs.* 1.48 ±0.9 in hormonally active ACC, p=0.76. No correlation of SOAT1 H-score was observed with Ki67, ENSAT stage, Weiss score and age at diagnosis neither in the adjuvant, nor in the advanced stage setting.

SOAT1 expression as factor of survival and response to mitotane treatment in ACC

In the adjuvant setting (Fig. 2A), we did not observe significant differences of RFS between ACC patients with low SOAT1 expression in comparison to those with high SOAT1 expression (median 22 months, range 1-153 vs. median 12 months, range 1.5-216 log rank p=0.12). When we only included patients with Ki67≥10% to analyse RFS, we did not observe significant differences between SOAT1 low and high expressing ACC either (log rank p=0.73). DSS (Fig. 2B) did not significantly differ between patients whose tumors expressed low levels of SOAT1 compared to those with high SOAT1 expression (median 51 months, range 1-252 *vs.* 31 months, range 2-216 log rank p=0.23). Similarly, in the group with advanced disease, no significant difference in PFS (Fig. 2C) between patients with low SOAT1 expression and those with high SOAT1 expression (median PFS 5 months, range 1-59 vs. median 4 months, range 1-25 log rank p= 0.66) was observed. Median DSS (Fig. 2D) was likewise not different in tumors with low vs. high SOAT1 (median 22 months, range 4-180 vs. 21 months, 2-83 months, log rank p=0.47). To increase numbers, we additionally analysed all patients together (Fig. S3A). Low SOAT1 expression was associated with a significantly longer median recurrence-/progression-free survival of 13 months (range 1 -153 months vs 8 months (range 1-216 months, log rank p=0.049). We did not observe a significant difference in DSS (Fig. S3B) between tumors with low SOAT1 vs high SOAT1 expression (median: 41 months, range 1 -252 vs. median: 28 months, range 2-216, log rank p=0.41) (22).

The proportion of tumors with low and high SOAT1 expression did not differ between patients in the adjuvant cohort without recurrence (low, n=44; high, n=30) and with recurrence (low, n=35; high, n=26) (Fig. 3A). Similarly, in the cohort with advanced disease, there were no differences between tumors with low and high SOAT1 regarding objective response to mitotane (low, n=6; high, n=4) *vs.* stable disease (low, n=6; high, n=7) and progressive disease (low, n=25; high, n=20), respectively (Fig. 3B).

We next aimed at multivariable adjustment for known clinical/histopathological ACC prognostic factors. In the adjuvant arm, univariate analysis revealed only a Ki67-Index <10% as significantly associated with improved DSS and RFS (Table 2). In patients with advanced disease the following factors were significantly associated with improved DSS: male sex, Ki67-Index <10% and preM-TTP >365 days. After multivariate analysis of all factors, including SOAT1 expression, only preM-TTP >365 days retained statistical significance (Table 3).

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SOAT1 expression is not related to mitotane plasma concentrations

We next examined the potential association of SOAT1 expression with mitotane plasma concentrations. Mitotane plasma levels after three months of treatment did not significantly differ between patients whose tumors showed high vs low expression of SOAT1 both in the adjuvant (median mitotane levels:10.3 mg/l *vs* 9.1 mg/l) and in the advanced disease setting (median mitotane levels: 11.7 mg/l *vs* 9.1 mg/l) (Fig. 4A). SOAT1 expression was not associated with mitotane plasma concentrations above 14 mg/l neither in the adjuvant (Fig. 4B) nor in the advanced disease arm (Fig. 4C). Similar results were observed after six months of mitotane treatment (median mitotane levels 14.2 mg/l *vs* 13 mg/l in the adjuvant group and 11.9 mg/l *vs* 12.8 mg/l in the group with advanced disease). When analyzing only patients reaching the mitotane target level of 14 mg/l after three months, significantly fewer patients with high SOAT1 expression responded to therapy (Fig. 4D) while this difference was no longer observed when considering the six months time point (Fig. 4E).

Median dose of mitotane intake was 4 g/daily (range 1-12 g) in the adjuvant arm and 5 g/daily (range 2-12 g/daily) in patients treated for advanced disease and did not significantly differ between the SOAT1 high and low expressing group (*p= 0.6* (adjuvant) and *p=0.4* (advanced disease)).

Discussion

Mitotane is the only approved drug for the treatment of ACC, however, objective response rates are only approximately 20% (5,6). In addition to its limited therapeutic potential, adverse events occur frequently and reliable markers predicting response to therapy are currently not established. Therefore, it is crucial to define a particular subgroup of patients that will take advantage from treatment and to avoid toxicity in patients unlikely to respond.

At present, this topic has been addressed only in a limited number of patients (13,14) and very recently a study demonstrated mitotane sensitivity only in a very specific sub-group of patients (27). Although mitotane has been used in the clinic for decades, its precise mechanism of action and molecular target remained unknown for decades, despite intense research including several different “omics” approaches (18-20,28). We have demonstrated that mitotane inhibits SOAT1, leading to ER-stress and cell death of adrenocortical cells (15). It was also shown that SOAT1 is predominantly expressed in adrenocortical cells, compared to cells of non-adrenal origin (15), possibly explaining the specific adrenolytic toxicity of mitotane. In addition, in glioblastoma, inhibition of SOAT1 has been proposed as a novel treatment (29,30).

In hepatocellular carcinoma high SOAT1 expression was associated with a worse prognosis (31) and has previously been described in prostate cancer as well (32). An adverse outcome of SOAT1 expression in ACC was recently demonstrated (33). These results suggest that the elevated expression of SOAT1 could be a prognostic feature of diverse cancers. In a small single center series of patients (n=25) with advanced ACC (15), we had previously shown that SOAT1 expression is associated with improved progression-free survival. This ENSAT multicenter retrospective study aimed at validating the value of SOAT1 as a histologic marker for mitotane response. Our results disprove our initial hypothesis, as no significant differences in response to mitotane treatment could be observed between ACC tissue samples with high and low levels of SOAT1 protein neither in an adjuvant setting nor in patients treated with advanced disease.

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Our study has the strength of a large collection of tissue samples from specialized ACC centers. SOAT1 expression was histologically determined in a centralized manner. All patients received mitotane monotherapy, no additional therapies were used during mitotane treatment. However, our study has several limitations. First, the clinical data and samples collection were retrospectively retrieved from twelve different and specialized ENSAT centers (11 European and one from Brazil) which likely is associated with different treatment strategies. This not only comprises surgery and medical treatment but also documentation and follow-up. Second, mitotane treatment itself is cumbersome and different dosing regimens are in use at different centers (34-36). In addition, patient-specific factors that are only partially understood lead to a high heterogeneity of mitotane plasma concentrations (37-39). Accordingly, mitotane plasma concentrations in our cohort after three and six months of treatment were highly variable. When considering only patients who reached mitotane plasma concentrations of >14 mg/L after three or six months, SOAT1 expression was not correlated to clinical response. Furthermore, established RNA or DNA markers predict prognosis in ACC but were not investigated in this work. Just to mention several miRNAs (40,41), and also the methylation status of several genes (42,43) were shown to be associated with prognosis in ACC.

The lack of an association of SOAT1 expression with survival endpoints and response implicates that additional target molecules different from SOAT1 may be relevant for its toxic effect in adrenal cortical cells. *In vitro*, SOAT1 expression was shown to not be a predictor as demonstrated in few ACC primary cultures (24) which would support the theory that additional targets might be of greater importance. One such potential mechanism includes inhibition of mitochondrial respiratory chain. The novel compound nevanimibe (previously known as ATR101) which has been developed as a new treatment for ACC has been shown to be a potent SOAT1 inhibitor by one group (44) but was also shown to inhibit mitochondrial respiration by a different group (45) similar to mitotane.

Importantly, we found pronounced heterogeneity of SOAT1 expression in approximately 20 % of tumor samples. It is conceivable that this tissue heterogeneity was not completely accounted for in the monocentric study by Ferreira Lacombe *et al.* (33) in which a tissue microarrays were used to evaluate SOAT1 expression whereas we used full sections. Relationship of SOAT1 with Ki67 index and cortisol secretion was demonstrated in the previous study but not in ours. However, in our study Ki67 value was provided by the various participating centers and thus a uniform analysis of this index is not guaranteed.

In an adjuvant setting, several other known factors such as resection status or Ki67 index (46), are important to predict tumor recurrence, since even after complete resection, recurrence rates are high (47-49). In line with previous studies, Ki67-index below 10% (Table 2) was significantly associated with a better DSS and RFS in our cohort of patients treated with mitotane in this setting. Our study could further not clearly demonstrate ENSAT stage to have prognostic value in this setting, where we observed only a trend toward a better RFS for patients with ENSAT stage I-II compared to those with stage III-IV as already showed in a previous study (27). This might be due to most patients were classified as either ENSAT tumor stage II or III and different centers might have looked for lymph nodes more or less thoroughly, which could result in a misclassification. Similarly, in advanced ACC, Ki67 index, resection status, mutational burden but also clinical factors like age or presence of symptoms, as well as preM-TTP have been identified to predict patient outcome independently of mitotane treatment (27,50-53). In our cohort of patients with advanced disease, Ki67-Index below

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10% was also associated with a better DSS (Table 3), which retained significance after multivariate adjustment, but was not observed for PFS in a univariate analysis (Table 3). This may be due to the relatively small cohort but is in line with a previous study in which only the DSS, but not the PFS correlated with a Ki67-Index below 10% in advanced ACC (5) In conclusion, in this multicenter study, we could not confirm SOAT1 expression to be a clinically useful marker to predict treatment response to mitotane.

Contributors:

The following scientists contributed tissue samples and clinical data in addition to those listed as co-authors:

Marcus Quinkler (Berlin), Masanori Murakami (Munich), Felix Beuschlein (Munich, Zurich), Harm R. Haak (Eindhoven), Marcin Motyka (Krakow), Vasileois Chortis (Birmingham) and Annamaria Colao (Naples).

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Table 1: Patient characteristics.

Parameters	adjuvant	advanced disease
Number of patients:	158	73
Age in years:	54.2 (17-83)	51 (16-80)
Sex:		
female	97 (61)	42 (58)
male	61 (39)	30 (42)
Tumor stage:		
I	10 (6.5)	2 (2.8)
II	88 (56)	19 (26.4)
III	52 (33)	11 (15.3)
IV	7 (4.5)	40 (55.6)
R status:		
n available	156	68
0	121 (77.6)	28 (41)
X	30 (19.2)	15 (22)
1	5 (3.2)	13 (19)
Ki67-Index:		12 (18)
n available	144	66
<10%	30 (21)	18 (27.3)
≥10%	114 (79)	48 (66.4)
Weiss score:		
n available	142	55
median (range)	6 (2-9)	6 (3-9)
Endocrine activity:		
n available	143	62
hormone excess	96 (67)	45 (72.6)
inactive	47 (33)	17 (27.4)
Months to mitotane start:	1 (0-3)	0 (1-3)
Mitotane concentration after 3 months:		
n available	132	
median (range)	9.05 (0-29)	10 (1-27)
Mitotane concentration after 6 months:		
n available	125	51
median (range)	13 (1-27)	12 (1-32)

Data is shown as number (percentage) or median (range). Hormone excess was considered as any form of autonomous adrenal hormone secretion.

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Table 2: Impact of SOAT1 expression and known prognostic parameters on RFS and DSS in the adjuvant (R0 or RX) cohort.

RFS	**univariate analysis**			**multivariate analysis**		
variables	**HR**	**95% CI**	***p***	**HR**	**95% CI**	***p***
Sex						
female (n=91)						
male (n=56)	1.13	0.72-1.76	0.60	1.30	0.77-2.19	0.33
Age						
<50 (n=90)						
≥50 (n=58)	0.78	0.49-1.23	0.29	0.72	0.40-1.29	0.27
Hormone excess						
Yes (n=90)						
No (n=43)	1.39	0.84-2.32	0.20	1.55	0.86-2.77	0.14
Tumor stage						
I+ II (n=90)						
III + IV (n=56)	1.51	0.97-2.34	0.07	1.54	0.90-2.62	0.11
Ki67						
<10 (n=28)						
≥10 (n=107)	3.810	1.64-8.84	0.002*	2.86	1.18-6.96	0.02
Mitotane levels 3 months (median:9.1 mg/l) n=122						
<9.1						
≥9.1	1.50	0.92- 2.44	0.11	-	-	-
Mitotane levels 6 months (median:13 mg/l) n=116						
<13						
≥13	1.02	0.62 - 1.67	0.95	-	-	-
SOAT1						
H-Score low: <2 (n=89)						
H-Score high: ≥2 (n=59)	1.42	0.91-2.21	0.12	1.07	0.61-1.85	0.82

DSS	**univariate analysis**			**multivariate analysis**		
variables	**HR**	**95% CI**	***p***	**HR**	**95% CI**	***p***
Sex						
female (n=81)						
male (n=53)	1.19	0.462-2.28	0.61	1.65	0.74-3.67	0.22
Age						
<50 (n=80)						
≥50 (n=55)	0.64	0.32-1.29	0.21	0.60	0.24-1.55	0.29
Hormone excess						
Yes (n=85)						
No (n=36)	1.52	0.68-3.40	0.31	1.48	0.58-3.79	0.42

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Tumor stage						
I + II (n=85)						
III + IV (n=48)	1.43	0.74-2.76	0.28	1.23	0.54-2.78	0.63
Ki67						
<10 (n=24)						
≥10 (n=99)	4.91	1.17-20.67	0.03*	3.60	0.80-16.24	0.10
Mitotane levels 3 months (median:9.1 mg/l) n=112						
<9.1						
≥9.1	1.52	0.76 – 3.06	0.24	-	-	-
Mitotane levels 6 months (median:13 mg/l) n=103						
< 13						
≥13	0.74	0.34-1.60	0.44	-	-	-
SOAT1						
H-Score low: <2 (n=81)						
H-Score high: ≥2 (n=54)	1.49	0.77-2.86	0.24	1.30	0.58-2.93	0.53

Hormone excess was considered as any form of autonomous adrenal hormone secretion. Abbreviation: preM-TTP, pre mitotane time to progression.

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Table 3: Impact of SOAT1 expression and known prognostic parameters on PFS and DSS in the cohort with advanced disease.

PFS	**univariate analysis**			**multivariate analysis**		
variables	**HR**	**95% CI**	***p***	**HR**	**95% CI**	***p***
Sex						
female (n=40)						
male (n=27)	0.75	0.44-1.27	0.28	0.81	0.43-1.53	0.51
Age						
<50 (n=29)						
≥50 (n=38)	0.84	0.50-1.42	0.52	0.73	0.36-1.50	0.40
Hormone excess						
Yes (n=43)						
No (n=14)	1.46	0.77-2.78	0.25	1.98	0.97-4.03	0.06-
preM-TTP						
<365 days						
≥365 days	0.37	0.18-0.72	0.004*	0.49	0.21-1.11	0.09
Ki67						
<10 (n=17)						
≥10 (n=45)	1.19	0.66-2.14	0.55	0.92	0.46-1.83	0.81
Mitotane levels 3 months (median:10 mg/l) n=58						
<10						
≥10	0.70	0.40-1.22	0.21	-	-	-
Mitotane levels 6 months (median:12.5 mg/l) n=48						
<12.5						
≥12.5	0.61	0.33-1.14	0.12	-	-	-
SOAT1						
H-Score low: <2 (n=37)						
H-Score high: ≥2 (n=30)	1.11	0.68-1.86	0.68	1.34	0.63-2.84	0.45

DSS	**univariate analysis**			**multivariate analysis**		
variables	**HR**	**95% CI**	***p***	**HR**	**95% CI**	***p***
Sex						
female (n=36)						
male (n=27)	0.48	0.26-0.92	0.026*	0.92	0.40-2.11	0.83
Age						
<50 (n=27)						
≥50 (n=36)	0.82	0.46-1.48	0.52	1.39	0.63-3.04	0.42
Hormone excess						
Yes (n=40)						
No (n=13)	1.04	0.52-2.07	0.92	1.20	0.52-2.80	0.67
Ki67						
<10 (n=14)						

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≥10 (n=45)	2.47	1.14-5.32	0.021*	1.83	0.73-4.60	0.20
preM-TTP						
<365 days						
>365 days	0.60	0.01-0.26	<0.001*	0.10	0.02-0.49	0.004*
Mitotane levels 3 months (median:10 mg/l) n=54						
<10				-	-	-
≥10	1.05	0.56- 1.98	0.88			
Mitotane levels 6 months (median:12.5 mg/l) n=45						
<12.5	0.62	0.30-1.27	0.19	-	-	-
≥12.5	0.62	0.30-1.27	0.19			
SOAT1						
H-Score low: <2 (n=35)						
H-Score high: ≥2 (n=28)	0.81	0.44-1.46	0.48	0.72	0.31-1.70	0.45

Hormone excess was considered as any form of autonomous adrenal hormone secretion.

Abbreviation: preM-TTP, pre mitotane time to progression.

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Figure legends

Figure 1: SOAT1 immunohistochemistry staining of full ACC FFPE sections. First column shows an overview of SOAT1 staining intensities within the same tumors (scale bars: 3mm). Second column shows 3x magnification of the representative slide in first column (scale bars: 700µm) and third column shows 20x magnification of the slide shown in column A (scale bars: 200µm) (A-C: SOAT1 H-score 3, inhomogeneous staining; D-F: SOAT1 H-Score 3, inhomogeneous staining; G-I: SOAT1 H-score 2, homogeneous staining, J-L: SOAT1 H-score 0, homogenous staining).

Figure 2: Kaplan-Meier plots of SOAT1 low and high expressing ACC. (A) Recurrence-free survival and **(B)** disease-specific survival of ACC patients in the adjuvant group. **(C)** progression-free survival **(D)** and disease-specific survival of ACC patients with advanced disease.

Figure 3: SOAT1 expression and treatment response. No significant differences regarding mitotane response between SOAT1 high and SOAT1 low expressing tumors were observed in the adjuvant arm **(A)**, nor in advanced stages **(B)**.

Figure 4: Correlation of SOAT1 expression and mitotane plasma concentrations. (A) In both arms, high SOAT1 expression was not correlated with higher mitotane plasma levels. Patients with high SOAT1 expression are not more likely to reach mitotane plasma levels above 14 mg/l not in the adjuvant setting **(B)**, nor in patients with advanced disease **(C)**. When only patients reaching the mitotane target level of 14 mg/l were analysed, high SOAT1 expression was significantly correlated with higher rates of recurrences after three months **(D)** which did not retain significance after six months **(E).**

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Figure 1

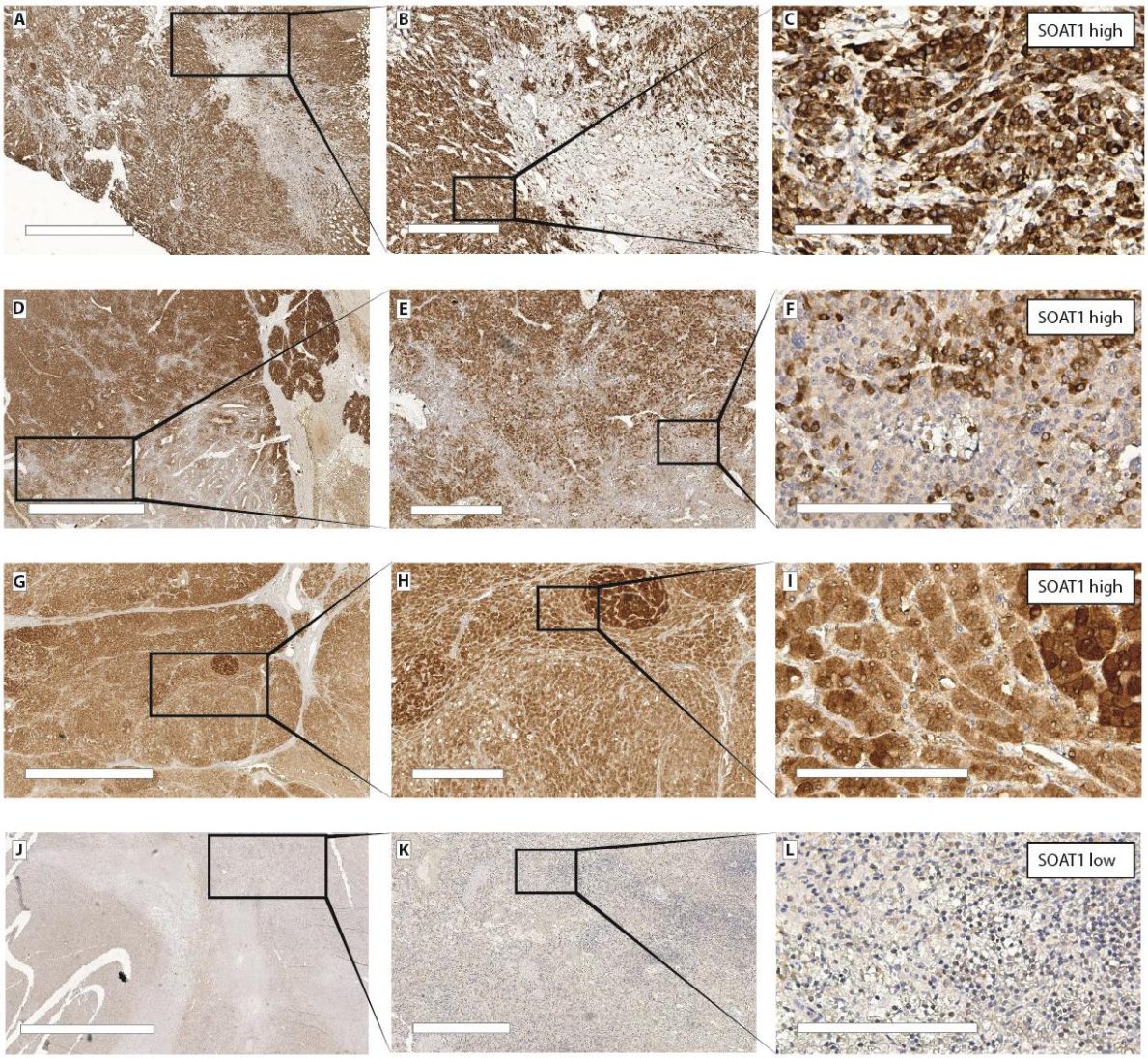

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Figure 2

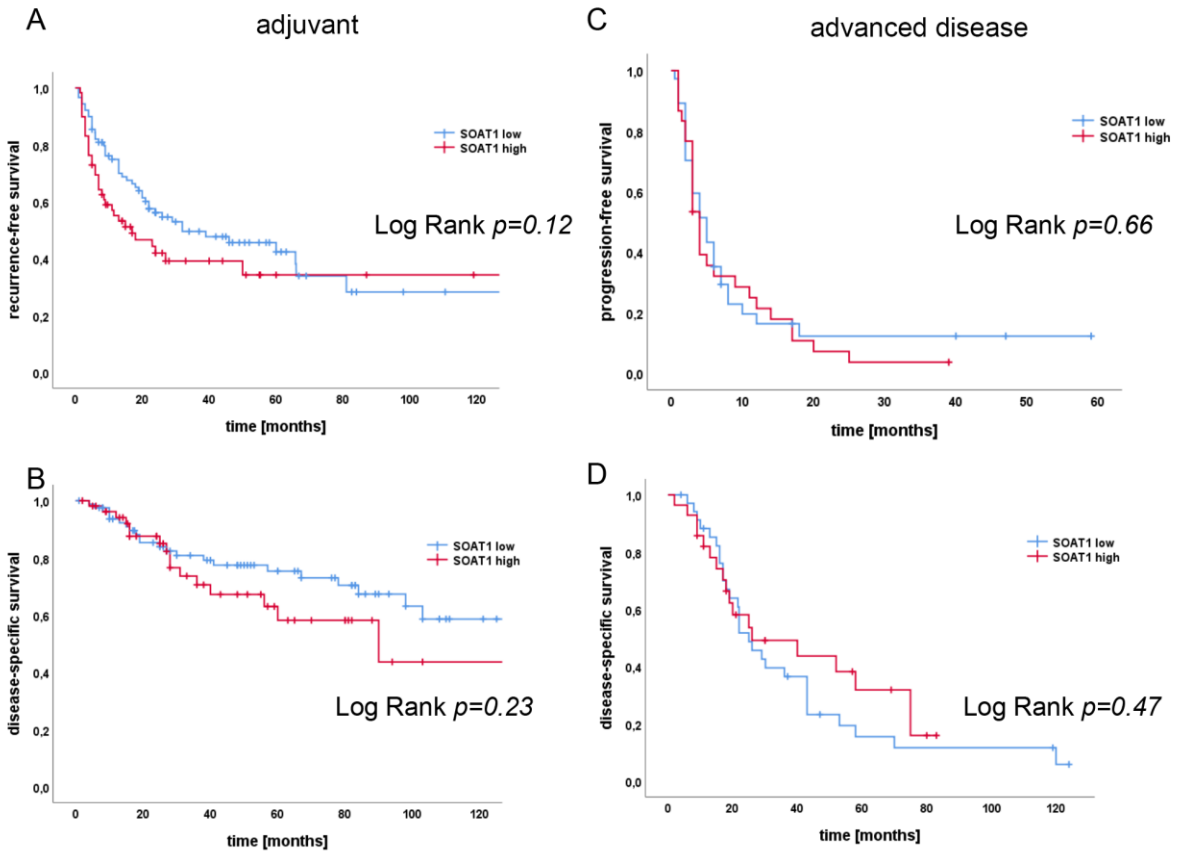

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Figure 3

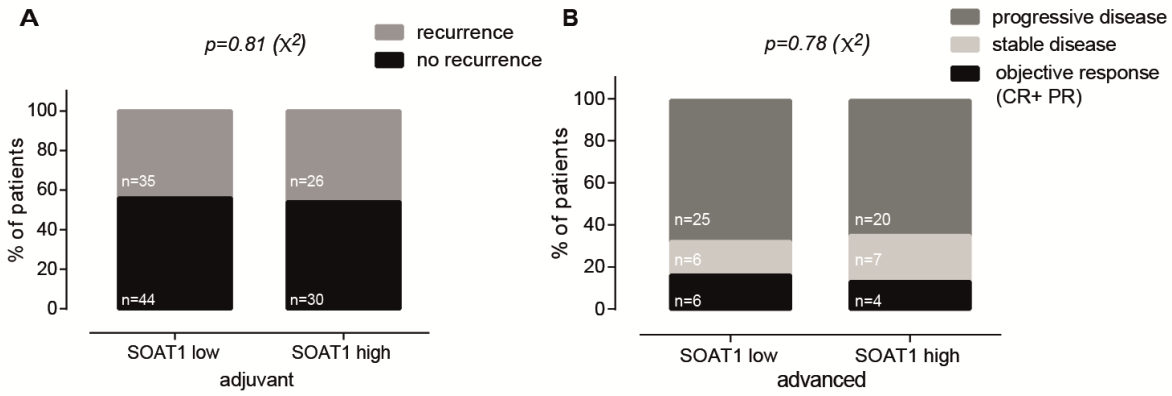

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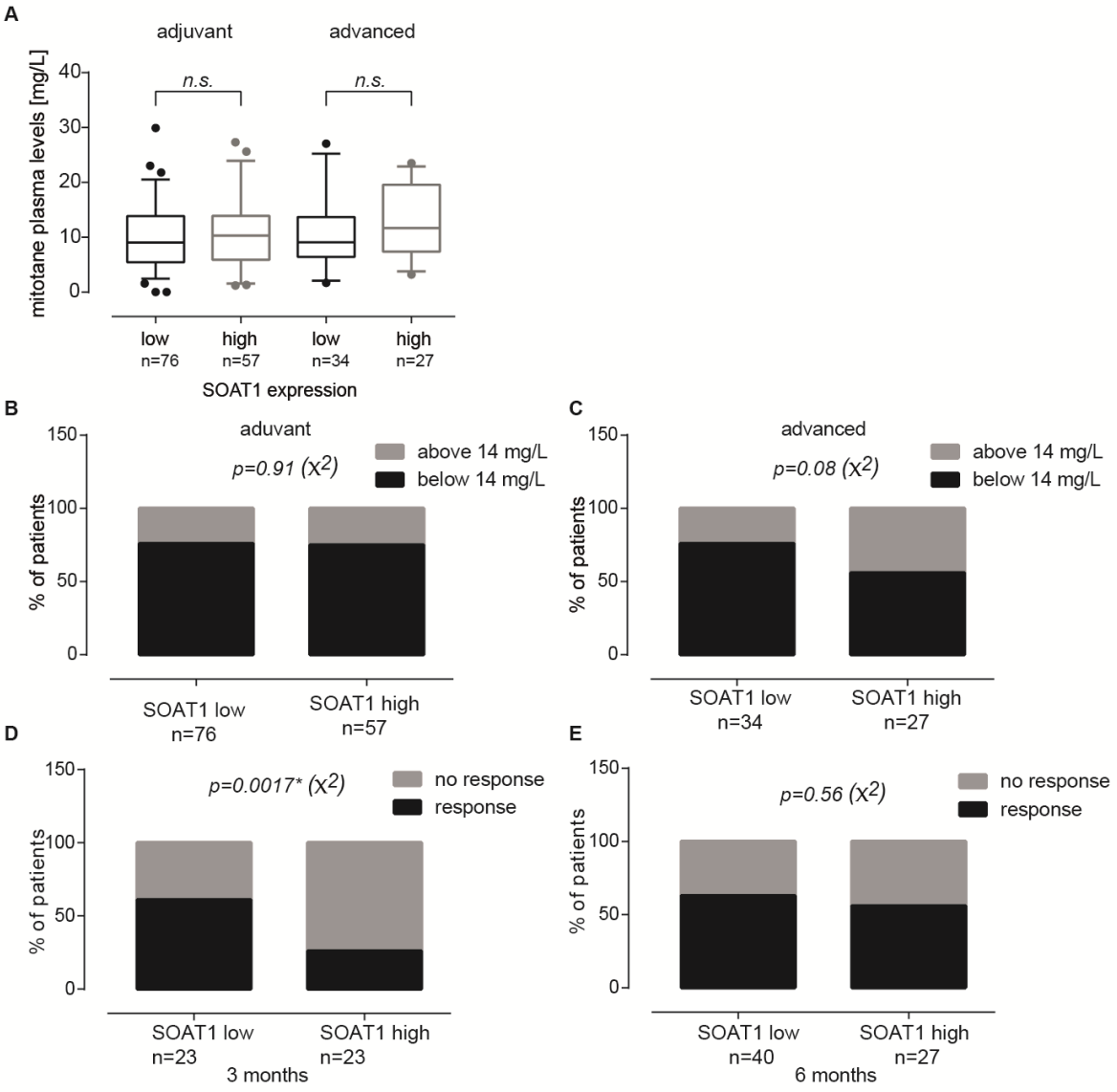
Figure 4



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