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Original Research

Mitotane treatment of adrenocortical carcinoma induces tumoural secretion of GDF-15: impact on poor prognosis and impaired responsiveness to immunotherapy

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Abstract

Purpose: Treatment options for adrenocortical carcinoma (ACC), where mitotane remains a mainstay of therapy, are unsatisfactory. Response rates of ACC to immune checkpoint inhibition (ICI) are disappointing, and immune cells are scarce in ACC. Growth/differentiation factor 15 (GDF-15) is a cytokine impairing tumoural immune infiltration. We here aimed to assess the value of serum GDF-15 for the prognosis of ACC and as a predictor of response to ICI.

Methods: GDF-15 was measured in serum samples of 151 patients and correlated with clinical data. Serum GDF-15 was analysed in a second cohort of 46 ACC patients who received ICI, including 14 responders. mRNA expression of GDF15 and genes related to immune response was quantified in 58 ACC tumour samples.

Results: We found GDF-15 induction in ACC cells and patients upon mitotane treatment. In ACC patients, serum GDF-15 concentration below the median was associated with significantly longer patient survival. GDF-15 levels in responders to ICI were significantly lower than in non-responders ($P = .0379$), and patients with low GDF-15 levels had a significant longer progression-free survival than patients with higher GDF-15 serum levels ($P = .036$). Expression of pro-inflammatory immune-related genes was lower in ACC tissue with GDF-15 expression above the median.

Conclusions: Mitotane increases GDF-15 levels and is associated with poor response to ICI. GDF-15 may mediate reduced infiltration with immune cells in ACC.

Keywords: tumour immune micro-environment, immune checkpoint inhibition, macrophage-inhibitory cytokine 1, adrenal cancer

Significance

Immune checkpoint inhibition (ICI) is only effective in approximately 15% of adrenocortical carcinoma (ACC) patients. Yet, there is no biomarker to identify responders prior to treatment. Mitotane remains the mainstay of ACC treatment. We studied a large cohort of ACC patients with and without mitotane and 46 patients receiving ICI. We discovered that mitotane therapy increases serum concentrations of the immunosuppressive cytokine growth/differentiation factor 15 (GDF-15). High serum GDF-15 is associated with shorter survival. High circulating GDF-15 at ICI start is associated with treatment failure and an anti-inflammatory gene expression profile in tissue. The combination of ICI with GDF-15 neutralising antibodies may be active in patients with ACC as currently successfully tested in other tumour entities.

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Introduction

Adrenocortical carcinoma (ACC) is a rare endocrine malignancy which affects approximately 1 per 1 million people per year,[1] and the majority of patients suffer from adrenal steroid excess.[2] Based on retrospective studies,[3] current guidelines recommend adjuvant treatment with mitotane in patients with intermediate and high risk of recurrence,[4] but mitotane is not beneficial in low-risk patients.[5] In advanced stages, mitotane is used as monotherapy or in combination with cytotoxic chemotherapy. Yet, the rate of objective tumour response to mitotane monotherapy is at best ~20% in selected patients[6] and adverse events are manifold and common. Even the combination of mitotane with platinum-based chemotherapy only leads to an objective response rate of about 25%.[7] Immune checkpoint inhibitors (ICI) have revolutionised the treatment of many cancer entities but not of ACC. The results of published studies are heterogeneous and objective response was observed in only 17 (13%) of 121 patients treated with different ICI across 5 clinical trials.[8-12] A similar response rate was confirmed in 2 large retrospective analyses.[13,14] In none of the trials, established response markers to ICI such as tumour mutational burden (TMB) or microsatellite instability (MSI) were associated with response. Serum markers of response to conventional treatment and immunotherapy have not been described in ACC.

We and others have reported low tumoural infiltration by T lymphocytes and natural killer (NK) cells in ACC, which is even lower in glucocorticoid-secreting tumours.[15,16] Nonetheless, no association was found between glucocorticoid secretion and response to ICI.[8-12] In other tumour entities, growth/differentiation factor 15 (GDF-15) has emerged as a promising biomarker for predicting ICI failure.[17] GDF-15 is a divergent member of the transforming growth factor β superfamily and acts as an endogenous immunosuppressive agent and hormone. Physiologically, GDF-15 is involved in feto-maternal tolerance.[18] Serum GDF-15 reduces appetite[19,20] and polymorphisms in the *GDF15* gene are linked to hyperemesis gravidarum.[21] Circulating GDF-15 levels increase with age, after injury, in states of inflammation and in the presence of malignant neoplasias. Mechanistically, GDF-15 was shown to inhibit activity of NK cells during systemic inflammation and to favour regulatory T cell (T_{reg}) differentiation.[22] It has been demonstrated that GDF-15 promotes tumour growth, impairs effective intratumoural immune cell infiltration and potently blocks immune checkpoint inhibitor activity.[23] Mitochondrial dysfunction, a hallmark of mitotane action,[24,25] is a known inducer of GDF-15.[26] Consistently, we have observed GDF-15 mRNA to be the single most highly upregulated gene in ACC cells after mitotane treatment.[27]

Given the common use of mitotane in the treatment of ACC, the low tumour infiltration with immune cells and the upregulation of GDF-15 by mitotane *in vitro*, we investigated the impact of mitotane on serum GDF-15 in ACC patients. Based on the literature, we hypothesised that GDF-15 is associated with poor prognosis and impaired response to ICI treatment.

Methods

Clinical cohorts

This retrospective study was conducted as part of the European Network for the Study of Adrenal Tumours (ENSAT) registry study that was approved by the ethics committees at the University Hospitals Würzburg (approval #88/11) and LMU Munich (approval #379-10) and conducted in accordance with the principles of the Declaration of Helsinki. All patients provided written informed consent.

Clinical and pathological data including sex, age at diagnosis, date of diagnosis, tumour stage according to the ENSAT staging system, hormone secretion, Weiss score, Ki-67 proliferation index, and mitotane plasma concentrations at the time point of GDF-15 measurement were collected. Hormone excess was defined as pathological glucocorticoid, androgen, aldosterone, or mixed steroid hormone secretion at diagnosis. Tumour mass was quantified as the number of involved organs and number and total maximum diameter of all tumour lesions using cross-sectional imaging from computer tomography (CT), 18-F-fluorodeoxyglucose (FDG) positron emission tomography (PET)-CT, or abdominal magnetic resonance imaging (MRI) at the time point of GDF-15 measurement.

Survival was determined from the time point of blood draw used to determine GDF-15 levels until ACC-related death or patient survival at the time point of last follow-up (September, 20, 2024).

Discovery cohort

Serum samples from 99 ACC patients before and 52 ACC patients after mitotane treatment were collected to study the impact of mitotane on GDF-15 serum levels. Serum of 42 adrenocortical adenoma (ACA) patients served as controls. ACC patients were consecutively recruited at the University Hospitals in Würzburg and Munich. The clinical data of these ACC patients are shown in Table 1 (refer to Table S1 for ACA patients).

Immune checkpoint inhibitor treated cohort

GDF-15 serum levels of 46 ACC patients who received ICI treatment on a compassionate use basis were determined in serum samples collected at the time of ICI initiation ± approximately 4 weeks. One patient with a pathogenic *CTNNB1* mutation and inconsistent data was excluded.

Tumour control rate was defined as complete response (CR), partial response (PR) and stable disease (SD) by clinical judgment in analogy to RECIST 1.1 criteria.[28] Progression was confirmed by cross-sectional imaging, survival as ACC-related death or patient alive at last follow-up (September, 20, 2024). The clinical data for this cohort are displayed in Table 2.

Quantification of GDF-15

GDF-15 concentration in serum was determined with the Quantikine ELISA Human GDF-15 Kit (SGD150, R&D Systems) in the discovery cohort and Roche Cobas ECLIA in the ICI treatment cohort. A previous study showed that results obtained by either method correlate well.[29] Healthy controls were measured with the Elecsys GDF-15 assay (Roche Diagnostics), raw data obtained by[29] and matched 1 to 1 by age and sex with respective patient samples. Healthy controls were apparently healthy individuals with no adrenal, nor other diseases.

Statistics

Descriptive statistics are given as median and range or SD; $P < .05$ was considered statistically significant. Group

Table 1. Clinical data of the ACC discovery cohort.

	Pre-mitotane	Post-mitotane
Patients—*n*	99	52
Age at diagnosis in years: median (range)	51 (19-80)	50.5 (19-74)
Age at blood draw in years: median (range)	53 (19-80)	52 (19-76)
Sex—*n* (%)		
Female	57 (58)	33 (63)
Male	41 (42)	19 (37)
ENSAT stage—*n* (%)	97	52
I	5 (5)	3 (6)
II	42 (43)	25 (48)
III	23 (24)	15 (29)
IV	27 (28)	9 (17)
R status—*n* {ENSATI-III} (%)		
0	53 (80)	28 (78)
X	6 (9)	4 (11)
1	6 (9)	4 (11)
2	1 (2)	0 (0)
Ki-67-index—*n* (%)		
<10	17 (23)	7 (19)
≥10	56 (77)	30 (81)
Endocrine activity—*n* (%)	66	34
Hormone secretion	43 (65)	19 (56)
No hypersecretion	23 (35)	15 (43)
GDF-15 levels (ng/mL) median (range)	0.81 (0.01-30.6)	2.17 (0.5-12.5)
TTP from blood draw (months): median (range) (*n* = 95) (*n* = 51)	45 (0-125)	34 (0-96)
Sum of tumour diameter (cm): median (range) (*n* = 93) (*n* = 46)	5.0 (0-53)	1.3 (0-53)
Mitotane level at blood draw (mg/dL) median (range) (*n* = 47)		14.3 (0.3-28.7)

Table 2. Clinical data of the ICI treated ACC cohort.

Patients—*n*	46
Age at diagnosis in years: median (range)	44.5 (13-78)
Age at blood draw in years: median (range)	49.5 (18-82)
Sex *n* (%)	
Female	28 (61)
Male	18 (39)
ENSAT stage—*n* (%)	
I	3 (7)
II	14 (32)
III	8 (19)
IV	18 (42)
ICI compound—*n* (%)	
Pembrolizumab	41 (89)
Nivolumab	4 (9)
Ipilimumab + nivolumab	1 (2)
Best response to ICI—*n* (%)	
CR	0 (0)
PR	7 (15)
SD	7 (15)
PD	32 (70)
Ki67-index—*n* (%)	
<10	4 (11)
≥10	32 (89)
Endocrine activity—*n* (%)	
Hormone secretion	26 (67)
Of which responders to ICI	8 (31)
No hypersecretion	13 (23)
Of which responders to ICI	5 (38)
GDF-15 levels (ng/mL) at start ICI (*n* = 46) median	4.2 (0.61-76.8)
TTP from blood draw (months): median (range) (*n* = 46)	3 (0-25)
Patients treated with mitotane before ICI: *n* (%)	46 (100)
Mitotane parallel to ICI: *n* (%)	6 (8)
Mitotane plasma levels at start ICI (mg/L) median (range) *n* = 34	1.9 (0-27.8)
Responders to ICI	0.91 (0-23.4)
Non-responders to ICI	2.85 (0-27.8)
Time (months) between end mitotane and start ICI: median (range)	4 (0-136)

comparisons were done as indicated for each result. Overall survival was defined as the time interval from blood sample collection to death or last follow-up. Recurrence-free survival in R0 resected patients was defined as the time interval between blood sample collection and the first occurrence of relapse. The Kaplan–Meier method and log-rank test were performed to estimate and compare event-free survival. Cox proportional hazards regression modelling was used for the identification of clinical factors that independently influence patients' survival. Proportional hazard ratio assumption was tested for time-dependent effects and Cox proportional hazards regression was only used when satisfied (>0.05). Known or potentially relevant prognostic factors (age, number of affected sites to resemble localised and metastatic disease and Ki-67 proliferation index) were analysed by univariate analysis. Since GDF-15 serum levels correlated with ENSAT stage, patients were stratified according to ENSAT stage before and during mitotane therapy. Receiver operating characteristics analyses were performed to calculate the area under the curve (AUC). To test for different confounders, patients were divided into subgroups (eg, measurable tumour mass) and sensitivity analyses were performed. Tumour control after immunotherapy was defined as either CR, PR, or SD and patients categorised into responders (tumour control at first staging) and non-responders (tumour progression at first staging or death prior to imaging). GDF-15 serum concentrations were determined around the start of ICI. The non-parametric Mann–Whitney *U* test was used to determine statistically significant differences.

Statistical analyses were performed using Prism (V.9.12, GraphPad Software Inc., La Jolla, CA, USA) and SPSS 27 (IBM Corporation, Armonk, New York, USA).

Nanostring nCounter gene expression analysis

Formalin-fixed paraffin-embedded (FFPE) ACC tissue sections (2-4 μm) mounted on slides were subjected to RNA extraction with the AllPrep DNA/RNA FFPE Kit (Qiagen). RNA quantity and quality were assessed by NanoDrop2000 (Thermo Fisher Scientific). Samples that met predefined quality standards were used for quantification of gene expression with NanoString nCounter. A customised panel (NanoString Technologies) consisting of 473 immune-related genes plus 11 reference genes was used according to the manufacturer's instructions. Analysis was performed by nSolver Analysis Software 4.0 (NanoString Technologies). For samples that passed imaging quality controls (percentage of fields of view (FOV) read > 75%, binding density 0.05-2.25, positive control linearity > 0.95, and positive control limit of detection > 2), raw gene counts were normalised to technical controls and housekeeping genes after positive control normalisation (geometric mean as normalisation factor). Normalised counts were log2 transformed and expression compared between

Table 3. Clinical data of ACC tissue samples analysed by the Nanostring Ncounter method.

	GDF-15 low	GDF-15-high
Patients—*n*	30	28
Age at diagnosis in years: median (range)	50 (18-74)	46 (20-77)
Sex—*n* (%)		
Female	21 (70)	16 (57)
Male	9 (30)	12 (43)
ENSAT stage—*n* (%)		
I	2 (8)	0 (0)
II	9 (35)	9 (35)
III	5 (19)	8 (30)
IV	10 (38)	9 (35)
R status—*n* {ENSAT I-III} (%)		
0	18 (95)	10 (59)
X	0 (0)	3 (18)
1	1 (5)	4 (23)
2	0 (0)	0 (0)
Ki-67 index—*n* (%)		
<10%	9 (32)	3 (12)
≥10%	19 (68)	22 (88)
Endocrine activity—*n* (%)		
Hormone secretion	23 (77)	21 (75)
No hypersecretion	7 (23)	7 (25)
Sum of tumour diameter (cm): median (range) ($n = 28$) ($n = 26$)	9 (4.5-18)	10 (3-22)

GDF-15 high and low groups were defined as below or above median GDF-15 mRNA expression.

groups of below and above median GDF-15 mRNA expression. For each comparison, the *P* value (1-tailed Student *t*-test) and false discovery rate (FDR) obtained by the Benjamini–Yekutieli method were calculated. Finally, genes were ranked on the basis of fold change (FC) and FDR setting ($FC \geq 2$ and $FDR < 0.1$). Bioinformatic analyses on gene-expression profiling were conducted by R Software v3.3.2. Clinical data of this tissue cohort are shown in Table 3. For GSEA pathway analysis, the WebGestalt analysis platform and the Reactome gene set were used (May, 16, 2024).

Results

Serum GDF-15 in ACC vs benign adrenal tumours

Tumoural GDF-15 secretion is a hallmark of tumour aggressiveness in many tumours. Physiologically, serum GDF-15 levels are below 1.035 ng/mL.[29] We found a trend for circulating GDF-15 to be lower in 42 patients with benign tumours (ACA) compared to 95 mitotane-naïve patients with ACC, yet within the physiological range of GDF-15 (0.65; range: 0.13-5.62 vs 0.81; range: 0.01-35.05 ng/mL, $P = .1$, Figure 1A). Patients with localised ENSAT stage I-III ACC had significantly lower GDF-15 serum concentrations compared to advanced ENSAT stage IV (0.6; range: 0.01-30.61; 1.65; range: 0.38-35.05; $P < .0001$; Figure 1B). For a subgroup of ACC patients ($n = 27$), the sum of tumoural diameters as a measure of tumour burden was available at the time point of blood sampling. Circulating GDF-15 concentrations correlated significantly with larger tumour mass ($r = 0.44$, Figure 1C). Age in ACA and ACC did not differ (ACA: 54.5 years vs ACC: 53.5 years, $P = .52$, Figure S1A) and serum GDF-15 in mitotane-naïve patients with ACC did not differ significantly between age groups either (cut-off: median 53 years; 0.73; range: 0.19-30.61 vs 0.90; range: 0.01-35.05 ng/L; $P = .34$; Figure S1B).

Steroid hormone synthesis involves discrete mitochondrial steps and GDF-15 secretion has been reported to be induced by mitochondrial stress. In mitotane-naïve ACC patients with hormonal data available at the time of diagnosis ($n = 64$), we found patients with hormonally active ACC to have significantly higher mean GDF-15 serum levels compared to patients with inactive ACC (3.1 ± 6.8 vs 0.9 ± 0.6 ng/mL; $P < .05$, Figure S1C). In ACA, we did not find significant higher mean GDF-15 serum levels in cortisol-producing adenomas compared to endocrine inactive adenomas with 1.2 ± 1.3 vs 0.7 ± 0.3 ng/mL; ($P = .31$, Figure S1D).

Mitotane induces GDF-15 secretion in vitro and in vivo

Next, we demonstrated a dose-dependent induction of GDF-15 expression in 4 ACC cell lines by mitotane treatment but not by other cytotoxic drugs (Supplementary Results and Figure S2).

Then we measured GDF-15 in ACC patients treated with mitotane (see Table S1 for clinical characteristics). We excluded patients with adjuvant mitotane and no tumour mass present at the time point of blood collection. Circulating GDF-15 levels during mitotane therapy ($n = 33$) were significantly higher compared to samples collected before initiation of mitotane treatment ($n = 25$, 2.0; range: 0.50-5.78 vs 0.6; range: 0.16-1.8 ng/mL; $P < .0001$, Figure 1D). We additionally analysed 40 paired samples of patients before and during mitotane therapy (Figure 1E) and observed an increase in GDF-15 serum levels in almost all samples investigated (prior mitotane treatment 0.66; range: 0.1-13.05 vs during mitotane treatment 2.3; range: 0.50-19.97 ng/mL, $P < .0001$). Mitotane plasma concentrations are known to vary widely, and some patients never reach therapeutically effective blood concentrations. In line with a dose-dependent effect of mitotane on GDF-15, mitotane plasma concentration and serum GDF-15 showed a strong positive correlation ($r = 0.58$, $P < .005$, Figure 1F). In addition, we analysed GDF-15 serum concentrations in ACC patients with adjuvant disease, before and during mitotane treatment. Prior mitotane treatment, GDF-15 levels were comparable to healthy controls in ACC patients (0.60 vs 0.61 ng/mL, respectively), yet increased significantly during mitotane treatment (2.0 ng/mL, $P < .0001$, Figure S3).

High GDF-15 levels are associated with reduced overall survival in ACC patients

GDF-15 is known to be up-regulated in different malignancies and high expression is associated with more unfavourable outcomes.[30,31] We therefore investigated the association of GDF-15 serum concentrations with survival in patients with ACC from the time when the blood sample was taken. To discriminate the actual impact of mitotane induced vs basal GDF-15 secretion, we analysed mitotane-naïve patients and patients treated with mitotane separately. Mitotane-naïve patients with basal serum GDF-15 below the median of 0.81 ng/mL had a significantly longer survival of 63 months compared to patients with high levels of GDF-15 (21 months, $P < .001$; Figure 2A). As we observed higher levels of GDF-15 in higher ENSAT stages, we stratified for ENSAT stage (ENSAT stage I-III vs ENSAT stage IV), in a sensitivity analysis and again observed a significant longer survival in patients with serum GDF-15 below the median before mitotane therapy (stratified

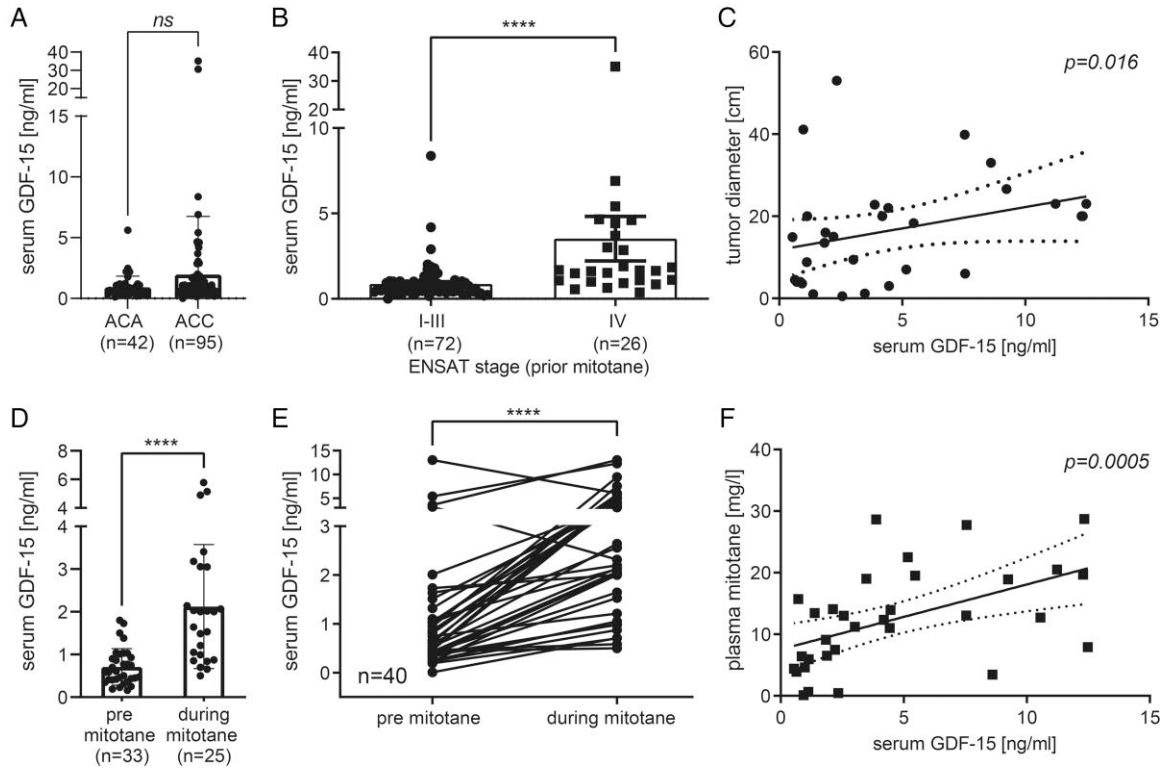

Figure 1. Serum GDF-15 in patients with adrenocortical carcinoma. Serum GDF-15 concentration in ACC compared to ACA (A) and in patients with ENSAT stage I-III compared to stage IV ACC (B). Correlation of serum GDF-15 and tumour burden at imaging ($n = 30$, Spearman r = 0.44 (C). Serum GDF-15 in ACC patients with tumour burden prior and during mitotane treatment (D). Serum GDF-15 in 40 matched ACC patients prior and during mitotane treatment (Wilcoxon matched-pair signed rank test, E). Correlation of serum GDF-15 with mitotane plasma concentration ($n = 32$, Spearman r = 0.58) (F). Mann–Whitney *U* test, ****$P < .0001$, ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma (mean ± SEM).

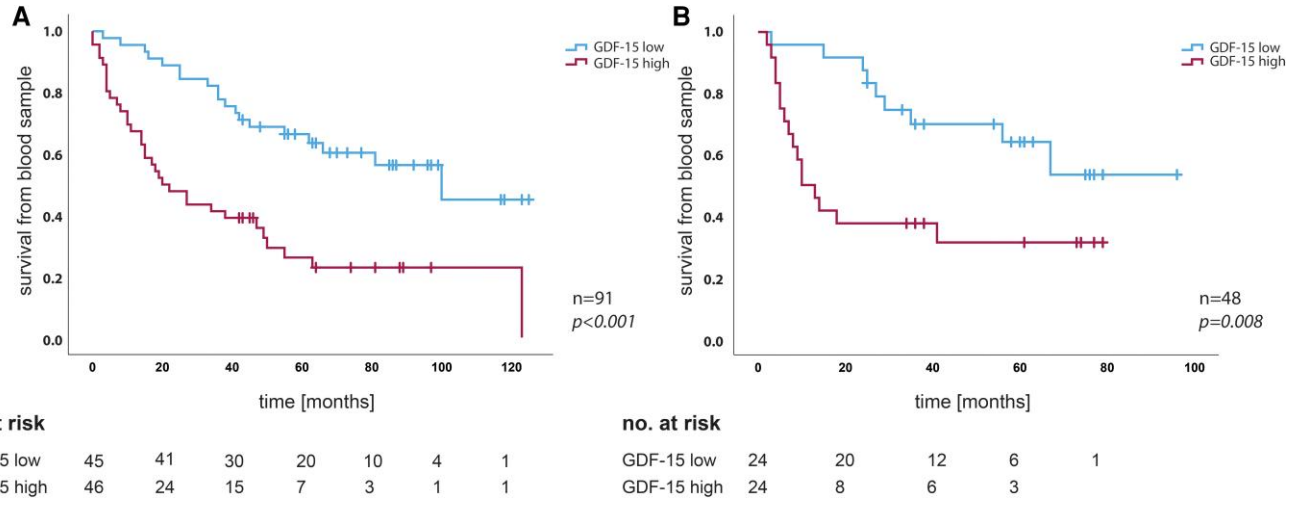

Figure 2. Survival of patients with ACC according to serum GDF-15. Kaplan–Meier survival plots of ACC patients before mitotane treatment (A, log-rank $P < .001$, cut-off median: 0.81 ng/mL) and during mitotane (B, log-rank $P = .008$, cut-off median: 2.17 ng/mL).

$P = .049$) (Figure S4A, B). In a univariate analysis, tumour burden (adjuvant, 0 affected sites; localised disease, 1 affected site; and metastatic disease, >1 affected site) and Ki-67 index were significantly associated with survival prior to mitotane treatment in line with their known prognostic role in ACC (Table 4). After adjustment for both factors in the group prior mitotane GDF-15 did not retain statistically significant with overall survival (HR: 1.47; 95% CI: 0.66-3.29; $P = .34$) (Table 4). Additionally, when only patients with tumour mass present were analysed, we did no longer find significant differences in overall survival in 34 patients before mitotane treatment (log-rank: $P = .288$), Figure S5A.

Patients with ACC who received mitotane and had GDF-15 serum concentrations below the median of 2.17 ng/mL survived for a median time of 55 months. Median survival of patients with high GDF-15 serum concentrations was only

Table 4. Impact of serum GDF-15 and known prognostic parameters on survival before mitotane treatment.

Pre-mitotane						
Survival	**Univariate analysis**			**Multivariate analysis**		
Variables	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Sex						
Female ($n = 58$)						
Male ($n = 41$)	0.64	0.36-1.13	0.13			
Affected tumour sites						
0						
1						
>1	2.39	1.66-3.45	<0.001*	2.29	1.48-3.55	<.001*
Ki67						
<10 ($n = 17$)						
≥10 ($n = 52$)	3.33	1.29-8.59	0.013*	3.56	1.17-10.84	.03*
Age						
<50 years ($n = 35$)						
>50 years ($n = 59$)	1.82	0.99-3.34	0.06			
GDF-15						
Low < 0.81 ng/mL ($n = 45$)						
High > 0.81 ng/mL ($n = 46$)	3.05	1.73-5.40	<0.001*	1.47	0.66-3.29	.34

Only significant parameters at univariate analysis were adjusted at multivariable analysis.
*P values are stated and significant P values additionally emphasised.

13 months ($P = .008$; Figure 2B). When stratified for ENSAT stage, we observed a significant longer survival in patients with serum GDF-15 below the median during mitotane therapy (stratified $P = .047$) (Figure S4C, D).

Due to non-satisfied hazard assumption, we did not perform hazard regression and multivariate analysis in patients during mitotane treatment. Yet, also here, log-rank test revealed a significant longer survival in patients with serum GDF-15 below the median ($P = .008$). When only patients with tumour mass present were analysed, we did no longer find significant differences in overall survival in 23 patients during mitotane treatment (log-rank: $P = .08$), Figure S5B.

Serum GDF-15 and response to immunotherapy

GDF-15 is an endogenous immunosuppressant[18] and leads to impaired T cell extravasation by inhibiting the lymphocyte function-associated antigen 1/intercellular adhesion molecule 1 (LFA-1/ICAM-1) axis in T cells.[23] It is well established that ICI efficacy depends on T cells infiltrating the tumour which prompted us to assess GDF-15 in ACC patients prior to treatment with ICI.

In the 46 ACC patients (Table 2) with serum samples available, GDF-15 serum concentrations ranged from 0.61 to 76.8 ng/mL and were significantly higher than in age- and sex-matched healthy controls (0.4 to 2.9 ng/mL). Median serum GDF-15 was 2.5; range: 0.64-12.99 ng/mL in responders vs 4.7; range: 0.61-76.80 ng/mL in non-responders ($P = .037$; Figure 3A). Responders to immunotherapy had a significantly longer progression-free survival, with all responders being still alive at manuscript submission ($P < .001$; Figure 3B). Furthermore, patients with GDF-15 serum levels below the median of 4.2 ng/mL at the start of ICI showed a significant longer progression-free survival compared to patients with high GDF-15 serum levels ($P = .036$) (Figure 3C). We calculated the AUC to discriminate between responders and non-responders and found an AUC of 0.69; $P = .038$ (Figure S6A). For mitotane plasma concentration, we found no significant value to discriminate responders and non-responders to ICI (AUC: 0.58, $P = .40$, Figure S6B). In this cohort, serum GDF-15 and tumour burden at imaging were not significantly intercorrelated (Figure S7A) but again mitotane plasma concentration significantly correlated with serum GDF-15 (Figure S7B).

In line with an immunosuppressive action of GDF-15 in the tumour immune micro-environment, we found a trend for lower expression of RNA encoding immune-related genes, such as CD3E, HLA-DRB1 and CXCL9 in 58 ACC tissue samples from treatment-naïve patients with high vs low expression of GDF-15 and GSEA pathway analysis revealed a significant downregulation of immune-related pathways in GDF-15 high samples (Figure 3D; Table S2).

Discussion

GDF-15 is an endogenous immunosuppressive cytokine upregulated in different tumour entities. It affects intratumoural immune cell infiltration. GDF-15 therefore reduces response to immune checkpoint blockade.[23] Most ACC are immunologically cold with minimal infiltration by lymphocytes and NK cells[15,16] and response to ICI is infrequent.[8-13]

Our study demonstrates that GDF-15 is elevated in serum of patients with ACC. We show that GDF-15 serum concentrations are significantly lower in patients who benefit from programmed cell death 1 (PD-1)-directed immunotherapy. We therefore suggest GDF-15 as a parameter worthwhile to investigate further for its value to inform selection of patients for anti-PD-1 immunotherapy. GDF-15 is readily measurable by a commercially available assay.[29]

Serum GDF-15 generally increases with tumour mass and is associated with poor prognosis in several cancer types.[18] In ACC, there are hardly any tumour markers for use in clinical routine. Current guidelines recommend tumour stage and tissue Ki-67 labelling index to inform treatment decisions.[4,32,33] Molecular tissue markers of prognosis have been developed but have not changed clinical practice yet.[34,35]

We here found no significant difference in serum GDF-15 between ACC without prior to treatment with mitotane vs ACA. We did not observe higher serum GDF-15 in steroid

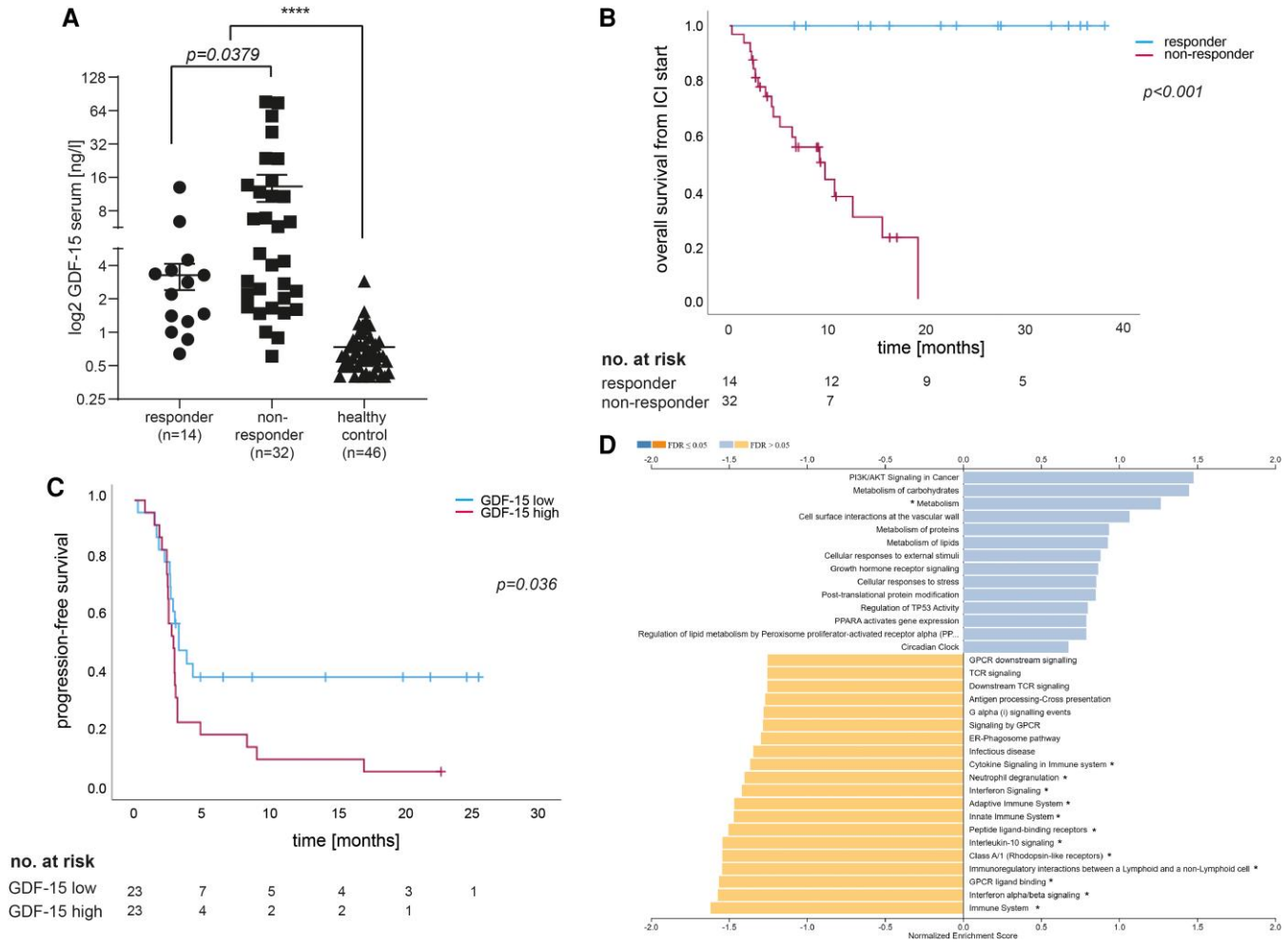

Figure 3. GDF-15 action in patients with ACC receiving immunotherapy. Serum GDF-15 in 46 ACC patients at the start of ICI treatment. Responders defined as CR, PR, SD. (CR, complete remission; PR, partial response; SD, stable disease) and 46 age- and sex-matched healthy controls (Mann–Whitney *U* test) (A). Kaplan–Meier survival plots of ACC patients with ICI (B), log-rank $P < .001$. Kaplan–Meier survival plots of ACC patients with ICI (cut-off median: 4.2 ng/mL (C), log-rank $P = .036$). Gene set enrichment pathway analysis of targeted mRNA expression data in ACC with *GDF-15* mRNA expression above the median. mRNA expression was analysed by Nanostring Ncounter panel in therapy-naïve patients with ACC. $*P < .05$ (D).

hormone-producing vs endocrine inactive ACA which might be due to the even smaller sample size of ACA.

In treatment-naïve patients with adrenal tumours, serum GDF-15 might appear to depend on tumour mass, which is usually higher in stage IV vs localised tumour stages. This is possibly the reason why GDF-15 does not retain statistical significance after adjustment for tumour burden. It is conceivable that the involvement of mitochondrial steroidogenic enzymes that are active even in cases without clinical hormone excess[36] leads to mitochondrial stress resulting in tumoural induction of GDF-15 in ACC. This is likely the reason why GDF-15 serum levels also increase in patients with no measurable tumour mass. Systemically, glucocorticoids suppress GDF-15 secretion as shown in patients with adrenal insufficiency,[37] which argue in favour of a tumoural origin of circulating GDF-15 in ACC patients.

Mitotane is the treatment standard for advanced ACC alone or in combination with chemotherapy. It is useful as an inhibitor of steroidogenesis and strongly perturbs mitochondrial integrity. At the molecular level, the GDF-15 promoter has been shown to comprise binding sequences for several transcription factors including C/EBP homologous protein (CHOP)[38] that in turn is strongly induced by mitotane.[27] Across all ACC cell lines tested, a striking induction of GDF-15 by mitotane but not by other cytotoxic chemotherapy drugs confirmed our previous observation (Figure S2B).

Mitotane-dependent tumoural induction of GDF-15 was reflected in serum of ACC patients treated with mitotane. The significant correlation of serum GDF-15 and plasma mitotane supports a direct effect of mitotane.

Given the known activity of GDF-15 in favouring immune cell exclusion[23] and in inducing regulatory T_{reg} cells,[22] patients with high GDF-15 likely have an immunologically unfavourable tumour micro-environment which may contribute to their poor outcome. For ACC it is known that metastases exhibit lower T lymphocyte infiltration than the corresponding primary tumours.[15] Standard mitotane therapy preceding resection of metastases may contribute to this immunologically cold tumour phenotype in ACC metastases and thus to the overall low response rate to PD-1 inhibition. In our ACC cohort of 46 patients who received ICI, responders had significantly lower GDF-15 blood levels. This has previously been shown in non-small cell lung cancer patients, where patients with low serum GDF-15 had significantly higher objective response rates to anti-PD-1 and its ligand (PD-L1) therapy than patients with high serum GDF-15.[17] Adverse immunological

effects of GDF-15 induction in ACC are further supported by lower expression of immune cell pathways in primary ACC tissues tumours with higher GDF-15 expression. For pathway analysis, the Nanostring nCounter panel of pre-selected genes and the limited number of available samples did not allow for a sufficiently powered analysis to statistically exclude possible false positives. Still, of 13 pathways with uncorrected *P* values of <.05, 10 are related to immune regulation or tumour-immune interaction, and 1 to metabolic effects known to be associated with GDF-15. As such a result could hardly be obtained through accidental false positive discoveries, these data further support an immunomodulatory role of GDF-15 in ACC.

Our study has some limitations. First, all samples were retrospectively analysed from prospectively collected biological specimens. Prospective studies are required for confirmation but will require long time periods to be completed in this orphan malignancy. Second, tissue samples were not available to directly study the impact of prior mitotane treatment on the tissue immune cell infiltration. In tissues post-mitotane treatment, we would have expected higher GDF-15 expression and likely greater differences in the expression of immune-related genes. Finally, only a small number of serum samples and data from patients treated outside of clinical trials with PD-1 inhibition were available and follow-up for some of these patients was short.

A strength of the study is the application of a validated and routinely available serum GDF-15 assay, and the high number of patients with sufficient follow-up available for the assessment of overall survival.

Beyond the validation of GDF-15 as biomarker in ACC, our study has important implications for the treatment of ACC. Although clinical trials have not found significant negative effects of mitotane on response rates to ICI, numbers of included patients were low.[8-11,13] Our finding of mitotane-induced GDF-15 in ACC raises the possibility that continuation of mitotane beyond progression may affect later therapy with ICI. While we found high serum GDF-15 to be associated with unresponsiveness to ICI, it is noteworthy that plasma mitotane concentration is not significantly associated with treatment outcome of ICI (Figure S6B). To disentangle the relevance of mitotane and serum GDF-15, a clinical trial is needed. However, studies in hepatocellular carcinoma bearing mice have demonstrated that anti-GDF-15 treatment could reduce the number of tumoural Treg cells in combination with PD-1 inhibition.[22] Similarly, anti-GDF-15 and anti-PD-1 therapy reduced the tumour burden and expression of FoxP3, a marker of Tregs, in a murine pancreatic cancer model while expression of granzyme B from activated cytotoxic T cells increased, indicating a more inflamed tumour micro-environment.[23] A study supporting the idea of clinically targeting GDF-15 to overcome resistance to ICI was very recently published. The authors combined the GDF-15 neutralising antibody visugromab with the anti-PD-1 antibody nivolumab and could demonstrate response to immune checkpoint inhibition in several solid tumour entities previously refractory to ICI.[39]

Targeting GDF-15 in mitotane-treated ACC may therefore enhance the efficacy of ICI treatment in ACC, similar to effects recently demonstrated in other malignant tumours.[39]

Conclusions

In conclusion, GDF-15 is a tumour-secreted anti-inflammatory cytokine released from ACC after mitotane treatment and is associated with poor prognosis. Serum GDF-15 may be used as a marker to select patients likely to benefit from immunotherapy. Most importantly, GDF-15 is a druggable target in combination, with immunotherapy of mitotane-treated ACC.

Supplementary material

Supplementary material is available at *European Journal of Endocrinology* online.

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Authors' contributions

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Conflict of interest: J.W. is a collaborator of CatalYm, a company developing a therapeutic GDF-15 antibody and

co-inventor of patents held by the University of Würzburg on anti-GDF-15 based therapies. J.W. and M.K. received travel support from CatalYm. K.C.W. is a collaborator of Roche Diagnostics and holds the following planned patents: EP 2 047 275 B1; US 8,951,742 B2, US 11,199,552 B2.

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