CLINICAL STUDY

Serum inhibin pro- α C is a tumor marker for adrenocortical carcinomas

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Abstract

Objective: The insufficient diagnostic accuracy for differentiation between benign and malignant adrenocortical disease and lack of sensitive markers reflecting tumor load emphasize the need for novel biomarkers for diagnosis and follow-up of adrenocortical carcinoma (ACC).

Design: Since the inhibin α -subunit is expressed within the adrenal cortex, the role of serum inhibin pro- α C as a tumor marker for ACC was studied in patients.

Methods: Regulation of adrenal pro- α C secretion was investigated by adrenocortical function tests. Serum inhibin pro- α C levels were measured in controls ($n=181$) and patients with adrenocortical hyperplasia ($n=45$), adrenocortical adenoma (ADA, $n=32$), ACC ($n=32$), or non-cortical tumors ($n=12$). Steroid hormone, ACTH, and inhibin A and B levels were also estimated in patient subsets. **Results:** Serum inhibin pro- α C levels increased by 16% after stimulation with ACTH ($P=0.043$). ACC patients had higher serum inhibin pro- α C levels than controls (medians 733 vs 307 ng/l, $P<0.0001$) and patients with adrenocortical hyperplasia, ADA, or non-adrenocortical adrenal tumors (148, 208, and 131 ng/l, respectively, $P=0.0003$). Inhibin pro- α C measurement in ACC patients had a sensitivity of 59% and specificity of 84% for differentiation from ADA patients. Receiver operating characteristic analysis displayed areas under the curve of 0.87 for ACC vs controls and 0.81 for ACC vs ADA ($P<0.0001$). Surgery or mitotane therapy was followed by a decrease of inhibin pro- α C levels in 10/10 ACC patients tested during follow-up ($P=0.0065$).

Conclusions: Inhibin pro- α C is produced by the adrenal gland. Differentiation between ADA and ACC by serum inhibin pro- α C is limited, but its levels may constitute a novel tumor marker for ACC.

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Introduction

Tumors of the adrenal gland are frequently detected on abdominal imaging studies (1, 2). Although the majority of adrenal neoplasms constitute non-functional adenomas, a subset of patients present with syndromes of hormonal excess or with malignancy (3, 4, 5). Adrenocortical carcinomas (ACCs) are rare tumors accompanied by a poor prognosis, especially in the presence of metastases (6, 7, 8). Determinants such as tumor size, imaging phenotype on computed tomography or magnetic resonance imaging, and serum adrenal steroid levels, particularly those of DHEA-S, have been applied to differentiate ACC from other adrenal neoplasms. These determinants, however, have limited sensitivity (9, 10). Thus, there is a clear need for additional diagnostic tools for differentiation between ACC and its benign counterparts. Also, given the limited value of steroid hormones as tumor markers in patients with established ACC, the availability of a

reliable serum marker could improve the diagnostic follow-up after surgery or medical therapy with mitotane or other chemotherapeutic agents.

Inhibins are dimeric peptide hormones belonging to the transforming growth factor- β superfamily of growth and differentiation factors (11). The inhibin α -subunit (encoded by *INHA*) has been implicated in adrenocortical tumorigenesis since gonadectomized *Inha* knockout mice develop ACCs (12). Several forms of the inhibin α -subunit are known to be produced by the human gonads. The inhibin α -subunit precursor contains three regions: a pro-region, an N-terminal region, and the mature C-terminal region called α C. In the presence of the inhibin β A- or β B-subunit, the α C-region can be linked to the β -subunit to form inhibin A or B respectively (13). During assembly, the α C-region can also bind to the pro-region, forming the 'free' inhibin α -subunit molecules, pro- α C and pro- α N α C (13, 14). These peptides are the most abundant serum inhibin forms and are thought to arise predominantly

in the absence of inhibin β -subunits (14, 15). Like the mature inhibins A and B, inhibin pro- α C has been linked to various forms of ovarian cancer (16, 17, 18).

The only detectable serum inhibin form in postmenopausal women is inhibin pro- α C, suggesting the existence of an extragonadal source of this free α -subunit form (19). Since physiological expression of *INHA* is confined to the ovary, testis, placenta, and adrenal cortex (20, 21), we hypothesized that serum inhibin pro- α C can be derived from the adrenal cortex. Therefore, we studied whether *in vivo* stimulation or inhibition of ACTH, the physiological regulator of adrenocortical *INHA* expression (22), alters serum inhibin pro- α C levels. Furthermore, given the role of the various inhibin forms as tumor markers in ovarian cancer and reports of *INHA* overexpression in human adrenocortical tumors (23, 24, 25), we investigated the possibility to use serum inhibin pro- α C as a tumor marker for adrenocortical neoplasms.

Materials and methods

Patient material

To obtain reference values for serum inhibin pro- α C levels, blood was collected from healthy blood bank donors. The total reference group included 111 men and 70 women (age range 20–70 years). For the study of *in vivo* regulation of adrenocortical inhibin pro- α C secretion, serum specimens were collected from patients who were evaluated for hypothalamus–pituitary–adrenal (HPA) axis abnormalities with 250 μ g synthetic ACTH_{1–24} (tetracosactide, before and after 30 min) intravenously, 750 mg metyrapone every 4 h orally (serum taken before and after 24 h), or before and after an oral dose of 1 mg dexamethasone (dexamethasone suppression test (DST)) overnight. Samples were included in the study if HPA axis responsiveness was within normal ranges, i.e. cortisol levels after tetracosactide or DST > 550 nmol/l or < 50 nmol/l, respectively, or 11-deoxycortisol levels > 350 nmol/l after metyrapone stimulation.

Serum samples were collected from patients who presented with an adrenal tumor or hyperplasia between 1999 and 2009 in the three participating centers. Samples were stored at -20°C . Tumors were classified on the basis of histopathologic evaluation. Adrenocortical tumors were designated as carcinomas if the van Slooten index was > 8 (26) or if a metastasized adrenal tumor was detected. In ten ACC patients, serum samples were also collected shortly after adrenal surgery or after starting mitotane therapy for metastasized disease. The study was conducted under the guidelines that had been approved by the medical ethics committee of the Erasmus Medical Center.

Determination of hormone levels

Inhibin pro- α C, A and B levels were measured by the commercially available enzyme-linked immunometric assay (Diagnostic Systems Laboratory, Webster, TX, USA). Serum levels of cortisol, progesterone, androstenedione, DHEA-S, and ACTH were measured using fluorescence-based immunoassays (Immulite 2000, Siemens Healthcare Diagnostics, Deerfield, IL, USA). Testosterone and estradiol levels were measured by coated tube RIA (Siemens Healthcare Diagnostics), DHEA levels by RIA (Diagnostic Systems Laboratory), and 17-hydroxyprogesterone and 11-deoxycortisol levels were estimated using previously described in-house RIAs (27). Local laboratory age- and sex-specific reference values were adopted for steroid levels. Intra- and inter-assay variabilities for the inhibin pro- α C assay were smaller than 8 and 10% respectively. For the DHEA-S assay, variation coefficients were smaller than 9% within assays and 11% between assays. Local assay reference levels are summarized in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article.

Statistical analysis

Normality of reference values was examined by D'Agostino and Pearson omnibus normality test. After log transformation, normality was obtained for all reference groups and 95% confidence intervals were calculated. Effects of adrenocortical function tests and tumor removal were evaluated by paired Student's *t*-tests. Differences between the groups of patients were estimated by Student's *t*-test or one-way ANOVA, followed by *post hoc* Tukey's multiple comparisons test. Pearson's correlation coefficients were calculated for associations between hormone levels; here, multiple testing was accounted for by Bonferroni correction. Analyses were performed by GraphPad Prism (version 5.01, GraphPad Software, La Jolla, CA, USA) and SPSS (version 15.0, SPSS, Inc., Chicago, IL, USA). Statistical significance was assumed at a two-sided *P* value lower than 0.05.

Results

Reference values of serum inhibin pro- α C

Blood donor samples were divided into groups of men ($n=111$), pre-menopausal women ($n=36$), and post-menopausal women ($n=34$). Age of 50 years was used as cutoff between pre- and post-menopause. In the post-menopausal group, three outliers (> 4 s.d. from mean) were excluded from further analysis. Overall, the median pro- α C value was 307 ng/l (range 17–1007 ng/l). Reference values of serum inhibin pro- α C were calculated as the 95% confidence intervals of the remaining samples and were as follows: 196–685 ng/l for men, 36–780 ng/l for pre-menopausal women, and 15–83 ng/l for post-menopausal women.

Figure 1 Effect of *in vivo* ACTH stimulation on serum levels of inhibin pro- α C. Samples were collected before and 30 min after 250 μ g tetracosactide intravenously to measure serum inhibin pro- α C levels in 15 patients with a normal HPA axis. * $P < 0.05$, paired *t*-test.

In vivo tests of adrenocortical function

Since expression of the inhibin α subunit is regulated by ACTH, we evaluated whether *in vivo* manipulation of

serum ACTH levels could affect serum inhibin pro- α C levels. Short-term adrenocortical stimulation through i.v. administration of tetracosactide in 15 subjects increased serum inhibin pro- α C levels by 16% ($P = 0.043$, see Fig. 1). Long-term ACTH stimulation through metyrapone ($n = 15$, +38%, $P = 0.15$) and overnight ACTH suppression with dexamethasone ($n = 8$, -16%, $P = 0.17$) did not significantly alter serum levels of inhibin pro- α C.

Adrenocortical pathology

Serum samples were obtained from patients with adrenocortical hyperplasia ($n = 45$), adrenocortical adenoma (ADA, $n = 32$), ACC ($n = 32$), or non-adrenocortical adrenal neoplasms ($n = 12$). Patient demographics and tumor characteristics have been summarized in Table 1. Patients were also divided based on gender and menopausal status. Female subjects were classified as post-menopausal when last menstruation was more than 1 year ago or serum FSH level was above 30 IU/l. When clinical data on menstrual cycle or FSH levels were missing, age above 50 years was considered to represent post-menopausal status. With respect to analysis of pro- α C levels, values from four pre-pubertal girls (ages at diagnosis: 11 months, 3, 6, and 9 years) were analyzed relative to levels in post-menopausal controls since it has been demonstrated that serum inhibin pro- α C reference values of these subgroups are comparable (28).

Table 1 Patient characteristics.

	Hyperplasia ^a	Adenoma (ADA)	Carcinoma (ACC)	Other ^b
Number of patients (<i>n</i>)				
Total	45	32	32	12
Males	11	13	9	5
Pre-menopausal women	18	12	6	1
Post-menopausal women ^c	16	7	17	6
Clinical syndrome (<i>n</i>)				
Cushing's syndrome	45	10	6	0
Virilization	0	2	4	1 ^d
Both	0	1	9	0
Feminization	0	0	2	0
Conn's syndrome	0	3	1	0
Non-functional	0	16	10	11
Age (years) ^e	45.9 $\pm$ 15.2	50.7 $\pm$ 15.7	52.5 $\pm$ 20.5	55.9 $\pm$ 15.2
Tumor size (cm) ^e	NA	4.5 $\pm$ 2.3*	10.9 $\pm$ 4.7*	NA
ENSAT 2008 classification (6)				
I	NA	NA	4	NA
II	NA	NA	6	NA
III	NA	NA	5	NA
IV	NA	NA	17	NA
Van Slooten index ^e	NA	2.7 $\pm$ 2.7	16.9 $\pm$ 5.8	NA

NA, not available. *Statistically significant difference, $P = 0.008$.

^aGroup composed of patients with Cushing's disease ($n = 25$), ectopic ACTH secretion ($n = 17$) or AIMAH ($n = 3$).

^bGroup composed of patients with adrenal tumors of primary non-cortical origin: pheochromocytoma ($n = 6$), metastasis ($n = 3$), ganglioneuroma, cyst and lymphoma (all $n = 1$).

^cFour prepubertal girls were analyzed as post-menopausal for comparison of age-related inhibin pro- α C levels.

^dPatient with a testosterone-secreting ganglioneuroma.

^eValues expressed as mean $\pm$ s.d.

Results of serum hormone measurements are shown in Table 2. Serum inhibin pro- α C levels were higher in patients with ACC than in controls ($P < 0.0001$) and also higher compared with patients with adrenal hyperplasia, ADA or non-adrenocortical adrenal neoplasms ($P = 0.0003$, Fig. 2A). Inhibin A was either not detectable or within normal ranges in all patients tested. Serum inhibin B levels were not different between patient groups, but were elevated in three ACC patients: 491 ng/l in a male patient (reference: < 400 ng/l), 194 ng/l in a 6-year-old girl, and 55 ng/l in a post-menopausal woman (both references: < 10 ng/l). Patients with ACTH-dependent adrenal hyperplasia had higher morning cortisol (Fig. 2B, $P = 0.0003$) and ACTH ($P = 0.005$) levels compared with patients with ADA and ACC. Serum steroid levels were not significantly elevated in ACC compared with ADA, but morning cortisol, androstenedione, and DHEA-S levels did show a pattern similar to that of inhibin pro- α C (Fig. 2). Compared with their gender- and age-specific reference values, four out of nine men (44%), three out of three children (100%), four out of six pre-menopausal women (67%), and eight out of 14 post-menopausal women (57%) with ACC had increased serum levels of inhibin pro- α C. High levels of serum inhibin pro- α C (i.e. 2.5 and 7.1 times the upper reference limit) were

demonstrated in two out of four patients with ACC European Network for the Study of Adrenal Tumors (ENSAT) stage I. Overall sensitivity of inhibin pro- α C serum levels for ACC was 59%, compared with 45% for DHEA-S levels ($P = 0.26$). Among 29 patients with ACC in whom both inhibin pro- α C and DHEA-S concentrations were measured, 12 had concomitantly elevated levels of both hormones, four had only elevated pro- α C levels, and one had only an increased level of DHEA-S. When comparing ACC with ADA patients, the specificities of the results of the pro- α C and DHEA-S assays were 84 and 92% respectively ($P = 0.36$). Positive predictive values of pro- α C and DHEA-S for the differentiation between ACC and ADA were 79 and 87% respectively ($P = 0.56$). Combining both measurements increased the positive predictive value to 92% ($P = 0.57$). Negative predictive values were 68% for pro- α C, 60% for DHEA-S, and 60% for the combination of both markers ($P = 0.71$). The highest level of inhibin pro- α C in a patient with ACC was 121 times the age- and sex-specific upper reference value, whereas elevations of androstenedione and DHEA-S were maximally 29 and 10 times the age- and sex-specific upper reference levels respectively.

Receiver operating characteristic (ROC) analysis of inhibin pro- α C in ACC patients vs controls showed areas

Table 2 Hormonal evaluation of patients with adrenal hyperplasia or neoplasms. Values are expressed as medians and ranges. Elevated level is positive if levels exceed local age- and sex-specific reference values, described in Supplementary Table 1.

	Hyperplasia	ADA	ACC	Other	P value ^a
Inhibin pro- α C (ng/l)	148 (8–5632) ^b	208 (0–8730) ^b	733 (31–18 957)	131 (18–1005) ^b	0.0003
<i>n</i> with elevated level/total <i>n</i>	7/45 (16%)	5/32 (16%)	19/32 (59%)	3/12 (25%)	
Inhibin A (ng/l)	0 (0–0)	0 (0–2)	0 (0–5)	ND	0.229
<i>n</i> with elevated level/total <i>n</i>	0/3 (0%)	0/13 (0%)	0/8 (0%)		
Inhibin B (ng/l)	96 (62–202)	216 (15–399)	150 (5–419)	142 (52–165)	0.454
<i>n</i> with elevated level/total <i>n</i>	1/5 (20%)	1/13 (5%)	3/9 (33%)	1/3 (33%)	
Morning cortisol (nmol/l)	679 (290–4348)	417 (159–821) ^c	436 (97–2050) ^c	447 (199–723)	0.0003
<i>n</i> with elevated level/total <i>n</i>	15/45 (33%)	2/31 (6%)	7/30 (23%)	0/12 (0%)	
Midnight cortisol (nmol/l)	638 (146–5116)	260 (42–617) ^c	318 (62–1661)	206 (81–800)	0.027
ACTH (nmol/l)	9.70 (0.55–217)	1.85 (0.55–4.40) ^c	0.98 (0.55–16.00) ^c	3.60 (1.30–13.30)	0.0051
Cortisol after DST ^e (nmol/l)	547 (94–4525)	114 (14–579)	339 (14–1760)	41 (29–332)	0.101
<i>n</i> with elevated level/total <i>n</i>	32/32 (100%)	10/11 (91%)	12/15 (80%)	1/3 (33%)	
Cortisolemia (nmol/24 h)	2520 (73–76 258)	1047 (272–1953)	716 (163–4709)	763 (288–1325)	0.029
<i>n</i> with elevated level/total <i>n</i>	38/44 (86%)	8/15 (53%)	10/25 (45%)	3/7 (43%)	
Progesterone (nmol/l)	2.1 (0.3–32.8)	0.7 (0.3–71.3)	2.3 (0.3–12.6)	0.6 (0.3–1.4)	0.359
<i>n</i> with elevated level/total <i>n</i>	4/11 (36%)	1/11 (9%)	7/19 (37%)	0/8 (0%)	
17OH-progesterone (nmol/l)	2.2 (0.5–16.1)	2.2 (0.2–71.3)	3.5 (0.8–31.0)	1.7 (0.8–3.6)	0.447
<i>n</i> with elevated level/total <i>n</i>	2/14 (14%)	2/13 (15%)	5/24 (21%)	0/9 (0%)	
11-Deoxycortisol (nmol/l)	29 (22–490)	23 (14–56)	34 (5–819)	24 (10–37)	0.222
<i>n</i> with elevated level/total <i>n</i>	3/7 (43%)	1/11 (9%)	7/23 (30%)	0/8 (0%)	
Androstenedione (nmol/l)	13.1 (5.4–581.0)	4.5 (0.5–21.3)	8.7 (1.6–287.0)	5.2 (0.6–12.6)	0.070
<i>n</i> with elevated level/total <i>n</i>	7/17 (41%)	4/27 (15%)	13/30 (43%)	1/10 (10%)	
DHEA (nmol/l)	20.8 (5.2–126.2)	7.7 (0.7–39.7)	22.9 (5.3–197.6)	16.0 (5.6–49.0)	0.087
DHEA-sulfate (μ mol/l)	4.7 (0.2–22.9)	1.1 (0.2–46.5)	4.1 (0.2–33.9)	1.7 (0.2–5.3)	0.034
<i>n</i> with elevated level/total <i>n</i>	10/22 (45%)	2/26 (8%)	13/29 (45%)	0/10 (0%)	
Estradiol (pmol/l)	147 (1–772)	101 (5–456)	170 (14–19 787)	88 (5–146)	0.453
Testosterone (nmol/l)	2.7 (0.8–14.1)	1.2 (0.1–10.1)	1.8 (0.3–24.6)	14.1 (0.3–22.1) ^d	0.025

ND, not determined.

^aOne-way ANOVA of all groups. *Post hoc* Tukey's multiple comparisons test revealed statistically significant differences compared with ACC^b, to hyperplasia^c or to ADA^d.

^eDST, 1 mg dexamethasone overnight suppression test.

Figure 2 Serum hormone levels in patients with adrenal pathology. Levels of (A) inhibin pro- α C, (B) morning cortisol, (C) androstenedione, and (D) DHEA-S were studied in patients with adrenocortical hyperplasia (Hyp), adrenocortical adenoma (ADA), adrenocortical carcinoma (ACC), and non-cortical adrenal tumors (other).

* $P<0.05$, ** $P<0.01$, *** $P<0.001$, one-way ANOVA followed by *post hoc* Tukey's multiple comparison test.

under the curves (AUCs) of 0.93, 0.75, and 0.88 for men, pre-menopausal, and post-menopausal women respectively. After Z-score transformation based on gender and menopausal status, inhibin pro- α C levels remained highly significantly elevated in ACC patients compared with all other groups ($P<0.0001$, Fig. 3A). Within male subjects, the pro- α C Z-scores were higher in ACC subjects than in all other subject groups ($P<0.0001$). For the female subjects, the ACC patients had higher Z-scores compared with the control and adrenal hyperplasia groups ($P=0.004$ for pre-menopausal and $P<0.0001$ for post-menopausal), but not relative to the adenomas (Fig. 3B). The combined ROC analysis of the Z-scores showed an AUC of 0.87 ($P<0.0001$, Fig. 3C) for the differentiation between ACC patients and control subjects. ROC analysis of serum inhibin pro- α C levels in ACC patients vs ADA patients yielded AUCs of 0.91, 0.70, and 0.67 for the three groups respectively; overall analysis after Z-score transformation gave an AUC of 0.81 ($P<0.0001$, Fig. 3C).

Treatment of ACC led to a decrease in serum inhibin pro- α C levels in all ten patients tested ($P=0.007$, Fig. 4A). Serum inhibin pro- α C and steroid concentrations restored to normal values in all five patients who underwent radical ACC resection, although two patients did subsequently develop lymph node metastases within 1 year after operation. Three patients underwent tumor-reductive surgery that led to a reduction of pro- α C levels in all. The presence of residual disease in these patients was accompanied by postoperative inhibin pro- α C levels that were still elevated compared with reference values. Serum steroid levels were normal in two out of three patients after incomplete resection. In addition, a decrease of pro- α C levels was also observed in two patients with metastasized ACC, 5 and 7 months after the initiation of mitotane. Mitotane therapy also diminished DHEA-S and other adrenocortical steroid levels in both patients, similar to the decline detected for inhibin pro- α C. This was reflected by radiological regression of multiple hepatic and pulmonary metastases in one patient, but was accompanied by progression of pulmonary and retroperitoneal lesions in the other.

In ACC patients, no significant relation was found between inhibin pro- α C levels and age, tumor size, or van Slooten index. In a combined group of patients with ADA or ACC, we found that pro- α C levels were correlated with serum levels of DHEA-S ($r=0.454$, $P<0.0001$, Fig. 4B), morning fasting cortisol ($r=0.391$, $P=0.002$), and midnight cortisol ($r=0.656$, $P=0.002$). Inhibin pro- α C levels were higher in patients with tumors causing steroid hormone overproduction compared with those with clinically non-functional tumors: 3202 ± 4841 vs 805 ± 1787 ng/l (mean $\pm$ s.d., $P=0.0065$) in patients with and without hypercortisolism and 4591 ± 5450 vs 842 ± 1950 ng/l ($P<0.0001$) in patients with and without hyperandrogenism respectively.

Figure 3 Inhibin pro- α C levels after adjustment for gender and menopausal status. (A) Inhibin pro- α C levels after Z-score transformation in normal subjects (NI) and patients with adrenocortical hyperplasia (Hyp), adrenocortical adenoma (ADA), adrenocortical carcinoma (ACC), and non-cortical adrenal tumors (other). *** $P < 0.001$, compared with ACC. (B) Mean Z-scores of inhibin pro- α C levels, stratified by gender and menopausal status. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$, compared with ACC. (C) ROC analysis of Z-scores of serum inhibin pro- α C levels. Patients with ACC ($n = 32$) were compared with control subjects ($n = 178$) or patients with ADA ($n = 32$).

Discussion

After inhibin α subunit expression was discovered in the human adrenal cortex (22), adrenal glands have been found to secrete inhibin-like immunoreactivity into the

circulation under the influence of ACTH (29, 30). Extracts of adrenal tumors were also found to contain inhibin-like immunoreactivity, which appeared to be increased in Cushing's adenomas (29). Subsequently, it was suggested that serum inhibin assays could also be used in the diagnosis of patients with adrenocortical pathology (31, 32). This is the first study demonstrating that serum levels of inhibin pro- α C are elevated in a subset of patients with ACC and may serve as a tumor marker for ACC.

In current clinical practice, imaging studies and assessment of the steroid hormone profile are important diagnostic tools for the preoperative differentiation between benign and malignant adrenocortical tumors. Due to overlap of tumor characteristics, this may be difficult especially for tumors with a diameter between 4 and 6 cm and for non-steroid secreting tumors (2, 3, 4, 5). In view of the increasing incidence of adrenal incidentalomas on abdominal imaging studies, additional diagnostic markers are needed for differentiation between various pathological entities. Assessment of steroid levels can be helpful as serum tumor markers to monitor the response to treatment in patients with ACC, but not all ACCs are hormonally functional (33, 34, 35, 36, 37). Furthermore, correlation with tumor burden has not been shown for adrenal androgens and the usefulness of steroid levels as adrenal tumor markers is restricted by the relatively low elevation above reference values.

The inhibin α -subunit is expressed in the zona reticularis of the human adrenal cortex, with some extension into the zona fasciculata (25). Adrenocortical inhibin β -subunit expression is low and exhibits a different zone-specific distribution pattern (23, 25), thereby reducing the possibility of formation of mature inhibin A or B (13). Inhibin pro- α C may therefore be expected to be the predominant inhibin form secreted by the adrenal cortex. In men and pre-menopausal women, the gonads are the main source of serum inhibin pro- α C, but the presence of inhibin pro- α C in serum of post-menopausal women suggests that the adrenal cortex also significantly contributes to its production (19). In spite of the gonadal contribution, ACTH can still modulate serum pro- α C levels. A similar response has been described for total inhibin-like immunoreactivity in hypogonadal men (29). On the other hand, we did not observe a change in serum inhibin pro- α C levels after chronic ACTH stimulation, as occurs in patients with pituitary or ectopic ACTH production or after metyrapone administration during 24 h. This could suggest the presence of adaptive mechanisms under long-term ACTH stimulation.

Following the discovery that the adrenal gland can secrete inhibin pro- α C and the role of inhibin forms as tumor markers for ovarian cancer, we now demonstrate that the majority of patients with ACC also have increased serum levels of pro- α C. The tumor suppressor role of the inhibin α -subunit, as detected in murine

models (12), therefore, does not apply to a subset of human ACCs. Serum levels of the inhibin pro- α C peptide were substantially higher in patients with ACC than in patients with benign adrenocortical disorders. The pro- α C form of inhibin thus constitutes a novel and specific serum tumor marker for ACC. In contrast, serum inhibin A and B levels did not differ between patient groups, although three ACC patients did have increased serum levels of inhibin B, as was previously described in two case reports (38, 39).

In contrast to inhibin pro- α C, serum cortisol and androgen levels, including DHEA-S, were not significantly different between ACC and ADA. Sensitivity and specificity of the inhibin pro- α C assay were comparable to those of DHEA-S. Although this study was not designed to detect significant differences in predictive values between these two diagnostic tests, inhibin pro- α C could have a more favorable sensitivity in contrast to a higher specificity of DHEA-S. The combined measurement of inhibin pro- α C and DHEA-S increased the positive predictive value for the detection of ACC to 92%, making concomitant elevation of both serum markers highly suspicious of malignancy. This suggests that the combined measurement of both serum markers could have additional diagnostic value. Inhibin pro- α C was increased in 25% of ACC patients with normal serum DHEA-S levels, making it the only serum tumor marker in these patients. Inhibin pro- α C measurement appears to be most discriminating in pediatric ACC patients, all of whom showed increased pro- α C levels, and in male subjects with adrenal enlargement. The discriminative power of inhibin pro- α C was found to be reduced in women, who form the largest subset of patients with ACC. As a consequence, the result of measurement of inhibin pro- α C has a low overall

sensitivity at 59%. Nonetheless, the magnitude of differences in serum pro- α C levels between the groups, particularly in male and pediatric subjects, underscores the potential diagnostic value of serum inhibin pro- α C as a serum marker for ACC.

Serum inhibin pro- α C levels appear to reflect tumor burden, falling drastically to normal values after radical surgery and also decreasing after tumor-reductive therapy. Although not correlated with tumor size in the entire group of patients, these levels seem suitable as a tumor marker for individual treatment success. The serum pro- α C levels detected are higher than those of adrenal androgens compared with their reference values, possibly leading to a broader range of sensitivity during follow-up.

The limitations of this study include the sample size of the patients with ACC. Using this multicenter approach, we obtained serum samples from 32 ACC patients, which, given the rare tumor incidence (40), comprises a large group. Controls were obtained from blood bank samples, leading to a predominance of male subjects, which is not representative of the gender-specific distribution of ACC (7). However, the currently described reference levels are highly comparable to the previously published reference values of the inhibin pro- α C assay (15), thereby validating this approach. The negative predictive value of inhibin pro- α C for the differentiation between ADA and ACC is moderate at 68%. This finding indicates that a normal serum pro- α C level is not informative in the presence of radiologically suspicious adrenal tumors and should not influence clinical decision making. Given that patients with ENSAT stage I ACC also displayed increased pro- α C levels suggests that the presence of elevated levels in patients with adrenal tumors of clinically uncertain behavior, such as small tumors, could reflect malignancy. This might constitute an additional argument for surgical intervention instead of surveillance.

This study was primarily designed to describe the characteristics of inhibin pro- α C as a serum marker for adrenal carcinoma. Future studies in larger patient groups comparing the predictive values, clinical applicability, and costs of serum inhibin pro- α C and DHEA-S and also diagnostic tools such as urinary steroid profiles by gas chromatography/mass spectrometry (41), are needed to determine the optimal test in patients with an adrenocortical disorder. With regard to follow-up, we only studied inhibin pro- α C levels after tumor surgery or chemotherapy. Whether pro- α C levels are also indicative for tumor recurrence or growth should be assessed in prospective studies, but the effect of tumor reduction on the inhibin α -subunit levels seems promising in this respect.

In conclusion, we describe serum inhibin pro- α C as a novel serum tumor marker for ACC. Inhibin pro- α C is secreted by the adrenal cortex and its levels are increased in the serum of ACC patients. Measurement of inhibin pro- α C, although hampered by a moderate

Figure 4 Association of inhibin pro- α C levels with ACC treatment and DHEA-S levels. (A) Treatment of ACC through radical resection (circles), incomplete resection (squares), or mitotane therapy (checkers) led to a normalization or reduction of serum inhibin pro- α C levels in ten out of ten patients. ** $P < 0.01$, paired t -test. (B) Association ($r = 0.45$, $P < 0.0001$) between serum inhibin pro- α C and DHEA-S levels in patients with ADA (gray circles, $n = 26$, $r = 0.46$, $P = 0.02$) or ACC (black squares, $n = 29$, $r = 0.41$, $P = 0.03$). Dotted line indicates maximum upper reference values: 780 ng/l for inhibin pro- α C and 17 μ M for DHEA-S.

sensitivity, might be a helpful diagnostic tool to discriminate between ACC and benign adrenal neoplasia in patients with normal steroid levels. Serum inhibin pro- α C has a high positive predictive value in combination with serum DHEA-S levels and might serve as a tumor marker for ACC during treatment follow-up.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJJE-11-0693>.

Declaration of interest

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

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Author contribution statement

J Hofland, R A Feelders and F H de Jong led the study design. R A Feelders, M N Kerstens, H R Haak, and W W de Herder provided serum samples and patient data. R van der Wal performed the hormone analysis. J Hofland and F H de Jong did the data analysis and drafted the original report. All authors had the opportunity for revision of the manuscript.

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