Clinical Chemistry 63:12
1836–1846 (2017)

A 13-Steroid Serum Panel Based on LC-MS/MS: Use in Detection of Adrenocortical Carcinoma

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BACKGROUND: Adrenocortical carcinoma (ACC) is a rare malignancy, with an annual incidence of 1 or 2 cases per million. Biochemical diagnosis is challenging because up to two-thirds of the carcinomas are biochemically silent, resulting from de facto enzyme deficiencies in steroid hormone biosynthesis. Urine steroid profiling by GC-MS is an effective diagnostic test for ACC because of its capacity to detect and quantify the increased metabolites of steroid pathway synthetic intermediates. Corresponding serum assays for most steroid pathway intermediates are usually unavailable because of low demand or lack of immunoassay specificity. Serum steroid analysis by LC-MS/MS is increasingly replacing immunoassay, in particular for steroids most subject to cross-reaction.

METHODS: We developed an LC-MS/MS method for the measurement of serum androstenedione, corticosterone, cortisol, cortisone, 11-deoxycorticosterone, 11-deoxycortisol, 21-deoxycortisol, dehydroepiandrosterone sulfate, pregnenolone, 17-hydroxypregnenolone, progesterone, 17-hydroxyprogesterone, and testosterone. Assay value in discriminating ACC from other adrenal lesions (phaeochromocytoma/paraganglioma, cortisol-producing adenoma, and lesions demonstrating no hormonal excess) was then investigated.

RESULTS: In ACC cases, between 4 and 7 steroids were increased (median = 6), and in the non-ACC groups, up to 2 steroids were increased. 11-Deoxycortisol was markedly increased in all cases of ACC. All steroids except testosterone in males and corticosterone and cortisone in both sexes were of use in discriminating ACC from non-ACC adrenal lesions.

CONCLUSIONS: Serum steroid paneling by LC-MS/MS is useful for diagnosing ACC by combining the measurement of steroid hormones and their precursors in a single analysis.

LC-MS/MS is recognized as the method of choice for low molecular weight analytes such as steroids in the clinical laboratory *(1–3)*. Among its advantages over steroid immunoassay is its superior analytical specificity and ability to measure multiple steroids in a single analysis *(4–8)*. To date, steroid paneling by LC-MS/MS has been applied to congenital adrenal hyperplasia screening *(9)*, investigation of polycystic ovary syndrome *(10)*, primary hyperaldosteronism subtyping *(11, 12)*, and subclinical cortisol-producing adenoma *(13)*.

Adrenocortical carcinoma (ACC)[7] is a rare malignancy of the steroid-producing adrenal cortex with an annual incidence of 1 or 2 cases per million *(14)*. Although associated with a number of familial syndromes, including Li–Fraumeni, Beckwith–Wiedemann, and Lynch syndromes and multiple endocrine neoplasia type 1, the majority of cases of ACC present sporadically, most frequently in the fifth or sixth decade of life and more commonly in women *(15, 16)*.

The diagnosis of ACC is challenging. Patients almost always present with tumors of ≥4 cm *(17, 18)*, but this only offers a clinical specificity of 61% *(19)*. Clinical evidence of steroid overproduction (e.g., Cushing's syndrome, androgen excess in females) is evident in <50% of cases *(20)*. Nonetheless, up to two-thirds of cases of ACC show biochemical evidence of hormone excess, with hypercortisolism most common *(15)*. The European Network for the Study of Adrenal Tumors recommends a biochemical workup for suspected ACC that includes serum cortisol (basal and postdexamethasone),

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Received June 26, 2017; accepted August 22, 2017.
Previously published online at DOI: 10.1373/clinchem.2017.277624

[7] Nonstandard abbreviations: ACC, adrenocortical carcinoma; DHEAS, dehydroepiandrosterone sulfate; IS, internal standard; IQC, internal quality control; PPC/PGL, phaeochromocytoma/paraganglioma; LLoQ, lower limit of quantification; ULoQ, upper limit of quantification; IQR, interquartile range; NFAA, nonfunctioning adrenal adenoma.

aldosterone (if hypokalemic or demonstrating arterial hypertension), 17-hydroxyprogesterone, dehydroepiandrosterone sulfate (DHEAS), androstenedione, testosterone, and 17β-estradiol (men and postmenopausal women). An alternative approach is the measurement of steroid metabolites in urine by GC-MS *(20–22)*. Two studies report clinical sensitivities of 90% and 100% and clinical specificities of 90% and 99% for GC-MS results in diagnosing ACC, respectively *(20, 22)*, and that the 11-deoxycortisol metabolite, tetrahydro-11-deoxycortisol, provides the greatest diagnostic yield. However, 11-deoxycortisol is rarely measured in serum and assays for this, and other steroid synthetic pathway intermediates such as 17-hydroxypregnenolone and pregnenolone, metabolites of which provide useful markers of malignancy in urine *(20)*, are not widely available.

Here we present an LC-MS/MS method for the paneling of 13 steroids in serum, which we assessed for its ability to differentiate samples from patients with ACC from those of other adrenal lesions in the setting of a tertiary referral center for adrenal pathology.

Materials and Methods

MATERIALS AND METHODS AND INSTRUMENTATION

The liquid chromatography (LC) instrumentation was an Aria Transcend TLX-II system, and the tandem mass spectrometer (MS/MS) used was a TSQ Vantage (both ThermoFisher Scientific). An Accucore^{TM} reversed-phase C18 column (RP-MS 100 × 2.1 mm i.d., 2.6-μm total particle size) fitted with a 0.5-μm precolumn filter was used (Fisher Scientific). Column temperature (40 °C) was maintained using a HotPocket (ThermoFisher). HPLC-grade methanol, acetonitrile, acetone, and 2-propanol were from Rathburn. Water was deionized in-house (18 mΩ, Elga). Androstenedione, cortisone, 11-deoxycortisol, 21-deoxycortisol, 11-deoxycorticosterone, pregnenolone, 17-hydroxyprogesterone, and 17-hydroxypregenenolone were from Steraloids. DHEAS was from ResearchPlus. Cortisol, corticosterone, progesterone, testosterone, testosterone-16,16,17-D_3, and formic acid were from Sigma-Aldrich. Androstenedione-2,2,4,6,6,16,16-D_7, cortisol-9,11,12,12-D_4, cortisone-1,2-D_2, corticosterone-2,2,4,6,6,17,21,21-D_8, 11-deoxycortisol-21,21-D_2, 21-deoxycortisol-2,2,4,6,6,21,21,21-D_8, DHEAS-16,16-D_2, DOC-2,2,4,6,6,17,21,21-D_8, pregenenolone-17,21,21,21-D_4, progesterone-2,2,4,6,6,17α,21,21,21-D_9, 17-hydroxyprogesterone-2,2,4,6,6,21,21,21-D_8, and 17-hydroxypregnenolone-21,21,21-D_3 were from CDN isotopes. Double charcoal-stripped human serum was from BBI solutions. Vacuette 4-mL Z Serum Separator Clot Activator and 3-mL K2 EDTA tubes were from Greiner Bio-One. External quality assurance (EQA) samples were from the United Kingdom External Quality Assurance Scheme (UKNEQAS).

CALIBRATOR, INTERNAL QUALITY CONTROL, AND INTERNAL STANDARD SOLUTIONS

Individual 1000-mg/L stock solutions for all analytes and internal standards (IS) were prepared in methanol and combined to prepare working solutions containing all analytes or IS for calibration, internal quality control (IQC), and IS purposes. To make the working solutions, appropriate volumes of stock solution were added to a 100 × 15 mm, 10-mL glass tube and then evaporated under nitrogen at 60 °C, followed by reconstitution in 1 mL of ethanol. Calibrator and IQC working solutions contained the following steroid concentrations: DHEAS, 2000 μg/mL; cortisol, 200 μg/mL; 17-hydroxypregnenolone, 160 μg/mL; 17-hydroxyprogesterone, 120 μg/mL; androstenedione, 80 μg/mL; pregnenolone, corticosterone, 11-deoxycortisol, 21-deoxycortisol, and cortisone, each 40 μg/mL; testosterone, 8 μg/mL; and 11-deoxycorticosterone, 4 μg/mL. These solutions were further diluted in ethanol as follows: 3 + 20 (v/v), 1 + 39 (v/v), and 1:199 (v/v). All 4 working solutions were used to make calibrator/IQC solutions by dilution in double charcoal-stripped serum (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol63/issue12). After thorough mixing and equilibration (24 h, 2–8 °C), calibrators and IQC were portioned (250 μL) into 1.5-mL polypropylene tubes (Eppendorf) and stored at −20 °C. The deuterated IS stock solution contained the following steroid concentrations: DHEAS-D_2, 25 000 μg/mL; cortisol-D_4, 2500 μg/mL; 17-hydroxypregnenolone-D_3, 2000 μg/mL; 17-hydroxyprogesterone-D_8, 1500 μg/mL; androstenedione-D_7, 1000 μg/mL; pregenenolone-D_4, corticosterone-D_8, 11-deoxycortisol-D_2, 21-deoxycortisol-D_8, and cortisone-D_2, each at 500 μg/mL; testosterone-D_3, 100 μg/mL; and DOC-D_8, 50 μg/mL. An IS working solution was prepared before each batch by dilution of 100 μL of the IS stock solution in acetonitrile to 20 mL.

CLINICAL SAMPLES

Samples (minimum volume 2 mL into an EDTA tube and 3 mL into a serum separator tube plus either random or 24-h urine samples) were collected by endocrine nurses from patients attending scheduled appointments in the Programed Investigation Unit at King's College Hospital NHS Foundation Trust. At this appointment, informed consent was obtained from each patient to allow biochemical testing (including steroid measurements) for clinical evaluation of their adrenal lesions in accordance with a Trust standard operating procedure for suspected adrenal cancer. All samples were collected between 9:00 and 11:30 AM. Samples were subsequently taken immediately to the laboratory for processing. EDTA and serum separator tubes were centrifuged at

2163*g* for 10 min, and serum and plasma were aliquoted and either analyzed immediately or stored frozen at −20 °C before analysis. Urine aliquots were acidified to a pH <2 for urine metanephrine analysis. Patients were categorized in the adrenal multidisciplinary meeting using combinations of biochemistry, radiology, and histology (if available). There were 10 ACC cases (all histologically proven) and 15 with phaeochromocytoma/paraganglioma (PCC/PGL, all histologically proven); 7 had adenoma with glucocorticoid excess; and 16 adrenal lesions demonstrated no biochemical evidence of adrenal cortical or medullary excess (NFAA group). Surgical cases were defined according to standard pathological criteria *(23–26)*. In nonsurgical cases, conventional imaging criteria were applied for stratification of benign or malignant neoplasms *(27)*.

ROUTINE BIOCHEMICAL ASSESSMENTS

Routine steroid immunoassays were serum cortisol, testosterone, progesterone, and 17β-estradiol (Siemens Centaur XPi); serum androstenedione and DHEAS (Siemens Immulite); and EDTA plasma aldosterone and renin mass (Diasorin Liaison). Serum 17-hydroxyprogesterone was measured at St. Thomas' Hospital, London by LC-MS/MS. We measured plasma metanephrines in EDTA plasma by LC-MS/MS *(28)* and urine metanephrines by HPLC with electrochemical detection (Chromsystems). Urine steroid profiling used GC-MS *(29)*.

SPECIMEN PROCESSING FOR LC/MS/MS

Portions of frozen calibrators, IQC, and unknown patient/EQA samples were thawed and mixed; then 250 μL was transferred into a 1.5-mL polypropylene tube. Subsequently, 250 μL of IS working solution and 500 μL of ice-cold acetonitrile were added, and tubes were then vortex-mixed for 30 s. Precipitated protein was pelleted by centrifugation (12 000*g*, 10 min), and the supernatant was transferred to a 10-mL glass tube containing 300 μL of deionized water (dH_2O). Ethyl acetate (1 mL) was added, and the tube was vortex-mixed for 5 min. Following centrifugation (161*g*, 1 min), the top organic layer was removed to a clean 75- × 10-mm, 4.5-mL glass tube. Extracts were evaporated to dryness under nitrogen at 60 °C and reconstituted in 200 μL of a 65 + 35 (v/v) mixture of dH_2O/methanol and transferred to an autosampler vial.

LC-MS/MS PROCEDURE

Extracts were injected (100 μL) onto the LC column at a flow rate of 0.40 mL/min. Mobile phases were (A) dH_2O and (B) methanol, each containing 0.1% (v/v) formic acid. The LC system was controlled using Aria MX (version 1.1, ThermoFisher). The gradient elution is summarized in Table 2 of the online Data Supplement. The total analysis time was 19.7 min, including column reequilibration. Eluent flow was diverted to waste for the first 3 min.

MS/MS was carried out using Xcalibur (version 2.2, ThermoFisher) in the positive mode using atmospheric pressure chemical ionization. Data were collected in high resolution (0.40 *m*/z full width at half maximum) in the multiple reaction monitoring mode, with 2 *m*/*z* transitions per analyte and 1 *m*/*z* per IS (see Table 3 in the online Data Supplement). Postanalysis processing used LC Quan^{TM} (version 2.6, ThermoFisher). For assay calibration, peak area ratios (analyte quantifier to IS) were used to construct calibration graphs, with lines fitted by linear regression. The intercepts were not forced through zero, and line weighting was applied (1/concentration).

METHOD VALIDATION

To validate the developed liquid–liquid extraction (LLE) LC-MS/MS assay, the recovery, linearity, and lower and upper limits of quantification (LLoQ and ULoQ) were determined in accordance with US Food and Drug Administration Center for Drug Evaluation and Research guidance for bioanalytical method validation.

To assess LLE recovery using ethyl acetate, 2 experiments were performed using IQC material. First, absolute recovery was evaluated by directly comparing analyte peak area from protein-precipitated samples that had undergone LLE with samples undergoing protein precipitation only. Second, relative recovery was assessed using IS-corrected peak area ratios in the same samples. To test linearity, charcoal-stripped serum was spiked with steroids at concentrations covering physiological and pathological ranges and tested in triplicate. LLoQ and ULoQ were defined for each analyte as the lowest concentration at which the imprecision (%CV) was <20% (LLoQ) or <15% (ULoQ), with the measured concentration within ±20% of the nominal value. Method precision was assessed using IQC material at 3 target values, either analyzed 6 times in 1 batch (intraassay precision) or in singlicate within 6 batches on different days (interassay precision). Matrix effects were assessed by the postcolumn infusion method *(30)* using an IS working solution infusion via a tee-piece during the analysis of extracted patient samples (n = 5), as well as monitoring IS intraassay precision during sample analysis. Two steroid stability experiments were performed using IQC material. First, freeze–thaw stability was assessed on samples undergoing freeze–thaw cycles on 3 consecutive days. Second, postextraction stability was evaluated in samples left either refrigerated (4 °C) or at room temperature for 7 days before analysis. In each case, analysis was performed against fresh calibrators.

Method comparison with cortisol, testosterone, progesterone, DHEAS, androstenedione, and 17-hydroxyprogesterone UKNEQAS samples was performed (n = 30 for each). Results were compared with the LC-MS/MS users group mean value for all steroids except progesterone. No UKNEQAS-registered labora-

tories perform progesterone analysis by LC-MS/MS, so results were compared with the all-laboratory immunoassay mean. Anonymized excess serum samples obtained in primary care were used to determine steroid reference ranges (n = 200).

STATISTICAL ANALYSIS

Statistical analysis was performed using Analyze-It® (version 4.65.3). Good method agreement was defined by (*a*) Deming regression analysis demonstrating a slope of approximately 1 with 95% CIs bracketing 1 with an intercept of approximately 0 (95% CI bracketing 0) and (*b*) the Altman–Bland plot giving a bias with a confidence limit spanning 0. Clinical data were found not to be normally distributed using the Shapiro–Wilk test. Pairwise comparisons were performed using Mann–Whitney *U*-tests, with post hoc Bonferroni correction. Data are reported as median and interquartile ranges (IQR). Values of $P < 0.05$ were defined as statistically significant.

Results

Chromatographic resolution of 13 steroids was achieved within 14.5 min (Fig. 1). This extended time was necessary to achieve baseline separation of the targeted isobaric steroids 21-deoxycortisol, corticosterone and 11-deoxycortisol, and 11-deoxycorticosterone and 17-hydroxyprogesterone.

Recovery of steroids after protein precipitation and subsequent LLE was assessed in both absolute and relative terms. For all steroids, absolute extraction recovery was >50%, with relative extraction recoveries, evaluated after IS correction, between 90% and 110% (see Table 4 in the online Data Supplement).

The developed method was linear over several orders of magnitude ($r \geq 0.99$) for all steroids (Table 1). We established ULoQs for each steroid that permitted measurement at the high pathological concentrations expected in ACC (Table 1), whereas the LLoQs were sufficiently low to allow quantification of most steroids in healthy individuals. Intraassay and interassay precisions were ≤10% for all steroids (see Table 5 in the online Data Supplement). No analytically significant ion suppression/enhancement was observed, as evidenced by infusion studies (suppression <15% for all steroids studied) and IS peak area precision <20% during analysis of extracted patient samples (Table 1). All steroids were stable through 3 freeze–thaw cycles, whereas extracted samples were stable at room temperature and at 4 °C for 7 days, with concentrations for all steroids within ±10% of the original value measured in each stability experiment. There was a good agreement between the developed method and EQA consensus values (see Fig. 1 in the online Data Supplement).

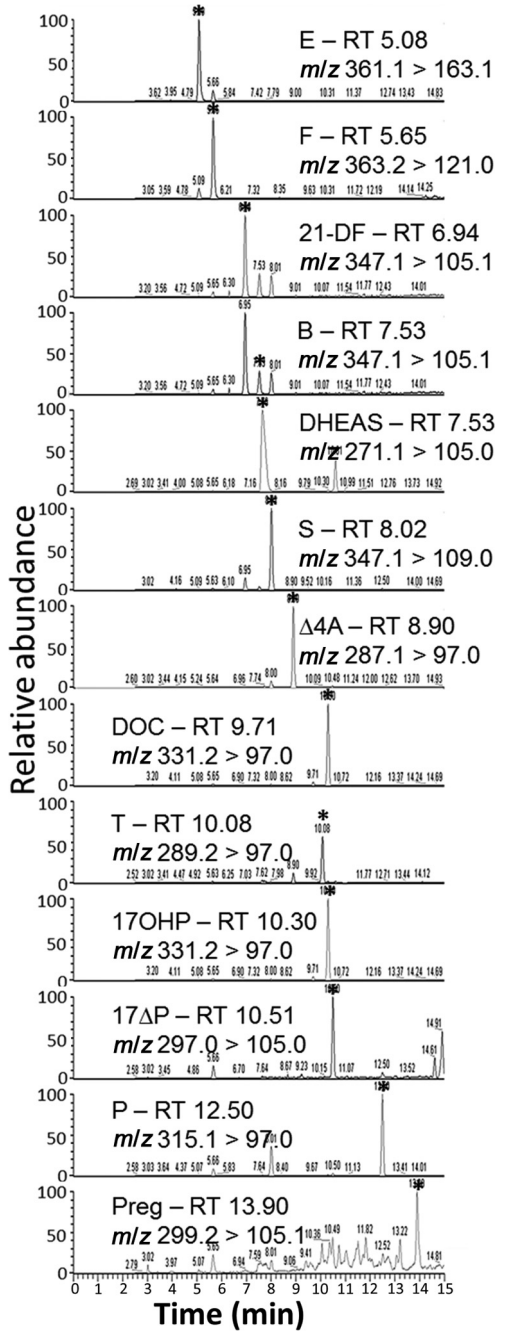

Fig. 1. Chromatographic resolution of steroids.

Single extracted ion chromatograms showing retention time (RT) and mass-to-charge (*m*/*z*) ratio of quantifier ion; analyte peak denoted by asterisk. B, corticosterone; DOC, 11-deoxycorticosterone; P, progesterone; Preg, pregnenolone; E, cortisone; F, cortisol; S, 11-deoxycortisol; 17OHP, 17-hydroxyprogesterone; Δ4A, androstenedione; 21DF, 21-deoxycortisol; T, testosterone; 17ΔP, 17-hydroxypregnenolone.

Table 1. Linearity, LLoQ, ULoQ, and IS precision.

	Calibration range, ng/mL	Recovery, %[a]	Linear regression y = ax + c (R)[b]	LLoQ, ng/mL	ULoQ, ng/mL	IS typical intrabatch CV, %
E	0–200	99 ± 9.8	y = 1.0063x − 0.2482 (0.99)	0.61	200	13.2
F	0–1000	98 ± 9.7	y = 1.0252x − 7.3743 (0.99)	1.25	1000	9.0
21-DF	0–200	102 ± 9.9	y = 1.0144x − 0.3055 (0.99)	0.12	200	16.2
B	0–200	101 ± 9.7	y = 1.0361x − 0.9363 (0.98)	0.25	200	13.1
S	0–200	99 ± 9.1	y = 0.9837x + 0.825 (0.99)	0.12	200	9.6
Δ4A	0–400	98 ± 8.6	y = 1.0053x − 1.3789 (0.99)	0.25	400	17.2
DHEAS	0–5000	101 ± 9.8	y = 0.9877x + 17.918 (0.99)	12.9	5000	7.4
DOC	0–10	99 ± 9.6	y = 1.0048x − 0.0279 (0.99)	0.1	150	13.9
T	0–20	100 ± 9.8	y = 0.9878x + 0.2064 (0.99)	0.05	60	6.7
17OHP	0–300	99 ± 8.6	y = 1.018x − 1.4606 (0.99)	0.38	600	13.3
17ΔP	0–400	101 ± 9.9	y = 1.0191x − 1.766 (0.99)	0.50	400	9.8
P	0–50	98 ± 8.8	y = 1.0077x − 0.2138 (0.99)	0.13	100	12.1
Preg	0–100	101 ± 9.5	y = 0.9961x + 0.0224 (0.99)	1.27	200	10.1

[a] Describes the percentage of measured vs expected concentration expressed as mean ± SD, n = 30, n = 3 for each concentration.
[b] y = ax + c; y, measured concentration; x, analyte peak area/IS peak area ratio.
B, corticosterone; DOC, 11-deoxycorticosterone; P, progesterone; Preg, pregnenolone; E, cortisone; F, cortisol; S, 11-deoxycortisol; 17OHP, 17-hydroxyprogesterone; Δ4A, androstenedione; 21DF, 21-deoxycortisol; T, testosterone; 17ΔP, 17-hydroxypregnenolone.

Demographic and clinical characteristics of the ACC, cortisol-producing adenoma, PPC/PGL, and NFAA groups are summarized in Table 2. Groups were well matched for age and sex, although the PCC/PGL group tended to be younger (Table 2). ACC cases presented with larger tumors. All patients with ACC and PPC/PGL underwent surgery, whereas only 57% of those with cortisol-producing adenoma and 12.5% of the NFAA group underwent more surgery.

For the ACC cases, 5 of 10 presented with clinical features of steroid hormone excess. Three females had signs of Cushing's syndrome, 1 female had androgen excess, and 1 male presented with uncontrolled hypertension. Of the remaining cases, 2 females presented with abdominal symptoms, 1 male with weight loss, and 1 male with hematuria; the initial clinical presentation was not documented for 1 male. Diagnostic workup of the ACC group using existing routine biochemical methods was standard and in line with European Network for the Study of Adrenal Tumors guidance. Urine steroid profiling was performed in 9 of 10 cases, and all profiles were consistent with ACC. Random cortisol concentration was increased in 6 of 10 cases, whereas cortisol failed to suppress to <1.8 μg/dL in 3 patients undergoing overnight dexamethasone suppression testing. Serum androgens were increased in 7 patients (increased testosterone in 2 females, androstenedione in 4 of 9 cases, and DHEAS in 5 of 9 cases). 17-Hydroxyprogesterone was increased in 6 of 9 cases tested. Progesterone was detected in 2 males (usually <1.6 ng/mL). The aldosterone/renin ratio was normal in all cases tested.

The non-ACC adrenal group was divided according to biochemical and radiological criteria: Overt biochemical glucocorticoid excess was defined by the failure of

Table 2. Demographics and clinical characteristics of the adrenal tumor patients.

	ACC group	Cortisol-producing adenoma	PPC/PGL	NFAA
Number	10	7	15	16
Sex, male/female	4/6	4/3	8/7	6/10
Age (range), years	59 (47–69)	68 (66–70)	50 (44–66)	62 (48–72)
Maximum diameter of tumor at time of serum collection, median (range), mm	100 (75–160)	40 (38–44)	56 (40–76)	17 (13–46)
Surgical removal of adrenal tumor (%)	10 (100)	4 (57)	15 (100)	2 (12.5)

cortisol to suppress to <5 μg/dL in the overnight dexamethasone suppression testing or a urine free cortisol concentration above the reference range (>71 μg/dL per 24 h). In these cases, radiology demonstrated lipid-rich pathology in 6 of 7 cases, with lipid-poor adenoma in the other case. In 15 patients with radiological features of PPC/PGL, catecholamine excess was confirmed by increased plasma and/or urine metanephrines. Patients were included in the NFAA group if they (*a*) were proven normal on histology or the mass was shown to be stable on imaging after >12 months follow-up and (*b*) had no clinical evidence of hormone excess, a normal aldosterone/renin ratio (or normal blood pressure), a normal overnight dexamethasone suppression test (cortisol <1.8 μg/dL), and normal plasma metanephrine and normetanephrine. In this group, radiology fell into 2 categories: Hounsfield units <10 or defined by a radiologist as a lipid-poor adenoma with no features of malignancy.

Comparison of LC-MS/MS steroid data between the ACC and non-ACC groups revealed striking differences (Table 3). Across the non-ACC adrenal lesion groups, only up to 2 steroid concentrations were increased above the reference ranges given in Table 3 in individual cases, whereas in ACC between 4 and 7 steroids were increased (median = 6 steroids). 11-Deoxycortisol was increased in all ACC cases (median, 6.2 ng/mL; IQR, 2.5–9.0; normal range, <0.9 ng/mL). Other steroids increased in ACC were androstenedione and DHEAS (6 cases), cortisol, pregnenolone, and 17-hydroxypregnenolone (5 cases), corticosterone (4 cases), and 17-hydroxyprogesterone, 11-deoxycorticosterone, and cortisone (3 cases). Testosterone was increased in 2 females and progesterone was detectable in 3 males with ACC (normally <0.13 ng/mL by LC-MS/MS). In 1 instance, 17-hydroxypregnenolone could not be reliably quantified because of the presence of an interfering peak. Steroid heterogeneity in ACC was demonstrated when data for each steroid in each ACC was plotted as the multiple of the median value calculated from the non-ACC adrenal lesion group (see Fig. 2 in the online Data Supplement).

Discrimination of the non-ACC adrenal lesion and ACC groups was possible using several steroids (Table 3). Whereas pregnenolone and 21-deoxycortisol could not be detected in the non-ACC adrenal lesion group, pregnenolone was measurable in 5 ACC cases and 21-deoxycortisol was detectable in 2 ACC cases. All other steroids except cortisone, corticosterone, and male testosterone showed significant increases in ACC when compared with non-ACC adrenal lesion groups. 11-Deoxycortisol and 17-hydroxypregnenolone provided the best discrimination between ACC and the non-ACC adrenal lesions (Table 3, Fig. 2). 17-Hydroxyprogesterone and androstenedione were increased in the ACC group, but the IQRs overlapped the normal reference ranges of these steroids (Table 3, Fig. 2). For DHEAS, 4 cases of ACC showed concentrations in the lower half of the reference range, whereas in the other cases its concentration was dramatically increased. The DHEAS concentration was lower in the cortisol-producing adenoma group than in the PPC/PGL and NFAA groups.

Because the values in ACC were so variable, the multivariate technique of principal component analysis was applied, both to the European Network for the Study of Adrenal Tumors-recommended sex-independent steroids (cortisol, androstenedione, DHEAS, and 17-hydroxyprogesterone) and to all sex-independent serum steroid panel steroids (all minus testosterone and progesterone). Both positive and negative correlations among variables after principal component analysis were observed in biplot graphs (Fig. 3). Using the European Network for the Study of Adrenal Tumors-recommended steroid measurements, all but 2 of the ACC patients were separated from the other adrenal lesions (Fig. 3A). When full panel data were included, complete separation was achieved (Fig. 3B). However, the ACC cases did not cluster, reflecting the heterogeneity of tumor steroid production, which is the hallmark of this disorder *(20)*.

Discussion

In this study we showed that serum steroid paneling by LC-MS/MS is a useful tool to discriminate ACC from other non-ACC adrenal tumor lesions. Previous practice for selection of biochemical investigations has been dictated by the clinical presentation, e.g., signs of cortisol or androgen excess. This only characterizes subpopulations of ACC; <50% of cases of ACC present with clinical symptoms of hormone excess *(20)*. In contrast, serum steroid paneling allows the investigation of adrenal masses more comprehensively by offering measurement of all major steroid biosynthetic intermediates. It is both the number of steroids increased and the marked increases of several synthetic intermediates without biological activity that appear particularly useful in discriminating ACC from other adrenal lesions, validating the paneling approach to adrenal mass investigation. The DHEAS concentration was lower in cortisol-producing adenoma than in other adrenal lesions, in keeping with previous observations *(13, 31, 32)*.

The cortisol precursor 11-deoxycortisol was most discriminating for differentiating ACC from non-ACC adrenal lesions, an observation consistent with previous studies demonstrating the usefulness of measuring its urinary metabolite tetrahydro-11-deoxycortisol by GC-MS *(20, 22)*. In blood, 11-deoxycortisol is known to be increased in benign and malignant adrenal tumors in children, although it was not reported whether 11-deoxycortisol discriminated benign from malignant disease *(33)*. That 11-deoxycortisol is such a useful marker suggests a critical change in 11β-hydroxylase activity in ACC. 11β-

Table 3. Comparison of serum steroid concentrations between ACC and non-ACC adrenal lesions.[a]

Steroid	Normal reference range	Steroid concentration, median (IQR), ng/mL				*P* value		
		ACC (n = 10)	Cortisol-producing adenoma (n = 7)	NFAA	PPC/PGL	ACC vs cortisol-producing adenoma	ACC vs PPC/PGL	ACC vs NFAA
T	M: 2.9–8.6	2.8 (2.1–3.5)	1.9 (0.9–3.6)	3.2 (2.3–4.3)	2.9 (2.3–3.6)	NS	NS	NS
	F: <1.70	0.35 (0.28–0.86)	0.07 (0.06–1.00)	0.15 (0.12–0.32)	0.17 (0.12–0.22)	NS	NS	NS
A4	<2.0	2.2 (1.5–3.5)	0.8 (0.7–0.9)	0.6 (0.5–0.8)	0.6 (0.4–0.9)	0.04	0.005	0.004
DHEAS	148–3852	6333 (861–10 159)	303 (215–513)	1176 (469–2116)	1222 (701–1547)	0.04	NS	NS
P	M: <0.13	3 cases: 0.25, 0.47, 0.31	<0.13 all cases	<0.13 all cases	<0.13 all cases	N/A	N/A	N/A
	F: <9.4	3 cases: 0.22, 0.25, 1.2	<0.13 all cases	<0.13 all cases	<0.13 all cases	N/A	N/A	N/A
17OHP	<1.7	1.17 (0.9–1.8)	0.7 (0.6–0.9)	0.6 (0.5–0.9)	0.7 (0.6–1.0)	0.01	0.003	0.004
Preg	<1.3	3.1 (2.1–6.8)	<1.3 all cases	<1.3 all cases	<1.3 all cases	N/A	N/A	N/A
17ΔP	<7.3	7.5 (3.4–19.3)	0.6 (0.5–0.7)	1.3 (0.8–1.9)	0.8 (0.6–1.6)	0.001	<0.0001	0.004
S	<0.9	6.2 (2.5–9.0)	0.7 (0.2–0.9)	0.3 (0.14–0.3)	0.2 (0.2–0.3)	0.004	<0.001	<0.001
21-DF	<0.12	3 cases: 0.12, 0.16, 0.16	<0.12 in all cases	<0.12 in all cases	<0.12 in all cases	N/A	N/A	N/A
DOC	<0.5	0.4 (0.3–0.6)	0.2 (0.2–0.3)	0.2 (0.1–0.2)	0.2 (0.1–0.2)	NS	0.01	0.002
B	1.2–20.5	7.0 (1.7–25.5)	2.6 (2.3–5.8)	2.4 (0.8–4.8)	2.4 (1.3–4.3)	NS	NS	NS
F	46–207	185 (118–270)	88 (84–148)	43 (35–81)	73 (65–137)	NS	0.05	0.004
E	7.1–27.9	21.0 (18.7–29.0)	15.1 (14.1–18.8)	14.9 (9.3–17.9)	15.0 (13.0–17.5)	NS	NS	NS

[a] Steroid concentrations expressed as median (IQR). $P < 0.05$ was defined as significant. NS, nonsignificant. Progesterone, pregnenolone, and 21-deoxycortisol were excluded from statistical analyses because of insufficient data points.
T, testosterone; A4, androstenedione; P, progesterone; 17OHP, 17-hydroxyprogesterone; Preg, pregnenolone; 17ΔP, 17-hydroxypregnenolone; S, 11-deoxycortisol; 21DF, 21-deoxycortisol; DOC, 11-deoxycorticosterone; B, corticosterone; F, cortisol; E, cortisone.

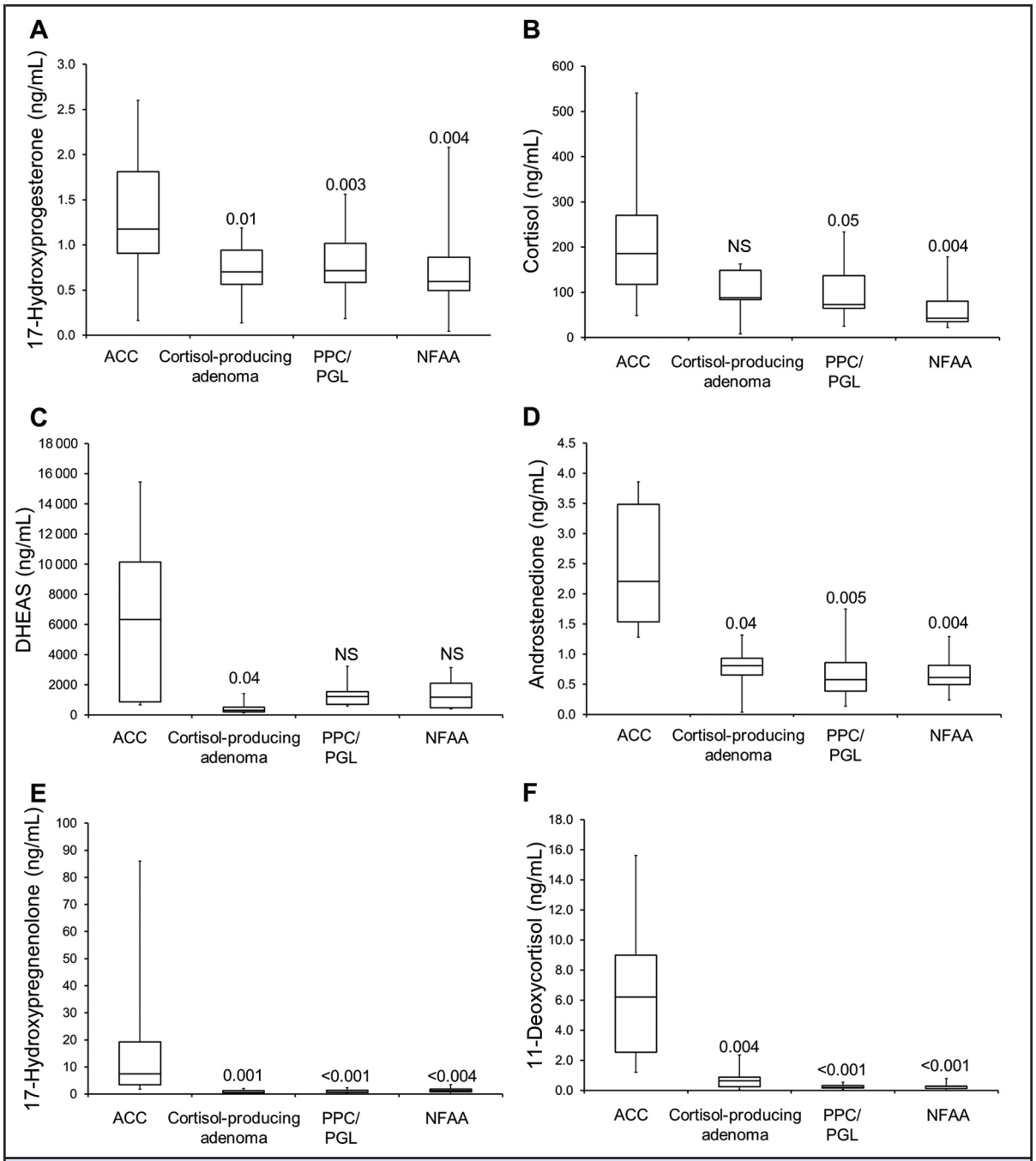

Fig. 2. Comparison of steroid concentrations between ACC and non-ACC adrenal lesions.

17-Hydroxyprogesterone (A); cortisol (B); DHEAS (C); androstenedione (D); 17-hydroxypregnenolone (E); and11-deoxycortisol (F). On each box-and-whisker plot, post hoc Bonferroni corrected *P* values are indicated. NS, nonsignificant.

Hydroxylase catalyzes 11-deoxycortisol conversion to cortisol within the inner mitochondrial membrane, under the control of corticotropin. Most of the other steroid pathway enzymes are located in the smooth endoplasmic reticulum. Disruption of mitochondrial oxidative phosphorylation is common in cancer, termed the "Warburg effect" *(34)*, so 11β-hydroxylase activity may be especially impaired in ACC. Alternatively, increased concentrations of steroid precursors could interfere with corticotropin release *(22)*. *CYP11B1* expression has been

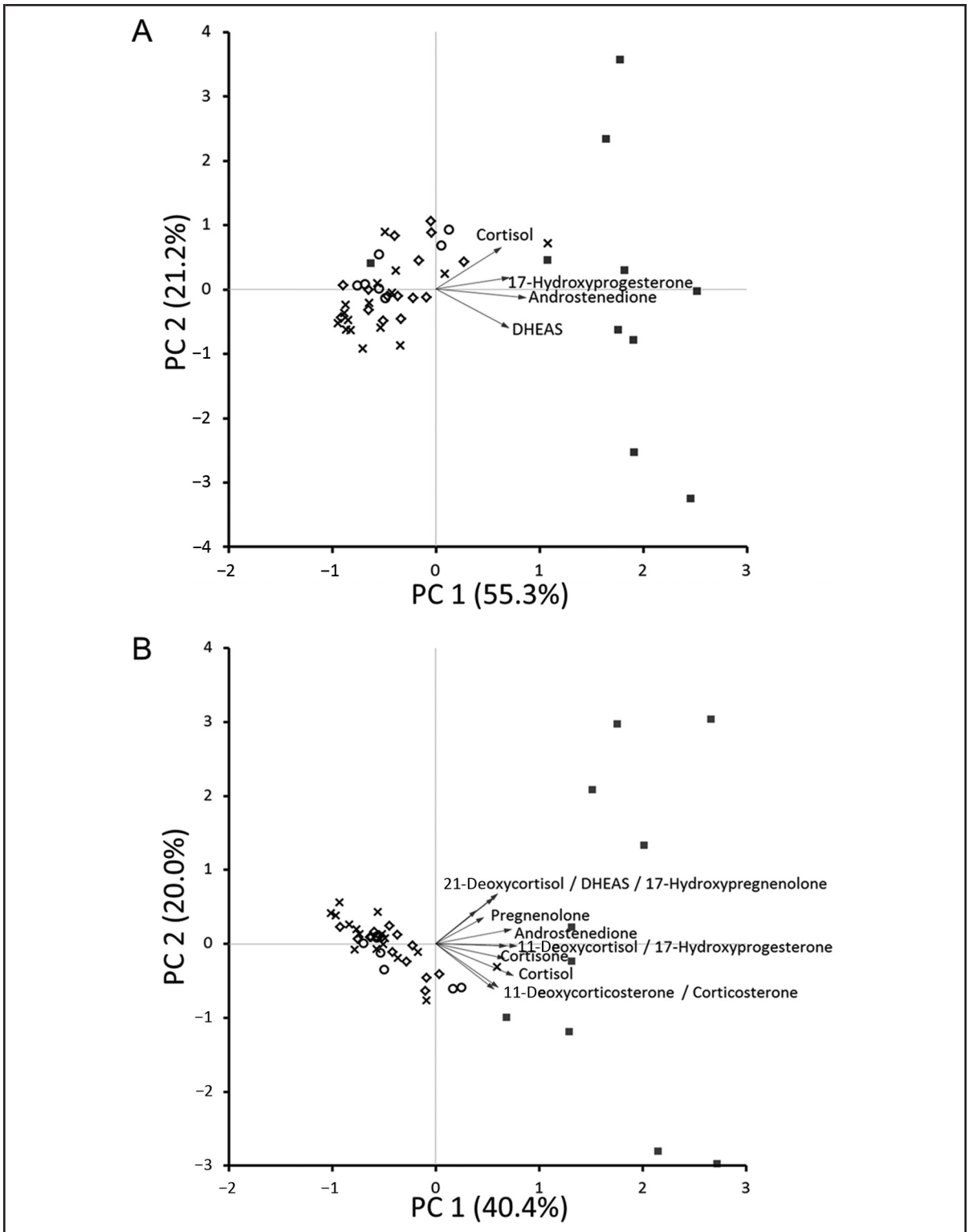

Fig. 3. Biplot analysis of steroids demonstrates full serum steroid panel discriminates ACC from other adrenal lesions.
European Network for the Study of Adrenal Tumors-recommended serum steroids (A) and serum steroid panel (B). Black square, ACC; open circle, cortisol-producing adenoma; black crosses, nonfunctioning adrenal adenoma; open diamonds, PCC/PGL.

shown to be downregulated in ACC, along with several other steroidogenic enzymes *(35)*. It may be that the heterogeneity of steroidogenesis observed in the current study is a reflection of variable loss of steroid synthetic pathway enzyme expression in each tumor. Whether this heterogeneity predicts pathological features or disease prognosis warrants further investigation.

Further studies are needed to evaluate the similarities and differences in qualitative and quantitative data produced by urine steroid profiling and serum steroid paneling. Quantification of serum pregnenolone and 17-hydroxypregnenolone were useful in ACC in the current study; however, the relative concentrations did not reflect the large amounts of their metabolites pregnenediol and pregnenediol often seen in urine. This discrepancy may be because these 3β-hydroxy-5-ene steroids are largely present in serum as sulfates, analogous to DHEAS. Other than DHEAS, the sulfated 3β-hydroxy-5-ene steroids are not measured by the LC-MS/MS method, but their sulfated metabolites are measured by GC-MS, as free compounds after enzymatic hydrolysis *(29)*. Nonetheless, the current study suggests that unconjugated pregnenolone and 17-hydroxypregnenolone are still useful ACC markers.

Urine steroid metabolite measurement may offer greater clinical sensitivity over single blood measurements because 24-h collections reflect steroid production throughout the day *(20)*. Nonetheless, accurate 24-h collections are often not easily obtained and may be inconvenient to patients. Serum steroid paneling by LC-MS/MS offers a viable alternative and may also be more easily interpretable for clinicians because it targets the smaller number of major circulating steroids rather than the large number of urinary steroid metabolites. In many institutions, plasma metanephrine measurement is favored for PCC/PGL exclusion in patients with large adrenal masses in which ACC is in the differential diagnosis. Combined plasma metanephrine and serum steroid panel measurements may be sufficient for the biochemical exclusion of ACC or PCC/PGL.

Further work is needed to clarify the effects of diurnal variation *(36)* and age and sex *(37)* on serum steroid paneling for ACC diagnosis. Our study used age-matched adrenal tumor groups with all samples collected in the morning to minimize these effects. Nonetheless, in most cases, concentrations of the most useful ACC markers exceed variations attributable to age, gender, or time of day; such increases are only otherwise encountered in forms of congenital adrenal hyperplasia *(9)*.

The inherent limitations of steroid immunoassays for adrenal tumor evaluation are demonstrated again in our study. Although progesterone was detected by LC-MS/MS in 2 patients who had tested positive by immunoassay, concentrations were much smaller. Pregnenolone and 17-hydroxypregenenolone sulfates are known 17-hydroxyprogesterone immunoassay interferences *(38)* and are potential progesterone immunoassay interferents. There was also evidence for interference in the androstenedione and 17-hydroxyprogesterone immunoassay results performed in the ACC cohort. Prediction of potential cross-reacting steroids is difficult because of steroid secretion heterogeneity in ACC.

In summary, LC-MS/MS serum steroid paneling offers a potentially important advancement in the clinical workup of patients with adrenal lesions by combining the measurement of both common and rarely measured steroids in a single analysis. It supports the published conclusions from urine steroid profiling that it is the increased concentrations of steroid synthetic pathway intermediates that best allow discrimination of ACC from non-ACC adrenal lesions.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: K.-M. Schulte, King's College Hospital.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: None declared.
Expert Testimony: None declared.
Patents: None declared.

Role of Sponsor: No sponsor was declared.

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