ISSN 2472-1972

Assay-Specific Spurious ACTH Results Lead to Misdiagnosis, Unnecessary Testing, and Surgical Misadventure—A Case Series

Loren Wissner Greene,[1,2] Eliza B. Geer,[3] Gabrielle Page-Wilson,[4] James W. Findling,[5,6,7] and Hershel Raff[7,8,9,10]

[1]Department of Medicine, New York University School of Medicine, New York, New York 10016; [2]Department of Obstetrics and Gynecology, New York University School of Medicine, New York, New York 10016; [3]Multidisciplinary Pituitary and Skull Base Tumor Center, Memorial Sloan Kettering Cancer Center, New York, New York 10065; [4]Neuroendocrine Unit, Department of Medicine, Vagelos College of Physicians & Surgeons, Columbia University, New York, New York 10032; [5]Endocrinology Center and Clinics, Medical College of Wisconsin, Milwaukee, Wisconsin 53051; [6]Department of Community Medicine, Medical College of Wisconsin, Milwaukee, Wisconsin 53051; [7]Department of Surgery, Medical College of Wisconsin, Milwaukee, Wisconsin 53226; [8]Department of Medicine, Medical College of Wisconsin, Milwaukee, Wisconsin 53226; [9]Department of Physiology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226; and [10]Endocrine Research Laboratory, Aurora St. Luke's Medical Center, Aurora Research Institute, Milwaukee, Wisconsin 53215

ORCiD numbers: 0000-0003-1796-3631 (L. W. Greene); 0000-0003-3722-1889 (E. B. Geer); 0000-0002-4064-1211 (G. Page-Wilson); 0000-0002-5128-8476 (H. Raff).

The proper clinical evaluation of pituitary and adrenal disorders depends on the accurate measurement of plasma ACTH. The modern two-site sandwich ACTH immunoassay is a great improvement compared with older methods but still has the potential for interferences such as heterophile antibodies and pro-opiomelanocortin (POMC) and ACTH fragments. We report the cases of five patients in whom the diagnosis or differential diagnosis of Cushing syndrome was confounded by erroneously elevated results from the Siemens ACTH Immulite assay [ACTH(Immulite)] that were resolved using the Roche Cobas or Tosoh AIA [ACTH(Cobas) and ACTH(AIA), respectively]. In one case, falsely elevated ACTH(Immulite) results owing to interfering antibodies resulted in several invasive differential diagnostic procedures (including inferior petrosal sinus sampling), MRI, and unnecessary pituitary surgery. ACTH(Cobas) measurements were normal, and further studies excluded the diagnosis of Cushing syndrome. In three cases, either Cushing disease or occult ectopic ACTH were suspected owing to elevated ACTH(Immulite) results. However, adrenal (ACTH-independent) Cushing syndrome was established using ACTH(AIA) or ACTH(Cobas) and proved surgically. In one case, ectopic ACTH was suspected owing to elevated ACTH(Immulite) results; however, the ACTH(Cobas) findings led to the diagnosis of alcohol-induced hypercortisolism that resolved with abstinence. We have concluded that ACTH(Immulite) results can be falsely increased and alternate ACTH assays should be used in the diagnosis or differential diagnosis of clinical disorders of the hypothalamic–pituitary–adrenal axis.

Freeform/Key Words: Cushing syndrome, immunoassay interference, diagnosis, adrenocorticotropin

Abbreviations: ACTH(AIA), Tosoh AIA; ACTH(Cobas), Roche Cobas; ACTH(Immulite), Siemens ACTH Immulite assay; DST, dexamethasone; IPS, inferior petrosal sinus; IPSS, inferior petrosal sinus sampling; IRB, institutional review board; LNSC, late night salivary cortisol; POMP, pro-opiomelanocortin; UFC, urinary free cortisol.

Received 18 January 2019
Accepted 15 February 2019
First Published Online 20 February 2019

April 2019 | Vol. 3, Iss. 4
doi: 10.1210/js.2019-00027 | Journal of the Endocrine Society | 763–772

The accurate measurement of plasma ACTH is necessary for the proper diagnosis, differential diagnosis, and monitoring of hypothalamic–pituitary–adrenal axis disorders, including Cushing syndrome, adrenal insufficiency, and congenital adrenal hyperplasia [1]. The development of the two-site "sandwich" immunometric assay for ACTH has greatly improved the sensitivity, specificity, and performance of this assay [2–5]. However, two-site immunometric assays are susceptible to interference, including the presence of heterophile antibodies and hormone fragments or precursors [4, 6–10].

A recent case series raised serious concerns about the performance of the Siemens Immulite ACTH assay [ACTH(Immulite)] [11]. The ACTH(Immulite) had yielded falsely elevated results, which had led to unnecessary testing and misdiagnosis [11]. Since 2012, we have been accumulating a series of cases highlighting serious issues with this assay. The purpose of the present report was to highlight this problem and provide endocrinologists with the clinical context and suggest a corrective course of action.

1. Case Series

Details of the 5 patients have been summarized in Table 1. The relevant laboratory specific-assay reference ranges have been provided parenthetically. The same method could have slightly different reference ranges depending on in which reference laboratory the assay had been validated and performed. The relevant ACTH immunometric assays reported were the ACTH(Immulite) (Siemens Healthcare Diagnostics, Tarrytown, NY) [12], Roche Cobas [ACTH(Cobas)] (Roche Diagnostics, Indianapolis, IN) [13], and Tosoh AIA [ACTH(AIA)] (Tosoh Bioscience, San Francisco, CA) [14]. The institutional review boards (IRBs) approved the present study. Patients 1, 2, and 3 provided written informed consent for specialty testing through Columbia University Medical Center (patient 1) and Memorial Sloan Kettering Cancer Center (patients 2 and 3). The NYU School of Medicine IRB and the Medical College of Wisconsin IRB determined that IRB approval and written informed consent were not required for patients 1, 4, and 5.

A. Patient 1

A 21-year-old woman had presented after evaluations by several other physicians. She reported a 20- to 23-kg weight gain after taking clomiphene alone or combined with dexamethasone for irregular menses with infertility. She had a history of hypertension, headaches, fever, insomnia, fatigue, abdominal pain, rashes, edema, early satiety, mild anorexia, and easy bruisability.

The early morning plasma ACTH(Immulite) levels were consistently elevated [122 and 203 pg/mL (reference range, 6 to 50) and 89 and 95 pg/mL (reference range, 6 to 58)]. The morning serum cortisol values were normal [12.5 μg/dL (reference range, 4.5 to 23.0) and 14.1 μg/dL (reference range, 4.0 to 22.0)], as were the late night salivary cortisol (LNSC) levels [<0.04 μg/dL (reference range, <0.09)]. Urinary free cortisol (UFC) values, measured using liquid chromatography–mass spectrometry/mass spectrometry, were elevated [54 μg/24 h (reference range, <45) and 98 μg/24 h (reference range, <50)] with elevated urine volumes (>2400 mL) and creatinine clearance [>131 mL/min (reference range, 80 to 130)], suggesting an overcollection of urine. Low-dose (1 mg) overnight dexamethasone (DST) suppressed morning serum cortisol to 0.8 μg/dL (reference range, <2.0). High dose (8 mg) overnight DST also resulted in suppressed morning serum cortisol (0.6 μg/dL) and an undetectable UFC. However, the corresponding plasma ACTH(Immulite) concentrations after low- and high-dose overnight DST remained elevated [83 and 84 pg/mL (reference range, 6 to 50), respectively]. The findings from normal-resolution (performed twice) and high-resolution pituitary MRI scans with and without gadolinium were unremarkable.

Because the elevated plasma ACTH results were inconsistent with the other clinical results, further evaluation of possible ACTH(Immulite) assay interference was undertaken. The result for the ACTH(Immulite) after pretreatment with a heterophile blocking reagent was 518 pg/mL,

Table 1. Summary of Cases

Case	Reason for Referral	Initial Adrenal Testing	ACTH(Immulite)	Imaging	Additional Testing and Procedures	Initial Diagnosis	ACTH (2nd Method)	Final Diagnosis
1	Weight gain Hypertension Fatigue Anxiety Bruising Irregular menses Infertility	Normal AM cortisol, elevated LNSC and UFC, positive LDDST Elevated ACTH (Immulite) after LDDST and HDDST	Elevated (122–203 pg/mL)	Normal pituitary MRI ×2 3rd MRI positive	IPSS ACTH(Immulite) positive (Table 2) Pituitary surgery (neoplasm ACTH staining negative)	Cushing disease	ACTH(Cobas) normal (16–38 pg/mL)	No Cushing syndrome
2	Striae Weight gain Moon facies Gestational diabetes	Elevated UFC, LNSC, LDDST	Normal (40 pg/mL) and elevated (62 pg/mL)	Pituitary MRI, 5-mm microadenoma Abdominal MRI, adrenal nodule DOTATE negative ×2	JVS ACTH(Immulite) no gradient IPSS ACTH(Immulite) no gradient Adrenal vein sampling Left adrenal vein cortisol > right Serum DHEAS low	Occult ectopic ACTH-dependent Cushing syndrome	ACTH(AIA) decreased (2–5 pg/mL)	Adrenal (ACTH-independent) Cushing syndrome
3	Fatigue Muscle weakness Hypertension Prediabetes Osteoporosis Renal calculi	Normal/elevated UFC Elevated LNSC Positive LDDST	Elevated (78–143 pg/mL)	Right adrenal nodule Pituitary MRI 1–2-mm lesion MIBG uptake in adrenal nodule DOTATATE normal	JVS ACTH(Immulite) elevated but no gradient IPSS ACTH(AIA) low and no gradient	Occult ectopic ACTH-dependent Cushing syndrome	ACTH(AIA) decreased (2–4 pg/mL)	Adrenal (ACTH-independent) Cushing syndrome
4	Weight gain Facial rounding Hirsutism Striae Supraclavicular fullness	Elevated UFC, LNSC Positive LDDST	Normal (17–21 pg/mL)	Possible 3-mm pituitary lesion Right adrenal mass (HU 40)		Cushing disease	ACTH(Cobas) decreased (1 pg/mL)	Adrenal (ACTH-independent) Cushing syndrome
5	EtOH abuse Cirrhosis Bruising Muscle atrophy Edema	Normal UFC Elevated LNSC Positive LDDST	Elevated (367–1031 pg/mL)	Pituitary MRI normal		Ectopic ACTH syndrome	ACTH(Cobas) decreased (7 pg/mL) ACTH(AIA) normal (14 pg/mL)	Ethanol-induced hypercortisolism

To convert ACTH to pmol/L, multiply by 0.2202.

Abbreviations: EtOH, ethanol; HDDST, high-dose DST suppression test; HU, Hounsfield units; IPSS, inferior petrosal sinus sampling; JVS, jugular venous sampling; LDDST, low-dose DST suppression test; MIBG, metaiodobenzylguanidine; UFC, urine free cortisol.

doi: 10.1210/js.2019-00027 | Journal of the Endocrine Society | 765

which differed by >50% from the untreated ACTH(Immulite) result (104 pg/mL), indicating the possible presence of heterophile antibody interference. This testing was repeated on a fresh plasma sample several months later, and the results were similar. Dilutions of plasma using the ACTH(Immulite) assay were linear. Elevated plasma ACTH(Immulite) results [122 pg/mL (reference range, 9.0 to 46.0)] had decreased to 27 pg/mL after treatment of the plasma sample with a 25% (w/v) buffered phosphate solution of polyethylene glycol 6000 (Sigma-Aldrich, St. Louis, MO) [15], consistent with the precipitation of interfering immunoglobulins. The plasma pro-opiomelanocortin (POMC) concentration [14.2 fmol/mL (reference range, 7 to 38)] was not suggestive of ectopic ACTH secretion [16].

Despite the normal low-dose DST described and the lack of evidence for cyclical Cushing syndrome, her symptoms and consistently elevated plasma ACTH(Immulite) levels led the patient to self-refer to yet another physician at another medical center, resulting in a third pituitary MRI scan with findings suggestive of a right superior pituitary microadenoma. This led to inferior petrosal sinus (IPS) sampling (IPSS) [17, 18] using the ACTH(Immulite) assay, which showed an IPS to peripheral gradient and lateralization to the left side (Table 2). Because the IPSS results ruled out ectopic Cushing syndrome and suggested the diagnosis of Cushing disease, pituitary surgery was performed. No adenoma was found, and the anatomic pathology report of the tissue removed (which included immunohistochemistry for GH, prolactin, ACTH, FSH, LH, and TSH) was consistent with normal anterior pituitary tissue.

Postoperative secondary adrenal insufficiency did not ensue. Her morning serum cortisol was 6.0 μg/dL (reference range, 6.7 to 22.6) and plasma ACTH(Immulite) level was 18.8 pg/mL (reference range, 7.2 to 63.3). At 1 week after surgery, serum cortisol was 11.8 μg/dL; however, the plasma ACTH(Immulite) level was again elevated (73 pg/mL). A pituitary MRI scan performed 2 months after pituitary surgery suggested a residual pituitary adenoma and/or postoperative changes. At 2 months postoperatively, the serum cortisol was normal 8.2 μg/dL (reference range, 4.5 to 23.0), but the plasma ACTH(Immulite) level was still elevated [79 pg/mL (reference range, 6 to 58)]. The serum cortisol continued to be normal at 5 months postoperatively [8.2 μg/dL (reference range, 4.5 to 23)], but the plasma ACTH(Immulite) level remained elevated [62 pg/mL (reference range, 6 to 58)]. When the possibility of issues with the ACTH(Immulite) assay was raised, the same sample was analyzed using the ACTH(Cobas) method and the level was found to be normal [35 pg/mL (reference range, 7.2 to 63)]. Commercial heterophile antibody blocking reagents failed to alter the ACTH(Cobas) results, and serial dilutions showed only a small deviation from linearity, indicating the absence of antibody interference with the ACTH(Cobas) assay. Additional follow-up data have confirmed that this patient did not have Cushing syndrome.

B. Patient 2

A 30-year-old woman had presented in her third trimester of pregnancy with violaceous abdominal striae. In retrospect, she noted weight gain and moon facies before conceiving, and she had developed gestational diabetes during her pregnancy. She delivered a healthy boy by

Table 2. Patient 1: IPSS ACTH(Immulite) Results

Time (min)[a]	Left Petrosal, pg/mL	Right Petrosal, pg/mL	Peripheral, pg/mL
Baseline	149 (2.8)	77 (1.5)	53
2	320 (5.3)	194 (3.2)	60
5	338 (2.6)	172 (1.3)	130
10	488 (7.3)	391(5.8)	67
15	459 (5.7)	231(2.9)	80

To convert pg/ml to pmol/L, multiply by 0.2202. Data in parentheses are IPS/peripheral ACTH(I) ratios.
[a]Represents time after injection of CRH.

cesarean section at 38 weeks. Subsequent testing confirmed the diagnosis of Cushing syndrome with markedly elevated UFC (537 μg/24 h) and LNSC [0.45 and 0.38 μg/dL (reference range, <0.09)] and a nonsuppressed 1-mg overnight DST serum cortisol concentration [20.6 μg/dL (reference range, <2.0)]. Random plasma ACTH(Immulite) concentrations were either high normal or elevated [40 and 62 pg/mL (reference range, <58)], consistent with ACTH-dependent Cushing syndrome.

A pituitary MRI scan showed a 5-mm microadenoma. The jugular venous sampling [19–21] plasma ACTH(Immulite) values did not demonstrate a central to peripheral gradient, with a baseline and CRH-stimulated peripheral plasma ACTH(Immulite) of 34 pg/mL and 38 pg/mL, respectively, and baseline and CRH-stimulated jugular vein ACTH(Immulite) level of 38 to 40 pg/mL and 39 pg/mL, respectively. Imaging for a potential ectopic ACTH using chest and abdominal MRI showed a 2.5 × 2.4-cm left adrenal nodule. The results of a DOTATATE scan were unremarkable, showing only physiologic uptake in the pituitary and adrenal glands. IPSS [17, 18] was performed at a different institution and, similarly, did not show a central to peripheral ACTH(Immulite) gradient, with an elevated 24-hour UFC on the day of the test (304 μg/24 h). The peripheral and IPS plasma ACTH(Immulite) levels were 30 to 60 pg/mL.

Owing to the normal or elevated basal plasma ACTH(Immulite) values in the setting of hypercortisolemia and the lack of a central to peripheral plasma ACTH(Immulite) gradient during jugular venous and IPSS without any anatomic evidence of an ectopic source of ACTH, she was given a provisional diagnosis of occult ectopic ACTH-dependent Cushing syndrome. She was treated with metyrapone for 9 months and improved clinically with normalization of the 24-hour UFC and LNSC values. A repeat DOTATATE scan was again negative. Adrenal vein sampling was conducted to assess for the possibility of an ACTH-producing adrenal lesion, which led to unexpected findings [22]. The plasma ACTH(Immulite) concentration in the inferior vena cava was within the reference range and was similar from both adrenal gland veins and the inferior vena cava (21 to 28 pg/mL), consistent with a non–ACTH-producing adrenal nodule. However, the serum cortisol was much greater from the left vs the right adrenal vein (211.0 vs 26.6 μg/dL, respectively), suggesting a cortisol-producing adrenal neoplasm. Furthermore, the serum dehydroepiandrosterone sulfate (DHEAS) level had been consistently low [22 μg/dL (reference range, 65 to 380) and 26 μg/dL (reference range, 31 to 228)], suggesting a primary adrenal etiology of Cushing syndrome.

While the patient was taking metyrapone, the plasma ACTH(Immulite) result was normal [37 pg/mL (reference range, <60)], with normal serum cortisol (11.6 μg/dL) and UFC (21 μg/24 h) levels. Still taking metyrapone, the plasma ACTH level using a different method [ACTH(AIA)] was subnormal [4.6 pg/mL (reference range, 7.4 to 64.3)], with a normal serum cortisol (10.3 μg/dL). Metyrapone was then discontinued, and the plasma ACTH(AIA) level was still low (2.4 pg/mL), but the serum cortisol and UFC had increased (18.5 μg/dL and 764 μg/24 h, respectively).

Because of the subnormal ACTH(AIA) results and findings from previous adrenal imaging studies, adrenal (ACTH-independent) Cushing syndrome was diagnosed, and the patient underwent unilateral adrenalectomy. The pathologic findings were consistent with a benign adrenal cortical adenoma. The postoperative serum cortisol was very low (0.5 μg/dL), consistent with secondary adrenal insufficiency. At the last follow-up examination, she was in remission and taking a physiologic oral hydrocortisone replacement, with marked weight loss and clinical improvement.

C. Patient 3

A 55-year-old woman had presented with fatigue and muscle weakness in the setting of hypertension, prediabetes, osteoporosis, and renal calculi. A previous abdominal CT scan had incidentally identified a 2.1-cm right adrenal nodule with enhancement characteristic for an adenoma. A subsequent evaluation revealed an elevated 1-mg DST serum cortisol (26.7 μg/dL) and variable UFC (22.4 to 132.0 μg/24 h). The plasma ACTH(Immulite) was elevated [78 pg/mL (reference range, <50) and 143 pg/mL (reference range, <58)], consistent with ACTH-dependent Cushing syndrome.

A pituitary MRI scan showed a possible 1- to 2-mm lesion but the finding was not definitive. Metaiodobenzylguanidine demonstrated uptake in the adrenal nodule, and DOTATATE showed only physiologic uptake in the pituitary and adrenal glands. Jugular venous sampling with CRH administration did not identify a central to peripheral gradient, with a peak stimulated jugular vein plasma ACTH(Immulite) of 124 pg/mL and peak peripheral plasma ACTH of 127 pg/mL. These findings were consistent with occult ectopic Cushing syndrome. However, using an alternate assay method, the plasma ACTH(AIA) was low [3.9 pg/mL (reference range, 7.4 to 64.3)], suggesting ACTH-independent Cushing syndrome.

Because of the discordant ACTH results, IPSS was performed with the ACTH(AIA) assay method, and the results were markedly and consistently less than the reference range (2 to 4 pg/mL). Repeat studies confirmed a subnormal plasma ACTH(AIA) level [3.3 pg/mL (reference range, 7.4 to 64.3)] and elevated LNSC (0.34 μg/dL and 0.41 μg/dL (reference range, <0.09)].

After 3 years of evaluations by multiple physicians, adrenal (ACTH-independent) Cushing syndrome was finally diagnosed, and unilateral adrenalectomy was performed. The pathologic examination findings were consistent with adrenal cortical adenoma. The postoperative serum cortisol was low (2.0 μg/dL), consistent with secondary adrenal insufficiency. At the last follow-up examination, she was in remission and taking an oral hydrocortisone replacement.

D. Patient 4

A 23-year-old woman had been referred for IPSS to confirm the suspicion of Cushing disease. The patient had experienced a rapid 14-kg weight gain during a 9-month period in a truncal distribution associated with facial rounding, hirsutism, supraclavicular fullness, and striking violaceous abdominal striae. She did not have hypertension or diabetes. She was not taking any medications.

Previous studies had showed an elevated LNSC [0.38 μg/dL (reference range, <0.11)] and UFC (127 μg/24 h (reference range, <45)] and a nonsuppressed overnight 1-mg DST serum cortisol level [14.4 μg/dL (reference range, <1.8)]. Previous basal plasma ACTH(Immulite) concentrations were within the reference range [17 and 21 pg/mL (reference range, 10 to 54)], which had suggested ACTH-dependent Cushing syndrome and had led to the referral for IPSS. A pituitary MRI scan showed a faint 3-mm area of diminished contrast enhancement in the left pituitary gland (seen on only one image). The basal morning serum cortisol was 17.8 μg/dL (reference range, 5 to 18).

The basal morning plasma ACTH was repeated with ACTH(Cobas) and was very low [1.0 pg/mL (reference range, 10 to 52)], with a subnormal serum DHEAS [5 μg/dL (reference range, 148 to 407)]. A peripheral CRH test (1 μg/kg ovine CRH intravenously) showed a very low basal ACTH(Cobas) concentration [1 pg/mL (reference range, 10 to 52)], which did not increase after CRH, suggesting ACTH-independent hypercortisolism. The basal serum cortisol was 17.3 μg/dL (reference range, 5 to 18) and also did not increase after CRH.

An abdominal CT scan showed a 2.5 × 2.2-cm right adrenal mass with a noncontrast Hounsfield unit of 40 and absolute contrast washout of 63%. The patient underwent successful laparoscopic right adrenalectomy, followed by secondary adrenal insufficiency. Her ACTH-independent hypercortisolism promptly resolved, and her hypothalamic–pituitary–adrenal axis had fully recovered by 18 months after surgery.

E. Patient 5

A 59-year-old woman had been referred for IPSS for ACTH-dependent hypercortisolism. She had a 25-year history of an alcohol abuse disorder (8 to 10 alcohol drinks daily), stage IV cirrhosis with portal hypertension and ascites, and chronic hyponatremia. In the previous year, she had lost 14-kg and had easy bruising, severe muscle atrophy and weakness, and substantial lower extremity edema. She was cachectic and appeared chronically ill. She had

excessive lanugo hair, multiple ecchymoses on her arms, severe proximal muscle weakness, and 2+ pretibial edema.

The LNSC was elevated [0.58 and 2.6 μg/dL (reference range, <0.14)]; however, the UFC was within the reference range [34 μg/24 h (reference range, <45)]. Overnight 1-mg DST yielded a nonsuppressed morning serum cortisol [8.9 μg/dL (reference range, <1.8)]. The previous plasma ACTH(Immulite) concentrations were markedly elevated [367 to 1031 pg/mL (reference range, 10 to 60)], which had led to the referral for IPSS. A pituitary MRI scan showed cerebral atrophy and cerebellar volume loss but a normal pituitary gland. Ectopic ACTH syndrome was suspected.

Before IPSS was performed, a peripheral CRH test (1 μg/kg IV) was performed initially, using the ACTH(Cobas) and ACTH(AIA) because the clinician was suspicious of the previously elevated ACTH(Immulite) results (Table 3). These results dramatically illustrate the problem we have demonstrated in our study. The ACTH(Cobas) and ACTH(AIA) responses to CRH were consistent with the blunted responses previously demonstrated in patients with alcohol-induced hypercortisolism [23]. These samples were then subsequently analyzed using ACTH(Immulite) and were markedly increased and inconsistent with the clinical diagnosis of alcohol-induced hypercortisolism. These findings confirmed the conjecture that the previous ACTH(Immulite) results were incorrect and made the likelihood of alcoholic-induced hypercortisolism very high. In anticipation of a possible liver transplantation, the patient discontinued all alcohol use. Nine months later, her late-night salivary cortisol levels were normal [0.09 and 0.08 μg/dL (reference range, <0.14)], confirming the diagnosis of alcohol-induced hypercortisolism [24].

2. Discussion

The present case series has illuminated and amplified serious issues with the ACTH(Immulite) assay [11]. Because many high-volume reference laboratories in the United States use this assay, we believe it is critical to inform physicians, especially endocrinologists and endocrine surgeons, of this ACTH assay problem. Since the assembly of our case series, multiple other instances of diagnostic confusion related to this assay have been brought to our attention, many of which resulted in prolonged and unnecessary endocrine testing and procedures for suspected Cushing syndrome, creating a potential patient safety problem. With the large series of patients recently reported and several previous case reports detailing spurious ACTH results with the ACTH(Immulite) assay [6, 8, 10, 11], we believe that our five patients are not isolated or unique and their cases represent a much larger problem, with many cases either unrecognized or unreported. Endocrinologists are very reliant on specialized test results. For several of our patients, the evaluation continued undaunted even when the laboratory findings were not concordant.

The documentation of heterophile antibody assay interference and/or the absence of a pituitary or ectopic source of ACTH did not deter the continued evaluation because many clinicians were persuaded by the inappropriately normal or elevated ACTH(Immulite) results. Repetitive and invasive differential diagnostic testing (*e.g.,* jugular vein sampling and

Table 3. Patient 5: Peripheral CRH Test Results (1 μg/kg Ovine CRH IV After Baseline Sample at 9:35 AM)

	Plasma ACTH (pg/mL)			
Time (min)	**ACTH(Cobas)**	**ACTH(AIA)**	**ACTH(Immulite)**	**Serum Cortisol (μg/dL)**
Baseline	7.3	14.2	835	16.8
15	11.2	23.7	914	21.2
30	12.3	22.1	879	22.4
60	14.1	26.9	825	25.3

To convert ACTH (pg/mL) to pmol/L, multiply by 0.2202; to convert cortisol (μg/dL) to nmol/L, multiply by 27.6.

IPSS) and expensive ^{68}Ga-DOTATATE positron emission tomography/CT testing ensued. Furthermore, in patient 1, the seemingly "positive" IPS/P gradients with the appropriate CRH stimulation ratios [17, 25] had seemed to confirm the pituitary location of ACTH hypersecretion, which, in turn, led to unnecessary pituitary surgery. In some cases, patients, disturbed by the persistently "abnormal" laboratory results, took it on themselves to pursue further endocrine testing and definitive treatment, which led to misdiagnosis and extensive unnecessary and expensive procedures. If the dramatically increased ACTH(Immulite) results in patient 5 during the peripheral CRH test (Table 3) were the only ACTH results available, this patient would have likely proceeded to have undergone unnecessary IPSS and perhaps even pituitary surgery. However, this did not occur because the assays were performed with ACTH(Cobas) and ACTH(AIA). This illustrates the important rule that IPSS should never be performed unless the diagnosis of ACTH-dependent Cushing syndrome is certain because normal subjects can have ACTH responses to CRH and ACTH IPS/P ratios that overlap with those of patients with Cushing disease [26, 27].

Donegan *et al.* [11] suggested that the presence of heterophile antibodies was responsible for some of the falsely increased ACTH(Immulite) results. The case of patient 1 confirmed that the ACTH(Immulite) is susceptible to heterophile antibody interference. However, heterophile antibodies are not always revealed by proprietary blocking reagents, leaving open the possibility that they could have been responsible for more incorrect ACTH(Immulite) results than previously thought [8]. Polyethylene glycol precipitation represents an alternative strategy for detecting immunoassay interference, because treatment of plasma and serum samples with polyethylene glycol has repeatedly been shown to precipitate immunoglobulins, including heterophile antibodies, as demonstrated in patient 1 [28].

Another possible cause is a substantial difference in the epitopes against which the two anti-ACTH antibodies used in the different immunometric assays are directed (Table 4). At first, we thought the correct results from the ACTH(Cobas) had been because it uses monoclonal, rather than animal polyclonal, antibodies and/or that the ACTH(Cobas) antibody epitopes are smaller and do not overlap. However, the ACTH(AIA) also uses animal polyclonal antibodies with larger epitopes, although they are shorter and nonoverlapping compared with the ACTH(Immulite) antibodies. In contrast to the ACTH(AIA), which uses only goat antibodies, the ACTH(Immulite) method uses both rabbit and mouse antibodies, which might make the method more susceptible to interference [7, 8]. We do not believe this phenomenon is related to biotin interference because that would artificially lower, rather than increase, the ACTH results [29].

Patients 2 and 4, who ultimately had a diagnosis of adrenal (ACTH-independent) Cushing syndrome, had had low serum DHEAS concentrations. This makes the diagnosis of ACTH-dependent Cushing syndrome very unlikely [30] and should have raised the specter of a spuriously elevated plasma ACTH, in particular, because these patients had a unilateral adrenal mass. Also, in patient 5, it would be highly unusual for a patient with ectopic ACTH with plasma ACTH concentrations >350 pg/mL to have normal UFC results [31].

In conclusion, despite substantial endocrine and imaging data suggesting otherwise, elevated ACTH(Immulite) results led to unnecessary testing and potentially harmful invasive

Table 4. N- and C-Terminal Antibody Epitopes (Inclusive Amino Acid Numbers) of the Different ACTH Assays

Assay	Monoclonal or Polyclonal	N-Terminal	C-Terminal
ACTH(Cobas)	Monoclonal	9-12	36-39
ACTH(Immulite)	Polyclonal	1-24 (rabbit)	18-39 (mouse)
ACTH(AIA)	Polyclonal	1-16 (goat)	24-39 (goat)

ACTH(Cobas) and ACTH(Immulite) antibody information provided by Mark A. Cervinski, PhD (Department of Pathology, Dartmouth-Hitchcock Medical Center) and reported with his permission; ACTH(AIA) antibody information provided by Barbara Petro (Tosoh Bioscience, Inc.) and reported with her permission.

procedures, including pituitary surgery. Because the ACTH(Immulite) assay is used by most clinical laboratories, this presents a substantial problem for the endocrine community. It is imperative that physicians know which ACTH assay is in use in their practice. We believe that an alternate ACTH assay such as the ACTH(Cobas) or ACTH(AIA) should be used in the diagnosis and differential diagnosis of patients with suspected disorders of pituitary-adrenal function.

Acknowledgments

The authors thank Drs. Joely Straseski (ARUP Laboratories), Mark A. Cervinski (Dartmouth-Hitchcock Medical Center), Barbara Petro (Tosoh Bioscience), and Sharon Wardlaw (Columbia University Medical Center, Neuroendocrine Unit) and the Mayo Clinical Laboratories and ARUP Laboratories for their invaluable assistance. The present study has been accepted in abstract form for a poster presentation at the Endocrine Society 2019 meetings.

Financial Support: The present study was supported by the Robert Wood Johnson Foundation Harold Amos Medical Faculty Development Program (grant 71951 to G.P.-W.) and the Aurora Research Institute (to H.R.).

Correspondence: Hershel Raff, PhD, Endocrine Research Laboratory, Aurora St. Luke's Medical Center, Aurora Research Institute, 2801 West KK River Parkway, MOB 260, Milwaukee, Wisconsin 53215. E-mail: hraff@mcw.edu.

Disclosure Summary: E.B.G. has received consulting fees from Strongbridge, Corcept, Pfizer and grants from Novartis Pharmaceuticals, Ionis Pharmaceuticals Inc., Strongbridge, and Chiasma. G.P.-W. has received consulting fees from Strongbridge. J.W.F. has received consulting fees from Corcept Therapeutics, and Novartis Pharmaceuticals and has been a research investigator for Corcept Therapeutics and Novartis Pharmaceuticals. H.R. has received consulting fees and a research grant from Cerium Pharmaceuticals. The remaining author has nothing to disclose.

References and Notes

1. Raff H, Sharma ST, Nieman LK. Physiological basis for the etiology, diagnosis, and treatment of adrenal disorders: Cushing's syndrome, adrenal insufficiency, and congenital adrenal hyperplasia. *Compr Physiol*. 2014;**4**(2):739–769.
2. Findling JW, Engeland WC, Raff H. The use of immunoradiometric assay for the measurement of ACTH in human plasma. *Trends Endocrinol Metab*. 1990;**1**(6):283–287.
3. Lindsay JR, Shanmugam VK, Oldfield EH, Remaley AT, Nieman LK. A comparison of immunometric and radioimmunoassay measurement of ACTH for the differential diagnosis of Cushing's syndrome. *J Endocrinol Invest*. 2006;**29**(11):983–988.
4. Talbot JA, Kane JW, White A. Analytical and clinical aspects of adrenocorticotrophin determination. *Ann Clin Biochem*. 2003;**40**(Pt 5):453–471.
5. Raff H, Findling JW. A new immunoradiometric assay for corticotropin evaluated in normal subjects and patients with Cushing's syndrome. *Clin Chem*. 1989;**35**(4):596–600.
6. Bolstad N, Kazaryan AM, Revheim ME, Distante S, Johnsrud K, Warren DJ, Nustad K, Edwin B. A man with abdominal pain: enough evidence for surgery? *Clin Chem*. 2012;**58**(8):1187–1190.
7. Bolstad N, Warren DJ, Nustad K. Heterophilic antibody interference in immunometric assays. *Best Pract Res Clin Endocrinol Metab*. 2013;**27**(5):647–661.
8. Grasko J, Willliams R, Beilin J, Glendenning P, Fermoyle S, Vasikaran S. A diagnostic conundrum: heterophilic antibody interference in an adrenocorticotropic hormone immunoassay not detectable using a proprietary heterophile blocking reagent. *Ann Clin Biochem*. 2013;**50**(Pt 5): 433–437.
9. Raff H, Findling JW, Wong J. Short loop adrenocorticotropin (ACTH) feedback after ACTH-(1-24) injection in man is an artifact of the immunoradiometric assay. *J Clin Endocrinol Metab*. 1989;**69**(3): 678–680.
10. Choy KW, Teng J, Wijeratne N, Tan CY, Doery JC. Immunoassay interference complicating management of Cushing's disease: the onus is on the clinician and the laboratory. *Ann Clin Biochem*. 2017; **54**(1):183–184.
11. Donegan DM, Algeciras-Schimnich A, Hamidi O, Young WF, Nippoldt T, Bancos I, Erickson D. Corticotropin hormone assay interference: a case series. *Clin Biochem*. 2019;**63**:143–147.
12. RRID. AB_2783635. https://scicrunch.org/resolver/AB_2783635.

13. RRID. AB_2783634. https://scicrunch.org/resolver/AB_2783634.
14. RRID. AB_2783633. https://scicrunch.org/resolver/AB_2783633.
15. Smith TP, Suliman AM, Fahie-Wilson MN, McKenna TJ. Gross variability in the detection of prolactin in sera containing big big prolactin (macroprolactin) by commercial immunoassays. *J Clin Endocrinol Metab*. 2002;**87**(12):5410–5415.
16. Page-Wilson G, Freda PU, Jacobs TP, Khandji AG, Bruce JN, Foo ST, Meece K, White A, Wardlaw SL. Clinical utility of plasma POMC and AgRP measurements in the differential diagnosis of ACTH-dependent Cushing's syndrome. *J Clin Endocrinol Metab*. 2014;**99**(10):E1838–E1845.
17. Findling JW, Kehoe ME, Shaker JL, Raff H. Routine inferior petrosal sinus sampling in the differential diagnosis of adrenocorticotropin (ACTH)-dependent Cushing's syndrome: early recognition of the occult ectopic ACTH syndrome. *J Clin Endocrinol Metab*. 1991;**73**(2):408–413.
18. Findling JW, Kehoe ME, Raff H. Identification of patients with Cushing's disease with negative pituitary adrenocorticotropin gradients during inferior petrosal sinus sampling: prolactin as an index of pituitary venous effluent. *J Clin Endocrinol Metab*. 2004;**89**(12):6005–6009.
19. Doppman JL, Oldfield EH, Nieman LK. Bilateral sampling of the internal jugular vein to distinguish between mechanisms of adrenocorticotropic hormone-dependent Cushing syndrome. *Ann Intern Med*. 1998;**128**(1):33–36.
20. Ilias I, Chang R, Pacak K, Oldfield EH, Wesley R, Doppman J, Nieman LK. Jugular venous sampling: an alternative to petrosal sinus sampling for the diagnostic evaluation of adrenocorticotropic hormone-dependent Cushing's syndrome. *J Clin Endocrinol Metab*. 2004;**89**(8):3795–3800.
21. Radvany MG, Quinones-Hinojosa A, Gallia GL, Wand GS, Salvatori R. Venous sampling for Cushing disease: comparison of internal jugular vein and inferior petrosal sinus sampling. *Endocr Pract*. 2016; **22**(9):1057–1061.
22. Hayes AR, Grossman AB. The ectopic adrenocorticotropic hormone syndrome: rarely easy, always challenging. *Endocrinol Metab Clin North Am*. 2018;**47**(2):409–425.
23. Wand GS, Dobs AS. Alterations in the hypothalamic-pituitary-adrenal axis in actively drinking alcoholics. *J Clin Endocrinol Metab*. 1991;**72**(6):1290–1295.
24. Findling JW, Raff H. Diagnosis of endocrine disease: differentiation of pathologic/neoplastic hypercortisolism (Cushing's syndrome) from physiologic/non-neoplastic hypercortisolism (formerly known as pseudo-Cushing's syndrome). *Eur J Endocrinol*. 2017;**176**(5):R205–R216.
25. Oldfield EH, Doppman JL, Nieman LK, Chrousos GP, Miller DL, Katz DA, Cutler GB Jr, Loriaux DL. Petrosal sinus sampling with and without corticotropin-releasing hormone for the differential diagnosis of Cushing's syndrome. *N Engl J Med*. 1991;**325**(13):897–905.
26. Raff H, Findling JW. A physiologic approach to diagnosis of the Cushing syndrome. *Ann Intern Med*. 2003;**138**(12):980–991.
27. Yanovski JA, Cutler GB Jr, Doppman JL, Miller DL, Chrousos GP, Oldfield EH, Nieman LK. The limited ability of inferior petrosal sinus sampling with corticotropin-releasing hormone to distinguish Cushing's disease from pseudo-Cushing states or normal physiology. *J Clin Endocrinol Metab*. 1993; **77**(2):503–509.
28. Sturgeon CM, Viljoen A. Analytical error and interference in immunoassay: minimizing risk. *Ann Clin Biochem*. 2011;**48**(Pt 5):418–432.
29. Samarasinghe S, Meah F, Singh V, Basit A, Emanuele N, Emanuele MA, Mazhari A, Holmes EW. Biotin interference with routine clinical immunoassays: understand the causes and mitigate the risks. *Endocr Pract*. 2017;**23**(8):989–998.
30. Dennedy MC, Annamalai AK, Prankerd-Smith O, Freeman N, Vengopal K, Graggaber J, Koulouri O, Powlson AS, Shaw A, Halsall DJ, Gurnell M. Low DHEAS: a sensitive and specific test for the detection of subclinical hypercortisolism in adrenal incidentalomas. *J Clin Endocrinol Metab*. 2017;**102**(3): 786–792.
31. Aron DC, Raff H, Findling JW. Effectiveness versus efficacy: the limited value in clinical practice of high dose dexamethasone suppression testing in the differential diagnosis of adrenocorticotropin-dependent Cushing's syndrome. *J Clin Endocrinol Metab*. 1997;**82**(6):1780–1785.