A Pitfall of Falsely Elevated ACTH: A Case Report and Literature Review

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Abstract
A 35-year-old woman with unintentional weight gain, hyperpigmentation of bilateral palms, and general fatigue was initially suspected of Cushing’s syndrome or adrenal insufficiency based on the isolated elevation of the plasma adrenocorticotropic hormone (ACTH) level (113.0 pg/mL) in the Siemens ACTH Immulite assay (ACTH [Immulite]). However, both of the diagnoses were excluded by screening tests including the overnight dexamethasone suppression test, the 24-hour urinary free cortisol excretion, and the ACTH stimulation test in spite of the consistent elevation of the plasma ACTH levels. We speculated that the existence of the immunoassay interference may be the underlying cause because the plasma ACTH level analyzed by the CIS Bio International ELSA-ACTH immunoassay (ELSA-ACTH) was within the normal range. After reviewing our case and several reported cases of falsely elevated plasma ACTH levels, we conclude that when discrepancy between clinical symptoms and laboratory measurements exists, medical practitioners ought to rely on formal diagnostic criteria rather than misleading laboratory results to avoid misdiagnosis or even unnecessary invasive testing and procedures. In addition, current methods for investigation and elimination of immunoassay interferences should be applied with caution due to variable efficacy and inevitable deviations.

Keywords
adrenocorticotropic hormone, adrenal insufficiency, Cushing’s syndrome immunoassay interference, heterophilic antibodies

Introduction
Accurate measurement of adrenocorticotropic hormone (ACTH) levels is essential for clinicians to diagnose and treat pituitary and adrenal disorders. Despite the wide use of 2-site immunometric assay for measuring serum ACTH concentrations, concerns have been raised in the recent years because several cases of misleading ACTH levels related to heterophilic antibodies have been reported.1,2 We report a case of isolated elevation of the plasma ACTH level, which was supposedly associated with heterophilic antibodies of the ACTH (Immulite), but failed to meet the diagnostic criteria of Cushing’s syndrome and adrenal insufficiency. Informed consent was obtained from the patient for publication of this case.

Case Presentation
A 35-year-old woman with a family history of type 2 diabetes mellitus complained of unintentional body weight gain of 10 kg per month last year and also suffered from hyperpigmentation of the bilateral palms and general fatigue. The initial laboratory tests showed hyperlipidemia (high-density lipoprotein [HDL] 42 mg/dL [reference range, >65 mg/dL], low-density lipoprotein [LDL] 173 mg/dL [reference range, <100 mg/dL]), and elevated plasma ACTH (113.0 pg/mL [reference range, 0.1-46.0]). The levels of other pituitary hormones were normal (Table 1). Thereafter, the patient was hospitalized for further evaluation of the pituitary-adrenal axis. Both the overnight dexamethasone suppression test and

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Received December 30, 2021. Revised April 23, 2022. Accepted May 8, 2022.

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24-hour urinary free cortisol excretion were incompatible with Cushing’s syndrome (Table 2). Moreover, the results of the stimulation tests of ACTH, thyrotropin-releasing hormone, and gonadotropin-releasing hormone, as well as magnetic resonance imaging (MRI) of the sellar turcica and abdominal sonography were all normal, indicating that adrenal insufficiency, hypothyroidism, and hypogonadism were less likely. The patient was discharged and started oral medications, including cortisone 25 mg once a day and atorvastatin 20 mg once a day (tapered to 10 mg once a day from June 21, 2019), with routine outpatient visits. However, the patient still complained of body weight gain (increased by 10 kg in 2 months), accompanied by persistent hyperlipidemia and elevation of ACTH (Figure 1). Hence, she was again admitted to the endocrinology ward for a further survey of possible adrenal insufficiency.

On admission, she was 165 cm in height and 81.3 kg in weight; her blood pressure was 126/83 mm Hg, with a regular pulse of 70/min. Hyperpigmentation of the bilateral palms was noted during the physical examination. The insulin tolerance test was incompatible with adrenal insufficiency (Table 2, Figure 2). Magnetic resonance imaging of the brain did not show pituitary gland abnormality, and bilateral adrenal glands were unremarkable on computed tomography scans of the abdomen and pelvis. Given the unsolved etiology of elevated serum ACTH level after serial examinations, the patient was advised to be tested again with ELSA-ACTH assay at the LEZEN Reference Lab, where her serum ACTH concentration was found to be normal (21.10 pg/mL [reference range, 10-60]). The patient has gradually recovered without further treatment.

**Discussion**

In this case, we initially ruled out Cushing’s syndrome and adrenal insufficiency, mainly based on the elevation of the plasma ACTH level, and Cushing’s syndrome was more plausible according to the clinical manifestations, including unintentional weight gain, general fatigue, and palmar hyperpigmentation. Nevertheless, both of the impressions were excluded as screening tests, including the overnight dexamethasone suppression test, 24-hour urinary free cortisol excretion, and ACTH stimulation test, were incompatible with them. Hence, it is doubtful whether isolated elevated plasma ACTH levels should be trusted.

To solve this problem, it is necessary to identify the factors affecting plasma ACTH levels. It has been reported that patients with major depressive disorder in some cases exhibited increased levels of plasma ACTH concentration. Also, prior oral intake of glucocorticoids should be carefully examined, given that they can acutely or chronically suppress hypothalamic-pituitary-adrenal function. Both conditions were excluded with high confidence in our case, after the patient’s medical records were comprehensively examined. Ectopic secretion of specific ACTH fragments and pro-opiomelanocortin by tumors may also increase the plasma ACTH level. However, this condition may be confirmed by gel exclusion chromatography, which can discriminate the components of plasma ACTH by molecular weights. Usually, there is a periodic occurrence of ACTH secretion in patients with primary adrenal insufficiency, congenital adrenal hyperplasia, and ACTH-dependent Cushing’s syndrome. Therefore, single equivocal results should not be relied on, and measurement should be conducted at different time points. Nevertheless, the results of hormone function tests and clinical images excluded the aforementioned diagnoses in our case. Laboratory test results of patients with adrenal disorders may disclose increased levels of ACTH due to their rapid response to stress. Consequently, blood samples should not be obtained by prolonged venipuncture or before acclimation of the patient to the hospital environment. The handling procedure of blood samples may also affect the quality or quantity of the plasma ACTH, given that the plasma ACTH may attach to glass surfaces and be cleaved by enzymes from blood cells at room temperature and hence become unstable. Therefore, the blood samples containing ACTH were drawn into plastic tubes with ethylenediaminetetraacetic acid (EDTA) and kept on ice until further manipulation. The results of hormone function tests and clinical images excluded the aforementioned diagnoses in our case. Laboratory test results of patients with adrenal disorders may disclose increased levels of ACTH due to their rapid response to stress. Consequently, blood samples should not be obtained by prolonged venipuncture or before acclimation of the patient to the hospital environment. The handling procedure of blood samples may also affect the quality or quantity of the plasma ACTH, given that the plasma ACTH may attach to glass surfaces and be cleaved by enzymes from blood cells at room temperature and hence become unstable. Therefore, the blood samples containing ACTH were drawn into plastic tubes with ethylenediaminetetraacetic acid (EDTA) and kept on ice until further manipulation of the plasma. It has also been demonstrated that several factors, including hemolysis, delayed centrifugation and separation of plasma, and the presence of excess EDTA, all erroneously decrease the concentration of ACTH. We argue that inappropriate handling of the blood samples was not the putative cause of the abnormal plasma ACTH, given that the analysis procedures were all performed by the qualified laboratory of our hospital.

It is essential to further elaborate the clinical cases including ours and the several previously published case series, because the patients in these cases were all proven to be normal despite the presence of a false elevation of the plasma

### Table 1. Hormonal Profile at First Outpatient Visit.

| Hormone                            | Measurement | Reference range       |
|-----------------------------------|-------------|-----------------------|
| ACTH (pg/mL)                      | 113.0       | 0.1-46.0              |
| Prolactin (ng/mL)                 | 18.5        | <25.0                 |
| Human growth hormone (ng/mL)      | 9.785       | 0.003-3.607           |
| IGF-1 (ng/mL)                     | 291.0       | 63.0-223.0            |
| Cortisol (µg/dL)                  | 5.40        | 5.00-23.00            |
| Renin (ng/mL/h)                   | 2.08        | 0.6-4.3               |
| Aldosterone (pg/mL)               | 159.9       | 68.0-173              |
| Testosterone (ng/dL)              | 13.0        | 15.0-70.0             |
| Estradiol (pg/mL)                 | 37.70       | 30.00-400.00          |
| FSH (mIU/mL)                      | 4.06        | 3.03-8.08             |
| LH (mIU/mL)                       | 4.99        | 1.80-11.78            |
| TSH (µIU/mL)                      | 1.54        | 0.25-5.00             |
| Free T4 (ng/dL)                   | 1.35        | 0.89-1.78             |

Abbreviations: ACTH, adrenocorticotropic hormone; IGF-1, insulin-like growth factor-1; FSH, follicle-stimulating hormone; LH, luteinizing hormone; TSH, thyroid-stimulating hormone.
Table 2. Investigation of Suspected Cushing’s Syndrome and Adrenal Insufficiency.

| Diagnostic test                  | Result                                                                 | Interpretation                                                                 | Outcome               |
|----------------------------------|------------------------------------------------------------------------|--------------------------------------------------------------------------------|-----------------------|
| 24-hour urinary free cortisol excretion | 193.60 µg/day (reference range, 20.90-292.30)                         | Less than 3 times the upper limit of normal                                       | Unlikely Cushing’s syndrome |
| Overnight dexamethasone suppression test | Plasma cortisol: 0.9 µg/dL                                            | Plasma cortisol < 1.8 µg/dL at 8-9 a.m. after 1 mg dexamethasone was given at 11 p.m. | Unlikely Cushing’s syndrome |
| ACTH stimulation test            | Plasma cortisol 30 minutes after 250 µg cosyntropin IM: 28.62 µg/dL    | Plasma cortisol > 16-18 µg/dL 30-60 minutes after 250 µg cosyntropin IM or IV    | Unlikely adrenal insufficiency |
|                                  | Plasma cortisol 60 minutes after 250 µg cosyntropin IM: 31.63 µg/dL    |                                                                               |                       |
| Insulin tolerance test           | Please refer to Figure 2                                               | Plasma cortisol > 18-20 µg/dL at 60, 90 minutes after insulin was given with serum glucose < 40 mg/dL | Unlikely adrenal insufficiency |

Abbreviation: ACTH, adrenocorticotropic hormone; IM, intramuscular injection; IV, intravenous injection.

Figure 1. Plasma ACTH levels of the present case.
Abbreviation: ACTH, adrenocorticotropic hormone.

Figure 2. Insulin tolerance test results of the present case.
Table 3. Summary of the Present Case and Similar Previous Reported Cases of Falsely Elevated Plasma ACTH Levels.

| Patient | Age  | Gender | Past history | Symptoms | Diagnosis | Initial ACTH level | ACTH immunoassay | Results of screening tests | Methods used to reveal assay interference | Additional testing and procedures |
|---------|------|--------|--------------|----------|-----------|--------------------|------------------|--------------------------|-----------------------------------------|--------------------------------------|
| Our case | 35   | Female | Family history of type 2 diabetes mellitus | Weight gain, bilateral palm hyperpigmentation, fatigue | Initial: Possible CS or AI Final: No CS/AI | 113.0 pg/mL (reference range 0.1-46.0 pg/mL) | ACTH (Immuli) | Normal 1 mg ODST | Change of the assay | MRI brain: No pituitary abnormality Normal 17α-hydroxyprogesterone |
| Case No. 1 (Greene et al) | 21   | Female | Use of clomiphene and dexamethasone | Weight gain, hypotension, fatigue, anxiety | Initial: Possible CS Final: No CS | 122-203 pg/mL (reference range 6-50 pg/mL) | ACTH (Immuli) | Elevated LNC and UFC | Use of HBT | Twice MRI brain: No pituitary abnormality Third MRI Brain: Right superior pituitary microadenoma IPSS ACTH (Immuli) positive Pituitary surgery (no neoplasm) |
| Case No. 3 (Donegan et al) | 59   | Male   | Unknown | Cold intolerance, hypertension, palpitations, low libido | Initial: Possible CS Final: No CS | 142 pg/mL (reference range 10-60 pg/mL) | ACTH (Immuli) | Normal cortisol and 24-hour UFC | Change of the assay | MRI brain: No pituitary abnormality |
| Case No. 6 (Donegan et al) | 39   | Female | Exogenous steroid use | Weight gain, acne, hirsutism, excess sweating | Initial: Possible CS Final: No CS | 21 pg/mL (reference range 10-60 pg/mL) | ACTH (Immuli) | Normal 1 mg ODST/UFC 2-day LDDST + CRH cortisol < 1, but ACTH elevated after suppression test | Use of HBT | MRI brain: No pituitary abnormality |
| Case No. 9 (Donegan et al) | 77   | Female | TSS for Rathke's cleft cyst | Unknown | Initial: Possible CS Final: No CD | 138 pg/mL (reference range 10-60 pg/mL) | ACTH (Immuli) | Normal cortisol | Change of the assay | MRI brain: No new abnormality |
| Case No. 11 (Donegan et al) | 46   | Male | Unknown | Muscle spasms, intermittent weakness | Initial: Possible AI Final: No AI | 115 pg/mL (reference range 10-60 pg/mL) | ACTH (Immuli) | Normal ACTH stimulation test | Change of the assay | NA |
| Case No. 12 (Donegan et al) | 46   | Male | Graves' disease, type 1 diabetes, ankylosing spondylitis | Unknown | Initial: Possible AI Final: No AI | 77 pg/mL (reference range 10-60 pg/mL) | ACTH (Immuli) | Normal ACTH stimulation test | Change of the assay | 21-hydroxylase antibody negative |
| Case reported in Morita et al | 49   | Female | Membranous nephropathy under oral prednisolone | Lethargy and nausea during tapering of prednisolone | Initial: Possible primary AI Final: No AI | 399.1 pg/mL (reference range 7.2-63.3 pg/mL) | Roche Elcsys ACTH | Normal ACTH stimulation test⁴ | Change of the assay Use of HBT | MRI brain: Normal adrenals and a slightly swollen pituitary Normal DHEA-S Negative rheumatoid factor Postive antinuclear antibodies |

Abbreviations: ACTH, adrenocorticotropic hormone; CS, Cushing's syndrome; AI, adrenal insufficiency; 1 mg ODST, 1 mg overnight dexamethasone suppression test; 24-hour UFC, 24-hour urinary free cortisol; ITT, insulin tolerance test; MRI, magnetic resonance imaging; LNC, late-night salivary cortisol; LDDST, low-dose dexamethasone suppression test; HBT, heterophile blocking tube; PEG, polyethylene glycol; IPSS, inferior petrosal sinus (IPS) sampling; CRH, corticotropin releasing hormone; TSS, transsphenoidal surgery; CD, Cushing's disease; NA, not applicable; DHEA-S, dehydroepiandrosterone sulfate. 

⁴Normal ACTH stimulation test, positive rapid ACTH stimulation test but negative prolonged ACTH stimulation test.
ACTH levels. This may be associated with the interference of the immunoassays (Table 3). The case No. 1 in Greene et al.\(^1\) and the cases No. 3, 6, and 9 in Donegan et al.\(^2\) were suspected of having Cushing’s syndrome, while the cases No. 11 and 12 in Donegan et al.\(^2\) were diagnosed with possible adrenal insufficiency. The medical history disclosed that the patient No. 1 in Greene et al.\(^1\) took clomiphene and dexamethasone for irregular menses with infertility, while the patients No. 6 and 9 from Donegan et al.\(^2\) respectively, took oral steroids and underwent surgery for Rathke’s cleft cyst. Furthermore, the patient No. 12 in the same study had type 1 diabetes, ankylosing spondylitis, and Graves’ disease that were treated with radioactive iodine.\(^{1,2}\) Otherwise, none of the patients had preexisting metabolic or endocrinological disorders. Similar to our case, laboratory tests including the overnight dexamethasone suppression test, the 24-hour urinary test of free cortisol and midnight plasma cortisol (or midnight salivary cortisol) excretion for screening Cushing’s syndrome, as well as the ACTH stimulation test for adrenal insufficiency were performed, and most of them showed negative results. However, the case No. 1 in Greene et al.\(^1\) showed elevated levels of late-night salivary cortisol, 24-hour urinary free cortisol excretion, and positive low-dose dexamethasone suppression test. Although neither of the patients were eventually diagnosed with Cushing’s syndrome or adrenal insufficiency, the patient No. 1 in Greene et al.\(^1\) underwent unnecessary clinical procedures, including repeated pituitary MRI scan, inferior petrosal sinus sampling, and pituitary surgery. Furthermore, the patients in our case and the patients No. 3, 6, and 9 in Donegan et al.\(^2\) only underwent MRI of the brain despite lacking evidence of endogenous hypercortisolism.

Next, we examine how clinical practitioners verify and cope with immunoassay interferences in these cases. It has been shown that heterophile antibodies, high-dose hook effect, and lipemia all contribute to the interference of immunoassays.\(^{2,9-10}\) Among the rare cases of the falsely elevated ACTH concentrations, the existence of heterophile antibodies in immunoassays has been widely discussed. Endogenous human autoantibodies, therapeutic antibodies, anti-animal antibodies, and rheumatoid factors can all be defined as heterophilic antibodies, as they often interact with the principal antibodies for assays and hence interfere with the quantification of the analyte.\(^{11}\) In the present case, measurement of the ACTH level was initially conducted via Siemens IMMULITE\(^6\) 2000 ACTH assay, a sandwich immunoassay consisting of a murine monoclonal ACTH capture antibody coupled to the antigen-coated plastic beads as the solid phase, as well as a rabbit polyclonal antibody coupled with alkaline phosphatase as the liquid phase. Previous studies have illustrated that the interaction between these antibodies and other unknown interference antibodies results in most of the doubtful plasma ACTH elevations analyzed using the ACTH (Immulite).\(^{1,2}\) Cisbio ELISA-ACTH assay used at the LEZEN Reference Lab was composed of an ACTH N-terminal and C-terminal specific monoclonal antibody, which were, respectively, coated on the ELSA solid phase and radiolabeled with iodine-125 tracer. The increased specificity of the ELISA-ACTH immunoassay yields more accurate plasma ACTH levels. Multiple lines of evidence have shown the methods for managing underlying interferences.\(^{2,4,15}\) Optimizing all inappropriate analytical procedures and repeating the plasma ACTH measurements can exclude preanalytical and analytical interfering factors. Laboratory technicians can also conduct the measurement with another test kit, especially when the initial one has been reported to produce misleading results. It was reported that after applying the ELISA-ACTH and Roche Cobas ACTH immunoassay for plasma analysis, the levels of plasma ACTH in our case and case No. 1 of the study by Greene et al.\(^1\) were within the normal range. However, Altawallbeh and Karger\(^{14}\) questioned the correctness of applying another immunoassay kit, given that this analysis method may also be subject to interferences. Due to the lack of comprehensive statistical investigations that compare erroneous rates of different commercialized immunoassay kits, medical practitioners could merely refer to reported cases and apply immunoassay kits that are relatively the least susceptible to interference. Moreover, practitioners can consider serial dilutions, and the existence of analytical interference may be proven by the lack of linearity after dilution. The blood samples of all the cases in Donegan et al.\(^2\) exhibited a nonlinear dose response when serial dilution was applied, indicating the presence of analytical interferences. Nonetheless, the linearity has retained after serial dilution in spite of the presence of heterophilic antibodies in the case of Morita et al.\(^4\) Hence, the authors suggested that heterophilic antibodies with strong binding capacities did not linearly decline with the dilution. This case emphasizes that serial dilution alone might not be sufficient to exclude the possibility of antibody interference. Among potential reagents capable of diminishing existing interferences, polyethylene glycol (PEG) is able to precipitate high molecular weight proteins and decrease interfering immunoglobulins, and commercially available heterophilic antibody blocking reagents are able to block both specific antibodies for human immunoglobulins and nonspecific ones including non-immuno globulins developed from sera of the species.\(^{2,4,15}\) The plasma of the patients in the case series of Donegan et al.\(^2\) (the patients No. 3 and 9) and Morita et al.\(^4\) was tested with PEG, and the findings were consistent with heterophile interference. The manuals of ACTH (Immulite) and ELISA-ACTH could not guarantee sufficient protection despite the addition of protective reagents that may minimize the risk of interference, such as...
the interference of human anti-mouse antibodies (HAMA). The efficacy of commercialized heterophile blocking reagents (heterophile blocking tube [HBT], Scantibodies Laboratory, Inc., Santee, CA, USA) was controversial, because they failed to eliminate interfering substances in the case series of Donegan et al. However, they successfully adjusted the plasma ACTH level in the case of Morita et al. Based on the study by Ismail, the repeated analysis using an alternative method, serial dilutions, or antibody blocking reagents may all cause a false sense of assurance provided that results are subjectively interpreted. Therefore, the existence of interfering antibodies could not be completely excluded, even with no abnormalities shown by the troubleshooting methods.

**Conclusion**

To conclude, our case demonstrates that the definitive etiology of hormonal derangement can be established only after clinical presentation, comorbidities, differential diagnosis, and laboratory testing results have all been considered. Endocrinology specialists should realize that diagnosis should be made based on clinical criteria rather than insufficient laboratory tests, because any measurements are prone to technical errors. Laboratory tests that are inconsistent with clinical findings should be scrutinized, and the application of alternative examination methods and integrative assessment is crucial for a correct diagnosis.

**Acknowledgments**

The authors would like to acknowledge Dr Yi-Hui Chen for assistance with statistical graphics and for the critical reading of the manuscript, the Tri-Service General Hospital, and the participants of our study.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**

The author(s) received no financial support for the research, authorship, and/or publication of this article.

**Ethics Approval**

Ethical approval to report this case was obtained from the Institutional Review Board of the Tri-Service General Hospital, National Defense Medical Center (TSGHIRB No.: A202005187).

**Informed Consent**

Written informed consent was obtained from the patient for her anonymized information to be published in this article.

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