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ORIGINAL

Falsely elevated plasma ACTH levels measured by the Elecsys assay related to heterophilic antibody in a case of secondary adrenocortical insufficiency

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Abstract. A 49-year-old woman with membranous nephropathy was referred to our hospital during the tapering of oral prednisolone, because of suspicion of primary adrenal insufficiency based on a plasma ACTH level of 399.1 pg/mL in the Elecsys assay and a serum cortisol level of 3.1 μg/dL. A rapid ACTH stimulation test revealed a suboptimal response, whereas a prolonged ACTH simulation test showed a sufficient increase in her urinary free cortisol. Also, big ACTH was not detected by gel exclusion chromatography. Therefore, we speculated that ACTH levels were falsely elevated due to some interference substances. Pretreatment of her plasma with either polyethylene glycol precipitation or a heterophilic blocking tube substantially reduced her ACTH values. When either the Immulite ACTH II or the TOSOH II ACTH was tried instead of the Elecsys ACTH, her plasma ACTH values turned out to be lower and appropriate for her clinical status. These results indicated that heterophilic antibodies interfered only with the Elecsys ACTH assay presumably by bridging the capture and tracer antibodies. To our knowledge, this is the first case in which the Elecsys ACTH assay yielded falsely elevated results. Regardless of the measurement system used, if there is a discordance between assay results and clinical findings, it should be considered to adopt additional procedures and/or another assay.

Key words: Adrenocorticotropic hormone (ACTH), Adrenal insufficiency, The Elecsys ACTH assay, Heterophilic antibody, Prolonged ACTH stimulation test

DESPITE advances in immunoassays, analytical errors by cross-reactions or non-specific reactions are not completely avoidable. We need to view laboratory results with caution when they are discordant with the clinical picture. In the field of endocrinology, falsely elevated levels of free thyroxine by anti-reagent or anti-analyte antibodies [1, 2] and falsely elevated PRL or TSH levels due to formation of macromolecular complexes (such as "macro-PRL" or "macro-TSH") by anti-analyte antibodies [2] are well known. In the case of ACTH, however, there are extremely few reports only on the Siemens Immulite ACTH and the Abbott Architect ACTH [3-10].

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Here we report a patient with secondary adrenal insufficiency, in whom plasma ACTH levels measured by the Roche Elecsys immunoassay were falsely elevated due to assay interference caused by heterophile anti-reagent antibodies. To our knowledge, this is the first case in which the Roche Diagnostic Elecsys ACTH assay yielded falsely high ACTH levels.

Case Presentation

The patient was a 49-year-old woman. Two years and nine months before, she developed nephrotic edema, which after renal biopsy turned out to be due to membranous nephropathy. Administration of prednisolone (PSL) was started at 40 mg/day. Thereafter, nephropathy remission was achieved and PSL dosage was gradually decreased. However, when the PSL was reduced from 5 mg/day to 4 mg/day at 14 months after initiating this drug, she began experiencing lethargy and nausea, which

worsened when the dosage was further decreased to 2 mg/day. On the basis of a plasma ACTH level of 399.1 pg/mL (87.8 pmol/L) (reference interval, 7.2 to 63.3 pg/mL) and a serum cortisol level of 3.1 μg/dL (85.5 nmol/L) (reference interval, 7.1 to 19.6 μg/dL), she was diagnosed as adrenal insufficiency, although neither hyponatremia nor hypoglycemia was observed. The PSL dosage was increased back to 5 mg/day and her symptoms were resolved. The cause of adrenal insufficiency was considered to be the suppression of endogenous cortisol production after the long-term glucocorticoid therapy. Although the reason for the elevated ACTH was unclear, it was assumed to reflect functional recovery at the pituitary, but not yet at the adrenal level. Because of her persistently high ACTH levels and malaise, which started again three months before referral, PSL was changed to a supraphysiological dose of hydrocortisone (HC). Then, she was referred to us and hospitalized for detailed evaluation of her pituitary-adrenal axis.

On admission, she was 157 cm in height and 52.2 kg in weight; her blood pressure was 120/64 mmHg, with a regular pulse of 80/min. There was no hyperpigmentation of the skin or mucosa. Blood biochemical results were as follows: plasma ACTH, 316.8 pg/mL (69.7 pmol/L); serum cortisol, 0.6 μg/dL (16.6 nmol/L); plasma renin activity, 0.5 μg/L/h; plasma aldosterone, 8.13 ng/dL (0.23 nmol/L); and serum dehydroepiandrosterone sulfate (DHEA-S), 27.30 μg/dL (0.74 μmol/L). There were no abnormalities in plasma catecholamine or the other pituitary hormone levels. The rheumatoid factor was negative, while antinuclear antibodies were positive at 1:2,560 in a speckled pattern. Magnetic resonance imaging showed normal adrenals and a slightly swollen pituitary. A rapid ACTH stimulation test with intravenous bolus injection of 250 μg synthetic ACTH (1–24) (Cortrosyn®: Daiichi Sankyo, Tokyo, Japan) revealed that the serum cortisol level did not increase sufficiently, *i.e.*, from 0.6 to 7.9 μg/dL (16.6 to 218.0 nmol/L), indicating adrenal insufficiency. By contrast, the same stimulus resulted in an appropriate response in the plasma aldosterone level from 8.13 to 23.70 ng/dL (0.23 to 0.66 nmol/L), which was incompatible with the diagnosis of primary adrenal insufficiency. Therefore, after her prescription was switched from HC to 0.5 mg/day dexamethasone, a prolonged ACTH stimulation test was performed, in which 0.5 mg ACTH (1–24)-zinc (Cortrosyn-Z®: Daiichi-Sankyo, Tokyo, Japan) was intramuscularly injected once a day over three consecutive days. The urinary free cortisol level, which was 6.6 μg/day (18.2 nmol/day) before ACTH (1–24)-zinc administration, increased to 3,108.3 μg/day (8,576.0 nmol/day) on the final day. This result ruled out the possibility of primary adrenal insufficiency [11].

Given these facts, we suspected that the immunoreactive but bioinactive ACTH molecules, such as "big ACTH," which result from incomplete cleavage of proopiomelanocortin (POMC), existed or that ACTH levels were falsely elevated owing to a substance in the specimen interfering with the ACTH measurements carried out thus far, all of which used the electrochemiluminescence Elecsys ACTH immunoassay and the Cobas 6000 analyzer (Roche Diagnostics, Basel, Switzerland).

In order to investigate these possibilities, the following procedures were tried. We examined the patient's and control plasma using the same Elecsys ACTH assay as follows: assaying samples in dilution, polyethylene glycol (PEG) precipitation, and absorption test with heterophilic blocking reagent. We also changed the assay of ACTH to another platforms. Finally, we performed gel exclusion chromatography to analyze the molecular size of immunoreactive ACTH in her plasma.

Assaying samples in dilution

For the dilution study, a plasma sample from another subject, whose ACTH level had been rendered undetectable by heat inactivation, was used as a diluent. We diluted her plasma at the various dilution rates and measured ACTH using the Elecsys ACTH assay. The scatter diagrams were made from the dilution rates and measured values, and the approximation straight lines and the coefficients of determination were calculated. In this study, the relationship between the dilution ratios and the measured values was linear ($R^2 > 0.99$) (Fig. 1).

PEG precipitation

The plasma was mixed well with an equal volume of 25% PEG fluid and centrifuged at 3,000 gravity for 5 minutes. The supernatant was measured for ACTH using the Elecsys ACTH assay. Percent of expected value was calculated as follows: [(concentration after treatment) × 2/(concentration before treatment)]. After the pretreatment with PEG, the ACTH values dropped substantially as compared to the results measured without any pretreatment (Table 1).

Absorption test with heterophilic blocking reagent

A heterophilic blocking tube (HBT) (Scantibodies Laboratory, Santee, CA, USA) was used as per the company's instruction manual. Apart from this, the analyses using adsorbent for removal of human anti-mouse antibodies (HAMA) were conducted. We mixed a plasma sample and 1 mg/mL of heterophilic blocking reagent-1 (HBR-1) (Scantibodies Laboratory, Santee, CA, USA) in a 1:1 volume ratio and incubated the mixture for 10 minutes at room temperature. After these pretreatments, the ACTH levels decreased dramatically, as compared to

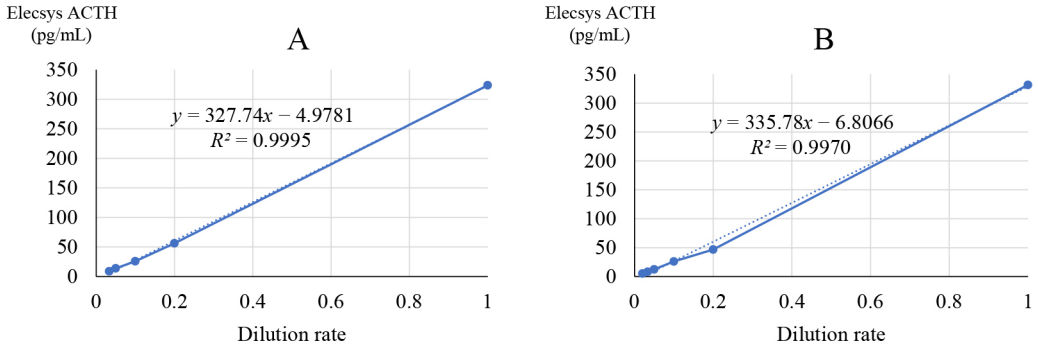

Fig. 1 Assaying samples in dilution

We diluted two plasma samples taken from our patient on different days (A and B) at the various dilution rates, and measured ACTH in the Elecsys assay. In both samples, the relationship between the dilution ratios and the measured values was linear.

Table 1 PEG precipitation and HBT processing in Elecsys ACTH

Sample	Roche Elecsys ACTH (Percent of expected value) pg/mL		
	Our case	Control 1*	Control 2*
Without any pretreatment	355.1	33.53	45.89
PEG precipitation**	103.7 (29.2%)	117.8 (351%)	121.1 (264%)
HBT processing	6.86 (1.93%)	29.29 (87.4%)	42.43 (92.5%)

* Control; plasma of healthy persons.
** As a correction, the value was multiplied by 2.

Table 2 Change of the assay to measure ACTH levels

a) Results of three different assays to measure ACTH levels without any pretreatment

Sample (Our case)	Roche Elecsys ACTH*	Siemens Immulite ACTH II** pg/mL	E-test TOSOH II ACTH***
1	348.1	31.5	Not available
2	276.0	Not available	49.6

* Roche Elecsys ACTH (ECLIA); reference interval, 7.2 to 63.3 pg/mL
** Siemens Immulite ACTH II (CLEIA); reference interval, 0.0 to 46.0 pg/mL
*** E-test TOSOH II ACTH (IEMA); reference interval, 7.7 to 63.1 pg/mL

b) Comparison of two different measurements of ACTH in absorption test of HAMA

Sample (Our case)	Roche Elecsys ACTH pg/mL	E-test TOSOH II ACTH
Without any pretreatment	392.0	51.0
Addition of HBR-1* (Percent of expected value)	55.4 (14.1%)	48.2 (94.5%)

* As a correction, the value was multiplied by 2.

the results measured without any pretreatment (Tables 1 and 2b).

Change of the assay to measure ACTH levels

We also tried another ACTH assay system, *i.e.*, the Siemens Immulite ACTH II [chemiluminescent enzyme immunoassay (CLEIA)] with Immulite 2000Xpi (Siemens Healthcare Diagnostics, Deerfield, IL, USA) and E-test TOSOH II ACTH [immuno-enzymometric assay (IEMA)] with AIA-2000 (Tosoh, Tokyo, Japan). Plasma ACTH values measured by the Siemens Immulite ACTH II and E-test TOSOH II ACTH were completely different from those by the Elecsys ACTH, but quite plausible in the light of the patient's clinical status (Table 2a). Moreover, the ACTH levels measured by E-test TOSOH II ACTH were quite consistent, regardless of whether the sample had been pretreated with HBR-1 or not (Table 2b).

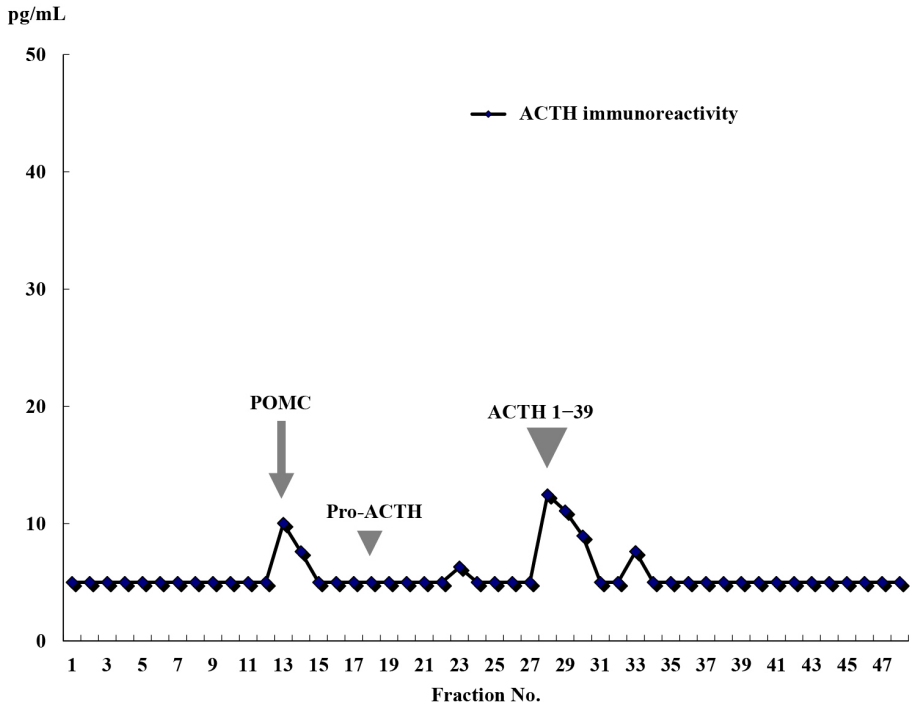

Fig. 2 Sephadex G75 gel chromatography of this patient's plasma using ACTH-RIA
Arrow indicates elution positions of POMC. Small arrowhead indicates where pro-ACTH should appear, if present. Big arrowhead indicates elution positions of ACTH (1–39). The peak at fractions 13–14 corresponded to the position of POMC but was considerably low. Therefore, it did not seem to be clinically significant.

Gel exclusion chromatography

To analyze the ACTH molecular size in her plasma, gel exclusion chromatography was performed. This procedure was described previously [12]. In brief, a 1 mL plasma sample was lyophilized, reconstituted with 0.5 mL of elution buffer (63 mM Na_2HPO_4, 13 mM ethylenediaminetetraacetic acid-2Na, 0.05% NaN_3, 0.1% bovine serum albumin, pH 7.4), applied onto a 50 cm Sephadex G75 superfine column (Amersham Biosciences Corp. Piscataway, NJ, USA), and eluted at a rate of 6 mL/hour. One-milliliter fractions were collected and subjected to radioimmunoassay (RIA).

The ACTH-RIA for each fraction was performed using rabbit antiserum against ACTH (1–24). The characteristics of the assay have been reported elsewhere [13]. In this study, each sample after chromatography was applied without dilution.

The result of this study is shown in Fig. 2. The peak at fractions 28–30 corresponded to the authentic ACTH (1–39). The peak at fractions 13–14 was considerably lower than that at the fractions 13–19 of high-molecular-weight forms of ACTH (so-called "big ACTH") presented by other cases of ectopic ACTH syndrome [14] or subclinical Cushing's disease [12], in whom a substantial amount of "big ACTH" was recognized. Accordingly, this peak at fractions 13–14 did not seem to be clinically significant and was regarded as non-specific.

From these observations, we concluded that heterophilic antibodies present in our patient's plasma interfered with only the Elecsys ACTH assay, resulting in falsely elevated ACTH levels. Thus, we decided to continue HC replacement, which was tapered thereafter. Her serum DHEA-S concentration gradually increased up to 38.60 μg/dL (1.05 μmol/L) at 7 months after discharge from hospital, which suggested at least partial recovery of ACTH secretion.

Discussion

It is extremely rare that falsely high or low ACTH levels by a substance in the specimen interfering with the ACTH measurements are confirmed. There have been only 23 such cases, to our knowledge [3-10] (Table 3). Of the 23 cases, 22 exhibited erroneously elevated ACTH levels. In some of these reports, the elevated ACTH levels measured on the Immulite platforms were proved to be false, either by pretreatment with heat-aggregated rabbit IgG, which is known to adsorb assay-interfering heterophilic antibodies [3], or by adoption of different assay platforms [4, 7-10].

In our case, the results of gel exclusion chromatogra-

Table 3 Previous reports of falsely high or low ACTH levels by a substance in the specimen interfering with the ACTH measurements

The reference number	Age/Gender	The final-diagnosis	Adopted ACTH assay method	Falsely high or low?	The examinations which revealed assay interference	Additional information
3	53/M	Low-grade focal pancreatitis (exclusion of ectopic ACTH producing tumor)	Siemens Immulite 2000	High	The pretreatment with heat-aggregated rabbit immunoglobulin	Nothing in particular
4	60/F	Cushing's syndrome due to bilateral nodular adrenal hyperplasia	Siemens Immulite 2000 Xpi	High	Change of the assay to measure ACTH levels	The use of HBT could not resolve this interference
5	36/F	Ectopic ACTH-dependent Cushing's syndrome caused by large gastric neuroendocrine carcinoma	Siemens Immulite 2000	Low	Assaying sample in dilution, HBT processing and change of the assay	Nothing in particular
6	33/F	Endocrinologically normal condition	Abbott Architect C8000	High	Assaying sample in dilution, HBT processing, PEG precipitation and change of the assay	Immunoassay interferences in the measurement of multiple hormones (TSH, ACTH, FSH, PTH, IGF-1, PRL, βhCG and Calcitonin) were proved
7	54/F	Post-hypophysectomy	Siemens Immulite 1000	High	Assaying sample in dilution and change of the assay	FSH and LH levels (Beckman Coulter DxI 800) were also falsely elevated The use of HBT revealed paradoxical over-recovery
8	54/F, 44/F, 69/F, 28/F	Incidentally discovered adrenal adenoma with subclinical hypercortisolism	Siemens Immulite 2000 Xpi	High	Assaying sample in dilution, HBT processing, PEG precipitation and change of the assay	Clinical study including the 437 consecutive patients with incidentally discovered adrenal adenomas
9	79/F, 71/F	Slight autonomous hypersecretion of cortisol by the adrenal nodule(s)	Siemens Immulite 2000	High	Assaying sample in dilution, HBT processing and change of the assay	Nothing in particular
10	43/F, 48/F, 59/M, 26/F, 46/F, 39/F, 42/F, 33/M, 77/F, 27/F, 46/M, 46/M	Cushing's syndrome due to the adrenal adenoma in 4 cases and endocrinologically normal condition in 8 cases	Siemens Immulite	High	Assaying sample in dilution, HBT processing, PEG precipitation and change of the assay	In all cases, clinically appropriate results were obtained by using the Roche Elecsys ACTH assay

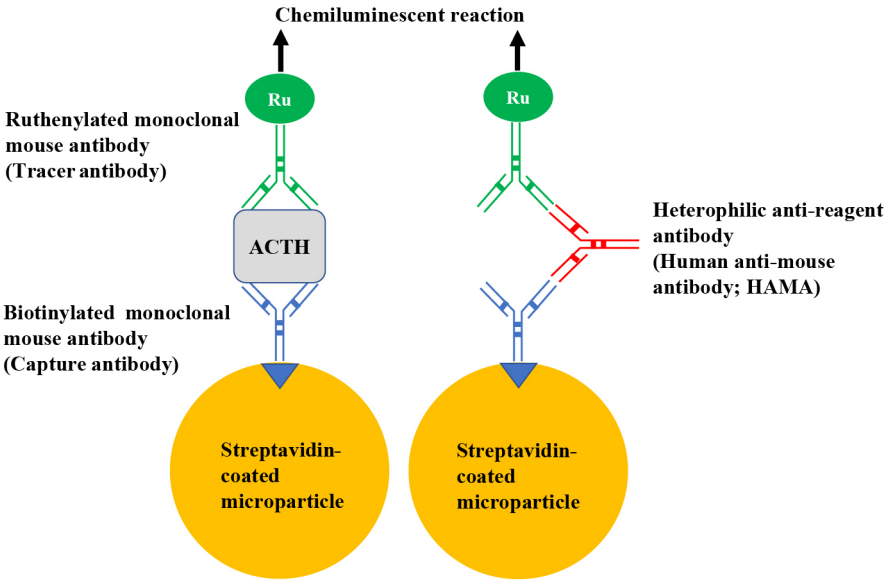

Fig. 3 Possible interference mechanism in the present case

Left panel shows the antigen-antibodies reaction in the normal specimen, and right panel indicates the cross reactions by the interference substance in our case. The heterophilic anti-reagent antibodies were presumed to bridge the capture and tracer mouse antibodies and to yield false results.

phy ruled out the possibility of the presence of big ACTH. Also, HBT pretreatment and other assay platforms revealed low ACTH levels, which were compatible with her clinical status. Therefore, we believe that the false elevations were caused by heterophile anti-reagent antibodies, which could bridge between capture and tracer antibodies (Fig. 3). The HBT contains a blocking reagent composed of specific binders which inactivate heterophilic antibodies, and is useful for detection of assay interference by such antibodies. Curiously, in plasma dilution examinations, the relationship between the measured values and the dilution rates was almost linear, which was quite unusual for a non-specific reaction caused by interfering antibodies [5]. Perhaps the heterophilic antibodies in this case might have a strong binding capacity that does not decline with dilution. Therefore, in order to exclude the possibility of antibody interference in exceptional cases like ours, it is important to not only carry out dilution examination [2], but also perform re-examinations using another assay and/or adopting additional procedures such as PEG precipitation and HBT treatment.

In all but one of the ACTH assay interference cases previously reported, the Siemens Immulite platforms were used [3-5, 7-10] (Table 3), whereas in the present case, the Roche Diagnostics Elecsys ACTH assay yielded falsely elevated ACTH levels, for the first time to our knowledge. The Siemens Immulite platforms adopt a murine monoclonal anti-ACTH antibody and a rabbit polyclonal antibody that is considered capable of reacting with unknown interference antibodies [9]. On the other hand, the Elecsys ACTH assay is an electrochemiluminescence method employing two monoclonal antibodies specific for ACTH (9–12) and for ACTH (36–39). This assay can be fully automatically performed in only 18 minutes and differs from the Siemens Immulite ACTH II and E-test TOSOH II ACTH in that it adopts mouse monoclonal antibodies for both capture and tracer. Perhaps, the interference substances in our case might be human anti-mouse antibodies (HAMA) that influence only the Elecsys ACTH assay (Fig. 3). It should be kept in mind that whatever measurement system is used, assay interference should always be suspected when there is a discordance between assay results and clinical findings. Furthermore, all the Elecsys systems, which are not limited to ACTH assay, are streptavidin/biotin-based immunoassays and use ruthenium as the luminescent material [15]. Method-specific interference caused by anti-streptavidin or anti-ruthenium antibodies has been described previously [15-19]. These interpositions tend to bring falsely decreased results in sandwich assays such as the Elecsys ACTH, and falsely elevated results in competitive assays [15, 19]. In our case, we compared the results of the Elecsys assays with those of other assays for LH, FSH, TSH, FreeT_3, FreeT_4 and cortisol, and found no differences (data not shown). Therefore, the possibility of the interference due to anti-streptavidin or anti-ruthenium antibodies in this case could be safely ruled out.

In our case, the prolonged ACTH stimulation test was

effective to definitively distinguish whether adrenal insufficiency was of primary or secondary origin (such as steroid withdrawal), because it is well known that cortisol secretion increases in response to continuous ACTH stimulation, only in secondary adrenal insufficiency. If the urine cortisol level increases two- to three-fold after continuous stimulation over three consecutive days, primary adrenal insufficiency is excluded [11]. Nowadays, however, the prolonged ACTH stimulation test is rarely performed because primary and secondary adrenal failure can usually be differentiated by basal ACTH measurement alone and ACTH (1–24)-zinc (long-acting synthetic ACTH used for continuous stimulation) is available only in the United Kingdom, Canada, Russia, Germany, Switzerland, Japan and so on. Nonetheless, the prolonged ACTH stimulation test may still be indispensable to confirm the correct diagnosis in unusual cases like ours.

This case teaches an important lesson that clinicians should be cautious about the possibility of technical errors in any measuring systems. When laboratory results and clinical findings are inconsistent, consideration of alternative examination methods and integrative assessment are of extreme importance for correct diagnosis.

Disclosure

All authors declare no conflicts of interest associated with this manuscript.

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