Wrong Biochemistry Results: Two Case Reports and Observational Study in 5310 Patients on Potentially Misleading Thyroid-stimulating Hormone and Gonadotropin Immunoassay Results

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Background: Immunoassays are used in almost all medical and surgical specialties, but they suffer from interference from proteins such as antibodies in some patients' sera. Such interferences are usually reported in the literature only as case reports after the introduction of a new assay.

Methods: We undertook a prospective observational study on 5310 patients for whom the common immunoassay tests for thyroid-stimulating hormone (TSH) and/or gonadotropins were requested. All TSH and gonadotropin results were critically assessed for a mismatch between the clinical details and analytical results to identify samples suspected of analytical unreliability. These were tested further by three approaches to screen for interference.

Results: From the 5310 sets of results, 59 patients' samples were identified as suspect and were tested further. Analytically incorrect results were found in 28 (0.53% of the total studied). The magnitude of interference varied, but in 23 of 28 patients (82%), it was considered large enough to have a potentially adverse effect on cost and/or the clinical care of these patients. Two cases, described in detail, illustrate the adverse effect of error on patient care and cost, and the second highlights the difficulties and limitations of current approaches for identifying interference and inaccuracy in immunoassays.

Conclusions: Because millions of TSH/gonadotropin tests are carried out in UK hospital laboratories alone, our data suggest that thousands of patients could be adversely affected by errors from interferences. Early identification of interference in cases with unusual results could be valuable.

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Laboratory results play a major role in guiding clinical decisions, and their integrity is subject to extensive quality-assurance procedures designed to minimize errors. However, clinicians may be unaware that laboratory data based on immunoassays are more prone to interference than are other routine tests *(1–4)*. Immunoassays are the main, and in many instances the only, analytical tools for measuring a wide range of analytes, such as hormones, tumor markers, cardiac troponin, therapeutic drugs, C-reactive protein, and microbial serology.

Interference from a component in the sample matrix, such as circulating antibodies, is specific to an individual patient, and these proteins have the potential to interfere in an unpredictable way with some (but not necessarily all) immunoassay tests performed on that patient, even when the same analyzer is used *(5)*. The inaccurate data may be reported as bona fide results because routine analytical quality-assurance checks are unable to detect such problems.

The reported prevalence of such interfering antibodies varies from 0.05% to >2% *(6–9)*. We report on our experience on the analytically, and in some cases clinically, significant inaccuracy of tests for three of the most commonly requested analytes, namely the pituitary glycoprotein hormones thyroid-stimulating hormone (TSH),[3] luteinizing hormone (LH), and follicle-stimulating hormone (FSH), in 5310 patients. As far as we are aware, this

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[3] Nonstandard abbreviations: TSH, thyroid-stimulating hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; TFT, thyroid function test; T₄, thyroxine; T₃, triiodothyronine; and CI, confidence interval.

is the first report describing a prospective observational study of the prevalence of potentially important interference in tests for these commonly requested analytes. These cases have been identified by a clinical-biochemical mismatch. We also describe two cases in some detail to highlight the insidious, unpredictable, and difficult nature of this problem.

Case Histories

CASE 1

A 48-year-old woman presented in April 1998 to her primary-care clinician with a history of tiredness, weight gain, and mental slowing. Her past medical history was unremarkable, but her mother was said to have "borderline low" thyroid tests. Initial thyroid function test results (TFTs; Abbott AxSYM) were 13.7 pmol/L free thyroxine (fT_4; reference interval, 10–25 pmol/L) and 22 mIU/L TSH (reference interval, 0.2–4.0 mIU/L). She was commenced on treatment with 25 μg/day thyroxine, but despite increasing the dose to 200 μg/day, there was no decrease in TSH (Table 1).

In July 1999, her TSH remained increased at 18.4 mIU/L, and the TFTs were reanalyzed by another assay method (Bayer ACS-180). The results confirmed the increased TSH (11.0 mIU/L) with a total T_4 of 132 nmol/L (reference interval, 50–140 nmol/L). She was referred to an endocrinologist. She appeared clinically euthyroid and vigorously denied any compliance problem. Her menstrual periods were regular.

Further investigations revealed normal full blood count and urea and electrolytes. TFTs at this time were 26.1 pmol/L fT_4, 4.9 pmol/L free triiodothyronine (fT_3; reference interval, 2.5–5.3 pmol/L), 30.7 mIU/L TSH (Abbott AxSYM) with negative thyroid peroxidase antibodies (Cambridge Life Sciences Ltd.), 139 nmol/L total T_4, 2.1 nmol/L total T_3 (reference interval, 1–2.5 nmol/L), and 20.2 mIU/L TSH (Bayer ACS-180). A trial of 250 μg of thyroxine was instigated, and the patient felt slightly better. Two months later, biochemical tests gave the following results: fT_4, 25.4 nmol/L; fT_3, 5.7 nmol/L; TSH, 23.9 nmol/L; sex hormone-binding globulin, 123 nmol/L (reference interval, 35–100 nmol/L); and thyroxine-binding globulin, 18.9 mg/L (reference interval, 13–27 mg/L).

Table 1. Case 1: effect on TFTs of increasing oral thyroxine dose.

| Date | Thyroxine dose, μg/day | Abbott AxSYM | |
		fT_4, pmol/L (9–25 pmol/L)[a]	TSH, mIU/L (0.2–4.0 mIU/L)[a]
June 1998	25	15.6	12.0
August 1998	50	17.0	8.8
October 1998	75	20.2	13.9
February 1999	100	17.9	27.6
April 1999	150	22.7	22.9
July 1999	200	27.5	18.2

[a] Reference interval in parentheses.

Further investigations were performed at her next clinical appointment and revealed normal pituitary computed tomography scan; FSH, 4.5 IU/L; LH, 86.7 IU/L; and a pituitary glycoprotein α-subunit concentration (<0.30 IU/L) within the reference interval. Three subsequent samples confirmed the same pattern, with FSH being consistently <10 IU/L and LH persistently >80 IU/L. The patient was then given a single dose of 2.0 mg of thyroxine orally, followed by serial hormone measurements over a period of 19 days. Despite clear peaks in serum fT_4 and fT_3 (exceeding the top of reference limits before gradually decreasing), the TSH remained increased throughout.

The possibility of primary hypothyroidism with TSH receptor defect or interference in TSH assays by endogenous antibodies was considered. The latter was confirmed by serial dilution in TSH-free serum, which demonstrated marked nonlinearity. The patient's thyroxine dose was decreased to 150 μg with no adverse effect, and further decreases followed.

During a period of ~2.5 years, this patient had 15 clinical consultations with her primary-care physician and hospital specialists, 77 laboratory tests, and a pituitary computed tomography scan. The patient was treated with a high thyroxine dose unnecessarily for more than 1 year. It appears that interfering antibodies in her serum affected the TSH and LH immunoassays but apparently not the FSH immunoassays. The exact nature of the interference has not been pursued.

CASE 2

An 87-year-old woman presented to her primary-care clinician with a history of tiredness and feeling low. Routine blood tests revealed normal full blood count, urea, electrolytes, and liver function tests, a fT_4 concentration of 12.3 pmol/L, fT_3 of 2.4 pmol/L, and TSH >100 mIU/L; thyroid peroxidase antibodies were negative. Reanalysis by a second method (Bayer ACS-180) demonstrated a total T_4 of 101 nmol/L, total T_3 of 1.7 nmol/L, and TSH of 8.2 mIU/L. The Bayer ACS-180 results were considered to be consistent with the clinical picture. The initial TSH value of >100 mIU/L by the Abbott AxSYM was therefore judged to be analytically suspect. The patient was not treated, and additional samples collected 6 and 8 months later gave TSH values of 48 and 34 mIU/L, respectively, by the initial method (Abbott AxSYM) but 5.3 and 3.6 mIU/L, respectively, by Bayer ACS-180. However, as part of the analytical evaluation, measurement of TSH was made by both analytical methods after serial dilution. The increased TSH value by the Abbott AxSYM (considered to be inconsistent with the clinical assessment) demonstrated excellent linearity; the lower value by the Bayer ACS-180 (considered to be acceptable on clinical grounds) revealed nonlinearity with a significant increase rather than decrease in concentration on dilution. No differences in the TSH results were

found before and after incubation with heterophilic blocking antibodies (SkyBio Ltd.).

The Bayer ACS-180 TSH result, although more compatible with circulating thyroid hormone concentrations as well as the clinical assessment, was considered to be analytically erroneous because it increased and was not linear on dilution; it therefore was disregarded. Despite excellent linearity by the Abbott AxSYM and good agreement before and after incubation with heterophilic blocking studies, the gross increase in TSH was also considered suspect and was also disregarded. In the absence of reference technology, TSH data by both methods were therefore considered insufficiently safe to be relied on for clinical decision-making. This patient's management, therefore, was clinically based aided by the assays of serum free and total T_4 and T_3.

Prospective Observational Study

AIM AND METHODS

The primary objective was to assess the impact of improved clinician/laboratory interface in identifying interference from a component in the sample matrix, such as endogenous antibodies, in routine samples submitted to a large clinical laboratory from primary- and secondary-care physicians. To achieve this, analytical results were reviewed in the context of clinical data provided on the test request card and/or additional information provided at the request of the laboratory consultant/director. Normal laboratory routine was therefore adhered to apart from even more rigorous clinical laboratory validation that was better than the usual laboratory/physician interface. Clinical laboratory validation was performed after all quality-assurance criteria had been met.

All patients' sera presented to our laboratory for TFTs and/or gonadotropins had results validated by one of us (A.A.A.I.). fT_4 and TSH were performed as first-line screens for TFTs, with additional tests initiated by the laboratory as deemed necessary. For gonadotropin requests, both LH and FSH were performed routinely. All analyses were performed by use of automated direct immunoassays.

The laboratory criteria for initiating the follow-up tests were as objective as possible and covered a variety of clinical-biochemical mismatches. These included (*a*) an endocrine profile inconsistent with the clinical summary; (*b*) an unexplained change from previously reported results; (*c*) a disproportionate increase in TSH relative to circulating serum fT_4 and fT_3; (*d*) a disproportionately low serum fT_4 concentration with normal TSH in ambulatory nonhospitalized patients on no medications; and (*e*) a disproportionate or persistent increase in one or both gonadotropins, not expected from the clinical summary. However, selection for follow-up studies was based on personal review of results and clinical information available to one of us (A.A.A.I.) rather than the strict application of an algorithm to identify mismatches.

PROTOCOL FOR ANTIBODY SCREENING

To establish the analytical integrity of the three tests used in this study in assessing interference, serum samples covering a wide range of analyte concentrations that were clinically and biochemically consistent were randomly selected. These were subjected to the following three tests, and the data were used to establish "laboratory reference ranges" of expected differences in samples assumed to be free of interference. Using the Altman–Bland plot (*10, 11*), we established the confidence intervals (CIs) of the differences for the three tests:

1. Dilution and parallelism. Samples were diluted with "analyte-free" sera to give separate twofold and fourfold (and eightfold if sufficient serum was available) serial dilutions. The undiluted and diluted samples were reanalyzed in the same run. Almost one-half of all samples, both with and without interference, had three rather than two serial dilutions.
2. Heterophilic antibodies blocking studies. Commercially available tubes (SkyBio Ltd) were used according to the manufacturer's instructions. The untreated and treated sera were then assayed in the same run.
3. Reanalysis using a different methodologic platform. Second-line analysis was made on a Bayer ACS-180 (Bayer PLC) after initial analysis on the Abbott AxSYM (Abbott Diagnostics Division).

Samples suspected of interference were routinely subjected to tests 1 and 2; test 3 was only used if both tests 1 and 2 were negative. This sequence was followed to use the two tests available locally before a sample was sent to another laboratory for analysis on a different analyzer.

STATISTICAL ANALYSIS

To simplify the statistical assessment of linearity in serially diluted samples, we back-calculated the analyte concentration after dilution by multiplying the analyte concentration by the dilution factor. The average value of the two or three diluted samples was then computed and paired with the concentration in the undiluted sample for statistical assessment. For each analyte, therefore, three sets of paired data were collated, namely (*a*) analyte concentration in undiluted sample vs average of dilutions; (*b*) analyte concentration before and after incubation with heterophilic blocking reagent; and (*c*) concentration of analyte obtained by two methodologic platforms. Paired data in serum samples with and without interference are shown in Fig. 1 for TSH. The three Altman–Bland plots were constructed by calculating the differences between each "paired result"; the signed difference was then divided by the mean value of the pair expressed as a percentage and then plotted. (The data on gonadotropins were similar to those for TSH apart from there being fewer patients identified with interfering antibodies; these plots are therefore not presented.)
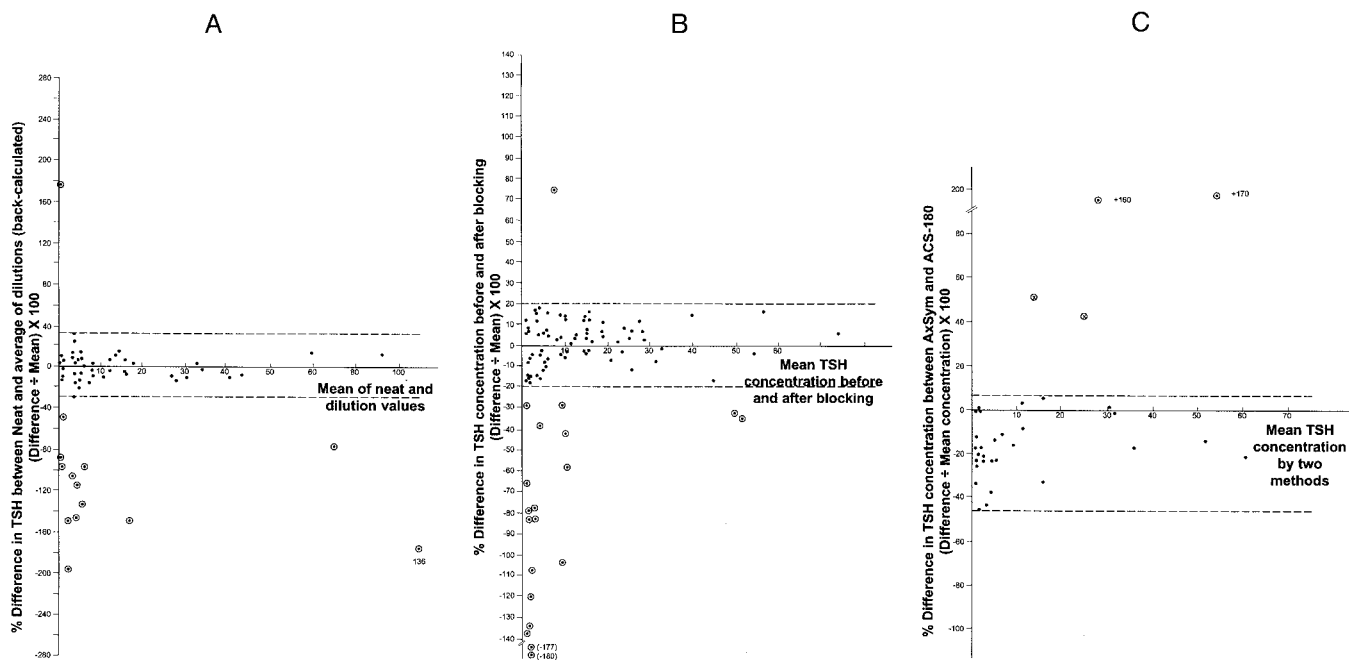
Fig. 1. Altman–Bland plots *(10, 11)* for TSH samples from patients with and without interference, showing the effect of serial dilutions *(A)* and heterophilic blocking antibodies *(B)* and comparison between Abbott AxSYM and Bayer ACS-180 data *(C)*.

■ are samples where interference was not suspected; ⊙ are those with interference. Data in panel C are from the two patients described as cases 1 and 2. The *horizontal dashed lines* represent the ranges encompassing all samples without interference.

Results

REFERENCE INTERVALS FOR DILUTION AND BLOCKING STUDIES

The investigation of samples considered to be free of interference provided the following reference intervals for dilution and antibody blocking studies. For the 41 samples used in the dilution study, the mean absolute difference was 8.9% (range, 0.0–27.1%; 95% CI, 6.8–10.9%). For the 66 samples used in the blocking study, the mean absolute difference was 8.2% (range, 0.84–20%; 95% CI, 6.8–9.5%).

The two assays used for TSH comparison had a mean bias of 19.5% (range, 5.8–48%; 95% CI, 13.4–25.5%) across a range of TSH values from 0.05 to 33.7 mIU/L.

INCIDENCE OF INACCURATE RESULTS CAUSED BY COMPONENTS IN THE SAMPLE MATRIX

After the identification of case 1 (not part of the prospective study), a total of 5310 patients' samples for TFTs and gonadotropins were assessed for potential interference in TSH, LH, and FSH immunoassays only, but not for circulating free thyroid hormones or gonadal steroids. For 282 patients, only gonadotropins were requested, and for 214 samples, both TFTs and gonadotropin requests were made. A total of 59 samples were considered to be "suspicious" during laboratory clinical validation and subjected to follow-up investigations. Twenty-eight of 59 were subsequently found to exhibit interference and, hence, inaccuracy. One of these 28 was case 2 described in detail above.

In addition to the data on the two cases described, summaries of laboratory data on the remaining 27 patients considered to have matrix interference are described in Tables 2 and 3. The clinical and biochemical details for Table 2 are provided in Table 1 of the data supplement (available with the online version of this article at http://www.clinchem.org/content/vol48/issue11/). A total of 22 of 28 patients (79%) showed interference in the heterophilic blocking studies. In 10 patients, significant changes in the hormone concentration were found with both dilution and antibody blocking follow-ups. However, some patients exhibited interference only in the antibody blocking test (12 patients) or only in dilution studies (6 patients). The serum from the patient we have described in detail as case 2 showed no change after incubation with the antibody blocking reagent and good linearity on dilution; however, it gave a different result with the alternative assay and was not linear on dilution in the second assay.

The magnitude of the interference varied from one patient to another (Fig. 1; also see Tables 1 and 3 in the data supplement); its impact on clinical management of individual patients was not investigated, being beyond the objective and scope of the present study. However, in 23 of 28 samples (82%), the interference was considered large enough to have a potentially adverse effect on cost and/or clinical care. None of the 28 patients had been treated with antibody-based drugs; the exact nature of the interference has not been pursued.

fT_4 and fT_3 were also measured in the samples treated

Table 2. Summary of the 25 cases with analytically suspect TSH results.[a]

Pattern	n	Age,[b] years	Sex, F/M	TSH,[b] mIU/L			Notes
				Initial	After blocking	On dilution	
Nonlinearity on dilution studies alone	5	52 (41–74)	4/1	1.2 (0.05–1.9)	1.2 (0.2–1.8)	7.2 (0.05–9.8)	TSH concentration increased in four patients but decreased in one
Abnormality in post-blocking studies alone	11	50 (9–69)	7/4	3.0 (0.42–88)	5.7 (0.8–61)	3.8 (0.4–89)	TSH concentration increased in nine patients but decreased in two
Abnormality in both tests	9	61 (37–77)	6/3	3.4 (0.05–42)	4.8 (0.24–99)	9.4 (0.44–254)	TSH concentration increased in both tests in all nine patients

[a] Full clinical and biochemical details are given in Table 1 in the data supplement (http://www.clinchem.org/content/vol48/issue11/).
[b] Values for age and TSH are medians (ranges).

with the blocking tubes. We found no examples of fT_3 or fT_4 being significantly different after blocking. Only TSH was measured using the second-line analyzer; we therefore have no information for fT_4 and fT_3 on a different assay platform or after serial dilutions.

Discussion

Identifying potential interference by a mismatch between clinical and biochemical data is important in planning the subsequent investigation and treatment of patients. We identified cases by clinical means and used three analytical screening tests to obtain evidence for interference. We used three different tests because one alone is inadequate; for example, linearity on dilution does not completely exclude the possibility of interference. It must be stressed that agreement in all three screening tests may not exclude interference, but the possibility of interference can be inferred by disagreement in one or more of these three relatively simple and inexpensive tests. The considerable costs and/or adverse clinical sequelae of not recognizing interference are well illustrated by case 1 as well as by

many other reported cases, some of which are quite dramatic [Ref. *(4)* and references therein].

Of the 59 patients suspected of interference, 28 showed interference leading to inaccuracy in TSH, LH, or FSH results, albeit of differing magnitude. On laboratory advice, clinicians took assay interference into account, and patients were managed with such limitations in mind as illustrated in case 2. Information on analytical interference (found or suspected) was also logged into patients' pathology files so that care can be taken in reviewing any future tests performed by any future immunoassay in that patient.

In the other 31 patients, the screening tests did not demonstrate that interference was present, although agreement in all three screening tests cannot be used to confidently exclude the possibility of interference. It is possible that the "negative" results for some of these 31 cases reflect shortcomings in the selection process used. However, comments were made on the laboratory reports pointing out the discrepancies that could be attributable

Table 3. Outcome of blocking and dilution studies in patients with suspected interference in LH/FSH profile.

Patient	Age/Sex	Initial laboratory result		Clinical summary and laboratory clinical validation	Outcome of blocking and dilution		Summary
		LH, IU/L	FSH, IU/L		Post-blocking LH/ FSH	Mean LH/FSH values from dilution	
26	50/F	11.0	99.0	Menopausal; persistently lower LH to FSH	LH, 36.0 IU/L FSH, 105 IU/L	LH, 21.5 IU/L FSH, 106 IU/L	Significant increases in LH after both blocking and twofold serial dilution. No significant change in FSH by both treatments. Interference in LH but not FSH.
27	42/F	84.0	4.4	Feeling hot; persistently disproportionate gonadotropin ratio	LH, 7.6 IU/L FSH, 7.9 IU/L	LH, 90.3 IU/L FSH, 4.6 IU/L	Blocking revealed a much lower LH and higher FSH, indicating interference in both, albeit more severe in LH. Good dilution linearity in both LH and FSH (twofold dilution).

to analytical inaccuracy and advising that care should be taken in interpreting these laboratory data.

The wider repercussion of interference in tests on clinical decision-making cannot be ignored. Consider the case of interference causing inaccuracy but still clearly increased TSH. Although this does not alter the diagnosis of hypothyroidism, it would confuse subsequent replacement therapy. In individuals with true hyperthyroidism, a similar interference would place TSH into the euthyroid range. In contrast, negative interference would suppress TSH to within the reference limits in patients with low circulating thyroid hormone concentrations, confusing the TFT profile with that of nonthyroidal illness. Moreover, interferences are not necessarily assay or analyte specific and are likely to affect other analytes measured by immunoassay in an unpredictable way in the same patient. In case 1, interference affected TSH and LH, but not FSH. This idiosyncratic interference within an individual has been reported only in a single previous case (5). It is therefore important to point out that although our studies were confined to pituitary glycoprotein hormones, it is highly likely that a similar effect could occur in an immunoassay of almost any other analyte, irrespective of its nature. Probably the most serious impact of interference on clinical decisions are immunoassays with clear "cutoff" limits, such as tumor markers (8, 12–15) and cardiac troponin (16–21).

Repeat analysis using a different platform was useful in highlighting interference in case 2 in whom the TSH results were grossly discrepant. However, in case 1, samples in two separate occasions were reanalyzed by another method. The TSH values were considered to be similar to the original results, and interference was not suspected because both pairs were increased, i.e., 18.4 and 30.7 mIU/L by AxSYM; the corresponding results by ACS were 11.0 and 20.2 mIU/L (reference values <4.0 mIU/L). However, when these pairs of results were compared with other samples free from interference measured by the two assays (Fig. 1C), it was clear that there was a discrepancy. This highlights that using results from two different assays to identify assay interference (test 3 in our screening protocol) requires background information about how the two methods usually compare with clinical samples free from matrix interference.

In our study a large number of cases (22 of 28, or 79%) showed identifiable interference in the antibody blocking studies, suggesting that matrix interference was caused by endogenous circulating antibodies. However, in 6 of 28 patients (21%), interference was demonstrated only by dilution, not by blocking studies, implicating other proteins or nonheterophilic/nontest interacting endogenous antibodies. The complex underlying mechanism(s) that cause(s) interference in immunoassays has been described in detail elsewhere [Ref. (4) and references therein].

Our study does not give information about which is the "best" single test to identify interference, but it does highlight that no single procedure can rule out interference. We suggest that all three approaches are used, with the sequence being influenced by the resources available to the individual laboratory. We believe that interference from endogenous antibodies is likely to worsen in the future because of the rapid emergence of "immune therapy" and the use of monoclonal antibodies in the diagnosis and treatment of a wide range of conditions [Ref. (4) and references therein].

In our practice we use "physiologic profiling", i.e., both fT_4 and TSH or LH and FSH combinations, rather than single analyte measurements. However, investigators using a single analyte, such as TSH or FSH, respectively, may take such limitation into account in developing their clinical protocol.

CONCLUSION

TSH and gonadotropins are among the most commonly requested tests by primary- and secondary-care physicians. Our study has shown that none of the three follow-up assays, i.e., doubling dilutions assay, blocking agents, and repeat analyses using different platforms, singular or in combination, could exclude interference with total confidence. If not recognized, interference could confuse patient management, and because the clinical sequelae may be subtle and less dramatic, interference may go unrecognized, leading to unnecessary treatment or undertreatment of many thousands of patients. Vigilance and better communication between clinician and laboratory staff could help ameliorate this insidious and highly unpredictable problem.

The use of "immunoglobulin-free" samples might provide more reliable immunoassay results because one cannot a priori predict either the extent or the nature of the antibodies causing interference. Because large-scale use of such an approach would require reengineering of immunoassay platforms, which is at present not feasible, closer clinician/laboratory interface remains a pragmatic approach to minimize this problem.

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