Interferences With Thyroid Function Immunoassays: Clinical Implications and Detection Algorithm

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ABSTRACT Automated immunoassays used to evaluate thyroid function are vulnerable to different types of interference that can affect clinical decisions. This review provides a detailed overview of the six main types of interference known to affect measurements of thyroid stimulating hormone (TSH), free thyroxine (T4) and free triiodothyronine (T3): macro-TSH, biotin, antistreptavidin antibodies, anti-ruthenium antibodies, thyroid hormone autoantibodies, and heterophilic antibodies. Because the prevalence of some of these conditions has been reported to approach 1% and the frequency of testing for thyroid dysfunction is important, the scale of the problem might be tremendous. Potential interferences in thyroid function testing should always be suspected whenever clinical or biochemical discrepancies arise. Their identification usually relies on additional laboratory tests, including assay method comparison, dilution procedures, blocking reagents studies, and polyethylene glycol precipitation. Based on the pattern of thyroid function test alterations, to screen for the six aforementioned types of interference, we propose a detection algorithm, which should facilitate their identification in clinical practice. The review also evaluates the clinical impact of thyroid interference on immunoassays. On review of reported data from more than 150 patients, we found that ≥50% of documented thyroid interferences led to misdiagnosis and/or inappropriate management, including prescription of an unnecessary treatment (with adverse effects in some situations), inappropriate suppression or modification of an ongoing treatment, or use of unnecessary complementary tests such as an I^{123} thyroid scan. Strong interaction between the clinician and the laboratory is necessary to avoid such pitfalls. ***(Endocrine Reviews 39: 830 – 850, 2018)***

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Immunoassay platforms are currently the method of choice in clinical laboratories for the measurement of thyroid function tests, notably owing to full automation, short turnaround time, and high specificity and sensitivity toward a large panel of heterogeneous molecules. However, immunoassays are vulnerable to different types of interference that can result in erroneous clinical decisions. The correct reporting of these interferences in clinical settings is essential and remains the responsibility of the clinical laboratory (1, 2). This task proves difficult because the interferences may be unique to an individual and change over time, inducing false-positive or false-negative results (3–6). Manufacturers are also aware of these interferences and are trying to limit their impact by developing different strategies (*e.g.,* by adding blockers) and by warning users via information provided in kit inserts (5). Despite these efforts, interferences still exist and need to be promptly recognized as such. Divergence between assay values and previous results obtained with the same test, as well as discrepancies with other biochemical parameters or clinical settings, are paramount in the suspicion and detection of an interference (3, 4). Good knowledge of clinical history is likewise of value because certain patients are more prone to developing an interference, be it because of recent immunization, transfusion, autoimmune disease, monoclonal therapy, or contact with pets.

In this review, we focus on interferences known to affect TSH, free T_4 (FT_4), and free T_3 (FT_3) as measured on immunoassay platforms. TSH and FT_4 are frontline parameters in the routine assessment of thyroid function, whereas FT_3 can complement the clinical workup in several specific situations (7, 8). Immunoassay technology remains the method of choice for thyroid hormone (TH) determination, with

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ESSENTIAL POINTS

- Every immunoassay is prone to interferences
- Divergence with previous results or discrepancy with other biochemical parameters or clinical settings are paramount in suspecting thyroid function assay interference
- The correct reporting of interferences is the responsibility of the clinical laboratory
- A single test rarely is sufficient to identify interferences
- At least 50% of all reported cases led to misdiagnosis and inappropriate management by the clinician
- Ongoing communication among biologists, clinicians, and manufacturers is essential to identify and prevent such interferences

major achievements made in the analytical field. The functional sensitivity of modern TSH assays has decreased from 1.0 mIU/L with first-generation immunoassays to 0.01 mIU/L with third-generation immunoassays, the influence of transport protein has been resolved by generalizing FT4 and FT3 determination assays (9, 10) and, more recently, major progress has been made in standardizing TSH and FT4 between immunoassays (11, 12).

Despite such achievements, immunoassays of thyroid function are still prone to numerous types of interference. Six main types of interference in thyroid function testing have been identified: (1) macro-TSH, (2) biotin, (3) antistreptavidin antibodies, (4) anti-ruthenium (-Ru) antibodies, (5) TH autoantibodies (THAAbs), and (6) heterophilic antibodies. Figure 1 depicts, in a simple way, their main sites of interference in two-site and competitive immunoassays.

In this systematic review, we describe the most essential thyroid interferences encountered in clinical laboratories, propose an algorithm for identifying them, and evaluate the clinical impact of these interferences. To this end, >100 articles published between 1981 and 2017 were reviewed.

Macro-TSH Interference

Macro-TSH is a large circulating form of TSH composed of monomeric TSH complexed with autoimmune anti-TSH antibodies. It can be detected on gel filtration chromatography (GFC) with a prevalence ranging from 0.6% to 1.6% (13–15). Absorption with Protein G Sepharose and chromatography studies have demonstrated that macro-TSH is mostly composed of IgG-bound TSH (13–18). Unlike TSH, which is a small bioactive hormone of 28 kDa easily filtered by the kidney, macro-TSH is a large molecule of at least 150 kDa that likely accumulates in the circulation, resulting in measurements indicating falsely increased TSH levels (13–17). Like macroprolactin (macro-PRL), macro-TSH is currently considered to be inactive. It is confined to the intravascular compartment because of its high molecular weight, and autoantibodies bound to TSH may prevent the activation of TSH receptors due to steric hindrance (13, 14, 19).

Currently, none of the available two-site immunometric assays used for TSH testing can completely discriminate macro-TSH from bioactive free TSH, even if some platforms are more sensitive to its presence (*e.g.,* Cobas analyzer, Roche Diagnostics, Rotkreuz, Switzerland) than others (*e.g.,* Architect analyzer, Abbott, Chicago, IL) (13, 15, 17, 20). Macro-TSH thus can lead to falsely high TSH results, the interpretation of which can be challenging for the clinician. The ideal immunoassay should detect only bioactive TSH and should not cross-react with macro-TSH. Yet, this ideal assay still does not exist (14–21).

Case reports in the literature typically show markedly elevated TSH with normal FT4 and FT3 levels (15–18). This biological feature is commonly encountered in subclinical hypothyroidism, as well as in less common situations such as malabsorption of L-thyroxine, use of certain drugs (*e.g.,* amiodarone, lithium), TSH resistance, biologically inactive TSH, and nonthyroidal illness during the recovery phase (8, 16, 22) (Table 1).

Mills *et al.* (15) used a cutoff of 10 mUI/L to suspect the presence of macro-TSH. A TSH concentration >10 mUI/L along with normal thyroid hormones could be proposed, therefore, to screen for the presence of macro-TSH. This cutoff is not perfect, however; some macro-TSH cases have been reported with only a slight elevation of TSH (*e.g.,* 5.1 and 9.0 mUI/L) (14, 25). Hence, interference should be suspected in a patient with isolated TSH elevation (typically markedly elevated), with THs in the upper half of the normal range, and without signs or symptoms of thyroid dysfunction.

The serum of the patient can be diluted with the diluent provided by the manufacturer. An increased recovery of diluted samples showing nonlinearity may be indicative of macro-TSH presence (16, 18, 25). It should be noted, however, that the dilution procedure is neither specific nor sensitive. Lack of parallelism can be encountered with other interfering antibodies (*e.g.,* heterophilic antibodies, rheumatoid factor, anti-Ru antibodies) (16, 17, 27, 28), and several studies have

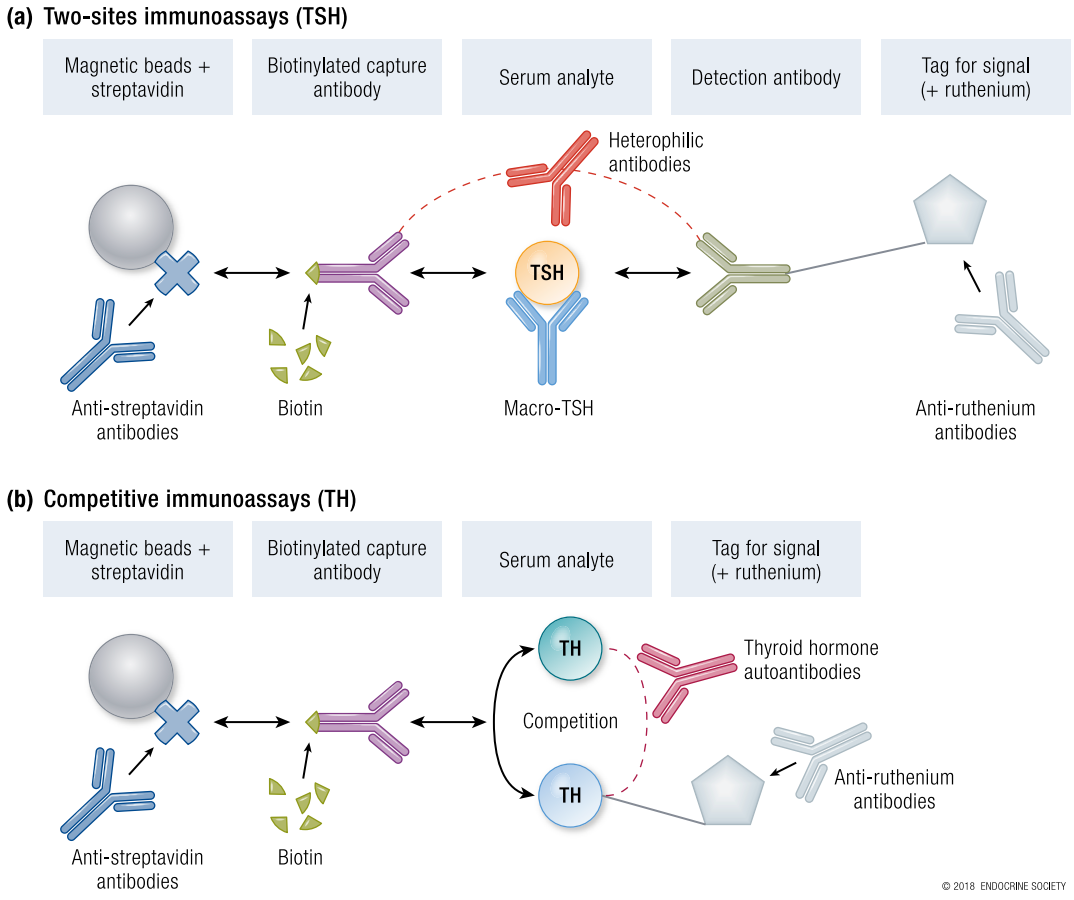


Figure 1. The six primary types of interference and the main sites affected in both two-site and competitive immunoassays.

shown a normal dilution pattern in the presence of macro-TSH (14, 23, 29).

The wide and easy use of the polyethylene glycol (PEG) precipitation procedure to screen for macroprolactinemia in hyperprolactinemic patients has also been transposed to macro-TSH detection (14). Multiple PEG precipitation procedures are available, with percent recovery typically performed. The presence of a high-molecular-weight interfering substance such as macro-TSH should be suspected if TSH recovery is low (30–34). Although several authors have used a 40% cutoff for macro-PRL (26–31), others have proposed a lower cutoff of <20% or <25% for macro-TSH (15, 16, 25). However, concern has been raised in the literature concerning the use of recovery calculation (35, 36). In 21 patients with substantial macroprolactinemia according to recovery calculation, nine cases of true hyperprolactinemia were confirmed on the basis of persistently high post-PEG prolactin concentrations (35). Therefore, recovery calculation can lead to mismanagement of thyroid conditions in certain patients, and normalization of hormone concentration after PEG precipitation should also be taken into account. In this context, the reference range provided by manufacturers cannot be used, because a fraction of ~25% of free analyte is coprecipitated upon PEG precipitation (37), and adjusted, post-PEG reference ranges must be established for each immunoassay, because the susceptibility to macrocomplexes varies between platforms (13, 15, 16, 18, 36–38). This approach may reasonably be used for macro-TSH screening. Indeed, Hattori *et al.* (14) found in some patients that the free and bioactive TSH levels may still be elevated in macro-TSH presence. These patients were likely to exhibit both macro-TSH and primary hypothyroidism and were treated as having such.

Even if the PEG precipitation procedure is convenient and may be used as a screening test for macro-TSH presence, an increase in globulin concentration can augment the fraction of precipitated TSH, thus leading to misclassification (14, 15). The preferred method for identifying macro-TSH remains GFC, and low recovery after PEG treatment should always be confirmed by GFC. Of 117 patients with low PEG recovery of TSH (<25%), only seven had evidence of high-molecular-mass TSH (>100 kDa) on GFC (14). Likewise, using GFC, another study confirmed macro-TSH presence in only three of 15 patients with low PEG recovery (<25%) (15). Nevertheless, GFC is costly, not widely available, and can confound macro-TSH with human anti-mouse antibodies (HAMAs) because HAMAs elute at the same position as

γ-globulin (>150 kDa) and are also precipitated after the PEG procedure. GFC confirmation testing, therefore, always should be performed along with screening for HAMAs (13, 14, 17). The possibility of a false-negative value due to near-complete dissociation of macro-TSH complexes on the chromatography column might account for rare cases in which low PEG recovery is associated with apparent absence of macro-TSH on GFC.

Incubating serum supposed to contain interference for 4 hours with a serum from a patient known to exhibit hypothyroidism (*i.e.*, high TSH levels) in a 1:1 ratio enables differentiation between macro-TSH and heterophilic antibody interference. Decreased recovery after incubation suggests excess TSH binding capacity (*i.e.*, macro-TSH), which is usually not found with heterophilic antibody interference (16, 18, 39).

Hormone replacement therapy (HRT) is indicated when TSH concentration is >10 mIU/L in subclinical hypothyroidism and >2.5 mIU/L if the patient is pregnant (40). However, several cases of macro-TSH described in the literature have been misclassified and the patients received inappropriate HRT (Table 1). Therefore, correct macro-TSH diagnosis is clinically important; HRT in patients with subclinical hypothyroidism is needed only in those with elevated free TSH concentrations (14). Transplacental transfer of macro-TSH has been documented as causing falsely elevated TSH in the neonate. The clearance of macro-TSH from the circulation takes several months and may have important consequences on neonatal screening programs (18, 24, 29). Interestingly, Hattori *et al.* (14) showed that L-thyroxine treatment decreased high TSH concentration due to macro-TSH from 499.4 to 90.5 mUI/L in a patient. Once the macro-TSH was identified by further investigations, the treatment was stopped and the TSH level returned to very high levels. This case report suggests that macro-TSH may respond to L-thyroxine (14), as may respond to dopamine agonist treatment (41). Therefore, the decrease of TSH after such treatment does not exclude the presence of macro-TSH.

Biotin Interference

Biotin (or vitamin H, B7, or B8) is a small (244.3-Da), soluble, essential decarboxylase enzyme cofactor synthesized by bacteria in the gut and directly bioavailable from food intake. Adequate intake has been evaluated to be 30 to 35 μg/d in adults and 5 to 25 μg/d in children (42–44). Recently, high biotin doses (100 to 300 mg/d) have been successful in the treatment of progressive multiple sclerosis in a pilot study and in a randomized, double-blind, placebo-controlled study (45, 46). Biotin is used in rare metabolic disorders (*i.e.*, biotinidase deficiencies and propionic acidemia at 10 to 40 mg/d) and is advertised as a dietary supplement for

Table 1. Macro-TSH Interferences as Reported in the Literature

Author (Ref.)	Analyte Affected (mIU/L)	No.	Clinical Consequence	Methodology Used To Detect Interference[a]
Spitz *et al.*, 1981 (23)	TSH ↑ (40–115)	1	TRH, levodopa, dexamethasone	**AC**, dilution test, **GFC**
Tamaki *et al.*, 1995 (24)	TSH ↑ (16.4–53.9)	3	ND	**Method comparison, binding of ^{125}I-labeled TSH, incubation with high TSH sample, GFC[b], AC**
Halsall *et al.*, 2006 (18)	TSH ↑ (213–308)	2	ND	**Method comparison, dilution test, adsorption of serum IgG, GFC, incubation with high TSH sample**
Mendoza *et al.*, 2009 (17)	TSH ↑ (38.1)	1	No	Method comparison, **dilution test**, blocking antibodies, **SEC**
Verhoye *et al.*, 2009 (25)	TSH ↑ (5.1–22)	3	Rx L-thyroxine (2 patients/3)	**PEG**, HBT, RF, **method comparison** (1 patient/3), **dilution test** (2 patients/3), **protein A absorption**, **GFC**
Sakai *et al.*, 2009 (26)	TSH ↑ (96–274)	1	ND	**PEG, method comparison, GFC, adsorption of serum IgG**
Rix *et al.*, 2011 (20)	TSH ↑ (≈50–103)	2	No	**PEG, method comparison, GFC**
Loh *et al.*, 2012 (16)	TSH ↑ (232)	1	No	**Method comparison**, **dilution test**, RF, **PEG, HBT, incubation with high TSH sample, GFC**
Mills *et al.*, 2013 (15)	TSH ↑ (10.2–33.6)	3	ND	**PEG, GFC, method comparison**
Hattori *et al.*, 2015 (14)	TSH ↑ (9.0–716)	11	Rx L-thyroxine (6 patients/11)	**PEG, GFC, adsorption of serum IgG**, dilution test

Abbreviations: AC, affinity chromatography; HBT, heterophilic blocking tube; ND, not determined; PEG, polyethylene glycol precipitation procedure; RF, rheumatoid factor; Rx, prescription of; SEC, size exclusion chromatography.

[a]Text in boldface indicates a test that was in favor of interference in the corresponding report.

[b]Not considered macro-TSH according to the definition found in the literature (>150 kDa).

alopecia or to improve nail and skin texture (≤20 mg/d) (47–52).

The high affinity of the noncovalent biotin-streptavidin interaction has been extensively used in two-site and competitive *in vitro* immunoassays as an immobilizing system (42, 53, 54). For example, in 2017, >50% of all immunoassays available in France were using this immobilization system to assess TSH, FT4, and FT3 values (55). Moreover, Holmes *et al.* (53) recently reviewed the current manufacturers' instructions for 374 methods used by eight of the most popular immunoassays and found that 59.1% were biotin based. Even if the prevalence of biotin interference is currently not known, the scale of the problem seems enormous given the high frequency of testing for thyroid dysfunction.

Interestingly, biotin has been reported to act as an interfering factor in certain immunoassay platforms (49, 54, 56, 57). In TSH sandwich assays, excess biotin displaced biotinylated antibody-antigen complexes from streptavidin-coated microparticles, resulting in falsely low TSH levels (as the assay signal is directly related to TSH concentration). In contrast, in competitive assays of FT4 and FT3, excess biotin caused overestimation of both hormones (as the signal is inversely proportional to hormone concentrations).

It is essential to note that the impact of biotin is directly related to the type of platform used (54, 58, 59). In Roche platforms, TSH, FT4, and FT3 may be affected by excess biotin. In Ortho Clinical Diagnostics platforms (Raritan, NJ), only TSH can be decreased because FT4 and FT3 do not use the biotin-streptavidin interaction. The opposite is true on Beckman Coulter Diagnostics platforms (Brea, CA), in which FT4 and FT3 can be elevated, whereas TSH is not affected (53, 54, 60). Interestingly, the Centaur FT4 platform (Siemens Healthcare, Erlangen, Germany) uses a preformed streptavidin-biotin complex not sensitive to the presence of biotin (53). Abbott and DiaSorin (Saluggia, Italy) immunoassays are also not affected by biotin, because the biotin-streptavidin immobilization system is not used for TSH, FT4, and FT3 measurements. Therefore, one of these last three platforms may represent the method of choice for indirectly identifying biotin interference.

The biochemical results obtained in patients taking biotin may erroneously affect the evaluation of thyroid status in different ways on different platforms. Hence, endogenous or exogenous hyperthyroidism may be suspected when hormones are measured on the Roche and Siemens platforms, subclinical hyperthyroidism or any other cause of isolated TSH diminution may be mistakenly diagnosed on the Ortho platform, and resistance to TH or drug interference (*e.g.,* amiodarone, heparin) may be evoked on the Beckman Coulter platform (8–22). It is crucial to bear in mind that the clinical presentation of hyperthyroidism may overlap with several features of neurometabolic disorders, conditions that are treated with high biotin doses (61). Furthermore, the setting can be even worse, because anti-TSH receptor antibodies may wrongly show up as positive due to the biotin presence (49, 54, 56, 58, 62–64).

The extent of biotin interference depends on several factors, such as sample volume (the lower the volume, the lower the biotin concentration), sandwich or competitive assays (excess antibody reagent in two-site immunoassays), one-step or two-step format, and wash or no-wash. Manufacturers often provide the biotin cutoff point above which interference may be observed. It remains difficult, however, to evaluate which daily doses these cutoffs correspond to. Moreover, these cutoffs have been determined *in vitro* and may thus not translate to *in vivo* conditions (54–62).

Biotin interference in immunoassays is not expected with normal dietary intake of biotin, with interfering doses varying from 1.5 to 300 mg/d as reported (56, 60, 63). Therefore, a practical and useful way to identify this interference is to address the question of whether the patient is taking biotin. The problem is that biotin is not always considered a medication or not necessarily documented on dietary supplements designed to improve the quality of hair, skin, or nails (52, 62, 63). If this information is missing or denied and a biotin interference still suspected, a dilution test with the manufacturer's diluent or a comparison with another method not using the biotin-streptavidin interaction can be used (46, 52, 54). A washout period may be advisable to be free of this interference. Several authors have reported different washout periods of 8 hours, 16 hours, 25 hours, 2 days, 3 days, or even more according to others, rendering the implementation of washout guidelines problematic (47, 49, 54, 56, 58, 59, 63, 65).

Use of streptavidin beads has been proposed to bypass the controversy about biotin washout periods (66–68). Briefly, the sample potentially containing biotin is incubated with streptavidin beads (recycled from the manufacturer's kits) and then reassayed on the same platform. If biotin is present, a substantial change from baseline is expected. This method avoids any interruption in biotin treatment and does not require a second blood sample.

Clear identification of biotin interference is important because this likely avoids unneeded repeated blood tests, referrals to specialists, delayed therapy, unnecessary imaging, stress for the patients, or the initiation of unsuitable treatments like methimazole (54, 57, 58, 61, 64, 69, 70) (Table 2).

It should also be remembered that THs are not the only parameters that can be affected by biotin. Other compounds such as troponin I, 25-hydroxyvitamin D, parathyroid hormone, estradiol, testosterone, vitamin B12, luteinizing hormone, and prostate-specific antigen may likewise be affected, with harmful clinical repercussions possibly ensuing (54–59, 72–75).

Anti-Streptavidin Antibody Interference

The prevalence of anti-streptavidin antibody interference affecting thyroid function tests has not been studied in the literature, to our knowledge, but it seems to be lower than that of biotin, because the number of published interferences is very low.

Streptavidin is a protein produced by *Streptomyces avidinii*. It has the ability to bind biotin with very high specificity and affinity (affinity constant, 10^{15}L/mol) (42). As mentioned in the preceding discussion of biotin interference, the biotin-streptavidin interaction has been extensively used in sandwich and competitive *in vitro* immunoassays as an immobilizing system (42–54). Like biotin, anti-streptavidin antibodies do cause interference that may result in disease mismanagement (Table 3).

Anti-streptavidin interference shares multiple features with biotin interference, because TSH tends to be low, whereas FT4 and FT3 levels are more likely to be elevated on platforms using biotin-streptavidin complexes. However, sandwich immunoassays (measuring TSH) seem to be less affected than competitive assays (measuring FT4 and FT3), and washout periods are thus not useful, because anti-streptavidin interference is endogenous and can persist for a long time (*e.g.,* at least 18 to 24 months) (54, 76, 77).

Challenging the patient's serum with another platform that does not use the biotin-streptavidin interaction (*e.g.,* DiaSorin, Abbott) represents a valuable test for identifying this interference. The PEG precipitation procedure and dilution test have also been useful for indicating this interference in the past (76–78). Incubation of the patient's serum with streptavidin-linked agarose offers another option, though this method is not widely available (76). Using the manufacturer's streptavidin beads, therefore, may be preferred in routine practice (66–68). Although these methods have been validated for biotin interference, they could likewise be transposed to the screening for anti-streptavidin antibodies (79). In all published cases, sending an aliquot to the manufacturer proved very effective for identifying the interference (76–79).

It should be noted that anti-streptavidin antibodies can interfere with anti-TSH receptor measurement and lead to a misdiagnosis of Graves' disease (77). In the five cases reported in the literature, two patients received antithyroid drugs. The first subject took this treatment of 3 months, whereas the other had symptoms of hypothyroidism (76, 77) (Table 3).

Anti-Ru Interference

The prevalence of anti-Ru interference has been estimated to range from <0.1% to 0.24% (80, 81). Ru (^{44}Ru) is a chemical element and rare transition metal belonging to the platinum group. It is mainly used as a chemical catalyst in electrical contacts, thick-film chip resistors, and platinum alloys. Ru may also be found in the food chain and clothing residues (82). In addition, Roche Diagnostics has extensively used Ru as a label in its immunoassays based on electrochemiluminescence technology. Applying a voltage to an electrode induces the chemiluminescence reaction: the Ru-$(bipyridyl)_3^{2+}$ and tripropylamine are excited and form Ru-$(bipyridyl)_3^{3+}$. The tripropylamine then acts as a reducing agent, enabling the Ru complex to return to its basal state with an emission of light [Ru-$(bipyridyl)_3^{2+*}$ → Ru-$(bipyridyl)_3^{2+}$]. The amount of light emitted during electrochemiluminescence is inversely proportional to the FT4 or FT3 concentration in the sample in a competitive assay and directly proportional to TSH level in a one-step sandwich assay, according to the manufacturer.

Ru interferences were first described in 2007 (80, 81). After the introduction of the FT3 assay by Roche Diagnostics, Sapin *et al.* (81) reported several observations of elevated FT3 concentrations not accompanied by the expected TSH suppression. Because the occurrence of such a thyroid function test pattern proves rare, the presence of an interfering factor was therefore investigated (80, 81). In 15 suspected interfering samples, two were slightly positive for anti-T3 antibodies, with seven sent to Roche for further analysis. Five of the seven samples were found to contain anti-Ru antibodies (bound to the ruthenylated anti-T3 antibody). The fact that only FT3 was sensitive to this interference may be accounted for by a lower Ru-labeled antibody concentration used in FT3 assays. Due to this interference, Roche Diagnostics in 2006 added a new blocking protein (free Ru crosslinkers) to FT3 assays, and Sapin *et al.* (81) found that this new formulation decreased the number of false-positive results in most samples.

The same year, Ando *et al.* (80) reported three similar cases concerning falsely high FT3 levels in three euthyroid patients. FT3 normalization occurred in two patients at the exact same time that Roche Diagnostics upgraded their FT3 assay, while producing second-generation tests designed to minimize nonspecific activity against the Ru crosslinker complex. The elevated FT3 values were actually due to nonspecific activity against the Ru crosslinker complex [tri$(bipyridyl)_3^{2}$]. Ruthenylated anti-T3 antibody and the Ru crosslinker complex may therefore be targets of anti-Ru antibodies (80, 81). PEG precipitation was useful for decreasing (or normalizing) the signal in these patients, suggesting that the interfering agent may be formed of immunoglobulins. In addition, using an alternative non-Ru method yielded lower FT3 results (80).

In 2009, Heijboer *et al.* (83) described anti-Ru interference in the FT4 assay in two euthyroid patients. Once again, comparison with a non-Ru method and collaboration with the manufacturer were decisive in further characterizing this interference.

"Anti-Ru interference may be more heterogeneous in their presentation than biotin, antistreptavidin antibodies, and macro-TSH interferences."

McKillop *et al.* (84) reported 33 cases of elevated FT4 values, which were challenged and proven to be normal with another method not using Ru. Interestingly, only one case of anti-Ru interference was reported after the release of the next-generation assay for FT4 at the end of 2008. The introduction of next-generation assays clearly reduced susceptibility to anti-Ru interference, though not in all cases (80, 81, 83, 84). The proposed mechanism of interference consisted of decreased signal owing to inhibited (FT3–anti-FT3) or (FT4–anti-FT4) binding, resulting in falsely elevated FT4 and FT3 concentrations (80–82).

In 2011, Buijs *et al.* (82) described that anti-Ru interference likely affected TSH in four patients. Analysis of these samples with an alternative method yielded completely normal results. Additional investigations performed by the manufacturer with a research conjugate less sensitive to anti-Ru antibodies demonstrated the presence of anti-Ru antibodies. Anti-Ru antibodies affecting T4 or T3 have been less frequently reported in the literature (27).

Although decreased TSH and/or elevated FT4 or FT3 levels were reported to occur more often (20 of the 22 cases reported), anti-Ru interferences may also induce elevated TSH and decreased FT4 or FT3 levels. We recently described falsely decreased FT4 and FT3 levels in a healthy 35-year-old woman (27), and Gessl *et al.* (28) reported a falsely elevated TSH value that could have been confused with macro-TSH. Anti-Ru interferences, therefore, may be more heterogeneous in their presentation than biotin, anti-streptavidin antibody, or macro-TSH interferences.

Although numerous methods have proven useful for detecting anti-Ru antibodies, a comparison with an alternative method that does not use the Ru label, along with dispatching an aliquot to Roche Diagnostics, appears optimal for fully characterizing these interferences. PEG precipitation has also been reported to be effective, but not in all situations (27, 85). The nature of the interfering agent has been identified as an anti-Ru antibody in several papers, whereas others have failed to clearly identify the source of the

Table 2. Biotin Interferences Affecting Thyroid Hormone Measurements as Reported in the Literature

Author (Ref.)	Analyte(s) Affected	No.	Manufacturer	Clinical Consequence	Methodology Used To Detect Interference[a]
Henry *et al.*, 1996 (47)	TSH ↓ FT4 ↑	1	Boehringer Mannheim	Delay in treating hypothyroidism	**Method comparison**
Kwok *et al.*, 2012 (60)	TSH ↓ FT4/3 ↑	1	Roche	No	**Dilution test, method comparison**
Wijeratne *et al.*, 2012 (49)	TSH ↔ FT4/3 ↑	2	Beckman	ND	HBT, **biotin withdrawal**
Barbesino *et al.*, 2016 (62)	TSH ↓ FT4 ↑	1	Roche	Scan with I^{123}	**Medication anamnesis, biotin withdrawal**
Minkovsky *et al.*, 2016 (69)	TSH ↓ FT4 ↑	1	Roche	Rx atenolol, scan with I^{123}	**Method comparison, HBT, biotin withdrawal**
Elston *et al.*, 2016 (56)	TSH ↓↔ FT4 ↑	1	Roche/Beckman[b]	No	**Biotin withdrawal**
Kummer *et al.*, 2016 (61)	TSH ↓ FT4 ↑	6	Roche	Rx antithyroid drugs (3 patients/6)	**Biotin withdrawal**
Seaborg, 2016 (71)	TSH ↔ FT4/3 ↑	1	ND	ND	**Medication anamnesis**
Bülow Pedersen *et al.*, 2016 (70)	TSH ↓ T4/3 ↑	1	Roche	Thyroid gland ultrasound	**Biotin withdrawal**
Batista *et al.*, 2017 (63)	TSH ↓ FT4/3 ↔	1	Ortho	Scan with I^{123}	**Biotin withdrawal**
Trambas *et al.*, 2017 (72)	TSH ↔ FT4/3 ↑	1	ND	ND	**Method comparison**
Willeman *et al.*, 2017 (65)	TSH ↓ FT4 ↑	1	Siemens	Delay in alemtuzumab administration	**Anamnesis of dietary habits**
De Roeck *et al.*, 2017 (58)	TSH ↓ FT4/3 ↑	1	Siemens	No	**Method comparison, biotin withdrawal**
Al-Salameh *et al.*, 2017 (64)	TSH ↓ FT4/3 ↑	1	Roche	Rx carbimazole 40 mg/d	**Method comparison, medication anamnesis**
Ranaivosoa *et al.*, 2017 (59)	TSH ↓ FT4↑	2	Roche[b]/Beckman	No	**Method comparison**, HBT, **dilution test, medication anamnesis, biotin withdrawal**

Abbreviations: HBT, heterophilic blocking tube; ND, not determined; Rx, prescription of.
[a]Text in bold indicates a test that was in favor of interference in the corresponding report.
[b]Underlined text corresponds to the Beckman manufacturer. The TSH is normal as measured with a Beckman analyzer.

Table 3. Anti-Streptavidin Antibodies Affecting Thyroid Hormone Measurements as Reported in the Literature

Author (Ref.)	Analyte(s) Affected	No.	Clinical Consequence	Methodology Used To Detect Interference[a]
Rulander *et al.*, 2013 (76)	TSH ↓ FT4 ↑	1	Rx methimazole, symptoms of hypothyroidism	**Dilution test**, HBT, HAMA blockers, **protein A-linked Sepharose, method comparison, aliquot to Roche Diagnostics**
Peltier *et al.*, 2016 (77)	TSH ↓ FT4/3 ↑	1	Rx thiamazole 10 mg for 3 mo	**Method comparison**, HBT, **PEG, aliquot sent to Roche Diagnostics**
Harsch *et al.*, 2017 (78)	FT4/3 ↑	1	ND	**Method comparison, aliquot sent to Roche Diagnostics**
Favresse *et al.*, 2017 (79)	TSH ↓↔ FT4/3 ↑	2	No	**Method comparison, dilution test**, HBT, **aliquot sent to Roche Diagnostics, streptavidin beads**

Abbreviations: HBT, heterophilic blocking tube; ND, not determined; Rx, prescription of.
[a]Text in bold indicates a test that was in favor of interference in the corresponding report.

interference (27, 86, 87). According to Zaninotto *et al.* (87), the observation that other hormones were not affected (*i.e.*, follicle-stimulating hormone, luteinizing hormone, and testosterone) suggested that interference was not due to anti-Ru antibodies. However, Buijs *et al.* (82) studied the influence of anti-Ru antibodies on other analytes, demonstrating their impact in α-fetoprotein, troponin, and progesterone assays, and Ando *et al.* (80) found a collateral effect on cholesterol. Therefore, we do not recommend ruling out the presence of anti-Ru antibodies solely based on the absence of effects on other analytes measured on the same platform. Screening for anti-Ru interference is crucial to limit unnecessary additional tests, referrals to endocrine units, or the prescription of inappropriate drugs (28, 80, 82, 85, 86) (Table 4).

TH Autoantibody Interference

Along with antibodies to thyroglobulin, microsomal thyroid peroxidase, and TSH receptor, THAAbs (mostly against T_4 and T_3) have similarly been described. Discovered in 1956, anti-T_4 and anti-T_3 THAAbs are the only ones that interfere in thyroid function tests (6, 88). For the most part, THAAbs are IgG isotypes with a polyclonal autoreactive response and are more prevalent in patients with autoimmune disorders (6, 89). Antibodies to thyroglobulin or thyroid peroxidase have been found in THAAbs-positive samples in up to 80% to 100% of cases (6, 90, 91). Although the prevalence of THAAbs in the general population is low [1.8% in the general population (92)], it increases up to 40% in autoimmune thyroid diseases (6–89). Screening for THAAbs should thus be performed in patients with autoimmune disorders if any interference is suspected (6). In addition, the real prevalence of THAAbs may be underestimated because they are not routinely assayed and can appear transiently in serum (93).

In the absence of THAAbs, the labeled tracer and free hormones in the sample compete for binding sites on the capture antibody. In the presence of anti-T_4 and anti-T_3 THAAbs, however, autoantibodies may bind to both the measured analyte and labeled tracer, thereby skewing the true concentration of THs (6). In one-step immunoassays, the patient's serum and labeled hormone analog are added to the reaction chamber at the same time and compete for the solid-phase antibody. The unbound material is then washed away, with only the bound analog measured. THAAbs bind to analogs because they are less available for competition. The signal, therefore, is reduced, yielding a falsely elevated hormone value (free and total hormone concentration), given that there is an inverse relationship between signal and analyte concentration (90–94). Assays in which there is no contact between the patient's serum and analog tracer (*i.e.*, two-step assays) are considered insensitive toward these autoantibodies (6, 90, 95, 98). Therefore, in theory, only one-step immunoassays are likely affected by THAAb interference [*e.g.*, Immulite 2000 and 2500 (Siemens Healthcare), Advia Centaur (Siemens Healthcare), Tosoh AIA 1800 (Tosoh, Tokyo, Japan)], and comparing these results against a two-step immunoassay [*e.g.*, Abbott AxSYM or Architect, Beckman DXI 800 or Access (Beckman Coulter), Immunotech radioimmunoassays, RIA-gnost (Cisbio Bioassays, Codolet, France)] is most probably the first valuable option. However, in practice, it should be noted that several one-step immunoassays are not sensitive toward THAAbs, whereas some two-step assays might be affected by their presence (90, 94, 96, 98). The nature and heterogeneity of the tracer, method of detection, and affinity of the antibodies may account for this phenomenon, at least to some extent (90, 94, 96, 98). If available, a comparison against equilibrium dialysis is the best choice (98, 99).

The treatment of serum with protein G (or protein A) Sepharose beads may likewise prove useful, given that THAAbs are primarily composed of IgG subclasses (6). The dilution test may likewise be used in some cases (100), yet it should not be used alone,

because several authors have reported that it may yield linear results (101). A more complex, specific method for identifying THAAbs is radioimmunoprecipitation (6, 89, 102). In short, the patient's serum is incubated with radiolabeled hormones (or analogs), while the immune complexes formed are precipitated with PEG. The radioactivity of the precipitate is then measured and compared against the total amount of radioactive label added (bound tracer divided by total tracer, reported as a percentage). Usually, 5% radioactivity is found in normal sera. Even if proven valuable in most cases, this method is not easy to perform, because it is based on using and detecting radioactivity. For this reason, several authors have proposed the much easier PEG precipitation method for assessing any posttreatment decreases in hormone levels (101).

THAAb interference may persist for several months or even years (94, 101, 103, 105). A relationship between the initiation or cessation of treatment (*e.g.*, methimazole for Graves disease) and the development or disappearance of THAAbs has been noted in the past (106). In patients known to exhibit THAAbs, TSH measurement provides the most reliable thyroid function test (94–98). As seen with other interferences, not recognizing THAAbs may lead to inappropriate diagnosis and treatment of presumed Graves' disease (97, 99, 103, 107) (Table 5).

Heterophilic Antibody Interference

The incidence of interferences due to heterophilic antibodies and HAMAs has been assessed at between 0.05% and 6% or more, depending on the assay and analyte considered (3, 108, 110). Concerning heterophilic antibodies against TSH, their incidence was found to be 0.4% in the largest prospective study to date, involving >5000 patients (111).

The definition of heterophilic, HAMAs, and human anti-animal antibodies (HAAAs) is imprecisely used in the literature and may thus be confusing (6, 39, 112). HAAAs are monospecific, high-affinity antibodies directed against animal epitopes from goats, rabbits, sheep, horses, or, more frequently, mice, whereas heterophilic antibodies are weak polyspecific antibodies (usually of low titer) formed early in the immune response prior to affinity maturation. They typically react with immunoglobulins derived from at least two species (113–116). Rheumatoid factor also belongs to this category because it reacts against the Fc region of human immunoglobulins, displaying

Table 4. Anti-Ru Interference Affecting Thyroid Hormone Measurements as Reported in the Literature

Author (Ref.)	Analyte(s) Affected	No.	Clinical Consequence	Methodology Used To Detect Interference[a]
Sapin *et al.*, 2007 (81)	FT3 ↑	5	ND	**Method comparison**, HBT, ^{125}I-T3 precipitation, **aliquot sent to Roche, anti-Ru blockers**
Ando *et al.*, 2007 (80)	FT3 ↑	3	TRH and radioiodine uptake tests (1 patient/3), higher dose of antithyroid drug (1 patient/3)	**Aliquot sent to Roche, PEG, method comparison**
Heijboer *et al.*, 2009 (83)	FT4/3 ↑	2	ND	**Method comparison**, HBT, RF, **aliquot sent to Roche**
McKillop *et al.* 2009 (84)	FT4 ↑	2	ND	**Method comparison, aliquot sent to Roche**
Buijs *et al.*, 2011 (82)	TSH ↓ and/or FT4/3 ↑	6	Scan with ^{123}I, thiamazole and L-thyroxine therapy (for 2 y) (1 patient/6), undertreated with L-thyroxine (2 patients/6) and ND (4 patients/6)	**Method comparison, dilution test**, mouse serum incubation, HBT, **aliquot sent to Roche**
Ohba *et al.*, 2012 (86)	FT4/3 ↑	1	Rx methimazole (malaise and increase in goiter)	**Method comparison, aliquot sent to Roche**, streptavidin beads, **GFC**
Gessl *et al.*, 2014 (28)	TSH ↑	1	Rx L-thyroxine	**Dilution test**, HBR, **PEG, aliquot sent to Roche, method comparison**
Zaninotto *et al.*, 2014 (87)	FT4/3 ↑	1	^{123}I scan	**Method comparison**, HBT, **PEG, aliquot sent to Roche, anti-Ig sera**, RF
Favresse *et al.*, 2017 (27)	FT4/3 ↓	1	No	**Method comparison, dilution test**, HBT, **PEG, aliquot sent to Roche**
Suarez Rivero *et al.*, 2017 (85)	TSH ↑	1	Increased L-thyroxine dosing for 6 wk	**Dilution test**, PEG, HBT, **aliquot sent to Roche, method comparison**

Abbreviations: HBT, heterophilic blocking tube; ND, not determined; RF, rheumatoid factor; Rx, prescription of.
[a]Text in bold indicates a test that was in favor of interference in the corresponding report.

Table 5. Thyroid Hormone Autoantibodies Affecting Thyroid Hormone Measurements as Reported in the Literature

Author (Ref.)	Analyte(s) Affected	No.	Clinical Consequence	Methodology Used To Detect Interference[a]
Stubb *et al.*,1990 (103)	FT4 ↑	1	Rx carbimazole (3 wk and undesirable effects)	**THAAb measurement, reverse-flow electrophoresis**
John *et al.*, 1990 (90)	FT4/3 ↑	8	ND	**Method comparison, THAAb measurement**
Iitaka *et al.*, 1990 (106)	FT4 ↑	2	Fluctuation in methimazole regimens (2 patients/2)	**THAAb measurement, immunoprecipitation**
Sugenoya *et al.*, 1991 (104)	FT3 ↑	1	ND	**THAAb measurement, acid-charcoal treatment, protein A chromatography, IgG purification and THAAb measurement**
Tokmakjian *et al.*, 1991 (105)	FT3 ↑	1	Decreased L-thyroxine (hypothyroid symptoms)	**THAAb measurement, method comparison**
Momotani *et al.*, 1992 (107)	FT4/3 ↑	2	Overtreatment with a saturated iodine solution and/or propylthiouracil	**THAAb measurement**
Crino *et al.*, 1992 (95)	FT3 ↑	1	TRH and T3 suppression tests	**Method comparison, THAAb measurement**
Zouwail *et al.*, 2008 (98)	FT4/3 ↑	1	ND	HBT, **THAAb measurement, method comparison**
van der Watt *et al.*, 2008 (97)	FT4 ↑	1	No	**Method comparison, THAAb measurement**
Massart *et al.*, 2009 (101)	FT4/3 ↑	1	ND	HBT, dilution test, **method comparison, THAAb measurement**
Beato-Vibora *et al.*, 2017 (100)	FT4 ↑	1	Reduction in L-thyroxine dose	**Method comparison, PEG, dilution test, THAAb measurement**
Lee *et al.*, 2017 (99)	FT4 ↑	1	RX methimazole	**Method comparison**, HBT, **THAAb measurement**
Srichomkwun *et al.*, 2017 (94)	FT4/3 ↑	1	ND	**Method comparison, extraction of T4 with alkalinized ethanol, THAAb measurement**

Abbreviations: HBT, heterophilic blocking tube; ND, not determined; Rx, prescription of.
[a]Text in bold indicates a test that was in favor of interference in the corresponding report.

cross-reactivity against animal antibodies (2). However, multiple definitions coexist. For example, Després and Grant (6) argued that HAMAs should be part of the heterophilic antibody definition, whereas rheumatoid factor differs; and Lippi *et al.* (117) claim that heterophilic antibodies comprise both "true" heterophilic antibodies and HAMAs. In daily laboratory practice, the term heterophilic antibody is typically used whenever one suspects a patient's sample contains antibodies that cause false results by binding to the assay antibodies (5). Knowledge of previous exposure is crucial, and theoretically the term heterophilic should be used when there is no evidence of prior exposure to a particular antigen, notably no previous diagnostic procedure or treatment involving animal immunoglobulins (5, 118). Several authors have, however, referred to HAAAs in the absence of animal immunoglobulin exposure, whereas others have referred to heterophilic antibodies, despite the source of exposure being well known (112–119). The situation may prove even more complex because, in several settings, heterophilic antibodies and HAAAs may be present together (112). For the sake of simplicity, in this review, we have considered true heterophilic antibodies and HAAAs together, because they may bring about similar types of assay interference.

Interference due to heterophilic antibodies may lead to falsely low or high analyte levels in one or more assay systems, depending on the interference site within the reaction. Although some cases of falsely low values due to heterophilic antibody interference have been described, falsely elevated values are more commonly reported in the literature (3). Furthermore, two-site immunoassays (typically TSH assays) are more sensitive toward heterophilic antibodies, whereas FT4 and FT3 assays are less prone to being affected by these interfering agents (6). In all 48 cases analyzed between 1981 and 2016, heterophilic antibodies resulted in falsely elevated analytes, most often TSH (95, 120, 127) (Table 6).

Comparison against an assay using other antibody species proved useful in 20 of 38 analyzed cases, whereas the dilution test indicated interference in 30 of 32 cases. The heterophilic blocking tube (HBT) test may also be used to overcome this interference. HBTs contain a blocking reagent composed of specific binders that inactivate heterophilic antibodies (137). Once the specific binder is bound to interfering

antibodies, the latter are no longer available to cause immunoassay interference (137). Of note is that the HBT test does not always show positive results in the presence of heterophilic antibodies (133, 134), thus the conclusions drawn by several authors likely are premature. For example, Ross *et al.* (133) claimed the interference observed in their cases (highly elevated TSH of 48 and 118mIU/L with normal FT4) was due

Table 6. Heterophilic Interferences Affecting Thyroid Hormone Measurements Reported in the Literature

Author (Ref)	Analyte(s) Affected	No.	Clinical Consequence	Methodology Used To Detect Interference[a]
Schaison *et al.*, 1981 (119)	↑ FT4	1	TRH and T3 administration (10 d)	**Dilution test, method comparison**, IE, ID, **rabbit serum and Ig, immunonephelometric assay**
Czernichow *et al.*, 1981 (114)	↑ TSH	14	^{123}I scan with or without TRH test (8 patients/14)	Method comparison, **dilution test, AC, ^{125}I-labeled rabbit IgG incubation (and PEG precipitation) (7 patients/14), rabbit Ig incubation**
Gendrel *et al.*, 1981 (128)	↑ TSH	7	^{123}I scan and TRH test (7 patients/7), L-thyroxine (up to 4 mo) (4 patients/7)	**Dilution test, rabbit serum**
Brennan *et al.*, 1987 (120)	↑ TSH	2	TRH test (2 patients/2) and L-thyroxine (increased doses and undesirable effects) (1 patient/2)	**Dilution test, method comparison, incubation with high TSH sample, mouse serum, AE**
Zweig *et al.*, 1988 (129)	↑ TSH	1	Rx L-thyroxine (increased doses)	**Dilution test**, method comparison, **mouse Ig**
Kahn *et al.*, 1988 (130)	↑ TSH	3	TRH test (2 patients/3) and L-thyroxine (3 patients/3)	**EP, different species Ig/sera, method comparison, RF, dilution test**, binding of ^{125}I-labeled TSH, **immunoabsorption with mouse IgG-1**
Harvey *et al.*, 1988 (131)	↑ TSH	1	Rx L-thyroxine (6 wk)	**Method comparison, mouse serum**
Wood *et al.*, 1991 (126)	↑ TSH	1	TRH test and L-thyroxine (2 mo)	**Mouse Ig**
Fiad *et al.*, 1994 (132)	↑ TSH	1	TRH test	Isoelectric focusing, EP, **method comparison, mouse Ig, different species sera, PEG**
Ismail *et al.*, 2002 (111)	↑ TSH	2	Rx L-thyroxine (for 18 mo) (1 patient/2)	**Method comparison, dilution test**, HBT
Santhana Krishnan *et al.*, 2006 (123)	↑ TSH	1	L-thyroxine (with undesirable effects) and propranolol	**Method comparison, mouse Ig**
Monchamp *et al.*, 2007 (112)	↑ FT4/3	1	ND	**Method comparison, dilution test**, sheep Ig, HBT, **AF**, RF
Sapin *et al.*, 2007 (81)	↑ FT3	2	ND	**Method comparison, HBT**, ^{125}I-T3 precipitation
Ross *et al.*, 2008 (133)	↑ TSH	2	ND	**Dilution test**, HBT, **method comparison, AC**
Chin *et al.*, 2008 (127)	↑ TSH, FT4/3	1	Discontinuation of antithyroid drugs	**Method comparison, dilution test, HBT, RF**
Ghosh *et al.*, 2008 (121)	↑ FT4	2	Rx carbimazole (1 patient/2)	**Method comparison**
Saleem *et al.*, 2009 (134)	↑ FT4	1	Discontinuation of L-thyroxine (symptoms of hypothyroidism)	**Method comparison, RF, protein A treatment**, HBT
Verdickt *et al.*, 2012 (125)	↑ TSH	1	Rx L-thyroxine (increased doses)	EP, **HBT, method comparison**
Morton , 2014 (122)	↑ TSH	1	Rx L-thyroxine (increased doses for 2 y)	**HBT**
Hattori *et al.*, 2015 (14)	↑ TSH	1	ND	**PEG, GFC, adsorption of serum IgG**, dilution test, **HAMA blockers**
Gulbahar *et al.*, 2015 (135)	↑ TSH	1	Rx L-thyroxine	**Method comparison, dilution test, HBT, RF, PEG**
Soleimanpour *et al.*, 2015 (124)	↑ TSH	1	Delayed diagnosis of thyroid storm	**HAMA blockers**
Revet *et al.*, 2016 (136)	↑ FT4	2	Decreased L-thyroxine doses (1 patient/2)	**Method comparison, adsorption of serum IgG**, HAMA blockers, HBT

Abbreviations: AC, affinity chromatography; AE, affinity extraction EP, electrophoresis; HBT, heterophilic blocking tube; ID, immunodiffusion Ig, immunoglobulin; IE, immunoelectrophoresis ND, not determined; RF, rheumatoid factor; Rx, prescription of.

[a]Text in bold indicates a test that was in favor of interference in the corresponding report.

to heterophilic antibodies, based on a negative HBT test and linear dilution pattern in one case out of two. However, these authors did not screen for macro-TSH which may have accounted for the interference. Recently, we observed a clear reduction in FT4 and FT3 concentrations after the HBT test, though this interference turned out to be due to an antibody against the streptavidin used as the immobilizing system in Roche assays (79). Therefore, care should be taken when interpreting HBT tests and we recommend not using this test alone.

The presence of heterophilic antibodies should be clearly indicated in the patient clinical file, because these interfering antibodies may persist for a prolonged time (*e.g.,* 4 to 12 months) (112, 114, 119). Heterophilic antibodies may pass the placenta, interfering with thyroid function tests in newborns (114, 119, 128).

Manufacturers have developed strategies to eliminate these interferences. These have included adding nonspecific animal immunoglobulins; heat-aggregated, nonspecific, murine monoclonal antibodies; and trace amounts of animal serum of the same species as that used in assay reagents, in addition to using $F(ab')_2$ fragments for the solid phase (5, 116, 129, 131). Although these strategies prove effective in most cases, several sera contain very high amounts of interfering antibodies that may still interfere in the assay (81, 116).

Other Interferences

Along with the aforementioned interferences that are the main focus of this review, other types of interferences exist and are briefly discussed in this section.

TH transport proteins variants

In humans, >99% of the total serum THs T3 and its precursor, T4, are bound to serum proteins: T4-binding globulin (TBG), transthyretin (TTR), and albumin. Of these proteins, TBG has the strongest affinity for TH, whereas human serum albumin (HSA) is the most abundant protein in plasma (138–140). Assays used for FT4 and FT3 are designed such that the equilibrium between T4 or T3 to their binding proteins is preserved and the amount of tracer displaced will therefore reflect the free hormone level rather the total hormone level (8). However, some situations alter this equilibrium. Patients known to have a genetic variant in TH transport proteins are clinically euthyroid but present spurious FT4 and FT3 results due to impaired affinity for THs (139, 140). These syndromes, therefore, could be considered interferences and need to be identified to avoid unnecessary treatments (139, 140). The use of ultrafiltration or equilibrium dialysis is recommended to overcome the problem, but these are only available in a restricted number of reference laboratories (140). Three different inherited defects of thyroxine-binding proteins are discussed in the following paragraphs. The effect of some displacing agents also is briefly presented (8).

HSA

Several genetic variants have been reported to alter the binding of T4 and T3 to HSA (139). Familial dysalbuminemic hyperthyroxinemia (FDH-T4) and hypertriiodothyroninemia (FDH-T3) are caused by mutations in the *ALB* gene (139). More specifically, mutations of Arg218 and Arg222 lead to FDH-T4 and mutations in Lys66 to the FDH-T3 (139). The prevalence of FDH has been reported to vary from 0.01% to 1.8%, depending on the ethnic origin (140). FDH-T4 patients are classically characterized by having an elevated concentration of total T4 level with a normal FT4 concentration and a normal physiological thyroid function when measured by ultrafiltration or equilibrium dialysis (139, 140). The TSH level is not affected, whatever the assay considered, and the response of TSH to TRH stimulation is normal (140–142). However, some assays may report a false increase in FT4 concentration (139, 140, 143, 144).

Techniques that minimize the disturbance between binding proteins and total hormones (*e.g.,* equilibrium or symmetric dialysis) returned results indicating normal free-hormone values in patients with FDH (139, 141, 143, 144). Competitive assays between T4 analog and unbound T4 can overestimate FT4 levels in patients with FDH, because the binding of T4 analog to albumin is enhanced (141). Cartwright *et al.* (141) tested four patients with FDH-T4 with eight different assays. As expected, the dialysis method showed normal FT4 results. Two-step assays are also suggested to be less sensitive to this interference because there is no contact between T4 analog and the serum albumin (141). However, Cartwright *et al.* showed that two-step methods could be affected (141–145). They also noticed that the FT4 concentrations measured with the Siemens Centaur one-step assay were less overestimated than in the two-step assays (141). Important variations across assays were observed and the Siemens Centaur, DELFIA (PerkinElmer, Waltham, MA), and Abbott methods were reasonably correlated with the dialysis method (141), and FDH syndromes must be confirmed by family studies and by molecular genetic testing (139).

Several cases of subtotal thyroidectomy and/or ^{131}I therapy, as well as unnecessary prescription of antithyroid drugs, have been reported in the literature (139). In 2005, a Danish study reported the case of a woman receiving thiamazole and who became pregnant. The patient decided to abort, given the risk of teratogenicity of the treatment and based on the recommendation of clinicians (146). It turned out that

the patient had an FDH-T4 syndrome. Clinicians should consider the possibility of FDH-T4 in euthyroid patients who have abnormal thyroid function tests results (139–142).

The FDH-T3 syndrome is associated with an increase of total T3 and a normal FT3 level in otherwise euthyroid individuals (139). Only few reports exist on the subject and the prevalence is unknown, although it must be rare (139).

TBG

At least 57 TBG variants exist (140). TBG defects are classified into TBG excess (prevalence of one in 25,000 people) and complete or partial TBG deficiency (prevalence of 1 in 15,000 and of 1 in 4000, respectively) (140–147). TBG defects are X-chromosome linked and are therefore fully expressed in male patients (140). TBG defects do not alter the metabolic state of the individual and do not cause thyroid disease. However, they produce alterations in total TH concentration in serum, whereas free TH levels remain unchanged. Hence, the suspicion of an inherited TBG defect should be raised when there are abnormal total T4 or T3 levels and a normal free TH. The absence of factors causing acquired TBG abnormalities should also be verified because they are much more frequent in clinical practice (148). However, the genetic analysis is mandatory to confirm the presence of inherited TBG defects (140).

TTR

At least 70 TTR mutations have been identified (140). Because of the low amount of T4 bound to TTR, not all variants will present abnormal binding affinity for T4 and this will lead to erroneous T4 measurements (140). Also, some variants are characterized by a decrease of binding affinity for T4 (*i.e.*, V30M, STTY, I84S), whereas others are characterized by an increase (euthyroid hyperthyroxinemia; *i.e.*, A109T, A109V, T119M, G6S). For example, the TTR variant A109T shares a similar presentation with the FDH-T4: elevated total T4 and a nonsuppressed TSH (139, 140). Some variants produce transient hyperthyroxinemia during nonthyroidal illness (140).

Drugs

As in patients known to have a genetic variant in TH transport proteins, some drugs also affect the equilibrium between T4 or T3 and their binding proteins, thus resulting in altered free TH concentrations (8). These displacing agents include aspirin, furosemide, carbamazepine, phenobarbital, phenytoin, nonsteroidal anti-inflammatory agents, phenylbutazone, and heparin (fractionated or unfractionated) (8, 149, 150). The artifactual hyperthyroxinemia due to heparin has been widely studied (8, 150, 151). The administration of heparin to healthy volunteers and subjects with hypothyroidism showed a rapid increase (2 to 15 minutes) in FT4 concentrations (up to fivefold) (151) due to the generation of nonesterified fatty acids. Detailed information about the mechanisms of this artifact has been discussed elsewhere (8, 150). *In vitro*, the generation of nonesterified fatty acids from triglycerides tends to increase during sample storage or incubation in heparin-treated patients (8). This artifact has been observed with different assays, including direct immunoassays, ultracentrifugation, and equilibrium dialysis (8), and it is especially present when laboratory methods require longer incubation periods at 37°C, in case of hypoalbuminemia, and when triglyceride concentrations are increased (152). Taking a blood sample at least 10 hours after the last heparin administration and analyzing the sample immediately thereafter can reduce this artifact. The assessment of total THs along with TSH and TBG appeared to be a valuable alternative to confirm the euthyroid status of such patients (22, 150). Other drugs are also known to alter TBG concentrations: Tamoxifen, raloxifene, estrogen, fluorouracil, clofibrate, heroin/methadone, and mitotane have been shown to increase serum TBG, whereas nicotinic acid, asparaginase, chronic glucocorticoid therapy, and androgens/anabolic steroids are recognized to inhibit TBG synthesis (8). These latter drugs generally result in changes in total hormones, whereas free THs are not impacted (8). The assessment of medication history is crucial, therefore, whenever tests of thyroid function are anomalous (150).

TSH variants

Drees *et al.* (153) identified 20 euthyroid individuals (19 South Asian and 1 Persian) with falsely undetectable biologically active TSH levels who had been wrongly diagnosed with hyperthyroidism. At least 7 of these 20 patients were treated with methimazole. Two other patients lowered their L-thyroxine therapy on the basis of the undetectable TSH results and on the recommendation of clinicians (153). In all patients, TSH had been determined with Siemens assays (namely, ADVIA Centaur TSH-3 Ultra, Immulite, Dimension, and Dimension Vista) (153). Use of other platforms (Abbott Architect, Beckman Coulter DxI, and Roche Modular E170) returned results indicating higher TSH concentrations, consistent with the clinical presentation for all patients (153). Therefore, this impact of the interference is assay dependent. Testing the serum with a method from another manufacturer should be considered when suspecting an erroneous TSH result (153). After further investigations, it appeared that a mutation in TSH-β (R55G) was responsible for discordance observed between TSH values obtained with Siemens immunoassays and other platforms (153). Authors hypothesized that this mutation may be responsible for altering an epitope on TSH, thus preventing the binding of monoclonal antibodies used by Siemens analyzers (153). Also,

moderately elevated TSH concentrations with abnormally glycosylated or bioinactive TSH isoforms have been described in cases of central hypothyroidism (154); in some exceptional cases, elevated TSH levels may result from mutations in the gene encoding the TSH β subunit (155).

Paraprotein

Paraproteins can also interfere in immunoassays by affecting the antibody binding (3). Luzzi *et al.* (156) observed a low TSH level on an AxSYM analyzer (Abbott) in a patient known to have an IgG κ paraprotein. FT4 and FT3 levels were within normal ranges and the patient was clinically euthyroid. The authors analyzed the same sample on the Immulite 2000 assay and found a normal TSH level. The presence of an interference was therefore highly suspected. The dilution test and the blocking experiment performed on the AxSYM analyzer returned normal TSH values. Immunoglobulins of the patient were also precipitated with ammonium sulfate and a serum electrophoresis performed on the concentrated immunoglobulins confirmed the presence of a sharp monoclonal peak in the γ region, consistent with the known IgG κ paraprotein. A serial addition of the concentrated immunoglobulins of the patient to a sample from a patient with a known TSH showed a clear decrease in TSH level. The mechanism of interference proposed is that the binding of an IgG κ paraprotein to the TSH assay (AxSYM) may have sterically blocked the binding of TSH. More recently, Imperiali *et al.* (157) identified a patient with two monoclonal bands (IgG λ and IgM κ) in whom a high TSH concentration (>100 mUI/L) on a DxC 880i platform (Beckman Coulter) was measured. This observation was discordant with a previous normal TSH value (0.59 mUI/L) obtained 6 months before, together with normal FT4 and FT3 values. The patient was also clinically euthyroid. Because the presence of an interference was suspected, the authors analyzed the samples with another method (Architect i2000SR; Abbott). No difference was observed after the HBT procedure. Interestingly, the disappearance of the monoclonal bands in electrophoresis was consistent with normalization of TSH levels.

Common Tests Used to Screen for Interferences in Current Immunoassays and Proposed Algorithm

Repeating the analysis with the same method is often performed as a first resort (97). A pipetting problem, inadequate washing, tracer aggregates, or bubbles may have generated incorrect results in several cases (5). After confirming the discordant result with a repeated analysis, several tests can be performed to rule out or identify the interfering agent. Of note, a negative test does not exclude an interference, whereas a positive result is likely be indicative of one.

Repeating the analysis with another assay method

Using another assay method has proven to be a good approach for detecting an interfering agent, with similar results usually interpreted as proof of "analytical authentication" (1, 158). Between-method differences often already hint at the interference source: A method using antibodies from different animal species points toward heterophilic interference, a different immobilizing system toward biotin or anti-streptavidin antibodies, a different detection system toward anti-Ru antibodies, and differences between one- *vs.* two-step immunoassays toward THAAbs. Measurement of THs by ultrafiltration, equilibrium dialysis, and tandem mass spectrometry may also be used as a valuable alternative (*e.g.,* FDH), even though such methods are not yet broadly available (11, 139, 141, 143, 144).

In addition, investigators must take into account any bias between two methods (1). If method 1 usually overestimates the analyte value by 35% in comparison with method 2, then a 35% increase may be expected in the absence of interference (158). A reversal or exaggeration of known biases may thus be indicative of interference (158). Exchange procedures with other laboratories using different techniques are, likewise, a good option.

"No single test is sufficient to identify interferences."

Doubling serial dilution

The dilution test is simple and relatively inexpensive, and provides rapid results when using current immunoassay platforms (159). An interfering agent can distort linearity and reduce parallelism in a doubling serial-dilution study with concentrations at one-half, one-fourth, and one-eighth. Assessing linearity or parallelism should not be visual. Using reference values at each dilution titer has proven the best solution (27, 159). Random inherent errors at each dilution point similarly should be taken into account; they are estimated at 10% in typical immunoassays (109, 159, 160). It should be mentioned, however, that the dilution test is not perfect. Only 60% of samples showing lack of parallelism or of linearity may actually be associated with endogenous antibody interference (159). Several interferences may likewise dilute without affecting parallelism (*e.g.,* macro-TSH). Therefore, this test should never be used alone. It must also be kept in mind that both FT4 and FT3 cannot be diluted with the manufacturer's diluent, because these assays are optimized for minimal disturbance of the endogenous equilibrium of free and bound hormone; this is why some diluents contain bovine serum albumin. Nevertheless, dilution of free hormones with 0.9% NaCl has been successful on the Beckman Coulter UniCel Dxl 800 for identifying interferences, with only minimal disturbance of the free- and bound-thyroxine equilibrium (161).

Adding blocking agents

Measurements before and after adding either native nonimmune serum or commercially available blocking antibodies are commonly used, as well. Although blocking antibodies are more expensive, they do increase the detection rate of interfering agents as compared with nonimmune sera (20% *vs.* 3%, respectively) (1). A normal result should not be used to exclude interference, given that 20% to 30% of cases prove to be insensitive toward this method. The commercially available HBT (Scandibodies Laboratories, Santee, CA) has simplified screening for heterophilic antibodies, representing a standardized approach (160). Briefly, 500 μL of the sample is added to the blocking tube that contains a pellet of blocking reagent. The tube is then gently mixed and incubated for 1 hour at room temperature. The sample is then retested and only a significant deviation from the initial result should be interpreted as heterophilic interference (109). HBT with lower sample volumes (*e.g.*, 250 μL) may prove useful at times for identifying high heterophilic antibody titers.

The combined use of the comparison method, dilution test, and blocking agents will identify antibody interference in approximately 90% of suspected samples (1). This assumption implies that no single test is sufficient to identify interferences.

Depleting interfering antibodies

The methods used for depletion or removal of interfering antibodies include precipitation, affinity extraction, and size-exclusion. A control sample should always be used alongside to ensure correct data interpretation (5, 97).

PEG, or the lesser used $(NH_4)_2SO_4$, precipitates proteins by lowering their solubility in plasma or serum. The PEG precipitation procedure has been successively used to screen for macro-PRL and macro-TSH (13, 14, 31, 32, 34). We strongly recommend using PEG 6000, given that it is currently the most widely used molecular form reported in the literature. Moreover, other compounds with a different molecular weight (*e.g.*, PEG 8000) have caused some biases (162). This method is used for macro-TSH as well as for all interferences involving an antibody (*e.g.*, THAAbs, heterophilic anti-Ru or anti-streptavidin antibodies) (27, 28, 77, 80, 87, 94, 97, 132, 135). When screening for macro-TSH the observation of a value within the post-PEG reference range should be preferred over the usual recovery rate to avoid misclassification. There is always coprecipitation of THs due to nonspecific binding; therefore, the determination of post-PEG reference values in healthy individuals is required. Clearly, these precipitation tests are without value

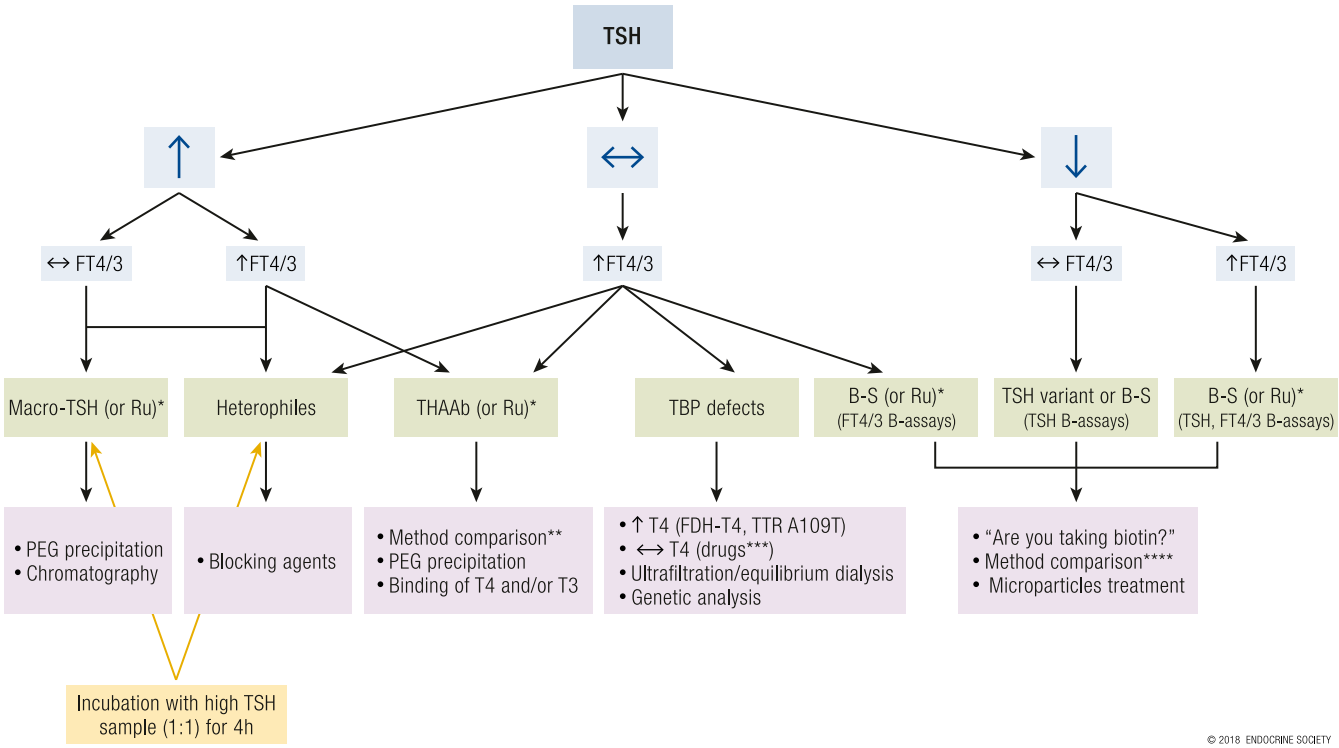


Figure 2. Proposed algorithm to screen for common thyroid interferences. B-S, biotin-streptavidin immobilization system (biotin or antistreptavidin interferences); Ru, ruthenium; TBP, thyroxine-binding proteins; *, only Roche platforms are affected and that method comparison with another platform not using the ruthenium label is advised; **, if available, a comparison against equilibrium dialysis represents the best choice; ***, *e.g.*, heparin (fractionated or unfractionated), furosemide, carbamazepine, or phenytoin; ****, assays not affected by biotin or antistreptavidin antibodies should be preferred.

when the interfering agent is not an antibody (*e.g.,* biotin).

Protein G or A columns (*e.g.,* Sepharose linked) can bind immunoglobulins with high affinity (5). Therefore, IgG could be depleted from plasma or serum and retesting the eluent would indirectly identify the interference, be it macro-TSH THAAbs, or anti-streptavidin or heterophilic antibodies. And specificity would thus be higher than with PEG precipitation (18, 24, 26, 76, 112, 114, 130, 133, 134, 136). Next, immunoglobulins could be eluted from the column without any denaturation with acidic buffers, enabling additional confirmation tests.

Size-exclusion methods prove likewise effective for separating interfering antibodies from analytes, given that antibodies exhibit larger molecular weights than most analytes (~150 kDa and ~950 kDa for IgG and IgM, respectively). GFC has been extensively used for macro-PRL and macro-TSH screening. Hattori *et al.* (14) have, however, warned that macro-TSH and HAMAs may display similar elution times. For this reason, these authors recommended screening for both HAMAs and macro-TSH. As already mentioned, incubating serum that possibly contains macro-TSH for 4 hours with an elevated TSH sample in a 1:1 ratio should render it possible to differentiate between macro-TSH and heterophilic antibody interference (16, 18, 39).

Other tests

Other tests have been successfully used, such as treatment with streptavidin beads (66–68); immunofixation and electrophoresis (156, 157); ammonium sulfate precipitation (156); incubation with a sample from a hypothyroid patient (with elevated TSH) (16, 18, 39); evaluation of T4-binding capacity after PEG precipitation for THAAb screening (102); Ru-blocking proteins (17); measurement of T4 and TBG to suspect FDH, THAAb, or the heparin artifact (6, 140, 150); molecular genetic testing for FDH (139); as well as heating to 70°C to 90°C (for heat-stable analytes only) (5).

Due to lack of time and high cost, routine screening for interference is not feasible in all samples (2). Therefore, only samples in which interference is suspected generally require additional tests to further characterize the interference. Two approaches coexist for identifying interferences. The first consists of routinely using the same sequence of tests (*e.g.,* method comparison, the dilution test, and HBTs), as proposed by Ismail *et al.* (111). The second takes advantage of the knowledge of the interfering pattern (*e.g.,* PEG precipitation to screen for macro-TSH when only TSH is elevated). Obviously, each laboratory will use tests according to its own resources and expertise. Several tests are easy to perform (*e.g.,* PEG precipitation, the dilution test, or HBTs), whereas others are the domain of specialized laboratories (*e.g.,* size-exclusion chromatography, affinity extraction). If these sophisticated tests are necessary, connecting with specialists will prove to be a great asset. Several examples in the literature have already shown that good communication with the manufacturers is essential to clearly identify the interfering agent (27, 76, 79, 81, 83, 85, 86).

It also proves vital not to report the results obtained with these tests, given that they do not reflect true concentrations and may thus be confusing for the clinician; this also applies to results from dilution tests or PEG precipitation (88, 118). Once the interfering agent has been found, a note should be added to the patient's file to avoid further misclassification. Inserting a recommendation into the laboratory information system may alert other laboratories about the interference (60).

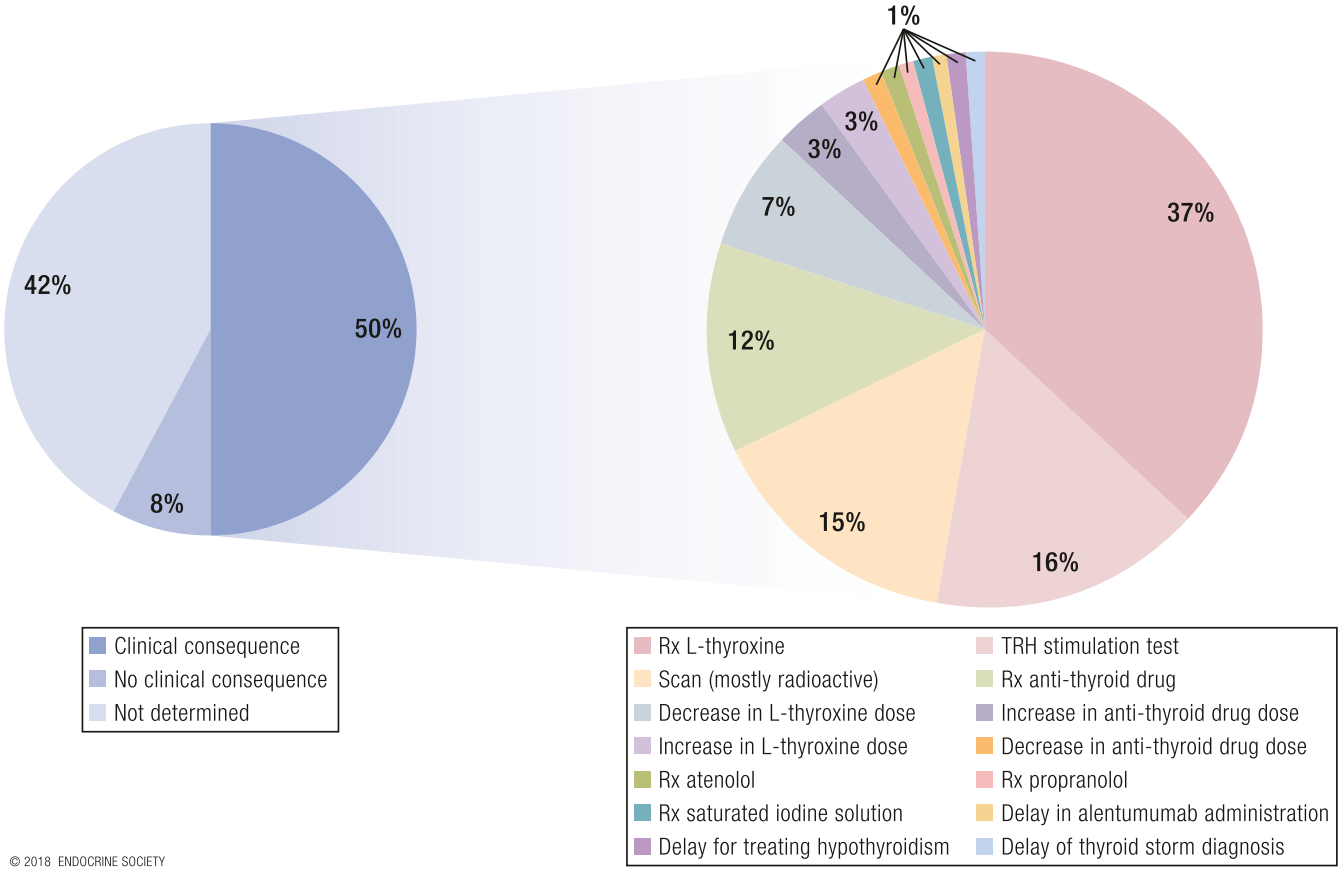
Figure 2 illustrates an algorithm we propose that takes all these factors into consideration and should facilitate the widespread identification of thyroid interferences.

Clinical Implications of Thyroid Interferences

For this review, we have evaluated the clinical impact of interference in thyroid function tests of >150 patients published between 1981 and 2017. Each patient case was classified as having (1) no clinical impact, (2) a negative clinical impact, or (3) a clinical impact not assessed. A negative clinical impact was defined as follows: (1) prescription of an unnecessary treatment for the patient (*e.g.,* L-thyroxine or antithyroid drugs), (2) delay in making the correct diagnosis, (3) an inappropriate halting or modification of ongoing treatment, and (4) superfluous use of other tests, including I^{123} thyroid scans or TRH stimulation tests. The stress caused, time lost, and additional costs arising from additional blood testing were not considered, because this information was missing in most published reports.

Based on this compilation, a negative clinical impact was observed in ~50% of cases. The most frequent clinical impact was the prescription of L-thyroxine (37%) followed by TRH stimulation tests (16%), thyroid scans (mostly radioactive; 15%), and the prescription of antithyroid drugs (12%). In several patients, a treatment was inappropriately initiated and the interference only discovered several years later. Undesirable effects from unnecessary treatments were similarly reported. The 50% figure is likely underestimated, given that 42% of case reports were unclear concerning this clinical issue. Only 8% of case reports noticeably mentioned

Figure 3. Clinical impact of interference in thyroid function tests of >150 patient cases published between 1981 and 2017. Note that only 8% of case reports mentioned that no harmful clinical consequences were encountered. Rx, prescription of.



that no harmful clinical consequences were encountered (Fig. 3). Finally, the theoretical possibility of having falsely normal thyroid function tests in a patient with real thyroid dysfunction should also not be underestimated, but it is likely that such cases often escape positive detection.

Conclusion

Interference in thyroid function testing should always be considered whenever clinical or biochemical discrepancies arise, with the interference pattern being essential to guide their identification. Several tests are available for screening and most of them are quite simple to perform and interpret. These tests include method comparison, dilution tests, and HBTs. It must be kept in mind that the reporting of these interferences is the responsibility of the clinical laboratory.

This review revealed that ≥50% of all cases reported were at first misdiagnosed and inappropriately managed by the clinician. The algorithm we propose may facilitate the widespread identification of thyroid test interferences. Ongoing communication with clinicians and manufacturers is of paramount importance to limit potential harmful consequences of these laboratory pitfalls.

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Abbreviations

FDH-T3, familial dysalbuminemic hypertriiodothyroninemia; FDH-T4, familial dysalbuminemic hyperthyroxinemia; FT4, free thyroxine; FT3, free triiodothyronine; GFC, gel filtration chromatography; HAAA, human anti-animal antibody; HAMA, human anti-mouse antibody; HSA, human serum albumin; HBT, heterophilic blocking tube; HRT, hormone replacement therapy; Macro-PRL, macroprolactin; PEG, polyethylene glycol; Ru, ruthenium; TBG, thyroxin-binding protein; TH, thyroid hormone; THAAb, thyroid hormone autoantibody; TTR, transthyretin.