

Interference Due to Heterophilic Antibody, Biotin and Thyroid Hormone Autoantibody

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Research Article

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Abstract

Purpose: Immunoassay is susceptible to interference by other substances in the serum. The main substances interfering with thyroid function testing include heterophilic antibody, biotin, thyroid hormone autoantibody, and Macro-TSH. We reported a patient with fraudulently elevated FT4 and TSH and described various common experimental methods used to explore the existence of substances interfering with thyroid function testing.

Methods and Results: FT4 and TSH were significantly lower when measured on the Architect platform (FT4: 7.09 pmol/L, TSH: 58.94 μ IU/ml). Polyethylene glycol precipitation showed lower FT4 and TSH, suggesting the presence of a high molecular weight interfering substance. Heterophile blocking tube study showed heterophilic antibody interfered with TSH detection. 125 I-hTSH binding study and radioimmunoprecipitation assay indicated that the patient didn't contain anti-TSH autoantibody. The new generation of Elecsys immunoassay kit indicated that biotin interfered with TSH detection. Radioimmunoprecipitation assay showed that all four kinds of thyroid hormone autoantibodies were positive. After reviewing 24 literatures, we provided the diagnostic strategy for investigation of interferences with thyroid function immunoassays.

Conclusion: We reported a case with falsely elevated TSH due to the combined action of heterophilic antibody and biotin and fraudulently elevated FT4 caused by thyroid hormone autoantibody. When there is a discrepancy between thyroid function and clinical manifestation, the presence of immunoassay interference with one or more indicators needs to be considered.

Introduction

Thyroid stimulating hormone (TSH) and thyroid hormone (TH) are important indicators to evaluate thyroid function and important basis for clinicians to diagnose and make clinical decision. When there is a discrepancy between thyroid function and clinical symptoms, immunoassay interferences need to be considered in addition to rare diseases such as Resistance to thyroid hormone syndrome (RTH), TSH-Secreting Pituitary Adenoma (TSH-oma). The main substances interfering with thyroid function testing currently include heterophilic antibody, biotin, thyroid hormone autoantibody (THAB), and Macro-TSH [1].

We reported a case with falsely elevated TSH due to the combined action of heterophilic antibody and biotin and fraudulently elevated free thyroxine (FT4) caused by THAB. Heterophilic antibody is a general term of a class of interfering antibodies that can be combined with the animal antibodies in immunoassay. It can replace the target substance by binding to the labeled antibody, resulting in pseudo high or low result. Biotin is a small molecule, soluble essential decarboxylase cofactor. When biotin is excessive, it can interfere immunoassay containing biotin. THAB is an autoantibody that binds to TH, resulting in a pseudo high level of free TH. In this paper, we described various common experimental methods used to explore the existence of heterophilic antibody, biotin and THAB and their interference in thyroid function detection.

Case Report

A 75-year-old woman presented to the outpatient department due to thyroid dysfunction. The patient has been suffering from Hashimoto's thyroiditis for 18 years (the results of thyroid function from 2002 to 2019 were shown in Figure 1a, and treated with levothyroxine (L-T4) and thiamazole, but had not taken thyroid related drugs in the past four years. She complained that her heart rate was fast and she had no other symptoms related to thyroid dysfunction. She had a history of family hereditary hypertension, hyperlipidemia and coronary heart disease, usually taking Valsartan and Hydrochlorothiazide Tablets, Nifedipine Controlled Released Tablets, Metoprolol Tartrate Tablets, Trimetazidine Dihydrochloride Tablets. No one else in the family had thyroid disease or autoimmune disease.

Thyroid function of the patient on November 6, 2019 were shown in Table 1. The patient's thyroglobulin antibody (TGAB) >4000 IU/ml (reference value, 0-115 IU/ml), anti-peroxidase antibody (TPOAB) >600 IU/ml (reference value, 0-34 IU/ml). The patient was negative for anti-thyroid stimulating hormone receptor antibody (TRAB) and rheumatoid factor (RF). Thyroid ultrasound was consistent with the diagnosis of Hashimoto's thyroiditis. This patient's TSH level exceeded the upper limit of systematic detection while free triiodothyronine (FT3) and FT4 level were within the normal range and there were no hypothyroidism symptoms, which attracted our attention. We reviewed her thyroid function examination from 2002 to 2019 (Figure 1a) and noted that FT4 and TSH were both higher than normal on several occasions between October 12, 2011 and November 6, 2019. Simultaneous elevation of FT4 and TSH can be seen in RTH, TSH-oma, and immunoassay interference. RTH is characterized by central and/or peripheral thyroid hormone resistance due to mutations in the thyroid hormone receptor gene, resulting in elevated FT4 and TSH[2]. We performed *TRBH* exome sequencing for her, and the results showed that no relevant mutant gene was detected, which ruled out RTH. TSH-oma secretes TSH autonomously that isn't regulated by negative feedback of thyroid hormones, leading to increases in TSH, FT4 and FT3, accompanied by symptoms of hyperthyroidism and optic chiasm compression (visual field defects or loss of vision) and/or compression of normal pituitary cells (anterior pituitary function deficits) [3]. The patient refused enhanced pituitary magnetic resonance and computed tomography examinations, so the differential diagnosis of TSH-oma could not be made. The patient didn't have symptom related to hyperthyroidism, nor did she have symptoms related to pituitary compression, so we believed that the simultaneous increase of TSH and FT4 caused by TSH-oma was less likely. Therefore, we turned our attention to immunoassay interference. We used the ADVIA Centaur XP immunoassay system (Siemens Healthcare Diagnostics, Munich, Germany) and the Architect TSH immunoassay (Abbot Diagnostics, Abbott Park, IL, USA) to redetect patient 's thyroid function, and the results were shown in Table 1. There was no significant difference in FT3 among the three immunoassays. FT4 detected on the Architect was lower than Elecsys and dropped below normal, while FT4 on the Centaur was higher than Elecsys and exceeded the upper limit of normal. TSH was detected at significantly lower values on both Architecture and Centaur than on Elecsys. The significant difference in FT4 and TSH between the three immunoassays couldn't be explained only by the analytical bias of different immunoassays, indicating a high likelihood of assay interference. In order to determine the true value of FT4 and TSH, polyethylene glycol (PEG) was used to precipitate interfering substance. After PEG precipitation, FT4 and TSH were

most similar to FT4 and TSH detected on Architect. According to the results of Architect and PEG precipitation, the patient was considered to be hypothyroidism and given L-T4 supplement therapy, starting from 25 ug/day. The patient was required to visit the clinic every month to check thyroid function and adjust the dosage of L-T4 (Figure 1b). At the same time, we collected the serum of patient to explore causes for the pseudo high FT4 and TSH. Unfortunately, due to the influence of 2019-nCoV, we only collected serum from patient at her visit on November 6, 2019 and July 15, 2019.

Table 1 The results of the procedures to investigate interfering substance resulting in falsely elevated TSH and FT4 ^a

	2019.11.06			2020.07.15		
	FT3/pmol/L	FT4/pmol/L	TSH/ μIU/ml	FT3/pmol/L	FT4/pmol/L	TSH/ μIU/ml
Elecsys ^b	3.29	12.7	>100	4.04	28.10	7.94
Architect ^c	3.78	7.09	58.94	4.37	14.63	4.64
Centaur ^d	3.52	56.22	72.36	4.11	45.38	4.24
PEG Precipitation	2.89	4.70	62.00	4.38	16.90	8.20
HBT	2.98	13.20	85.90	4.07	27.10	7.84
New generation Elecsys kit ^e	-	-	67.6	-	-	6.84

a: Thyroid function was reassayed using Elecsys assay for all the procedures, except for Architect and Centaur and New generation Elecsys.

b: Reference interval of Elecsys assay, FT3 (3.1-6.8 pmol/L) FT4 (12.0-22.0 pmol/L), TSH (0.27-4.2 μIU/ml).

c: Reference interval of Architect assay, FT3 (2.63-5.7 pmol/L) FT4 (9.01-19.05 pmol/L), TSH (0.35-4.94 μIU/ml).

d: Reference interval of Centaur assay, FT3 (2.3-6.3 pmol/L) FT4 (10.3-24.5 pmol/L), TSH (0.35-5.5 μIU/ml).

e: The tolerance of the new generation Elecsys kit to biotin is 1200ng/ml. TSH reference value (0.27-4.2 μIU/ml).

Methods And Results

The thyroid function was redetected after incubating with heterophile blocking tube(HBT) (Scantibodies, Santee, CA)[4], and the results were shown in Table 1. FT4 showed no significant change and TSH significantly decreased (from >100 to 85.90 μ IU/ml), which indicated that heterophilic antibody did interfere with TSH detection. After incubating with HBT, the TSH value was still higher than TSH detected on Architect and detected after PEG precipitation, and we suspected that there were other substances that interfered with TSH detection. In order to investigate whether the interfering substance was anti-TSH autoantibody, 125 I-hTSH binding study was carried out [5]. The serum of the patient was incubated with 125 I-hTSH (Beijing North Institute of Biotechnology Limited Company, Beijing, China), and the mixture was precipitated with PEG. The radioactivity of the precipitate was detected. The difference in radioactivity between patient and controls was small (patient: 7314cpm, controls: 6401 ± 778 cpm), which indicated that the patient didn't contain anti-TSH autoantibody. In order to determine whether TSH was interfered by biotin, we used a new generation of Elecsys immunoassay kit (Batch No: 08429324) to detect TSH (Table 1). There was a significant difference between TSH detected by the old and new generation of Elecsys immunoassay kit (from >100 to 67.6 IU/mL), which indicated that biotin did interfere with TSH detection and resulted in the pseudo high TSH. Radioimmunoprecipitation assay (RIA) was performed to determine whether THAB interfered with FT4 detection [6]. Serum was incubated with 125 I-T3/ 125 I-T4 (Beijing Fury Runze Biotechnology Co., Ltd, Beijing, China) and precipitated with Protein G (Merck KGAA, Darmstadt, Germany)/anti-human IgM-Agarose (Sigma-Aldrich, Saint Louis, USA), and the radioactivity of the precipitate was detected (Table 2). The results showed that all four kinds of THAB were positive, and the titer of anti-T4IgG was highest.

Through a series of experiments, we found that it was heterophilic antibody and biotin that led to the increase of TSH, and THAB that resulted in the increase of FT4. After 9 months of treatment, the patient's TSH gradually approached the normal value on July 15, 2020 (Fig. 1b). There was slight difference in TSH detected before and after PEG precipitation or between the three platforms (Table 1). Incubating with HBT and redetecting on new generation of Elecsys immunoassay kit demonstrated that TSH detection was no longer affected by heterophilic antibody and biotin. The FT4 of the patient was still significantly different in the three platforms. The results of RIA (Table 2) showed that anti-T4IgG was still positive, but the titer was lower than that on November 6, 2019, and the other three antibodies all turned negative. The decrease or even disappearance of immunoassay interference after treatment may be related to no more exposure to relevant antigens and recovery of immune disorders.

Table 2 Titer of various THAB

	Anti-T3IgG ^f	Anti-T3IgM	Anti-T4IgG	Anti-T4IgM
2019.11.06	4.42	7.14	31.14	4.29
2020.07.15	3.68	3.62	19.27	3.29
Reference value ^g	0-4.40	0-4.56	0-3.97	0-4.23

f: Percentage of anti-T3 IgG = (precipitation of $^{125}\text{I-T3}$) / (total $^{125}\text{I-T3}$)

g: The reference values were provided by another article, which has not yet been published.

Discussion

Immunoassay is the preferred method for the detection of thyroid function in clinical laboratory, which has the advantages of high level of automation, short turnaround time, high specificity and sensitivity. However, immunoassay is susceptible to interference by other substances in the serum, including heterophilic antibody, biotin, THAB, and Macro-TSH, leading to falsely high or low result. A review analyzed more than 150 literatures and found that at least 50% of the reports recorded misdiagnosis and/or mistreatment by clinicians due to interference in thyroid function detection[7]. According to the patient's thyroid function from 2002 to 2019 (Figure 1a), we suspected the interference with TSH and FT4 detection should have started in October 2011. We didn't have the patient's serum at that time, so we couldn't prove our speculation. Interference led to the simultaneous increase of TSH and FT4, so that doctor couldn't give an accurate diagnosis, resulting in a long time without effective treatment for patient. When TSH > 10 IU/ml, the mortality rate of cardiovascular system will increase with the increase of TSH[8]. In this case, the patient had not received effective treatment for more than ten years, which had adverse effect on the prognosis of coronary heart disease. Interference with thyroid function detection have a great impact on the diagnosis and treatment of patients.

Heterophilic antibody is autoantibody that can bind to animal antibody or human antibody. Depending on the cause of antibody production, heterophilic antibody can be divided into the following three categories. Heterophilic antibody produced after the injection of an animal antibody for therapeutic or diagnostic purposes is called a human anti-animal antibody. Human anti-mouse antibodies (HAMAs) cause the most frequent interference due to the antibodies used in most testing reagents are mouse antibodies. Heterophilic antibody that bind to the Fc segment of human IgG is called rheumatoid factor (RF), which is common in patients with rheumatoid arthritis [9]. RF can also cross-react with animal antibodies due to the homology between human antibodies and some animal antibodies in the Fc segment. The rest interfering antibodies with affinity to animal antibodies are found in patients without known exposure to animal antibodies, which are called heterophilic antibodies. The prevalence of interference caused by heterophilic antibody ranges from 0.05 to 6%, depending on the assay and test reagent [10-12]. Sandwich immunoassay (typical TSH assays) is more sensitive to heterophilic antibody, whereas FT4 and FT3 assays are less susceptible to these antibodies[13]. A study of 5,000 subjects showed that heterophilic antibody interfered with TSH detection in 0.4% of cases[14]. Heterophilic antibody will imitate target objects in binding to the capture antibody and detection antibody in the kit, resulting in higher results in the sandwich method and lower results in the competition method. PEG precipitation, doubling serial dilution and using another assay method can be used as screening methods. Incubating with HBT can determine the existence of heterophilic antibody, but negative result can not completely exclude heterophilic antibody.

Biotin (vitamin B7) is an essential cofactor of carboxylases involved in fatty acid metabolism, leucine degradation, and gluconeogenesis, and is usually ingested through food. When a patient ingested an excess of biotin, this excess biotin and the biotin in the kit will competitively bind to streptavidin. Depending on whether the assay is a competitive or sandwich assay, this excess biotin may result in a higher or lower test result. The TSH detection method of Elecsys immunoassay is the sandwich assay (Figure 2A), and the interference of biotin will lead to the pseudo low TSH (Figure 2B). Doubling serial dilution and using another assay method without biotin and streptavidin beads adsorption can be used as detection methods for biotin interference. Roche diagnostics has recently updated its TSH assay to overcome biotin interference. The tolerance of the new TSH kit to biotin increases from 25ng/ml to 1200ng/ml. Comparing TSH results detected on the old and new generation Elecsys TSH kit can be used as a new method to identify biotin interference. Previous reports showed that biotin can cause low TSH and lead to the wrong diagnosis of hyperthyroidism [15-17]. In our case, however, biotin caused high TSH level, contrary to previous reports. It has been described that heterophilic antibody can interfere with detection by interacting with biotin [18]. Based on this, we speculated that the high TSH caused by biotin might be due to the interaction between heterophilic antibody and biotin (Figure 2-D). The TSH detection method of Elecsys immunoassay was the sandwich assay (Figure 2-A). When biotin and heterophilic antibody independently interfere with TSH detection, TSH is pseudo low and high, respectively (Figure 2-B, C). When excessive biotins and heterophilic antibody were present, the heterophilic antibody was primarily fixed to the magnetic bead by biotin, and then directly combined with detection antibody or combined with the biotinylated capture antibody-TSH-detection antibody complex, resulting in high pseudo TSH results. In the new generation Elecsys kit, the biotin blocking antibody bound to the excess biotin, so that the heterophilic antibody and biotin couldn't bind to magnetic beads, thus eliminating the interference. In addition to the new generation Elecsys kit, PEG precipitation could also prove that biotin and heterophilic antibody interfered with detection together. If biotin and heterophilic antibody independently interfered with the detection, the TSH result after PEG precipitation should be similar to TSH detected after heterophilic antibody blocking, which was not consistent with the result of this case. When biotin and heterophilic antibody interacted, PEG precipitation removed the heterophilic antibody and the biotins bound to the heterophilic antibody, so that the TSH was not affected by the two interfering substances (Table1).

A: Sandwich assay: capture antibody immobilizes TSH to the beads by biotin-streptavidin interaction, and ruthenium-labeled antibody is immobilized to the beads by binding to TSH.

B: Biotin: when the serum contains an excess of biotin, the biotins bind to streptavidin on the magnetic beads, making the biotinylated capture antibody unable to be fixed to the magnetic beads, resulting in low TSH concentration.

C: Heterophilic antibody: heterophilic antibody imitates TSH in binding to capture antibody and detection antibody, resulting in the high TSH concentration.

D: Biotin and heterophilic antibody: heterophilic antibody is fixed to the magnetic bead through biotin, and the remaining epitopes can be connected with the detection antibody and biotinylated capture antibody-TSH-detection antibody complex, resulting in high TSH.

THAB, an autoantibody that can bind to TH, includes four types: anti-T3IgG, anti-T3IgM, anti-T4IgG, and anti-T4IgM, among which IgG type antibody is most common. In 1972, Ochi Y immunized rabbits with slightly denatured thyroglobulin (TG) and those rabbits successfully produced THAB [19]. Subsequently, Benvenga S found that THAB was the first antibody to appear after thyroid fine-needle puncture [20], and the positive rate of antithyroglobulin antibody (TGAB) in THAB-positive individuals was 80-100%[7]. It is generally believed that the leakage of TG in the thyroid is the cause of THAB production. The occurrence of THAB is closely related to autoimmune diseases. the prevalence of THAB in the normal population is 1.8%[21],and in thyroid autoimmune diseases is 20%-40%[22,23]. Whether THAB interferes with the detection depends on the detection method used[24]. In addition, autoantibody titer, affinity, specificity and other immunological characteristics determine the degree of interference[13]. When thyroid hormones are detected using a one-step method (e.g., Elecsys, Centaur), THAB can bind to labeled thyroid hormones and lower labeled thyroid hormones will be detected after elution, resulting in a pseudo high thyroid hormone concentration. In the two-step method (such as Architect), all serum components are removed through a cleaning step before the tracer is added, leaving the test reagent undisturbed by substances in the serum. PEG precipitation, serum dilution test and changing detection platform can be used as screening methods for THAB, and RIA is the diagnostic method for THAB. What properties of THAB can determine whether to interfere with detection remains to be further studied.

Macro-TSH, a high-molecular-weight form of TSH, can bind to both protein A and protein G in most cases, leading to the proposal that Macro-TSH is composed of TSH and immunoglobulin G (IgG)[25,26]. ¹²⁵I-hTSH binding study has verified that IgG binding to TSH is anti-TSH autoantibody[5]. Due to the slower clearance of the high molecular mass TSH from the circulation, Macro-TSH can result in falsely high serum TSH concentrations. The prevalence of Macro-TSH ranges from 0.6 to 1.62%[26-28]. Different two-site immunoassays use different antibodies, and different antibodies have different affinity for Macro-TSH, which makes different two-site immunoassays have different sensitivity to Macro-TSH[27]. PEG precipitation, doubling serial dilution and using another assay method can be used as screening methods. ¹²⁵I-hTSH binding study and gel filtration chromatography (GFC) can be used as diagnostic methods, in which GFC is the gold standard.

In conclusion, we reported a case that FT4 and TSH were simultaneously interfered, in which TSH detection was interfered by heterophilic antibody and biotin, and FT4 was interfered by THAB. When there is a discrepancy between thyroid hormone and TSH or thyroid function and clinical manifestation, the presence of immunoassay interference with one or more indicators needs to be considered. Figure 3 provided the diagnostic strategy for investigation of interferences with thyroid function immunoassays.

Declarations

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Conflicts of interest: None of the authors has any conflict of interest to declare.

Availability of data and material: Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in References.

Code availability: None

Authors' contributions: haiqing zhang, jiajia ni and long yu contributed to the conception of the study; performed the experiment; jingyi li and li zhang contributed significantly to analysis and manuscript preparation; qingqing yang, chunjia kou and shuqi li performed the data analyses and wrote the manuscript; Guoyu tian, xueying liu, yuyao wang and huizhi zhou helped perform the analysis with constructive discussions.

Ethical approval: This study was approved by the Biomedical Research Ethic Committee of Shandong Provincial Hospital. (LCYJ;NO.2019-145)

Consent to participate: We informed the patient verbally about the study and obtained the patient's consent.

Consent for publication: Written informed consent for publication was obtained from all participants.

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Figures

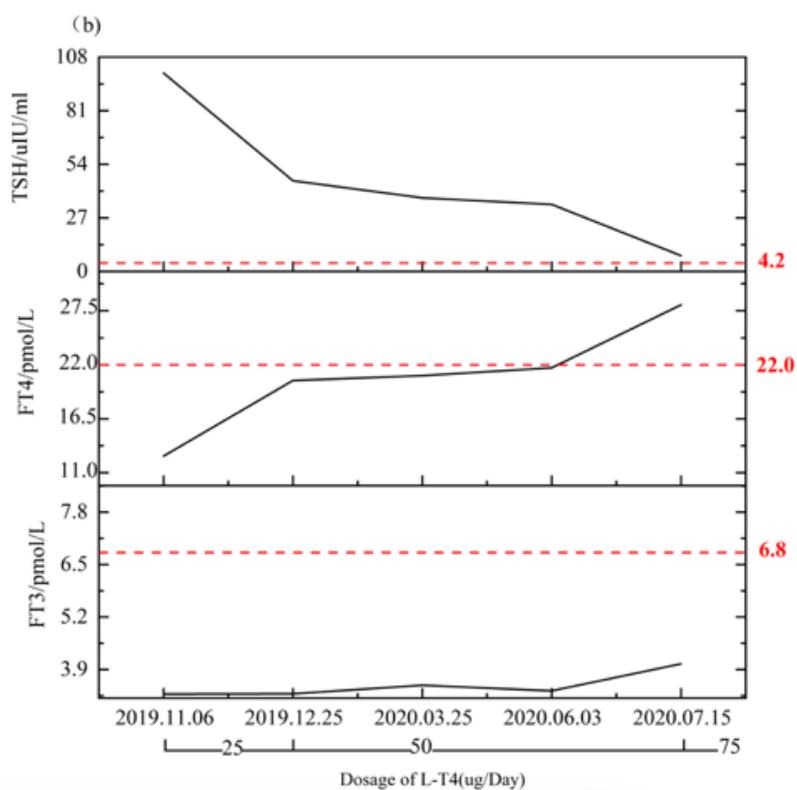
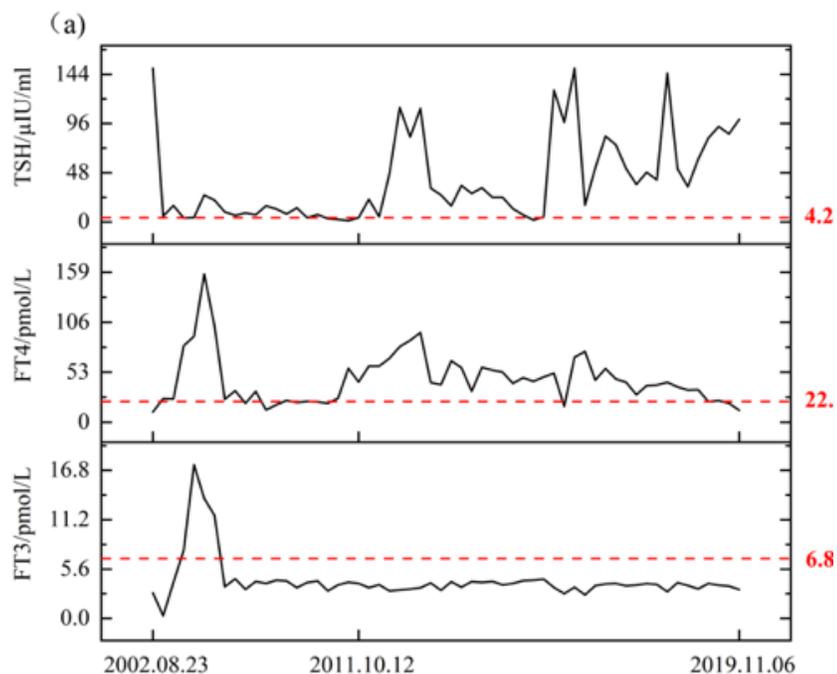


Figure 1

Thyroid function from 2002 to 2019 (a) and 2019 to 2020 (b) and L-T4 dosage adjustment from 2019 to 2020. Thyroid function was detected on the Elecsys immunoassay, and the lines parallel to the horizontal axis represented the upper limit of TSH, FT4 and FT3 reference values successively from top to bottom.

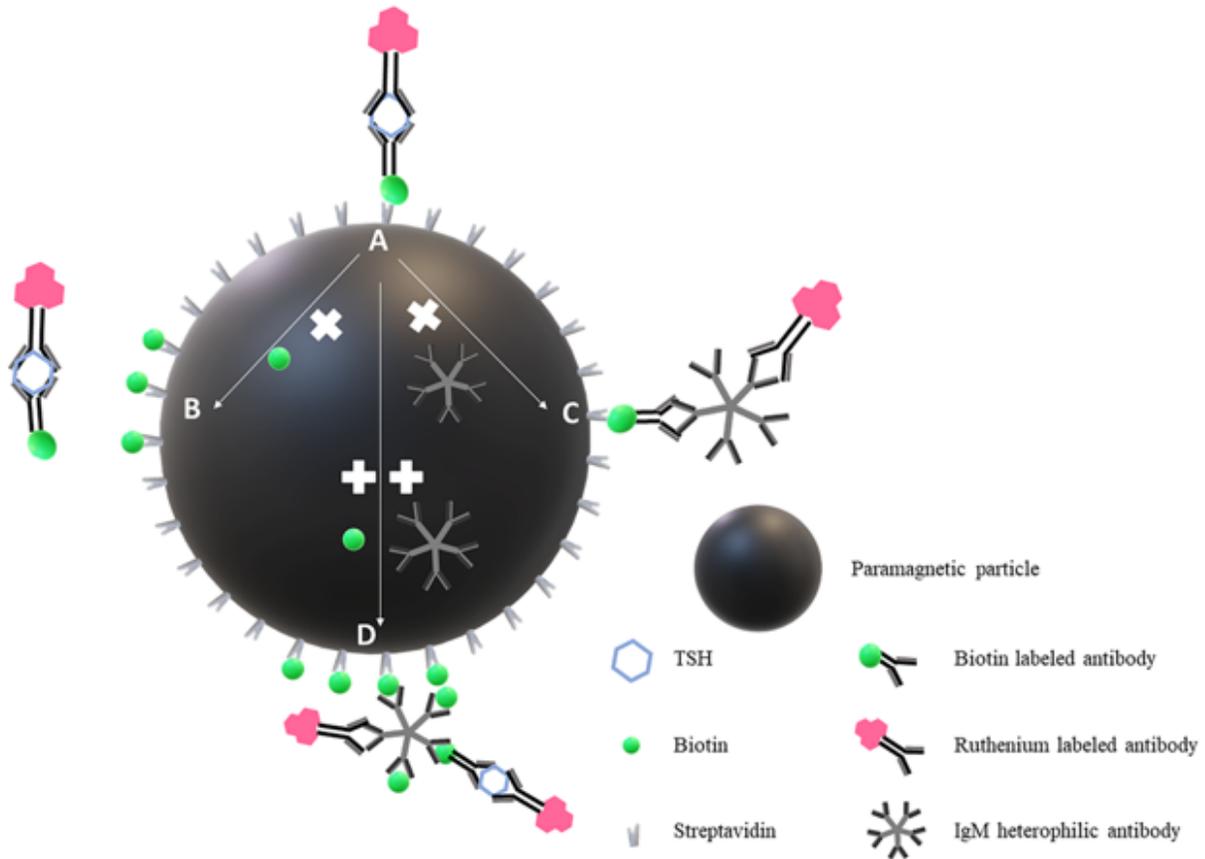


Figure 2

Assay setup and proposed method of interference. A: Sandwich assay: capture antibody immobilizes TSH to the beads by biotin-streptavidin interaction, and ruthenium-labeled antibody is immobilized to the beads by binding to TSH. B: Biotin: when the serum contains an excess of biotin, the biotins bind to streptavidin on the magnetic beads, making the biotinylated capture antibody unable to be fixed to the magnetic beads, resulting in low TSH concentration. C: Heterophilic antibody: heterophilic antibody imitates TSH in binding to capture antibody and detection antibody, resulting in the high TSH concentration. D: Biotin and heterophilic antibody: heterophilic antibody is fixed to the magnetic bead through biotin, and the remaining epitopes can be connected with the detection antibody and biotinylated capture antibody-TSH-detection antibody complex, resulting in high TSH.

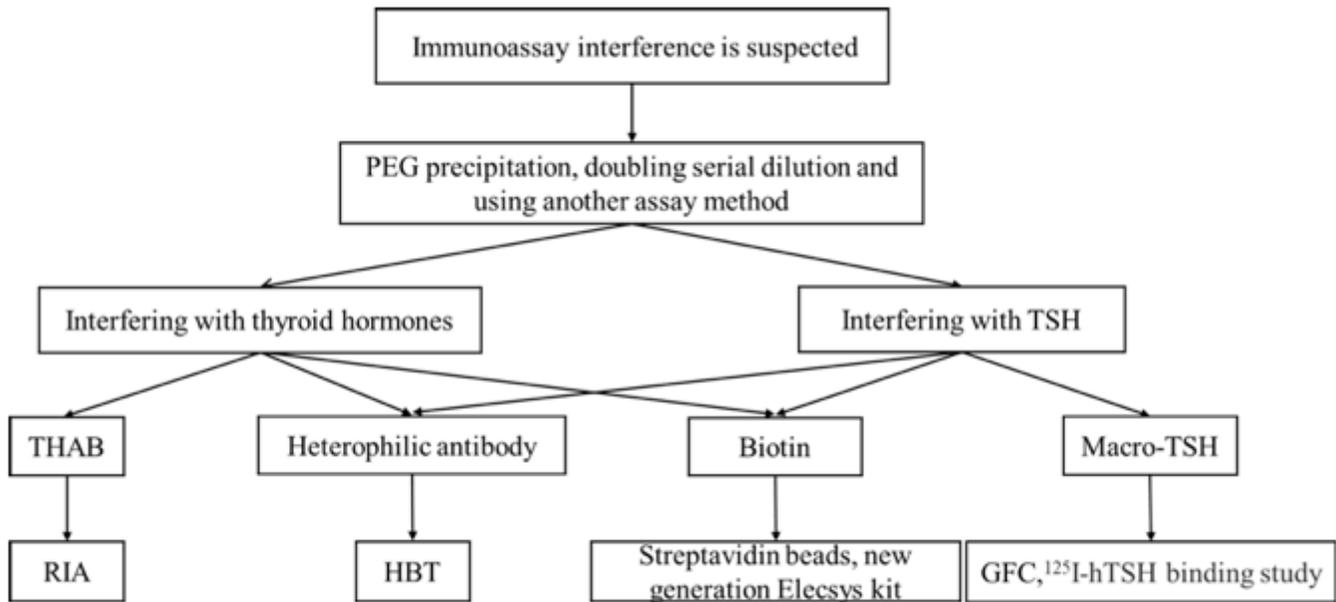


Figure 3

Diagnostic strategy for investigation of interferences with thyroid function immunoassays