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Research Article

Interpretation of heterophile-blocking tube results in thyroid function testing using analytical change limits: An exploratory study of clinically discordant cases

Rana Turkal[1], Ali Yaman[1], Tulay Cevlik[1], Hulya Gozu[2], Onder Sirikci[1,3], Goncagul Haklar[1,3]

[1]Biochemistry Laboratory, Marmara University Pendik Training and Research Hospital, Istanbul, Türkiye
[2]Division of Endocrinology, Department of Internal Medicine, Marmara University Faculty of Medicine, Istanbul, Türkiye
[3]Department of Biochemistry, Marmara University Faculty of Medicine, Istanbul, Türkiye

Abstract

Objectives: This study aimed to evaluate an analytical framework for interpreting heterophile-blocking tube (HBT) results in thyroid function tests (TFTs) by comparing HBT-associated changes in thyroid-stimulating hormone (TSH), free thyroxine (fT4), and free triiodothyronine (fT3) with analyte-specific analytical change limits (ACLs), documenting transitions across laboratory reference intervals, and comparing changes across the three analytes measured in the same treated aliquot.

Methods: This single-center, prospective, exploratory observational study enrolled 41 adults whose TFT results were discordant with their clinical presentations. Untreated and HBT-treated aliquots of the same serum specimen were analyzed in the same run. Signed percentage changes were compared with ACLs of ±2.77 × the analytical coefficient of variation (CV_a) (12.47% for TSH, 15.84% for fT3, and 14.40% for fT4), and paired concentrations were compared using the Wilcoxon signed-rank test with Holm adjustment. Changes in the three analytes from the same treated aliquot were compared, and the sensitivity of the classification to the imprecision estimate was examined.

Results: Median signed changes were -0.45% for TSH, -6.47% for fT3, and -2.11% for fT4; only fT3 showed a significant paired reduction (adjusted p=0.0038). The ACL was exceeded in 8/41 (19.5%) fT3, 1/39 (2.6%) TSH, and 2/41 (4.9%) fT4 results. The rates of TSH and fT4 exceedance were close to those expected from analytical variation alone (p=0.86 and p=0.61, respectively), whereas fT3 exceeded the limit approximately four times more often (p=0.0008). fT3 changes showed no significant correlation with concurrent changes in TSH or fT4, clustered in specimens with elevated pre-HBT fT3 levels, and remained more frequent at every assumed CV_a ranging from 1.8% to 8%. Threshold-supported normalization occurred in four fT3 results and in none of the TSH or fT4 results.

Conclusion: An imprecision-based ACL combined with reference-interval transition provides a transparent criterion for HBT interpretation. HBT-responsive changes occurred predominantly in fT3, whereas TSH and fT4 in the same aliquots were largely unaffected, arguing against a nonspecific pretreatment effect. A change exceeding the ACL is compatible with, but does not by itself prove, heterophile antibody interference and should be interpreted together with the complete thyroid profile, the clinical context, and, where necessary, independent confirmatory testing.

Keywords: Endocrinology, heterophile antibodies, immunoassay, thyroid function tests

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Automated immunoassays are the methods of choice for thyroid function testing, with thyroid-stimulating hormone (TSH) and free thyroxine (fT4) as the primary parameters and free triiodothyronine (fT3) as a complementary test. Nevertheless, thyroid immunoassays remain susceptible to analytical interference from macro-TSH, biotin, anti-strepta-

Address for correspondence: Rana Turkal, MD. Biochemistry Laboratory, Marmara University Pendik Training and Research Hospital, Istanbul, Türkiye
Phone: +90 216 625 45 45 **E-mail:** ranaturkal@yahoo.com **ORCID:** 0000-0002-0042-7261

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vidin and anti-ruthenium antibodies, thyroid hormone autoantibodies, heterophile antibodies, and rheumatoid factor [1–4]. The 2026 European Thyroid Association (ETA) guideline emphasizes that susceptibility to interference is assay-specific and recommends that endocrinologists and laboratory specialists be familiar with the assay used for a particular patient and its limitations [5, 6].

Heterophile antibodies are endogenous immunoglobulins with low-affinity, polyspecific binding to animal immunoglobulins, including assay reagent antibodies; by bridging or blocking capture and detection antibodies, they may produce falsely increased or decreased results. They are distinguished from specific human anti-animal antibodies that develop after exposure to animal-derived immunoglobulins, although immunization, transfusion, autoimmune disease, monoclonal antibody therapy, animal contact, or infection may raise suspicion [1, 2, 7, 8]. Because interference depends on assay architecture, reagent antibodies, and blocking systems, the same sample may yield markedly different results across different platforms, and interference cannot be predicted from clinical findings alone [2, 9, 10]. Reported frequencies range from approximately 0.05% to 6%, depending on the assay, analyte, population, and criteria used to define interference; unrecognized interference in thyroid testing may prompt unnecessary investigations and inappropriate treatment [2, 7, 9].

Suspected interference can be investigated by remeasurement using an alternative assay, serial dilution, polyethylene glycol (PEG) precipitation, or treatment with a heterophile-blocking reagent [11–13]. Heterophile-blocking tubes (HBTs) are a practical option for routine laboratories, but the response to blocking varies according to the interfering antibody, blocking reagent, and assay design; an unchanged result does not exclude interference, and a change does not identify the interfering substance [1, 11]. The 2026 ETA guideline lists HBT pretreatment among the recommended investigative tools, describing it as effective for heterophilic antibody interference but limited to known heterophilic interactions and cautioning that results may be assay-dependent and that false positives may occur; however, it does not specify how large a post-HBT change must be to be regarded as meaningful [5]. Responses to blocking reagents are assay-specific, and HBT-related artifacts have been described for certain assay designs [14–16]. Objective analytical criteria for interpreting HBT results therefore remain limited.

Recognition of interference depends on communication between clinicians and laboratory specialists when the thyroid function test (TFT) pattern is internally inconsistent or does not fit the clinical picture [5, 13, 17, 18]. Published blocking studies have used assay-imprecision-based or empirically derived thresholds [19, 20] or group-level paired tests alone [21]. We therefore evaluated an analytical framework for interpreting HBT results in clinically selected patients with discordant TFTs by comparing changes in TSH, fT4, and fT3 after HBT treatment with analyte-specific analytical change limits and documenting reference-interval transitions.

Materials and Methods

Study population

This single-center, prospective, exploratory observational study was conducted over a 10-month period, during this period, 17,989 TFT panels were performed for 8,299 unique patients evaluated at the Endocrinology Outpatient Clinic of Marmara University Pendik Training and Research Hospital. Forty-one adults were selected by endocrinologists for HBT evaluation because their TFT results were discordant with their clinical presentation; HBT testing was requested on a case-by-case basis, and patients were not systematically screened or consecutively enrolled. Eligibility required at least one of the following: an internally inconsistent TFT profile, biochemical findings that did not explain the clinical presentation, absence of the expected biochemical response to treatment, or hormone results incompatible with the suspected thyroid disorder. Case-based selection reflects the setting in which HBTs are used in practice, as current guidance recommends considering assay interference when TFTs are incongruent with the clinical picture or with each other [5]. This study was approved by the Marmara University Faculty of Medicine Ethics Committee (Date: 05.09.2025, Decision No.: 09.2025.25-0760), and all participants provided informed consent.

Clinical questionnaire

A standardized questionnaire administered at the Biochemistry Laboratory documented demographic characteristics, occupation, transfusion history, allergic disease, dietary habits, pet ownership or other animal exposure, chronic diseases (including autoimmune, thyroid, rheumatological, and cardiovascular diseases), reason for admission, recent infections, vaccinations, medication use, dialysis, and previous therapies, in line with recommendations for clinical review in patients with suspected assay interference [2, 5]. The questionnaire was descriptive; the study was not designed to test associations between exposures and HBT responses.

Sample collection and preliminary assessment

Venous blood was collected into gel-containing serum separator tubes, allowed to clot for 30 minutes, and centrifuged at approximately 2190×g for 10 minutes. Each serum specimen was visually inspected, and lipemia, icterus, and hemolysis (LIH) indices were measured using a Beckman Coulter AU5800 analyzer (Beckman Coulter, Brea, CA, USA); all 41 specimens were within the normal category for all three indices, and none were excluded. Before HBT testing, calibration status, internal quality-control results, reagent status, analyzer flags, and sample identification were reviewed; internal quality control was within acceptance limits, and satisfactory performance for TSH, fT3, and fT4 was confirmed through the Randox International Quality Assessment Scheme (RIQAS). HBT testing was undertaken only when no preanalytical, analytical, or postanalytical error explained the discordance.

Heterophile-blocking procedure and thyroid hormone measurements

Heterophilic Blocking Tubes (part No. 3IX762, Scantibodies Laboratory, Santee, CA, USA) contain a proprietary lyophilized reagent with binders intended to inactivate heterophile antibodies; because the reagent is lyophilized, the procedure adds no diluent. In accordance with the manufacturer's instructions, 500 µL of serum was transferred into an HBT, mixed by five inversions, and incubated for 60 minutes at 18–28°C. Paired untreated and HBT-treated aliquots from the same specimen were analyzed in the same run using a UniCel DxI 800 analyzer (Beckman Coulter). TSH was measured using a two-site sandwich chemiluminescent immunoenzymatic assay (Access HYPERsensitive hTSH), whereas fT3 and fT4 were measured using competitive chemiluminescent immunoenzymatic assays (Access Free T3 and Access Free T4) [22]. HBT-treated results were used solely for within-pair comparisons and were not reported as patient results.

Because HBT-related over-recovery has been reported for Access assays whose solid phase consists of goat anti-mouse antibody-coated particles, attributable to murine components of the blocking reagent [15, 16], assay architecture was verified using the manufacturer's instructions for use [22]. The HYPERsensitive hTSH assay uses paramagnetic particles coated with goat anti-mouse immunoglobulin G (IgG) carrying mouse monoclonal anti-hTSH complexes and therefore has the design feature for which HBT-related over-recovery has been described. The Free T3 assay uses a monoclonal anti-T3–alkaline phosphatase conjugate with streptavidin-coated particles and a biotinylated T3 analog, whereas the Free T4 assay uses a biotinylated mouse monoclonal anti-T4 antibody with streptavidin-coated particles; neither free-hormone assay uses a goat anti-mouse solid phase. TSH results after HBT treatment were therefore examined specifically for over-recovery.

Laboratory reference intervals were 0.34–5.60 mIU/L for TSH, 2.60–4.37 ng/L for fT3, and 0.61–1.12 ng/dL for fT4. The TSH reportable range was 0.015–100 mIU/L (functional sensitivity, 0.015 mIU/L; lower values were reported as <0.015 mIU/L); the measuring intervals were approximately 0.88–30 ng/L for fT3 and 0.25–6.0 ng/dL for fT4.

Assessment of changes following HBT treatment

For each analyte, the effect of HBT treatment was expressed as the signed percentage change: 100×(HBT-treated−untreated)/untreated. To identify changes exceeding analytical imprecision, an analyte-specific analytical change limit (ACL) was calculated at the two-sided 95% level as $ACL_{95}=1.96\times\sqrt{2}\times CV_a=2.77\times CV_a$, where CV_a is the analytical coefficient of variation and $\sqrt{2}$ accounts for the imprecision of two measurements [23, 24]. Because the paired measurements represented an in vitro comparison of aliquots from the same specimen, within-subject biological variation was not included, and a conventional reference change value was not applied.

Laboratory-derived between-day CV_a values (4.50% for TSH, 5.20% for fT4, and 5.72% for fT3; cumulative internal quality-control data from two control levels) were used, yielding ACLs of ±12.47%, ±14.40%, and ±15.84%, respectively. Between-day imprecision comprises the within-run component together with between-run and between-day components and is therefore expected to be at least as large as within-run imprecision [23]; the resulting ACL may consequently have yielded relatively conservative thresholds for same-run comparisons, such that classification would tend toward undercalling rather than overcalling threshold exceedance, although imprecision varies with concentration and was not separately established for HBT-treated serum. The manufacturer's stated within-run CV_a values at control concentrations within and above the reference intervals are 2.5–3.1% for TSH, 1.8–2.7% for fT4, and 2.6–5.1% for fT3 and are higher only at the lowest control level of each assay [22]; the sensitivity analysis described below (1.8–8%) encompasses all of these values. An absolute change exceeding the ACL was classified as exceeding the change expected from analytical variation alone and is termed an HBT-responsive change because HBT treatment does not identify the interfering substance.

Exploratory cross-analyte and sensitivity analyses

Because TSH, fT3, and fT4 were measured in the same HBT-treated aliquot, a pretreatment effect common to the entire aliquot (volume displacement or gross matrix alteration) would be expected to affect all three analytes. Comparisons across analytes therefore address a nonspecific pretreatment effect and complement an HBT-treated control group. Three post hoc analyses were added during peer review and are reported as exploratory. First, observed ACL exceedance for each analyte was compared with the nominal 5% rate of a two-sided 95% limit using the one-sided exact binomial test; this nominal rate applies only if the CV_a used reflects the imprecision of the paired measurements under the study conditions. Exceedance was compared across analytes using Cochran's Q test for related proportions in the 39 patients with quantifiable pairs for all three analytes, with an exact p value obtained by permutation within patients. Second, Spearman correlation was used to assess whether percentage changes were related across analytes within specimens and whether the fT3 change was related to the pre-HBT fT3 concentration; fT3 changes were also summarized according to whether pre-HBT fT3 exceeded the upper reference limit, and the paired fT3 comparison was repeated after excluding specimens exceeding the ACL. Third, ACL classification was recalculated for assumed CV_a values of 1.8–8%.

Statistical analysis

Continuous variables were summarized as the median (interquartile range [IQR]). Paired pre- and post-HBT comparisons for the three analytes were the primary analyses and were performed using the two-sided asymptotic Wilcoxon signed-rank test, with Holm adjustment for the three comparisons; a

Table 1. Comparison of thyroid function test results before and after heterophile-blocking treatment

Analyte	Evaluable pairs, n	Pre-HBT, median (IQR)	Post-HBT, median (IQR)	Signed change, %, median (IQR)	Paired Wilcoxon P	Holm-adjusted P[b]	Analytical threshold exceeded, n/N (%) [95% CI]	Post-HBT normalization with ACL exceedance, n/N (%) [95% CI]
TSH (mIU/L)	39[a]	2.36 (1.57–5.06)	2.34 (1.52–4.75)	-0.45 (-3.71 to +1.74)	0.2519	0.2519	1/39 (2.6%) [0.5–13.2%]	0/39 (0.0%) [0.0–9.0%]
fT3 (ng/L)	41	3.66 (3.28–5.00)	3.56 (3.15–3.96)	-6.47 (-13.91 to +1.08)	0.0013	0.0038	8/41 (19.5%) [10.2–34.0%]	4/41 (9.8%) [3.9–22.5%]
fT4 (ng/dL)	41	1.18 (0.95–1.55)	1.15 (0.91–1.53)	-2.11 (-5.77 to +2.59)	0.0389	0.0778	2/41 (4.9%) [1.3–16.1%]	0/41 (0.0%) [0.0–8.6%]

Data are presented as median (interquartile range). Signed percentage change was calculated as 100 × (post-HBT – pre-HBT)/pre-HBT. Paired P values were obtained using the two-sided asymptotic Wilcoxon signed-rank test and adjusted for the three analytes using the Holm method. Analytical change limits (ACLs) were defined as ±2.77 × CV_a: 12.47% for TSH, 15.84% for fT3, and 14.40% for fT4. Post-HBT normalization with ACL exceedance required both movement from outside to within the corresponding reference interval (TSH, 0.34–5.60 mIU/L; fT3, 2.60–4.37 ng/L; fT4, 0.61–1.12 ng/dL) and an absolute percentage change exceeding the corresponding ACL. Wilson score 95% confidence intervals were calculated for the proportions reported in the final two columns. For n/N, n denotes the number of pairs meeting the specified criterion and N the number of evaluable pairs. [a]: TSH paired concentration and percentage-change analyses included 39 quantifiable pairs because two pairs were reported as <0.015 mIU/L both before and after HBT treatment; [b]: A two-sided Holm-adjusted p<0.05 was considered statistically significant. ACL: Analytical change limit; CI: Confidence interval; CV_a: Analytical coefficient of variation; fT3: Free triiodothyronine; fT4: Free thyroxine; HBT: Heterophile-blocking tube; IQR: Interquartile range; TSH: Thyroid-stimulating hormone.

Holm-adjusted p<0.05 was considered statistically significant. ACL exceedance and reference-interval transitions were descriptive secondary outcomes. Post-HBT normalization with ACL exceedance was defined as a transition from outside to within the reference interval accompanied by an absolute change exceeding the ACL; Wilson score 95% confidence intervals were calculated for proportions. TSH pairs reported as <0.015 mIU/L both before and after treatment were excluded from paired concentration and percentage-change analyses but retained for reference-interval transition analyses; zero differences were excluded from the signed-rank calculation. Analyses were performed using Python 3.12.13 with NumPy 2.3.5 and SciPy 1.17.0; figures were generated using Matplotlib 3.10.8.

Results

Study cohort

The clinical and exposure characteristics of the 41 patients are summarized in Appendix 1. The median age was 41 years (IQR, 31–59; range, 22–74), and 27 patients (65.9%) were female. Multiple-analyte abnormalities were the most frequent pre-HBT pattern (16 patients, 39.0%), followed by isolated fT4 abnormalities (10, 24.4%); all three analytes were within the reference intervals in 7 patients (17.1%), whereas isolated fT3 and isolated TSH abnormalities were observed in 5 (12.2%) and 3 (7.3%) patients, respectively. Known thyroid disease was recorded in 20/38 (52.6%), animal contact in 12/40 (30.0%), and recent upper respiratory tract infection or gastroenteritis in 7/40 (17.5%) of patients with available information.

Cohort-level paired comparisons and specimen-level ACL exceedance

Paired fT3 and fT4 results were available for all 41 patients; TSH percentage-change analyses included 39 pairs because both results for patients 30 and 34 were <0.015 mIU/L (Table 1). Median TSH was 2.363 mIU/L (IQR, 1.565–5.064) before and 2.343 mIU/L (IQR, 1.523–4.747) after treatment; the median signed change was -0.45% (IQR, -3.71% to +1.74%; Holm-adjusted p=0.2519). One of 39 pairs (2.6%) exceeded the TSH ACL (+12.66%), with both measurements within the reference interval. Median fT4 decreased from 1.18 ng/dL (IQR, 0.95–1.55) to 1.15 ng/dL (IQR, 0.91–1.53), representing a median change of -2.11% (IQR, -5.77% to +2.59%); the difference was significant before (p=0.0389) but not after Holm adjustment (p=0.0778). Two of 41 pairs (4.9%) exceeded the fT4 ACL, neither with normalization. Median fT3 decreased from 3.66 ng/L (IQR, 3.28–5.00) to 3.56 ng/L (IQR, 3.15–3.96), representing a median change of -6.47% (IQR, -13.91% to +1.08%), which was within the fT3 ACL; the reduction remained significant after adjustment (p=0.0013; Holm-adjusted p=0.0038). Eight of 41 pairs (19.5%) exceeded the fT3 ACL (patients 1, 3, 4, 22, 26, 32, 33, and 40; Fig. 1): seven decreases ranging from -16.3% to -38.6% and one increase of +35.6% (patient 22).

Reference-interval transitions

Individual fT3 trajectories are shown in Figure 2, and the eight patients with threshold-exceeding changes are described in Table 2. Four patients (patients 1, 3, 4, and 40) moved from above to within the fT3 reference interval; patients 26 and 32 remained within the interval, patient 33 remained above it, and patient 22 remained below it. Evaluated independently of the threshold, post-HBT normalization occurred in five fT3 results (12.2%; patients 1, 3, 4, 7, and 40) and two fT4 results (4.9%; patients 15 and 34), but in no TSH result; patient 7 reached the fT3 upper reference limit with a change (-5.6%) within the ACL. Thus, four fT3 results (9.8%) fulfilled both the normalization and threshold criteria (Table 1), whereas neither fT4 normalization exceeded the ACL, and two additional fT4 results moved from within to outside the interval.

Exploratory cross-analyte and sensitivity analyses

Under a two-sided 95% ACL, approximately 5% of pairs are expected to exceed the limit by chance, provided that the imprecision estimate reflects the paired measurements under

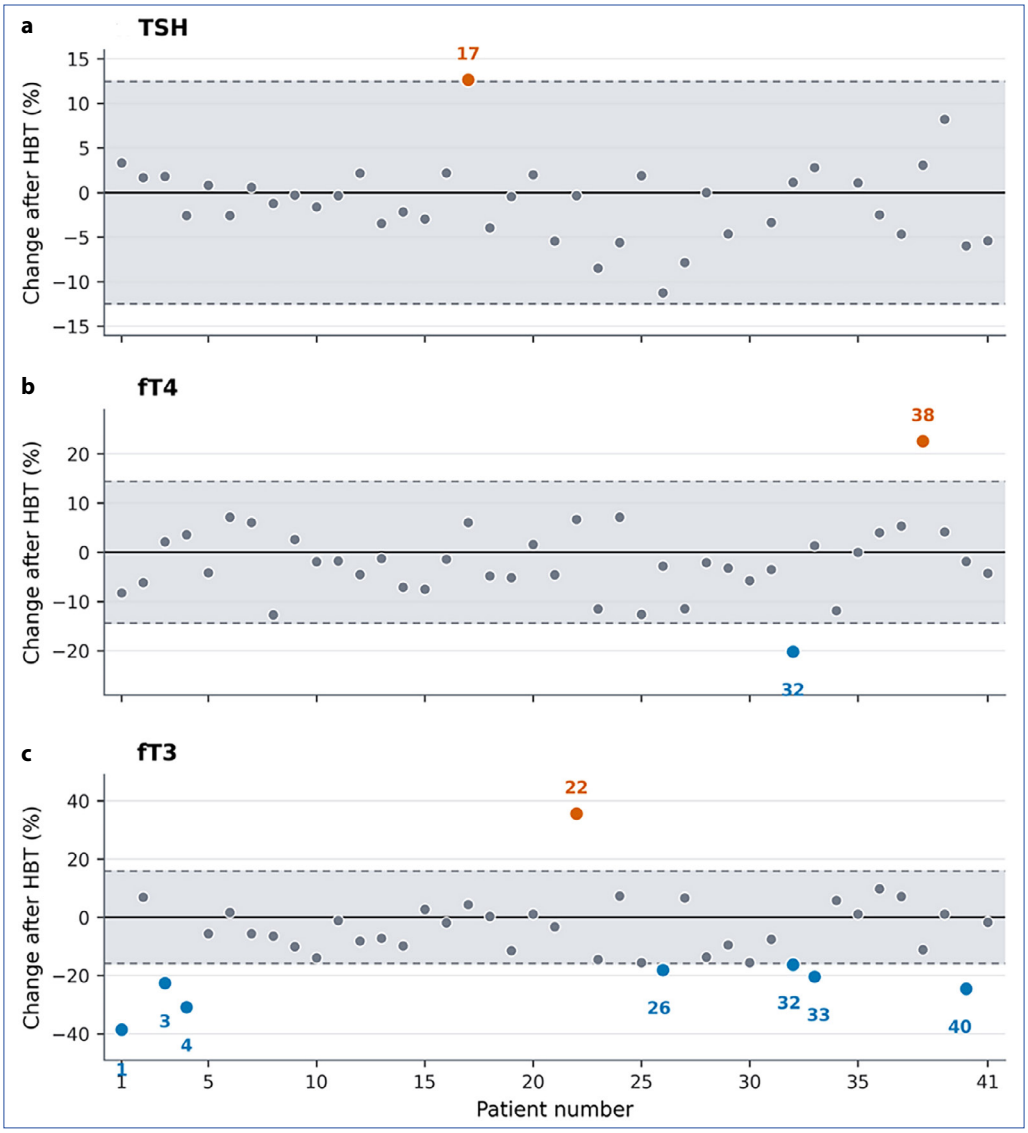

Figure 1. Percentage changes in thyroid function test results following heterophile-blocking treatment. Panels show the percentage changes in (a) thyroid-stimulating hormone (TSH), (b) free thyroxine (fT4), and (c) free triiodothyronine (fT3) for the 41 patients. Signed percentage change was calculated as 100 × (post-HBT − pre-HBT)/pre-HBT. The shaded bands represent the analyte-specific analytical change limits (ACLs), calculated as ±2.77 × CV_a. The CV_a values were 4.50% for TSH, 5.20% for fT4, and 5.72% for fT3, yielding ACLs of ±12.47%, ±14.40%, and ±15.84%, respectively. Gray points represent changes within the corresponding ACL, whereas orange and blue points represent increases and decreases exceeding the ACL, respectively. Patient numbers are shown for results exceeding the corresponding ACL. Percentage changes in TSH were not calculated for patients 30 and 34 because both their pre-HBT and post-HBT results were reported as <0.015 mIU/L. Under a two-sided 95% ACL, approximately 5% of points (about two per panel) are expected to fall outside the band by chance, provided that the imprecision estimate reflects the paired measurements under the study conditions.

ACL: Analytical change limit; CV_a: Analytical coefficient of variation; fT3: Free triiodothyronine; fT4: Free thyroxine; HBT: Heterophile-blocking tube; TSH: Thyroid-stimulating hormone.

the study conditions. Observed exceedance was close to this nominal rate for TSH (1/39, 2.6%; p=0.86) and fT4 (2/41, 4.9%; p=0.61), but not for fT3 (8/41, 19.5%; p=0.0008) (Table 3). Exceedance differed significantly across the three analytes in the same 39 patients (Cochran's Q=8.60, df=2, p=0.014; exact p=0.018): 29 patients showed no exceedance, seven exceeded the fT3 ACL only, one exceeded both the fT3 and fT4 ACLs, one exceeded the fT4 ACL only, and one exceeded the TSH ACL only. No systematic increase in TSH was observed: 23 of 39 quantifiable pairs decreased and 15 increased, and the single pair exceeding the TSH ACL remained within the reference interval. Percentage changes were not significantly correlated

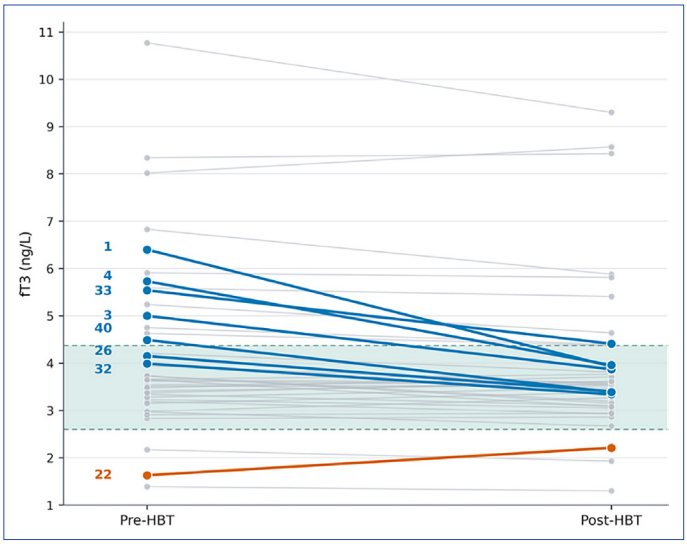

Figure 2. Individual changes in free triiodothyronine concentrations following heterophile-blocking treatment. Each line connects the pre-HBT and post-HBT fT3 measurements of an individual patient. The shaded band represents the laboratory reference interval (2.60–4.37 ng/L). Eight patients whose absolute percentage changes exceeded the fT3 ACL of 15.84% are highlighted and labeled. Blue lines indicate decreases and the orange line indicates an increase following HBT treatment; patients whose changes remained within the ACL are shown in gray. Among the eight highlighted patients, patients 1, 3, 4, and 40 showed post-HBT normalization with ACL exceedance, moving from above to within the reference interval. Patients 26 and 32 were already within the reference interval before HBT treatment, patient 33 remained above the upper limit, and patient 22 remained below the lower limit.

ACL: Analytical change limit; fT3: Free triiodothyronine; HBT: Heterophile-blocking tube.

across analytes within specimens (fT3 vs. fT4, ρ=0.25, p=0.11; fT3 vs. TSH, ρ=-0.10, p=0.56; fT4 vs. TSH, ρ=0.17, p=0.29). In the eight specimens with fT3 changes exceeding the ACL, concurrent TSH changes ranged from -11.2% to +3.3% (all within the TSH ACL), and fT4 changes ranged from -20.2% to +6.7%; only patient 32 also exceeded the fT4 ACL.

The fT3 change was inversely related to the pre-HBT fT3 concentration (ρ=-0.45, p=0.003). Among 14 specimens with pre-HBT fT3 above the upper reference limit, the median change was -12.5%, and 5 (35.7%) exceeded the ACL, whereas among 27 specimens with fT3 within or below the reference interval, the median change was -1.9% (paired p=0.10), and 3 (11.1%) exceeded the ACL. Among the 22 specimens within the reference interval that did not exceed the ACL, the median change was -0.4% (p=0.22). After exclusion of the eight ACL-exceeding specimens, a small residual decrease in fT3 persisted (n=33; median, -3.2%; IQR, -9.9% to +1.7%; p=0.02), similar in magnitude to the overall fT4 change. Across assumed CV_a values of 1.8–8% (ACL, 5.0–22.2%), fT3 exceedance ranged from 30/41 to 5/41 and was higher than TSH (9/39 to 0/39) and fT4 (19/41 to 1/41) exceedance at every assumption (Table 3); with the manufacturer's within-run CV_a range for fT3 (2.6–5.1%), 22 to 11 fT3 pairs would have exceeded the ACL.

Discussion

The principal finding was that changes exceeding the analyte-specific ACL after HBT treatment occurred predominantly in the fT3 assay: 8 of 41 pairs (19.5%) showed an absolute fT3 change greater than 15.84%, four (9.8%) both exceeded this limit and moved from above to within the reference interval, and fT3 was the only analyte with a significant paired difference after Holm adjustment. The ACL was exceeded in only 1 of 39 TSH pairs and 2 of 41 fT4 pairs, with no threshold-supported normalization, close to the approximately 5% expected for a two-sided 95% limit when no effect is present, provided that the imprecision estimate is appropriate, whereas fT3 exceedance was approximately four times higher. A response to blocking may thus be confined to one component of the thyroid profile even when all three analytes are measured in the same specimen on the same platform, consistent with the known dependence of antibody interference on assay format, reagents, and blocking strategy [1, 2, 5]. Current guidance recommends HBT pretreatment but provides no criterion for determining when a post-HBT change is analytically meaningful [5], and the criteria used in published blocking studies are heterogeneous, as discussed below. A limit derived in advance from the laboratory's own imprecision is transparent and transferable across laboratories, and the observed exceedance for analytes without a detectable HBT effect offers a simple, albeit assumption-dependent, plausibility check of the threshold.

The frequency of fT3 responses must be interpreted in the context of patient selection. Only 41 of 8,299 patients (0.49%) underwent HBT evaluation and were selected because their results were discordant with the clinical picture or with the remainder of the profile, in accordance with current recommendations [5]; the exceedance rate in this group therefore cannot be used to estimate interference in the general outpatient population, which would require a large unselected series [9, 25]. In a much larger study using Roche electrochemiluminescence assays, Imamura et al. [25] confirmed interference in 53 of 83,044 fT3 measurements (0.064%), compared with 11 of 124,609 TSH measurements (0.009%) and 6 of 116,012 fT4 measurements (0.005%). Although the populations, platforms, algorithms, and definitions differed, both studies identified fT3 as the most frequently affected analyte; the fT3 interferences characterized by Imamura et al. [25] were mainly attributed to idiotype or anti-streptavidin antibodies, supporting a broader susceptibility of fT3 measurement rather than a specific predominance of classical heterophile antibodies.

The predominance of fT3 responses is unlikely to be explained by assay format alone: sandwich assays such as TSH are susceptible to antibody bridging, whereas competitive free-hormone assays may be affected by endogenous antibodies, altered binding proteins, tracer interactions, and other method-specific factors [1, 2]. In competitive assays, an endogenous antibody may bind the assay antibody, tracer or analog, or analyte itself and may therefore produce falsely low or falsely high results in the same assay; a nonuniform direc-

Table 2. Clinical and biochemical characteristics of patients with fT3 changes exceeding the analytical change limit after HBT treatment

Patient no.	Age, years	Recorded clinical context	Pre-HBT fT3, ng/L	Post-HBT fT3, ng/L	Signed change, %	fT3 reference-interval transition	Post-HBT TFT profile
1	31	Toxic multinodular goiter; methimazole discontinued	6.40	3.93	-38.6	Above to within[a]	TSH remained below the reference interval
3	31	Palpitations; propranolol use	5.00	3.87	-22.6	Above to within[a]	fT4 remained above the reference interval
4	35	Preoperative evaluation; previous HBT-responsive troponin result and bird exposure	5.73	3.96	-30.9	Above to within[a]	All three TFT results were within their respective reference intervals
22	74	Thyroid nodule; cardiovascular and metabolic comorbidities	1.63	2.21	+35.6	Below to below	fT4 remained above the reference interval
26	22	Previous fT4 elevation	4.15	3.40	-18.1	Within to within	All three TFT results remained within their respective reference intervals
32	23	Persistent TSH elevation; previous gastric cancer treatment	3.99	3.34	-16.3	Within to within	TSH remained above the reference interval; fT4 change (−20.2%) also exceeded the fT4 ACL
33	44	History of hypothyroidism; levothyroxine discontinued; microadenoma	5.54	4.41	-20.4	Above to above	TSH and fT4 remained above their respective reference intervals
40	33	Isolated fT3 elevation	4.49	3.39	-24.5	Above to within[a]	All three TFT results were within their respective reference intervals

[a]: Post-HBT fT3 normalization with ACL exceedance was defined as a transition from above to within the fT3 reference interval (2.60–4.37 ng/L), accompanied by an absolute percentage change exceeding the fT3 ACL of 15.84%. Post-HBT normalization refers only to the fT3 result and does not imply clinical resolution. Concurrent TSH changes in these eight specimens ranged from -11.2% to +3.3% (all within the TSH ACL). ACL: Analytical change limit; fT3: Free triiodothyronine; fT4: Free thyroxine; HBT: Heterophile-blocking tube; TFT: Thyroid function test; TSH: Thyroid-stimulating hormone.

tion of change, as observed here (seven decreases and one increase), is thus expected rather than anomalous [1, 11]. Method dependence is well documented: Külz et al. [26] found that discordant fT4 and fT3 results varied among platforms, whereas total T3 remained within the reference interval in 70 of 71 samples; Cheng et al. [27] obtained TSH values ranging from 5.52 to <0.015 mIU/L for the same sample on four platforms, with markedly different responses to a blocking reagent; and Khoo et al. [28] found that fT4 assay susceptibility to familial dysalbuminaemic hyperthyroxinaemia, which was greatest for Beckman Access, depended on assay conditions rather than architecture. Because interference is method-specific and current guidance recommends that laboratories understand the behavior of the assays they use [5, 6], platform-specific data are required, and published HBT data for thyroid assays on the DxI/Access platform are scarce. Whether the predominance of fT3 responses reflects a particular vulnerability of the DxI fT3 assay, whose reagent formulation includes goat, bovine, and avian proteins and a streptavidin–biotin solid phase [22], remains uncertain and requires cross-platform evaluation.

Patient-level findings demonstrate why threshold exceedance and reference-interval transition should be evaluated separately. Of the eight patients with threshold-exceeding fT3 changes, four moved into the fT3 reference interval, but the complete profile normalized in only two; of the remaining four, two were within the interval before and after treatment, one remained above it, and one increased but remained below it (Fig. 2, Table 2). Conversely, patient 7 reached the fT3 upper reference limit without exceeding the ACL, and two fT4 results normalized without exceeding the fT4 ACL, whereas two others moved outside the interval. Neither criterion alone confirms interference or establishes clinical significance.

No generally accepted decision rule exists for blocking experiments. Emerson et al. [19] classified a result as discrepant when |Z|, calculated from within-run imprecision, exceeded 4; Preissner et al. [20] used an empirical cutoff of 3 standard deviations from the mean percentage difference after HBT treatment. Our ACL of $2.77\times CV_a$ is the two-sided 95% limit for the difference between paired measurements [23, 24]; because its basis and nominal cutoff differ from those criteria, response rates across studies should not be compared directly. Because the between-day CV_a was derived from quality-control data accumulated across runs and days, it may have yielded relatively conservative thresholds for same-run comparisons: with the manufacturer's within-run CV_a range for fT3, the number of ACL-exceeding fT3 results would have been 11–22 rather than 8, and the excess of fT3 over TSH and fT4 persisted at every CV_a assumption between 1.8% and 8%. Thus, although the absolute number of ACL-exceeding results depends on the imprecision estimate used, the excess

Table 3. Exploratory cross-analyte and sensitivity analyses of analytical change limit exceedance

A. Observed versus expected ACL exceedance in the same HBT-treated aliquots

Analyte / subgroup	Exceeding ACL, n/N (%)	Nominal 5% of N	Exact binomial P	Signed change, %, median (IQR)
TSH	1/39 (2.6)	2.0	0.86	-0.45 (-3.71 to +1.74)
fT4	2/41 (4.9)	2.1	0.61	-2.11 (-5.77 to +2.59)
fT3	8/41 (19.5)	2.1	0.0008	-6.47 (-13.91 to +1.08)
fT3, pre-HBT fT3 > upper reference limit	5/14 (35.7)	0.7	—	-12.5
fT3, pre-HBT fT3 within or below reference interval	3/27 (11.1)	1.4	—	-1.9
fT3, within reference interval and not exceeding ACL	—	—	—	-0.4 (n = 22)
fT3, excluding ACL-exceeding specimens	—	—	—	-3.2 (-9.9 to +1.7)

B. Sensitivity of ACL classification to the assumed analytical coefficient of variation

Assumed CV_a	ACL (±%)	TSH exceeding, n/39	fT4 exceeding, n/41	fT3 exceeding, n/41
1.8%	5.0	9	19	30
2.6%	7.2	5	9	22
3.0%	8.3	3	7	19
4.0%	11.1	2	7	14
5.0%	13.9	0	2	12
5.1%	14.1	0	2	11
Laboratory between-day CV_a (4.50/5.20/5.72%)	12.5/14.4/15.8	1	2	8
7.0%	19.4	0	2	6
8.0%	22.2	0	1	5

Part A: the nominal proportion of pairs exceeding a two-sided 95% ACL in the absence of an effect is 5%, provided that the imprecision estimate reflects the paired measurements under the study conditions; P values are from the one-sided exact binomial test against 5% and are exploratory. Cochran's Q test for exceedance across the three analytes in the 39 patients with quantifiable pairs for all analytes: Q = 8.60, df=2, p=0.014 (exact permutation p=0.018); joint pattern: no exceedance in 29, fT3 only in 7, fT3 and fT4 in 1, fT4 only in 1, TSH only in 1. Spearman correlations of percentage changes across analytes: fT3 vs fT4, ρ=0.25 (p=0.11); fT3 vs TSH, ρ=−0.10 (p=0.56); fT4 vs TSH, ρ=0.17 (p=0.29). Part B: ACL=2.77 × assumed CV_a; the same assumed CV_a was applied to all three analytes except in the laboratory between-day row. Manufacturer's stated within-run CVs: TSH 2.5–3.1%, fT4 1.8–2.7%, fT3 2.6–5.1% at control concentrations within and above the reference intervals, and up to 6.6% at the lowest control level [22]; the 1.8–8% range encompasses all of these values. ACL: Analytical change limit; CV_a: Analytical coefficient of variation; df: Degrees of freedom; fT3: Free triiodothyronine; fT4: Free thyroxine; HBT: Heterophile-blocking tube; IQR: Interquartile range; TSH: Thyroid-stimulating hormone.

of fT3 exceedance over TSH and fT4 was present at every imprecision estimate examined. Our results also illustrate why a significant paired test should not be taken as evidence of interference: the cohort-level fT3 reduction was significant, yet its median magnitude (-6.5%) was well within the ACL, and only eight specimens exceeded the applied ACL. A significant group-level test can also arise from a small systematic shift present in every sample without any specimen being individually discrepant. Specimen-level criteria referenced to analytical imprecision [19] or to an empirically derived distribution of changes [20] are therefore required.

An HBT-associated change exceeding the ACL is compatible with endogenous antibody interference but does not confirm heterophile antibody interference or identify the interfering substance; blocking reagents do not neutralize every heterophile or human anti-animal antibody, and no single investigation can fully characterize interference [2, 5, 11]. Candidate mechanisms for HBT-responsive fT3 changes on this platform include heterophile and human anti-animal antibodies (the manufacturer notes that human anti-goat antibodies may interfere with the Free T3 assay [22]), thyroid hormone autoantibodies, and anti-idiotype antibodies; the present study cannot distinguish among them. Confirmatory approaches include remeasurement using a differently designed platform, total T3 measurement, and, for TSH, PEG precipitation or immunoglobulin depletion; equilibrium dialysis or ultrafiltration with mass spectrometry provides a more independent assessment of free hormones [29, 30], whereas serial dilution and PEG precipitation are less suitable for fT3 and fT4 because they disturb the bound–free equilibrium [2, 5, 11]. For suspected fT4/fT3 interference, the ETA guideline recommends an alternative assay, total hormone measurement, or referral to a specialist laboratory, while acknowledging that local availability may be limited [5]. The present study was designed to evaluate the HBT procedure as used in routine practice on the laboratory's single immunoassay platform; confirmation using a differently designed platform was outside the study protocol, and no alternative thyroid immunoassay or total T3 method was available at our institution. Although a liquid chromatography–tandem mass spectrometry system was available, direct free-hormone measurement requires prior separation of the free fraction by equilibrium dialysis or ultrafiltration, and no validated method of this kind had been established. The previous HBT-responsive troponin

result of patient 4, whose fT3 decreased by 30.9%, supports the clinical suspicion of an antibody-mediated mechanism in that patient but does not constitute independent analytical confirmation of the fT3 change (Table 2).

The questionnaire findings are descriptive: animal contact and recent infection were reported by 30.0% and 17.5% of patients with available information, respectively, but without a comparison group and with only eight threshold-exceeding fT3 results, the study could not assess whether any exposure was associated with interference. Heterophile reactivity occurs in individuals without recognized exposure [1, 7], so a negative exposure history does not exclude interference.

Although the study did not include a control group of samples without suspected interference subjected to the same HBT procedure, an internal reference was available within the study material. Preissner et al. [20] treated their entire study cohort and used the distribution of changes itself as the reference; a related approach was used here, with TSH and fT4 measured in the same treated aliquots serving as internal references. In our study, the cross-analyte comparisons addressed a nonspecific effect common to the entire aliquot: TSH and fT4 measured in the same treated aliquots exceeded their ACLs at rates close to the nominal rate, with a median TSH change of -0.45%, arguing against a volume or gross matrix effect of the tube. The clustering of fT3 changes in specimens with elevated pre-HBT fT3 is consistent with interference where it was clinically suspected, although this association is descriptive because percentage change is related to the baseline value. The small residual fT3 decrease after excluding the ACL-exceeding specimens (median, -3.2%) was similar to the overall fT4 change and remained within the ACL; it may represent subthreshold interference, a minor assay- or pretreatment-related effect, or both. HBT-related over-recovery has been described for Access assays with goat anti-mouse-coated particles [15, 16], and a marked HBT-associated increase in luteinizing hormone on the DxI 800 was recently reported [21]. Of the three assays used here, only the HYPERsensitive hTSH assay has this design feature, and no systematic increase in TSH was observed; the fT3 and fT4 assays use streptavidin–biotin solid phases, so this particular mechanism is unlikely to explain the fT3 findings, although other reagent-related artifacts cannot be excluded. HBT applicability must therefore be verified assay by assay, and an HBT-treated control series on this platform is needed.

The strengths of the study include the paired same-specimen, same-run design, which excluded biological and between-run variation; the standardized blocking procedure; consistently applied analyte-specific limits evaluated alongside reference-interval transitions and individual trajectories; and the availability of TSH and fT4 results from the same treated aliquots. The main limitations are the small, single-center cohort; use of a single immunoassay platform; the absence of a separately treated control group, addressed in part by cross-analyte comparisons within the same treated aliquots; the absence of independent confirmation; reliance on between-day rather than within-run CV_a; and incomplete questionnaire data. Where the data permitted, the influence of these limitations was examined rather than merely acknowledged through cross-analyte comparisons and sensitivity analysis. Without independent confirmation, the findings are more appropriately described as HBT-responsive changes compatible with endogenous antibody interference than as evidence of heterophile-antibody positivity, and they do not permit estimation of the prevalence of interference in the outpatient population or on the DxI platform.

In conclusion, in this clinically selected cohort, an imprecision-based analytical change limit combined with reference-interval transition provided a transparent criterion for interpreting HBT results. HBT-associated changes exceeding the limit occurred predominantly in the fT3 assay, whereas comparable responses were uncommon for TSH and fT4 in the same treated aliquots. This pattern identifies fT3 on the DxI platform as the analyte for which HBT results most often require further work-up and raises the possibility of method-specific susceptibility of fT3 measurement under the conditions studied, but it does not establish that every threshold-exceeding change was caused by a heterophile antibody or establish the mechanism or prevalence of interference. HBT treatment should remain one component of a broader interference investigation and should be interpreted in conjunction with the complete thyroid profile, clinical context, and, where necessary, independent confirmatory testing; close communication between clinicians and laboratory specialists remains essential when thyroid results are internally inconsistent or do not fit the clinical course.

Disclosures

Ethics Committee Approval: The study was approved by the Marmara University Faculty of Medicine Ethics Committee (no: 09.2025.25-0760, date: 05/09/2025).

Informed Consent: Informed consent was obtained from the participants.

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Appendix 1. Clinical history and exposure characteristics of patients evaluated for suspected immunoassay interference (n=41)

Characteristic	Value[a]
Age, years, median (IQR) [range]	41 (31–59) [22–74]
Female sex	27/41 (65.9%)
Male sex	14/41 (34.1%)
Known thyroid disease	20/38 (52.6%)
Thyroid medication use	17/38 (44.7%)
Non-thyroid medication use	22/38 (57.9%)
Upper respiratory tract infection or gastroenteritis within the previous 6 months	7/40 (17.5%)
Animal contact	12/40 (30.0%)
Allergic disease	5/41 (12.2%)
Influenza vaccination	5/41 (12.2%)
Previous blood or blood-product transfusion	2/41 (4.9%)

[a]: Data are presented as median (interquartile range) [range] or n/N (%), where N represents the number of patients with available information. Clinical history and exposure variables were summarized descriptively. HBT: heterophile-blocking tube; IQR: Interquartile range.