Thyroglobulin Assay Interferences: Clinical Usefulness of Mass-Spectrometry Methods

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Abstract

Context: Thyroglobulin autoantibodies (TgAbs) affect thyroglobulin immunometric assays (TgIMAs), causing falsely low results. Conversely, heterophilic antibodies (HAs) may cause falsely elevated results. Thyroglobulin (Tg) measurements by mass spectrometry (MS) resist antibody interference. The most effective use of TgIMA/TgMS in the evaluation of Tg remains unclear.

Objective: The objective of this work was to study the usefulness of TgMS vs TgIMA in the presence of Tg measurement interference by HA and TgAb.

Methods: In 163 thyroid cancer patients, Tg was postoperatively measured by TgIMA and TgMS. When TgIMA was elevated and TgMS undetectable, HA was assessed by serial dilution and pretreatment with HA blocking reagent. TgIMA and TgMS were compared in TgAb-positive patients with well-characterized clinical status.

Results: 6 out of 45 cases with TgIMA > 1 ng/mL had undetectable TgMS. HA interference was confirmed by serial dilution and HA blocking reagent addition. In TgAb-positive cases, TgIMA and TgMS were highly correlated (R² = 0.86). In patients with structural disease and TgAb, TgIMA and TgMS were detectable in 6/19 patients, and 9/19 cases, respectively. The TgMS concentration range in those discrepant cases ranged from 0.5 to 2.0 ng/mL. Hence, the presence of TgAb was associated with inappropriately reduced Tg concentrations with both TgIMA and TgMS.

Conclusion: HA cause falsely elevated TgIMA with undetectable TgMS with significant frequency. TgMS can be used to rule out HA interference. Albeit resistant to TgAb in vitro, TgMS detects little Tg in patients with TgAb and structural disease. Hence, TgAb may reduce Tg concentrations in vivo. The implication is that no assay design may be able to overcome this problem. TgMS may not detect structural disease in TgAb-positive patients.

Key Words: thyroid cancer, thyroglobulin, thyroglobulin antibody, TgAb, heterophilic antibody, immunometric assays, mass spectrometry

Abbreviations: HA, heterophilic antibody; LOQ, limit of quantitation; MS, mass spectrometry; NSD, No structural disease; PREABL, preablation; RIA, radioimmunoassay; SD, structural disease; Tg, thyroglobulin; TgAb, thyroglobulin autoantibody; TgIMA, thyroglobulin immunometric assay.
In this article, we also study and compare the effect of TgAbs on Tg concentrations measured simultaneously by TgIMA and TgMS in a well-characterized group of patients to better understand the respective roles of these 2 methods in the monitoring of patients with thyroid cancer.

Patients and Methods

Patients

The electronic medical record of the main hospitals of the Mass General Brigham Healthcare system were queried for patients who, between the beginning of 2014 and the end of 2020, had at least 1 blood test for TgIMA, and, additionally, 1 for TgMS. We selected patients who had the 2 types of tests no more than 1 month apart from each other to avoid changes in Tg concentrations due to changes in clinical condition. Only 1 pair, the earliest available, was selected for each patient. The medical record for each patient was reviewed to assess the history of their thyroid cancer, related pathology, and imaging. Patients who had undergone an incomplete thyroidectomy were excluded. Each patient was assigned to 1 of 3 clinical statuses listed below.

1. Preablation (PREABL). These patients had their Tg tested before or at the time of their radioactive iodine ablation. This group included post-thyroidectomy patients who never had RAI treatment and had no evidence of structural disease.
2. No structural disease (NSD). These patients had previously undergone RAI ablation and had no evidence of persistent disease by imaging or pathology.
3. Structural disease (SD). These patients had previously undergone RAI ablation and had strong or definitive evidence of persistent or relapsing cancer. At the time of the Tg tests, all patients with SD either had abnormal neck nodes, biopsy proven or iodine avid neck or distant metastases, fluorodeoxyglucose-avid lesions on positron emission tomography scan or highly suspicious lesions on a computed tomography scan. Clinical details on this group are provided in Table 1.

In order to analyze the significance of discrepant results in the 2 Tg assays and establish true and false positives and negatives, the assignment to clinical groups was based on imaging and pathology data alone, independently of measured Tg concentrations. While this approach may lead to some selection biases, we think that by identifying a rigorously defined group (SD) in which Tg would be expected to be detectable with all likelihood, we have at the very least established a reasonable gold standard for true positive and false negative results in the Tg assays.

Assays

All TgIMAs were run with the Beckman Access Tg immunoassay (Beckman Coulter, Inc.). The limit of quantitation (LOQ) for this assay is 0.1 ng/mL. All TgMS assays were run with the Mayo Clinic Laboratories assay as previously described [9, 22]. The LOQ of the TgMS assay was 0.5 ng/mL. For statistical and plotting purposes all values <LOQ were computed as 0.5 × LOQ for each assay. Over the years a variety of Food and Drug Administration–approved thyroglobulin autoantibody (TgAb) immunoassay methods were employed. Table 2 describes the various assays for TgAbs used in this study. The cut-off provided with each method was utilized to label patients as TgAb positive or negative. When patient sera from 2 different dates were considered, the patient was categorized as TgAb positive if either 1 of the 2 tests were positive. Only 1 case had such a discrepancy, in which the 2 TgAb measurements were run with different assays. Because of the different LOQ between Tg assays, TgIMA with detectable results of >0.5 ng/mL were considered concordant with TgMS results of >0.5 ng/mL.

Table 1. Location of structural disease in the SD group

| Disease location       | No. of patients |
|-----------------------|-----------------|
| Neck lymph nodes      | 17              |
| Lung                  | 11              |
| Bone                  | 5               |
| Thyroid bed           | 4               |
| Mediastinum           | 3               |
| Liver                 | 2               |
| Brain                 | 1               |

Several patients had SD in multiple locations, accounting for the difference between the sum of column 2 and the number of patients in the SD group (n = 38).
patients were female, and 35 were male. The mean age was 48.8 years. Ninety-three patients were classified in the NSD group, 32 in the PREABL group, and 38 in the SD group. One hundred and one patients (62%) were TgAb positive, and 62 (38%) were TgAb negative, reflecting the fact that clinicians were likely to order both TgIMA and TgMS tests in patients thought to have TgAb interference.

**Overall Assay Concordance**

The correlation between TgIMA and TgMS was excellent ($R^2 = 0.91$ for the whole series, Fig. 1). In the 163 patients examined there was a set of 45 patients with detectable TgIMA, and a set of 44 patients with detectable TgMS. There were 9 cases out of 45 with detectable TgIMA who had undetectable TgMS, representing 5.5% of the whole cohort and 20% of the TgIMA-detectable group. There were also 9 different cases of patients with TgMS $\geq 0.5$ ng/mL who exhibited TgIMA $<0.5$ ng/mL. All but 2 were TgAb positive. In the 2 TgAb-negative cases, the TgIMA was $<0.5$ ng/mL, but not undetectable.

**HA Interference**

Of the 9 cases that exhibited detectable TgIMA and undetectable TgMS, 3 cases were HA positive. These 3 cases were previously described [5] and were all recaptured by our search, demonstrating the accuracy of our retrieval methodology. Of the 6 additional cases, 3 had TgIMA concentrations $>1.0$ ng/mL, and therefore deemed to be clinically relevant for this study [1]. These 3 cases were subjected to additional studies to rule out HA interference. In 2 of these cases where TgIMA was $>1.0$ ng/mL, serial TgIMA dilutions were nonlinear, and in all 3 cases the addition of HBT resulted in a drastic decrease in the TgIMA concentrations (Fig. 2). Review of the records for these 3 cases indicated that the treating physicians were considering cross-sectional imaging in 1 case, ongoing close monitoring in the second case, and empirical radioactive iodine treatment in the third case. All the discrepant cases had been classified either in the PREABL or NSD groups. Only 1 of these cases was TgAb positive. There were no TgIMA-positive/TgMS-negative cases in the SD group. In the 3 additional patients with undetectable TgMS, TgIMA was measured at 0.5, 0.6 ng/mL, and 1.0 ng/mL. In the first 2 cases the discrepancy was considered not to be clinically relevant, as the TgIMA concentration was very close to the TgMS LOQ. In the third case, review of the chart revealed that all previous and following TgIMAs had been measured at 0.2 ng/mL, suggesting that HA interference was unlikely.

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### Evaluation of Tg Heterophile Interference

Tg heterophile interference was studied in sera with TgIMA concentrations not significantly different from observed TgMS concentrations (Fig. 3). Twelve out of 101 (11.8%) patients in this group had detectable and comparable Tg in both assays. Evaluation of Tg Heterophile Interference

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assays (Fig. 3). There was only 1 TgAb-positive patient with detectable TgIMA, and undetectable TgMS, who was also HA positive. In the presence of TgAb, TgMS was detectable in 20 out of 101 (19.8%) of patients, including all 12 patients with detectable TgIMA. In the 8 TgAB-positive patients with detectable TgMS and undetectable TgIMA, the TgMS range was low, 0.6-3.4 ng/mL. This may be reflective of suppression of TgIMA in the presence of TgAb. Of these patients, 3 were in the SD group, 2 were in the NSD group, and 2 were in the PREABL group.

In the SD group, 17 out of 19 TgAb-negative patients had TgIMA >1.0 ng/mL, and TgMS was >1.0 ng/mL in 18 out of 19 cases. One case had undetectable Tg with both assay methods. In contrast, only 5 out of 19 (26.3%) TgAb-positive SD patients had TgIMA >1.0 ng/mL and 7 out of 19 (36.8%) TgAb-positive SD patients had TgMS >1.0 ng/mL. The TgMS range in these 2 SD patients with TgIMA ≤1.0 ng/mL and TgMS >1.0 ng/mL was low, 1.2 and 2.0 ng/mL respectively, despite imaging evidence of extensive metastatic spread to the lungs.

**Discussion**

In patients with thyroid cancer, a detectable Tg after total thyroidectomy and radioiodine ablation is thought to indicate occult disease [24], and often leads to patient anxiety, expensive imaging, and, sometimes, unnecessary treatment with radioactive iodine [25].

In this retrospective series, 20% of thyroid cancer patients (all clinical status groups) with detectable Tg by TgIMA and 16% of those with TgIMA >1.0 ng/mL had undetectable Tg by TgMS. In all cases with TgIMA >1.0 ng/mL and undetectable TgMS, TgIMA concentrations dropped significantly after the use of HBT, ascribing the cause of the TgIMA elevation to HA interference [26]. The prevalence of HA interference in this population was 3.6%. Upon review of the medical record, there was no evidence of recurrent or persistent thyroid cancer in any of these 6 discordant cases. The incidence of HA in our cohort appears to be higher than that previously reported. Giovannella et al [7] reported a 1% prevalence in a large study, but the test for HA was not limited to sera with TgIMA >1.0 ng/mL. Preissner et al [26] described a 3% prevalence in sera with TgIMA >1.0 ng/mL, but there was no detailed clinical characterization of patients. The higher prevalence in our retrospective study may reflect an increased suspicion for interference from the ordering physicians, since only patients in which both TgIMA and TgMS had been measured were included [9]. Even if this is an overestimation of the prevalence of HA interference, our data reflect a real-world clinically relevant situation, in which clinicians may have found the TgIMA result inconsistent with the clinical presentation and therefore questioned it. Our data show that in patients with unexplained elevated TgIMA, the prevalence of HA is significant. This interference can be easily detected by comparison of TgIMA with TgMS results. TgMS methods employ trypsin digestion of all serum proteins, including HA, and...
thus are immune to HA interference. Since TgMS is more expensive and time-consuming and less sensitive, it is not recommended as a first-line test for Tg [20]. However, commercial TgMS assays from several laboratories are available to clinicians.

Our study confirmed that in the presence of TgAb, many patients with SD will have undetectable Tg almost as often with TgMS as with TgIMA [16, 18]. In any given serum, the Tg concentration measured with TgIMA was closely correlated with TgMS in both TgAb-negative ($R^2 = 0.94$) and TgAb-positive sera ($R^2 = 0.86$). In addition, the few TgAb-positive sera with undetectable TgIMA in which TgMS was detectable, the Tg concentration was low, even in the presence of extensive structural disease. Thus, with regard to TgAb interference, TgMS assessment provided marginal benefit.

How can assays with substantially different designs be similarly affected by TgAbs? TgAbs clearly affect the ability of TgIMAs to efficiently measure Tg, as demonstrated by recovery studies [12, 14]. In contrast, TgMS assays, have been reported to provide approximately 100% recovery of Tg added to TgAb-positive sera, because of the digestion of immunoglobulins in the serum [9, 20, 22]. Azmat et al proposed that undetectable TgMS in patients with structural disease could be explained by genetic variations in the Tg polypeptide used by TgMS. Our data, benefitting from a large series of paired TgIMA/TgMS tests, do not support this interpretation, as it would result in discrepancies between TgIMA and TgMS, which we have not observed. The other interpretation that some tumors may have lower Tg secretion would require that such tumors would be found only in the TgAb-positive patients [21]. Some studies have suggested that Tg can be measured by RIA in TgAb-positive patients with structural disease [18]. However, TgAbs can result in low level false positive results in TgRIA [27]. Thus, the specificity of detectable TgRIA concentrations in the presence of TgAbs should be validated by demonstrating undetectable Tg in TgAb-positive patients who have undergone successful treatment for thyroid cancer. Such validation has not been presented to date. Our results suggest that TgAb negatively affects Tg concentrations in vivo, before the serum reaches the test tube [14, 28, 29]. According to this interpretation, TgAb would result in reduced Tg from some patients’ circulation in vivo, and would not artifically reduce Tg levels in vitro only. One plausible mechanism is that the clearance of circulating Tg is accelerated by the presence of TgAbs. This hypothesis could be tested by in vivo kinetic tests employing radiolabeled Tg. The TgAb effect appears to be variable, and many TgAb-positive patients have measurable Tg concentrations by both TgIMA and TgMS.

This study provides 2 main conclusions. First, given the non-negligible and clinically relevant prevalence of HA in patients with elevated TgIMA in our study, we propose that Tg should be measured by TgMS in all patients with clinically significant but unexplained TgIMA elevations. If TgMS is confirmed to be elevated, then the patient can be submitted to appropriate further testing and clinical evaluation. If TgMS is negative, then confirmation of HA interference by serial dilutions and using heterophilic antibodies blockers should be performed. We do recognize that TgMS may not be commercially available in some areas. In those cases, if the clinical suspicion for HA is strong enough, then serial dilutions and

Figure 2. Experiments showing HA interference in 3 of the 6 new cases with high TgIMA and undetectable TgMS. The graph shows the nonlinear effect of dilutions on TgIMA concentrations in 2 out of 3 cases, a marked reduction in TgIMA levels after the addition of HBT in all 3 cases, and undetectable TgMS in all 3 cases.
pretreatment HBT should be run directly. Alternatively, Tg could be measured by RIA, a method thought to be less prone to HA interference. Switching to another IMA employing different antibodies is another possible strategy, as HA interference may vary between different antibody systems. However, this would require an in depth acquaintance with the different assays by the ordering clinicians, as selecting an assay with a similar antibody source would not solve the problem. We should also note that other interferences, not mediated by HA, can occur. However, all discrepancies in our series were proven to be caused by HA.

Secondly, this study demonstrates that when TgIMA is undetectable in patients with structural disease and TgAb TgMS is also often undetectable, or very low. Since the 2 tests are radically different in design, we propose that this finding is due to the actual absence of Tg in most TgAb-positive sera, whether in patients with structural disease or free of disease. This interpretation has important consequences on the development of the next Tg assays, as it implies that this phenomenon may not be eluded by any assay design. It is important that clinicians are aware that an undetectable Tg by TgMS, in the presence of TgAbs cannot be considered reassuring. Our findings suggest that in patients with TgAbs, the measurement of Tg may provide an underestimation of the tumor burden with any Tg assay design due to in vivo reduction of Tg concentrations.

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**Authors Contributions**

G.B. contributed by designing the study, collecting and analyzing the data, writing the manuscript. A.A.S. contributed by designing and performing the heterophilic antibody experiments, writing the manuscript. J.B. contributed by designing and performing the heterophilic antibody experiments, writing the manuscript.

**Disclosures**

G.B. reports no conflicts of interest related to this paper. A.A.S. reports no conflicts of interest related to this paper. J.B. reports no conflicts of interest related to this paper.

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**Figure 3.** Individual TgIMA and TgMS pairs in the study population according to TgAb status and to clinical status. Starred symbols indicate sera with proven HA interference, white rounded symbols indicate sera with detectable TgMS and undetectable TgIMA. Large proportional (to n) diamonds indicate sera with undetectable Tg in both assays. Shaded areas indicate concentrations that fall below the LOQ for each respective assay. A dotted line represents at the clinically meaningful Tg cutoff level of 1.0 ng/mL.
Data Availability
Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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