Review article

Harmonization of Thyroid Hormone Testing; A Complex Challenge to Improve Patient Outcomes

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Thyroid diseases are extraordinarily complex endocrine conditions. Hyperthyroidism, hypothyroidism, and thyroid cancer is diagnosed and monitored using thyroid hormone (TH) laboratory testing. Treatment decisions adhere to published clinical practice guidelines that rely heavily upon laboratory measurements such as thyroid stimulating hormone (TSH), thyroxine (T4), free thyroxine (FT4), and thyroglobulin (TG). The immunoassays for TH testing utilize various methodologies dependent upon specific reagent manufacturers. This presents a challenge for laboratories to provide TH measurements that are dependable and consistent. This challenge has led to initiatives for the standardizations and harmonization of TH testing. TH immunoassays are complex methods that are highly susceptible to interferences such as heterophile antibodies, binding proteins, and anti-reagent antibodies. In addition, there is a lack of appropriately established reference intervals (RI) for TH laboratory values. Factors such as pregnancy, age, sex, and geographical location complicate the standardization of TH RIs. Harmonization and standardization for TH testing is challenging, however, it is clear that patients with thyroid disease would benefit from these initiatives.

Keywords: Thyroid hormones; Thyroid testing; Harmonization; Standardization

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Introduction

Thyroid testing is among the highest volume assays utilized across the global healthcare network.[1] Medicare costs for thyroid stimulating hormone (TSH) have been reported at $469 million per year. Approximately 59 million TSH and 18 million free thyroxine (FT4) tests performed are performed annually with TSH among the top 25 laboratory tests performed in four out of five hospitals.[1,2] FT4, TSH, along with thyroglobulin (TG) immune-assays play an important role in the diagnosis and management of thyroid diseases.[3] The availability of numerous testing platforms and diagnostic methods for TH testing creates a complex challenge in establishing harmonization across manufacturer's and standardizing reference intervals. In laboratory medicine, harmonization of laboratory testing refers to achieving equivalent results with the same interpretation irrespective of the procedure used, the unit or reference interval applied, and when and/or where a measurement is made.[4] Additionally, standardization involves the attainment of harmonization through traceable methods of laboratory values to primary reference material.[2]

Over the last decade, organizations such as the American Association for Clinical Chemistry (AACC) and the College of American Pathologists (CAP) have led the effort to harmonize laboratory results by establishing the International Consortium for Harmonization of Clinical Laboratory Results (ICHCLR).[4] In addition to the work for ICHCLR, thyroid-specific organizations such as the Partnership for the Accurate Testing of Hormones (PATH) are attempting to improve standardization and harmonization among thyroid immunoassays.[5] The lack of standardization and harmonization prevents clinical laboratories from assessing the accuracy and reliability of reference materials.[5] Additionally, systemic bias or disparity in interferences among the different immunoassays has been observed when a single patient is analyzed with multiple methodologies leading to misinterpretations.[6] Thyroid immunoassays are among the many laboratory tests that are lacking uniformity through standardization and harmonization. Harmonizing results for thyroid tests would lead to the development of standardized treatment guidelines, improve the accuracy of clinical diagnostics, and reduce the number of medical errors improving patient outcomes.

Thyroid Hormones and Disease

THs regulate cellular differentiation, growth, and metabolism in every tissue in the body.[7] The hypothalamic-pituitary-thyroid axis regulates thyroid hormone levels. The pituitary gland produces TSH, which stimulates the production of T4 and 3,3'5-triiodothyronine (T3).[7,8] Given the importance of THs in cellular growth and metabolism, TH testing, specifically TSH, FT4, and TG levels are important for the diagnosis and management of thyroid diseases such as hyperthyroidism (excessive thyroid hormone production), hypothyroidism (reduction in thyroid hormone production), and thyroid cancer. Studies have shown 4.6% of individuals in the United State (U.S.) aged 12 years or older have hypothyroidism while 1.2 % have hyperthyroidism.[9] Despite the prevalence of thyroid disease, the U.S. Preventative Task Force recommends thyroid testing only in the presence of symptoms and risk factors and advises against using it as a screening method.[10]

Patient's presenting with symptoms of hypothyroidism include hair loss, weight gain, dry skin, constipation, fatigue, and depression.[11] Hashimoto's thyroiditis is an autoimmune form of hypothyroidism that restricts FT4 production, resulting in an increased production of TSH.[10,11] Hyperthyroidism symptoms include weight loss, palpitations, heat intolerance, fatigue, tremors, and exophthalmos.[12] Graves' disease (GD), toxic multinodular goiter (TMNG), and toxic adenoma (TA) are examples of hyperthyroidism. The excess production of thyroid hormone inhibits the release of TSH.[10] It is also important to note that conditions such as GD produce autoantibodies that can complicate the

interpretation of TH values. Symptoms associated with hyperthyroidism and hypothyroidism mimic other conditions resulting in patients failing to recognize the symptoms of thyroid disease. This can delay the diagnosis and treatment for serious conditions such as thyroid cancer.

Thyroid cancer prevalence has increased substantially accounting for approximately 2.1% of all cancer diagnoses and ranked as the 9th leading cancer in 2020, worldwide.[13,14] Common types of thyroid cancer include papillary, follicular, and medullary, with mortality rates at 20 years averaging 1% - 2%, 10% - 20%, and 25% - 50% respectively.[15] Over 90% of endocrine malignancies include a thyroid cancer diagnosis.[16] Thyroid cancer is the most common diagnosis among adolescents and adults, and is the seventh most common in females.[16] Studies have shown an increased risk of thyroid cancer in individuals who are diagnosed with benign thyroid nodules, adenoma, and goiter.[17] While TSH and FT4 immunoassays provide information for diagnosing hyperthyroidism and hypothyroidism, thyroid cancers are detected through imaging of suspected nodules or goiters and confirmed with fine needle aspiration cytology.[13] TG is secreted in small amounts by the thyroid gland in healthy individuals but is often elevated in papillary and follicular thyroid cancer.[16] TG immunoassays become an important piece for monitoring and treating thyroid cancer. With the increasing incidence of thyroid cancer, it is important to educate patients on the importance of routine preventative care, which includes routine TH testing.

Routine care and treatment for thyroid disease is important because TH regulates the metabolism throughout the body. Treatment of hypothyroidism has historically been through prescribing the synthetically produced exogenous form of T4, levothyroxine.[10] While the U.S. Food and Drug Administration has approved the use of generic versions of levothyroxine, the brand-name drug is the treatment of choice for endocrinologists and professional organizations.[10] Hyperthyroidism disorders such as GD, TMNG, and TA can lead to a condition called thyrotoxicosis. Treatment for each of the disorders is dependent on the diagnosis and include thyroidectomy, ß-blockers, radioactive iodine, or antithyroid drugs.[18] Thyroid cancer treatment for most low-risk cancers involves surgical removal including lobectomy and total thyroidectomy.[15] Active surveillance is a viable alternative to traditional surgical treatment options for low-risk differentiated thyroid cancer.[16]

Monitoring thyroid disease is difficult and often specific to the patient or the stage of the disease. Factors such as, sex, and pregnancy can dictate need and frequency for thyroid laboratory testing. For example, monitoring non-pregnant patients with hyperthyroidism requires testing TSH levels at intervals of six to eight weeks until within the reference range, then every six to twelve months, pending no change in clinical status.[18,19] This is significantly different than monitoring thyroid cancer.

Thyroid cancer, like other cancers, has a possibility of recurrence. Monitoring the disease is an essential step for managing patient care. Imaging and laboratory testing methods are used to monitor disease following partial or complete removal of the thyroid. The presence of or increase in TG post-surgery may be evidence of the recurrence of thyroid cancer.[20] To reduce recurrence and improve monitoring of TG levels, post-surgical radioactive iodine ablation of residual thyroid tissue is an option.[20] Regardless of what TH is measured, the monitoring of each analyte plays a significant role in determining the type of patient care required.

Clinical Utility of Thyroid Immunoassays

TSH Immunoassays

TSH immunoassays are extremely sensitive and specific, making it the most utilized laboratory test for the initial diagnosis of thyroid disease.[23] TSH immunoassays are classified as first to third generation according to the limit

of detection or improvement in the sensitivity.[8,24] Historically, competitive, and non-competitive immunoassays are utilized for TSH. Competitive first-generation immunoassays use polyclonal antibodies while non-competitive second and third-generation immunoassays utilize monoclonal antibodies.[25] The third-generation immunoassays are available on various automated laboratory analyzers. The automated methods are primarily two-site sandwich immunoassays that detect labeled antibodies specific to TSH epitopes.[26] In addition to improving sensitivity (0.01–0.02 μIU/mL) for TSH testing, the evolution to third-generation immunoassays can differentiate hyperthyroid, euthyroid, and hypothyroid conditions.[24-26]

Despite the progress made with improving sensitivities in newer-generation testing, comparability between different manufacturer methods exist. The International Federation of Clinical Chemistry (IFCC) Working Group on Thyroid hormones demonstrated that method-related variations exist in thyroid immunoassays.[27] The variations indicate potential issues with RIs in TSH testing. As defined by the Clinical Laboratory Standards Institute (CLSI), RIs are ranges derived from healthy individuals within a definitive percentage measurement of 95%. The RIs for TSH are calculated within a percentage of measurements from between the 2.5th - 97.5th percentile.[28,29] Most laboratories follow this recommendation for all diagnostic assays.

While following CLSI recommendations for establishing RIs through analysis of healthy individuals, there are additional factors to consider. Factors such as additional patient conditions, age, sex, ethnicity, and regional iodine intake should all be included when establishing valid RIs for thyroid testing.[30] For example, the 2017 American Thyroid Association (ATA) guidelines recommend using RIs of population and trimester-specific TSH based on a population without known thyroid disease and in an area with premium iodine distribution.[31] More recent developments in establishing RIs utilize a patient-personalized approach. Personalized RIs are established by comparing the patient's laboratory test results with their individual RIs as opposed to population-based methods.[32] Regardless of the method utilized, the need for consistent and dependable TSH results and RIs remains to accurately diagnose and treat thyroid disease.

In addition to the RI challenges, limitations associated with TSH immunoassay methodologies include non-analyte-specific interferences and analyte-specific interferences.[33] Non-analyte interferences include heterophile antibodies (HAb), anti-reagent antibodies, and streptavidin-biotin.[33,34] HAb present in individuals can falsely elevate TSH values. Anti-reagent antibodies (ARA) and streptavidin-biotin found in patient serum can block the functionality of assay-specific reagents, interfering with TSH results. Analyte interferences include TSH autoantibodies which can falsely elevate test results and TSH variants, including nine separate variants detected by TSH immunoassays.[34] Additional limitations include medications such as steroids, the timing of administering levothyroxine as well as season and diurnal TSH variations.[33] Immunoassay manufacturers continue to develop methods to limit the effect of interfering substances and understand the fluctuation in hormones that can alter the accuracy of TSH results.

FT4 Immunoassays

In addition to TSH, FT4 laboratory testing plays a significant role in the diagnosis and treatment of thyroid disease. Approximately 98.98% of T4 is protein bound, leaving 0.02% of FT4 for detection and quantification in immunoassays.[35] The testing methods for FT4 have similar characteristics as TSH immunoassays. Diagnostic screening for thyroid disease commonly includes TSH and FT4 in tandem, contributing to laboratory test utilization problems. FT4 testing is recommended only as a follow-up to abnormal TSH values.

FT4 testing methods also vary in design. While direct method liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays

are available, automated indirect competitive immunoassays are the most popular for measuring FT4.[35] These methods deploy either one-step, labeled antibody, or two-step principles. One-step methods are easily automated and are competitive immunoassays using labeled hormone analogs. This limits interaction with binding protein thyroid hormones and hormones found in a patient's sample for a solid-phase anti-hormone antibody. The FT4 in a patient's sample competes with solid-phase hormone for the labeled antibody creating a measurement from a function of the fractional occupancy of hormone-antibody binding sites in the reaction mixture. A final washing step in the procedure creates an inversely proportional measurement of the FT4.· The two-step method, also known as back-titration, harnesses immobilized T4 to isolate a portion of total T4 from a diluted patient sample followed by a washing step, allowing for an inversely proportional calculation of FT4.[34,36]

Like TSH, RIs for FT4 are method dependent because of calibration biases and rely heavily on population-specific statistics such as patient conditions, ethnicity, age, and sex.[30,35] Guidelines for RI calculation methods are published by international organizations such as the ATA. However, data indicates that most laboratories do not follow the recommenddations.[31]

FT4 immunoassays are affected by non-analyte-specific limitations in testing which include protein interferences, thyroxine-binding globulin (TBG) excess or deficiency, pregnancy, familial dysalbuminemic hypothyroxinemia (FDH), transthyretin-associate hypothyroxinemia (TAH), HAb, and ARA. Protein interferences include the presence of paraproteins and abnormal immunoglobulins. Congenital TBG excess or deficient quantities can also cause interference with FT4 immunoassays.[34]

FT4 is monitored closely in patients who are pregnant due to the prevalence of thyroid disease. Evidence has shown that the thyroid gland becomes stressed during pregnancy. Thyroid disease, because of pregnancy, is incredibly more complicated than hypo or hyperthyroidism. Pregnancy interferences are method-related due to existing standardization differences and method sensitivity to the decreasing amounts of albumin present during gestation.[34] However, protein-specific FT4 immunoassay interferences are not unique to patients who are pregnant.

Additional interfering proteins include binding proteins thyroxine-binding globulin, transthyretin, and serum albumin. Each of the binding proteins are important in the proper storage and transport of TH. Presence of these proteins maintains appropriate levels of available TH to prevent a deficiency in the accessible substrate, loss of iodine, and other thyroid-related clinical factors.[37] Additionally, the autosomal mutation caused by FDH and TAH alters the structure of the binding proteins resulting in falsely elevated FT4.[34] This is due to the immunoassay's reliance upon the binding proteins present in human serum. Comparably, FT4 immunoassays also depend upon antibodies to perform measurements.

HAb, specifically autoimmune antibodies such as rheumatoid factor can interfere with FT4 immunoassays. Capture antibodies for measuring FT4 are unsuccessful because the assay is unable to differentiate them from HAb. This type of interference is reduced from 2 – 5 % by adding a HAb blocker reagent.[34] While this reduction improves performance, interference from HAb remains clinically significant.

In addition to HAb, human serum can also contain interfering ARA that target frequently used FT4 immunoassay reagents such as ruthenium or streptavidin.[34] For example, exposure to the bacterium *Streptomyces avidinii* produces an antibody that competes with streptavidin reagent, leading to falsely elevated FT4 levels.[38] There is a likelihood that human serum contains interfering ARA not yet discovered. These unknown factors complicate the diagnosis of thyroid diseases.

TG Immunoassays

TG immunoassays have evolved much like the methodologies used for detecting TSH and FT4. Improving sensitivity for TG is of particular

importance for the treatment and monitoring of thyroid cancer in patients following a thyroidectomy. The quest for sensitive detection methods with limited interferences for TG is essential. This has led to multiple testing methodologies that include chemiluminescent immunoassay (CLMIA), radioimmunoassay (RIA), and immunometric (IMA) technology.[39]

CLMIA methodologies utilize a luminescent labeled molecule that produces detectable radiation of light. This method performs measurements on analytes such as albumin and TG.[40] TG CLMIA methodologies are available in clinical laboratories.

RIA utilizes radioisotope-labeled antigens that compete with TG in a patient's sample for binding to a high-affinity TG antibody.[14] Although TG-RIA are standardized against BCR®457 certified reference material (Merck KGaA, Darmstadt Germany) from the European Commission Institute for Reference Materials, the method demonstrates unacceptable sensitivity. TG-RIA does however offer improved performance in the presence of interfering polyclonal antibodies.[39]

Similar to TSH and FT4 testing, first-generation LC-MS/MS provides reliable and consistent test results but due to expensive equipment accompanied by the need for highly trained laboratory personnel has limited availability.[39] In comparison, IMA is a sandwich or two-site methodology that uses two binding antibodies termed capture and detection antibodies. First, the highly specific binding antibodies are added to the patient sample, attaching to TG epitopes, followed by the detection antibodies to form a 'sandwich' where the automated process can detect and measure TG.[41] This methodology is highly popular internationally among testing laboratories as a tool for monitoring patients with thyroid cancer.

TG is primarily monitored as a tumor marker for differentiated thyroid cancer patients post-thyroidectomy, making the process for establishing RIs different from other THs.[42] As previously indicated, the production of TG occurs in response to the stimulation of the TSH receptor by TSH.[14] After two years of age, TG levels fall in the same range as adults but because most testing occurs post thyroidectomy or lobectomy, RIs become irrelevant. Individualized RIs based on thyroidectomy versus lobectomy procedures are preferred for determining appropriate TG levels.[34]

Immunoassays for TG measurements present several limitations which include heterophile and autoantibody interference. As previously mentioned with TSH and FT4 immunoassays, heterophile antibodies cause interferences, even in the presence of a low quantity of heterophile antibodies. The prevalence of autoantibodies represents a significant amount of the interferences associated with TG immunoassays. Most interestingly, elevated levels of autoantibodies may not interfere while low levels have a remarkable effect.[34,39] To limit interferences caused by autoantibodies, testing laboratories implement tandem testing to include TG antibody (TGAb) immunoassay. For example, in some laboratories, TG testing begins with an assay for TGAb via IMA. When the TGAb measurement is below the detectable limit, then TG testing is performed by a sensitive second-generation IMA. If TGAb is detected, specimens are assayed by RIA or LC-MS/MS because each is resistant to TGAb. However, some studies indicate that there is no diagnostic advantage to using TG LC-MS/MS versus an immunoassay methodology.[34]

Harmonization and Standardization Challenges

Improving and providing high quality patient care is essential for healthcare organizations. Laboratory services are essential to the delivery of high-quality patient care. There is an overwhelming consensus that the harmonization of laboratory results can play a significant role in improving patient outcomes.[4] The prevalence of thyroid disease is significant and oftentimes is accompanied by signs and symptoms that can be difficult to

detect.[21] Laboratory testing utilized by physicians and endocrinologists for the detection and management of thyroid disease should be accurate and reliable.[4] In the presence of harmonization, clinical practice guidelines would become more uniform, allowing for more consistent and appropriate healthcare decisions for the treatment of patients with thyroid disease.[4]

An additional consideration that affects the harmonization of a laboratory test is the variability of reference intervals (RIs) which are impacted by the patient population, test methodology, and the laboratory performing the assay. Clinical laboratories establish RIs for each thyroid immunoassay. However, there are circumstances where laboratories solely depend on manufacturer RIs. Manufacturer RIs are established by gathering data on populations either regionally or nationally.[22] This poses a problem for providers because of the variability that exists between patient populations in distinct locations. This can compromise a health care provider's ability to make sound clinical decisions regarding a patient's diagnosis and treatment options.

When considering the massive effort required to accomplish standardization and harmonization, the implications for manufacturers, governing bodies, and clinical laboratories are significant. For example, before implementation of standardization in laboratory practice, considerations are necessary to meet applicable regulatory requirements.[35] Thyroid disease is prevalent worldwide, and many countries have clinical practice standards for disease diagnosis and management. Standardization will require newly published guidelines and provider buy-in for thyroid hormone testing. International laboratory organizations, general and specialty healthcare providers, laboratory professionals, nursing professionals, and patient-centered organizations will require substantial educational time to prepare for any standardization efforts.[35]

Large-scale initiatives for change require a tremendous amount of time for planning before implementation. Standardization and harmonization initiatives are designed to improve patient outcomes but there are concerns about the potential increased risks for patient safety during a change in laboratory test values and RIs.[35] Laboratory reports are a vital resource for assisting providers with interpreting laboratory values. During the transition to standardized thyroid immunoassays, laboratory administrators in the clinical laboratories performing tests will need to design laboratory reports in a manner that prevents confusion. For example, administrators could provide customized patient reports including pre- and post-standardization results and RIs.[35] Quality management systems in the laboratory are designed to assess pre-analytic, analytic, and post-analytic processes in the laboratory, including changes in laboratory operations that may lead to non-conforming events.[43] Like other healthcare services, clinical laboratories are looking for ways to mitigate costs in the services provided. Standardization and harmonization for each clinical laboratory must be evaluated to determine the most cost-effective way to implement the processes. Keeping in mind not only reagent costs but also the time contributed to the planning, implementation, and monitoring of the change.

Discussion

Thyroid diseases are complex conditions that require a tremendous amount of endocrine scientific expertise to treat patients. This expertise includes interpreting TH laboratory test values. What is clear about TSH, FTA and TG immunoassays is that there is a multitude of methods commercially available. For example, RIA and IMA for TG are performed in clinical laboratories worldwide. The development of numerous different testing methodologies has improved analytical sensitivities and limited interferences. While developing new methods provides accurate and reliable laboratory values, there are additional implications to consider.

The overwhelming consensus suggests that TH testing lack of standardization and harmonization of the immunoassays contributes to inconsistencies in the diagnosis and monitoring of thyroid disease.[44] It is important to understand that while standardization and harmonization terms are often used interchangeably, each is uniquely different. It is not always the case that standardizing thyroid immunoassays will lead to harmonized thyroid test results and standardized reference methods are not always necessary for harmonization of thyroid test results.[45] The paramount objective in thyroid immunoassay testing standardization is the harmonization of test results so laboratory values from the same sample are interchangeable no matter the testing personnel, laboratory, or methodology.[45,46] Experts have recommended three steps for the standardization and harmonization to achieve equivalent results between methods including 1) the use of reference methods and materials for creating a reference system, 2) utilizing the reference system for the development of calibrating measurement procedures, and 3) evaluate the correlation of laboratory values throughout each method to verify the uniformity of patient results from patient care and research settings.[47] Overall benefits for reaching this objective include improved monitoring of disease progression, proper utilization of TH testing, and development of evidence-based practice guidelines.[46]

There are limitations associated with achieving standardization and harmonization in the laboratory. First, standards with known International System of Units (SI units) are not available for TSH and FT4 immunoassays. The IFCC approved an international conventional reference measurement procedure for FT4. However, because of the intricacies associated with TSH immunoassays, reference measurement procedures have not been developed.[44] This further emphasizes the need to harmonize thyroid hormone testing.

Second, establishing standardized RIs for FT4 and TSH immunoassays is a complex issue. As mentioned previously, laboratories are inconsistent in establishing dependable RIs, making it extremely difficult for providers to correctly interpret laboratory values. While an FT4 RI procedure exists, an attempt to establish a procedure for TSH is seen as unlikely.[5] The IFCC Committee for Standardization of Thyroid Function Tests (C-STFT) considered the more logical approach to harmonization as opposed to standardization. The C-STFT follows the International Organization for Standardization process for traceability. The C-STFT completed a multi-assay comparison with untreated and clinical specimens concluding that harmonization was feasible.[5] Harmonization is possible when immunoassay manufacturers are allowed to individually adjust calibrators using previously established target means from another method of comparison with similar sample types. The C-STFT believes this may allow manufacturers the ability to develop consistent RIs.[5]

Third, there is a wide variety of interferences associated with TH immunoassays including autoantibodies, reagent antibodies, and binding proteins. For example, due to the high prevalence of autoantibodies present in patients treated for differentiated thyroid cancer, experts recommend that all TG testing be performed with an TG antibody measurement.[14] Considering these interferences, along with the absence of known SI units and the complexity with establishing RIs, it is understandable why initiatives for reaching harmonization have yet to be accomplished.

Conclusion

Thyroid disease is prevalent worldwide. Hyperthyroidism, hypothyroidism, and thyroid cancer diagnosis relies heavily upon TH testing that is sensitive, accurate and precise. Accuracy or trueness of laboratory values means results produced are close to values derived from referenced methods. Precision or repeatability means the degree to which a laboratory can produce the same values.[46] Considering the definitions along with the wide variety of

methodologies available for TH testing emphasizes the importance of standardization and harmonization. RIA, IMA, and LC-MS/MS methods are all subject to interferences that can be further complicated by the nature of the thyroid disease.

Professional groups such as the ATA and C-TFT recognize the limitations associated with thyroid testing and are guiding efforts for standardization and harmonization.[5,31] While there are organized efforts to produce comparable results and appropriate RIs, achievement of these efforts remain unsuccessful. Endocrine specialists, clinical laboratories, and assay manufacturers must collaborate to successfully implement standardization and harmonization. Harmonizing thyroid laboratory test results is important to assist health care providers in making the best treatment decisions to improve patient care.

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