**ASSAY OF THYROID HORMONE AND RELATED SUBSTANCES**

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**ABSTRACT**

This chapter reviews how improvements in the sensitivity and specificity of thyroid tests [total and free thyroid hormones (T4 and T3), TSH, thyroid autoantibodies (TRAb, TPOAb, and TgAb) and thyroglobulin (Tg)] have advanced the detection and treatment of thyroid disorders. The strengths and limitations of current methodologies [Radioimmunoassay (RIA), Immunometric assay (IMA) and Liquid Chromatography/Tandem Mass Spectrometry (LC-MS/MS)] are discussed, together with their propensity for analyte-specific and non-specific interferences relating to analyte heterogeneity (TSH, TgAb and Tg), analyte-specific autoantibodies (T4Ab, T3Ab, TSHAb and TgAb) and interferences from heterophile antibodies (HAb) or assay reagents such as Biotin and Rhuthenium. Currently, between-method differences preclude establishing universal thyroid test reference ranges. However, collaborations between the International Federation of Clinical Chemistry (IFCC), the committee for the standardization of thyroid function tests (C-STFT), and the in-vitro diagnostic (IVD) industry are now focused on eliminating these between-method differences.

**INTRODUCTION**

Figure 1 shows the timeline for improvements in the sensitivity and specificity of thyroid test methodologies made over the last 60 years (1). In the 1950s the only thyroid test available was an indirect estimate of the serum total (free + protein-bound) thyroxine (T4) concentration, using the protein bound iodine (PBI) technique (2). Early technological advances in radioimmunoassay (RIA) (3-6), immunometric assay (IMA) (7-11), and most recently, liquid chromatography-tandem mass spectrometry (LC-MS/MS) methodologies (12-14) have progressively improved the sensitivity and specificity of thyroid tests. Currently, most thyroid testing is made on serum specimens using automated IMA methodology to measure total thyroid hormones (TT4 and TT3), estimate free thyroid hormones (FT4 and FT3) (13,15,16), and measure TSH (13) and thyroglobulin (Tg) (14,17). Automated IMA methodology is also used to detect autoantibodies that target the TSH receptor (TRAb) (18-20), the thyroid peroxidase enzyme (TPOAb) (21), and the thyroglobulin protein (TgAb) (22-24). When indicated, the thyroid hormone binding proteins thyroxine binding globulin (TBG), transthyretin (TTR)/prealbumin (TBPA), and albumin can also be measured (25-27). The IFCC and CDC continue their efforts to encourage test manufacturers to identify the causes of, and reduce the magnitude of, between-method variability in thyroid hormone and TSH measurements (12,28-33). Isotope-dilution liquid chromatography/tandem mass spectrometry (ID-LC-MS/MS) has become the reference measurement procedure (RMP) for total thyroid hormone measurements (28) and free hormone (FT4 and FT3) measurement in equilibrium dialysates (15,28,34,35). TSH methods are now being re-standardized to the new International Reference Preparation (81/615) and harmonized to the all-method mean (13,33,36). Although serum Tg can now be detected by LC-MS/MS as tryptic peptides (37-42), the clinical value relative to the expense of this technique is still debated (14,41,43). Thus, despite technical improvements in sensitivity, specificity and standardization, the problem of substantial between-method variabilities remains for all tests (13,14,28,30,32,33,35,44-46). Establishing universal thyroid test reference ranges that would apply to all methods, by removing current between-method biases, would greatly benefit healthcare systems worldwide. Current guidelines for managing pregnant (47,48) and non-pregnant patients with hypothyroidism (49-52), hyperthyroidism (53,54), thyroid nodules (55), or differentiated thyroid cancers (DTC) (17,23,56-60) are also referenced.

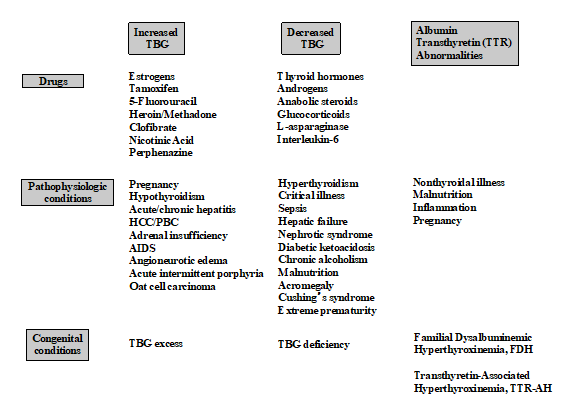
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**Figure 1. Timeline for the Major Technical Advances in Thyroid Testing. The figure shows the development of increasingly more sensitive TSH tests: first generation, (1G), second generation (2G), and third generation (3G), and advances in the methodologies used to measure total thyroid hormones (TT4 and TT3), indirectly estimate free thyroid hormones (FT4 and FT3), directly measure FT4, and measure the thyroid autoantibodies TPOAb, TgAb, and TRAb and Thyroglobulin (Tg). From reference 1.**

**TOTAL THYROID HORMONE MEASUREMENTS (TT4 and TT3)**

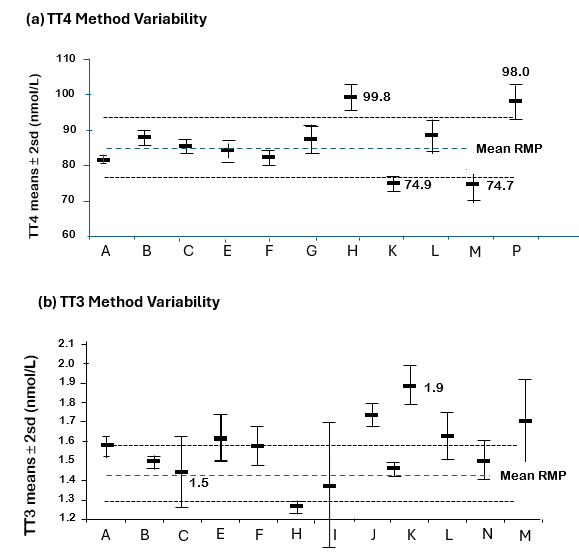
Thyroxine (T4) circulates 99.97 percent bound to the plasma proteins, primarily TBG (60-75 %) but also transthyretin TTR/TBPA (15-30 %) and albumin (~10 %) (25,26,61). In contrast 99.7 % of Triiodothyronine (T3) is bound to TBG (26,61). The total (free + protein-bound) thyroid hormones (TT4 and TT3) circulate at nanomolar concentrations that are considerably easier to measure than the free hormone moieties (FT4 and FT3) that circulate in the picomolar range (62). Serum TT4 methods have evolved over the past five decades from protein-bound iodine and competitive protein binding tests (2,63) to non-isotopic immunometric assays and most recently, isotope dilution tandem mass spectrometry (ID-LC-MS/MS) methods (13,64,65) (66). Since total thyroid hormone concentrations are influenced by conditions that change the binding protein concentrations (Figure 2), the measurement of the free thyroid hormone is considered more clinically reliable (13).



**Figure 2. Conditions that Influence Thyroid Hormone Binding Proteins. From references 25, 27 and 61.**

Total thyroid hormone methods typically require the inclusion of inhibitors, such as 8-anilino-1-napthalene-sulphonic acid to block hormone binding to serum proteins and facilitate the binding of thyroid hormone to the antibody reagent(s) (67). T3 concentrations are ten-fold lower than T4, so measuring T3 has always presented a greater sensitivity and precision challenge than measuring T4. Currently both TT4 and TT3 are measured by immunometric assays performed on automated platforms using enzymes, fluorescence, or chemiluminescent molecules as signals (11,13,62).

Between-method variability among eleven TT4 and twelve TT3 immunoassays are shown in Figure 3 (28) from sera from healthy individuals and compared with values reported by isotope dilution tandem mass spectrometry (ID-LC-MS/MS) - the reference measurement procedure (RMP) that uses primary T4 and T3 standards for calibration (13,28). Although most methods fell short of the optimal 5 percent goal established by the C-STFT, 4/11 TT4 assays agreed within 10 percent of the reference, whereas most TT3 assays exhibited a positive bias that would necessitate re-standardization (28,68,69). Thus, as would be expected, TT4 assays are more reliable than TT3 assays. However, variability persists likely resulting from matrix differences between calibrators and patient sera, the efficiency of the blocking agent employed, and reagent lot-to-lot variability (13,69-72).

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**Figure 3. (a) TT4 and (b) TT3 Between-Method Variability. The figure shows the variability among 11 TT4 (A-P) and 12 TT3 (A-M) methods (shown as assay means ±2 sd) relative to the RMP for the method. For the assays differing >10% from the RMP mean, the numerical value of the mean is listed (28).**

**TT4 and TT3 Reference Ranges**

The problem of between-method differences in TT4 and TT3 measurements (Figure 3) is compounded by the continued use of non-SI units by some countries. TT4 reference ranges have approximated 58 to 160 nmol/L (4.5-12.5 µg/dL) for more than four decades. However, in euthyroid pregnant women there is an approximate 2-fold rise in TBG concentrations by mid-gestation that produce a steady TT4 increase beginning in the first trimester and plateauing at approximately 1.5-fold pre-pregnancy levels by mid-gestation (73-75). As a result, some have suggested that the non-pregnant TT4 reference range be adjusted by a factor of 1.5 when assessing thyroid status in the latter half of gestation (47,73,74,76). TT3 reference ranges generally approximate 1.2 - 2.7 nmol/L (80 –180 ng/dL) (77), but as shown in Figure 3, TT3 displays more between-method variability than TT4 (69,78).

**FREE THYROID HORMONE TESTS (FT4 and FT3)**

In accordance with the free hormone hypothesis, it is the free thyroid hormone fractions (0.02 *%* of TT4 and 0.2 % of TT3) that exert biologic activity at the cellular level (79) and protein-bound hormone is considered biologically inert. Since binding-protein abnormalities are highly prevalent (Figure 2) (25,27,61), free hormone measurements (FT4 and FT3) are preferable to total hormone (TT4 and TT3) (13,15). However, the measurement of free hormone concentrations independent of protein-bound hormone remains technically challenging (13,15,80). This is especially the case for FT3, because FT3 immunoassays are more susceptible to interference by free fatty acids and drugs present in the circulation, prompting many laboratories to prefer a TT3 over a FT3 assay (13). FT4 and FT3 fall into two categories – **direct** methods that employ a physical separation of free from protein-bound hormone and indirect free hormone **estimate** tests (16).

**Direct FT4 and FT3 Methods**

Direct free hormone methods have employed equilibrium dialysis (ED) (13,81,82), ultrafiltration (83-85), or gel filtration (86) to separate free hormone from the dominant protein-bound moiety. The IFCC has now established equilibrium dialysis, isotope dilution, liquid chromatography, tandem mass spectrometry (ED ID-LC-MS/MS) using primary calibrators as the RMP for FT4 measurements (13,32,87-89). Specifically, equilibrium dialysis of serum is performed under defined conditions before measuring FT4 in the dialysate by ID-LC-MS/MS (12,34,35). Manufacturers are recommended to use this RMP to recalibrate their FT4 immunoassay tests (13). However, even direct methods that employ equilibrium dialysis or ultrafiltration to separate free from protein-bound hormone are not immune from technical problems relating to dilution, adsorption, membrane defects, temperature, the influence of endogenous binding protein inhibitors, fatty acid formation, and sample-related effects (13,80,82,90). Because direct free hormone methods are technically demanding, inconvenient, and expensive, they are typically only readily available in reference laboratories and most clinical laboratories use FT4 and FT3 estimate tests - immunoassay “sequestration” methods (see below). However, a direct free hormone test can be especially useful for evaluating thyroid status when immunoassay values appear discordant with the clinical presentation and/or the TSH measurement (15,91). All current FT4 and FT3 estimate tests remain binding-protein dependent to some extent (69).

EQUILIBRIUM DIALYSIS (ED)

Early equilibrium dialysis methods used I131 and later I125 labeled T4 tracers to measure the free T4 fraction, that when multiplied by a total hormone measurement gave an estimate of the free hormone concentration (81). Subsequently, symmetric dialysis in which serum was dialyzed without dilution (or employing a near-physiological medium) was used to overcome dilution effects (82). By the early 1970s higher affinity T4 antibodies (>1x1011 L/mol) and high specific activity T4-I125 tracers were used to develop sensitive RIA methods that could directly measure FT4 and FT3 in dialysates and ultrafiltrates (83,92). Subsequent improvements have involved employing more physiological buffer diluents and improving the dialysis cell design (82,92). More recently, isotope-dilution liquid chromatography/tandem mass spectrometry (ID-LC-MS/MS) (93) has been used to measure FT4 in ultrafiltrates (94) and dialysates (13,32,35,36,87,95,96).

ULTRAFILTRATION METHODS

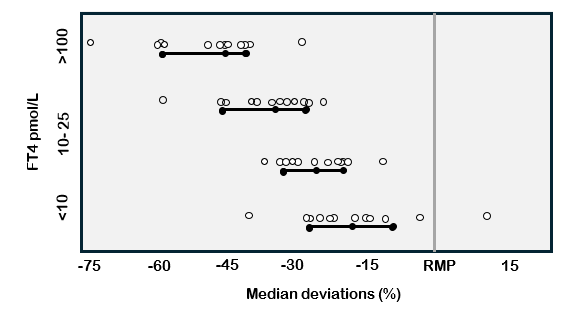
Ultrafiltration has also been used to remove protein-bound T4 prior to LC-MS/MS measurement of FT4 in the ultrafiltrate (97). Direct FT4 measurements employing ultrafiltration are sometimes higher than those made by equilibrium dialysis, because ultrafiltration avoids dilution effects (98). Moreover, ultrafiltration is not influenced by dialyzable inhibitors of T4-protein binding that can be present in conditions such as non-thyroidal illness (NTI) (90). However, ultrafiltration can be prone to errors when there is a failure to completely exclude protein-bound hormone and/or adsorption of hormone onto the filters, glassware, and tubing (99). In addition, ultrafiltration is temperature dependent such that ultrafiltration performed at ambient temperature (25°C) will report FT4 results that are 67 percent lower than ultrafiltration performed at 37°C (97). However, FT4 concentrations measured by ID-LC-MS/MS following either ultrafiltration at 37°C or equilibrium dialysis usually correlate (100).

GEL ABSORPTION METHODS

Some early direct FT4 methods used Sephadex LH-20 columns to separate free from bound hormone before eluting the free T4 from the column for measurement by a sensitive RIA. However, because of a variety of technical issues, assays based on this methodologic approach are not currently used (62).

**Indirect Free T4 and Free T3 Estimate Tests**

The first free hormone estimate tests were free hormone “indexes” (FT4I and FT3I) – a correction of the total hormone concentration for the influence of binding proteins assessed either using a direct TBG measurement or a binding-protein estimate (uptake) test (101,102). Current free hormone estimate tests are typically automated immunoassays that employ an antibody to sequester a small amount of the total hormone that is purportedly proportional to the free hormone concentration (13,15). Both index tests (FT4I and FT3I) and FT4 and FT3 immunoassays are typically protein-dependent to some extent and may under- or overestimate free hormone when binding proteins are grossly abnormal (80,103-105). As with TT4 methods, current FT4 immunoassays have significant between-method variability and biases (relative to the RMP) that far exceed the biological FT4 variation (Figure 4) (13,28,69). Recalibrating methods against the RMP has been shown to significantly reduce biases (32). It is hoped that manufacturers will continue to work to eliminate between-method biases and establish reference intervals that would apply to all methods (106).



**Figure 4. FT4 Between-Method Variability in FT4 Immunoassays. This figure shows deviations in FT4 measurements made by 13 different immunoassays relative to the reference measurement procedure (RMP = ED-ID-LC-MS/MS) (89).**

TWO TEST INDEX METHODS (FT4I AND FT3I)

Free hormone indices (FT4I and FT3I) are unitless mathematical calculations made by correcting the total hormone test result for the influence of binding proteins, primarily TBG (107). These indexes that have been used for more than 50 years require two separate tests to estimate free hormone (80). The first test involves the measurement of total hormone (TT4 or TT3) whereas the second test assesses the binding protein concentration by either a direct TBG immunoassay (103), a Thyroid Hormone Binding Ratio (THBR) or “Uptake” test (102), or an isotopic determination of the free hormone fraction (80,108).

*TBG Immunoassays*

Data has been conflicting concerning whether indexes that employ THBR in preference to a direct TBG are diagnostically superior (109). Free hormone indexes calculated using TBG measurement (TT4/TBG) may offer improved diagnostic accuracy over THBR when the total hormone concentration is abnormally high (i.e. hyperthyroidism), or when drug therapies interfere with THBR tests (110). Regardless, the TT4/TBG index is not totally independent of the TBG concentration, nor does it correct for **a**lbumin or **t**ransthyretin binding protein abnormalities (figure 2) (104).

*Thyroid Hormone Binding Ratio (THBR) / "Uptake" Tests*

The first "T3 uptake" tests developed in the 1950s employed the partitioning of T3-I131 tracer between the plasma proteins in the specimen and an inert scavenger (red cell membranes, talc, charcoal, ion-exchange resin, or antibody) (111-113). The "uptake" of T3 tracer onto the scavenger provided an indirect, reciprocal estimate of the TBG concentration in the specimen. Initially, T3 uptake tests were reported as percent uptakes (free/total tracer). Sera with normal TBG concentrations typically had approximately 30 percent of the T3 tracer taken up by the scavenger. During the 1970s methods were refined by replacing I131-T3 tracers by I125-T3 with a calculation of the hormone uptake based on the ratio of isotopic counts between the absorbent, and total minus absorbent counts. Results were expressed as a ratio with normal sera having an assigned value of 1.00 (108). Historically, the use of T3 as opposed to T4 tracer was made for practical reasons relating to the ten-fold lower affinity of TBG for T3 versus T4, facilitating a higher percentage of T3 tracer binding to the scavenger, thereby allowing shorter isotopic counting times. Because current methods use non-isotopic proprietary T4 or T3 "analogs", counting time is no longer an issue and current tests may use a "T4 uptake" approach - which may be more appropriate for correcting for T4-binding protein effects. Differences between T3 and T4 "uptakes" have not been extensively studied (114). Although all THBR tests are to some degree TBG dependent, the calculated FT4I and FT3I usually provides an adequate correction for mild TBG abnormalities (i.e. pregnancy and estrogen therapy) (73,102,103,115) but may fail to correct for grossly abnormal binding proteins (26) seen in euthyroid patients with congenital TBG extremes (103,104,116), familial dysalbuminemias (62,105,117-119), thyroid hormone autoantibodies (120-122), or medications that directly or indirectly influence thyroid hormone binding to plasma proteins (13,62,104,123).

*Isotopic Index Methods*

The first free hormone tests developed in the 1960s were indexes calculated from the product of the free hormone fraction, measured isotopically by dialysis, and TT4 measured by PBI and later RIA (81). These early isotopic detection systems were technically demanding and included paper chromatography, electrophoresis, magnesium chloride precipitation, and column chromatography (81,124-126). The free fraction index approach was later extended to ultrafiltration (83,85) and symmetric dialysis (127), the latter measuring the rate of transfer of isotopically labeled hormone across a membrane separating two chambers containing the same undiluted specimen. Ultrafiltration and symmetric dialysis had the advantage of eliminating dilution effects that influenced tracer dialysis values (82,128). However, free hormone indexes calculated using an isotopic free fraction were not completely independent of the TBG concentration and were influenced by tracer purity and the buffer matrix employed (92,129).

*Clinical Utility of Two-Test Index Methods (FT4I and FT3I)*

In the past some have favored the two-test FT4I approach for evaluating the thyroid status of patients with abnormal binding protein states like pregnancy or NTI (73,82). However, the continued use of these FT4I tests remains controversial (130). Until FT4 immunoassays are re-standardized to remove biases (13,69), FT4I remains a useful confirmatory test when binding proteins are abnormal or for diagnosing central hypothyroidism (69).

**Free Thyroid Hormone Immunoassay Methods (FT4 and FT3)**

Currently, most free hormone testing is made using automated FT4 and FT3 immunoassays (62,131). These immunoassays are based on "one-step", "labeled antibody" or "two-step" principles (80). For more than twenty years controversy has surrounded the standardization and diagnostic accuracy of these methods, especially in pathophysiologic conditions associated with the binding protein abnormalities such as pregnancy (15,73,131). These assays are subject to variability due to polymorphisms, drug interactions, high free fatty acid (FFA) levels, or thyroid binding inhibitors such as those present in non-thyroidal illness (NTI) (11,30, 62, 69, 90, 99,104,105,121,132). Studies of the inverse FT4/TSH log/linear relationship have emphasized the need to evaluate each method with clinical specimens containing abnormal binding proteins (94,133,134). Currently, most FT4 and FT3 immunoassays display significant negative or positive biases that exceed the intra-individual biological variability (12,13). As shown in Figure 4, all but one of the FT4 immunoassays tested had a negative bias relative to the FT4 RMP. Although the IVD industry is being encouraged to recalibrate their free hormone immunoassays against the RMP to reduce between-method biases (13, 28, 69, 87,135), implementation of a global re-calibration effort has been delayed by cost as well as practical, educational, and regulatory complexity.

ONE-STEP FT4 AND FT3 METHODS

The “one-step” approach uses a proprietary labeled hormone analog, designed for minimal interaction with thyroid hormone binding proteins, that competes with hormone in the specimen for a solid-phase anti-hormone antibody in a classic competitive immunoassay format (15,62,80). After washing away unbound constituents, the free hormone concentration should be inversely proportional to the labeled analog bound to the solid support. Although conceptually attractive, the diagnostic utility of the one-step approach has been shown to be dependent on the degree that the analog is "inert" with respect to binding proteins (80,94,133,134).

LABELED ANTIBODY FT4 AND FT3 METHODS

Labeled antibody methods are "one-step" methods that use a labeled antibody in preference to a labeled hormone analog. The free hormone in the specimen competes with solid-phase hormone for the labeled antibody and is quantified as a function of the fractional occupancy of hormone-antibody binding sites in the reaction mixture (15,62,80,136). The labeled antibody approach is used as the basis for several automated immunoassay platforms because it is easy to automate and considered less binding-protein dependent than the labeled analog approach, since the solid phase hormone does not compete with endogenous free hormone for hormone binding proteins (15,80,137-139).

TWO-STEP, BACK TITRATION FT4 AND FT3 METHODS

The two-step approach was first developed by Ekins and colleagues in the late 1970s (79,113). Two-step methods typically employ immobilized T4 or T3 antibody (for FT4 and FT3 immunoassays, respectively) to sequester a small proportion of total hormone from a diluted serum specimen without disturbing the original free to protein-bound equilibrium (62,80). After removing unbound serum constituents by washing, a labeled probe (originally 125-I T4, or more recently a macromolecular T4 conjugate) is added to quantify unoccupied antibody-binding sites that are inversely related to the free hormone concentration - a procedure that has been referred to as "back-titration (80).

CLINICAL UTILITY OF FT4 AND T3 IMMUNOASSAY MEASURMENTS

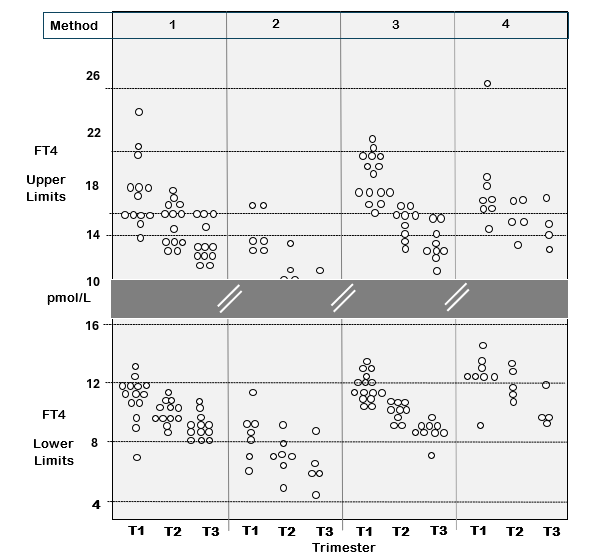
Current reference ranges for FT4 and FT3 immunoassays are method-dependent because of calibration biases that preclude establishing a universal reference range that would apply across methods (13,68,86). These biases are evident for FT4 immunoassay methods shown in Figure 4. Most FT4 methods give diagnostically reliable results when binding proteins are near-normal, provided that a method-specific reference range is employed (68). However, both TT3 and FT3 immunoassays tend to be inaccurate in the low range (77,139) and have no value for diagnosing or monitoring treatment for hypothyroidism (52,140). However, FT3 measurements can be useful for diagnosing or confirming unusual cases of hyperthyroidism. Most FT4 methods give diagnostically reliable results when binding proteins are near-normal, provided that a method-specific reference range is employed (69). However, both TT3 and FT3 immunoassays tend to be inaccurate in the low range (78,140) and have no value for diagnosing or monitoring treatment for hypothyroidism (52,141), although FT3 measurements can be useful for diagnosing or confirming unusual cases of hyperthyroidism.

*Ambulatory Patients*

FT4 and FT3 tests are used in preference to TT4 or TT3 measurements because they have better diagnostic accuracy for detecting hypo- and hyperthyroidism in patients with abnormal thyroid hormone binding proteins (figure 2). FT4 typically serves as a second-line test for confirming primary thyroid dysfunction detected by an abnormal TSH, but is the first-line test when thyroid status is unstable (early phase of treating hypo- or hyperthyroidism); in the presence of pituitary/hypothalamic disease (when TSH is unreliable); or when patients are taking drugs such as dopamine or glucocorticoids that are known to affect TSH secretion (10,104,110,142). Mild "subclinical" thyroid dysfunction is characterized by a TSH/FT4 discordance (abnormal TSH/normal FT4) reflecting the intrinsic complex nature of the inverse log/linear TSH/FT4 relationship (8,10,143) - a relationship that is modified by age and sex (144,145). Thus, small changes in FT4, even within normal limits, are expected to produce a mild degree of TSH abnormality - between 0.05 and 0.3 mIU/L (with subclinical hyperthyroidism) and 5 and 10 mIU/L (with subclinical hypothyroidism). An unexpected TSH/FT4 discordance if confirmed, should prompt an investigation for interference with FT4, TSH or both tests (91,146,147). FT4 interference can result from severe binding protein abnormalities such as congenital TBG excess or deficiency (26,62,103,148,149), dysalbuminemias (105,150-152), thyroid hormone autoantibodies (147,153-155), or drug interferences (62,104,123).

*Pregnant Patients*

Current reference ranges for FT4 immunoassays are method-dependent because of calibration biases that precludes establishing a universal reference range that would apply across methods (Figure 4) (156,157). This between-method variability has profound effects on the setting of the FT4 reference range for pregnancy (Figure 5). As with non-pregnant patients, TSH is the first-line test to use for assessing thyroid status during pregnancy (48,158). However, FT4 measurement is needed for monitoring anti-thyroid drug treatment of hyperthyroid pregnant patients who have an undetectable TSH. The question whether an isolated low FT4 during pregnancy is a maternal or fetal risk factor, remains controversial (159,160), although some studies suggest that low FT4 may be a risk factor for gestational diabetes and fetal complications (161-163). Non-pregnant FT4 reference ranges do not apply to pregnancy since FT4 progressively declines as gestation progresses, necessitating the use of a trimester-specific reference ranges (73,158,164,165). Setting universal trimester-specific FT4 reference ranges is currently hampered by the between-method differences shown in Figure 4 and 5 (69,156,165), compounded by the differences related to ethnicity (166-170), iodine intake (171-173), smoking (174), and BMI (145,166). Establishing institution-specific trimester-specific reference ranges from the 2.5 to 97.5 percentiles by recruiting at least 400 pregnant patients (170) is not practical for most institutions. After the proposed re-standardization of FT4 methods against the RMP the feasibility of establishing universal trimester-specific reference ranges will improve (13,69,135). However, binding protein effects will remain, and population-specific factors will still have to be considered.



**Figure 5. Between-Method FT4 Variability Impacts Thyroid Testing in Pregnancy. The figure shows the upper and lower FT4 reference limits (2.5–97.5%) from 43 published studies of FT4 measurements made in each trimester of pregnancy by four different methods: Abbott (1), Beckman (2), Roche (3) and Siemens (4). The data shows the expected trend for higher FT4 in the first trimester, resulting from thyroidal human chorionic gonadotropin (HCG) stimulation which is maximal in early pregnancy. The data is re-drawn with permission from reference 156.**

*Hospitalized Patients with Nonthyroidal Illnesses (NTI)*

The diagnostic performance of current FT4 methods has not been evaluated in hospitalized patients with NTI where the severity of illness, binding protein inhibitors, and drug therapies can negatively impact the reliability of both thyroid hormone and TSH testing (10,30,62,90,122,132,181-183). Three categories of hospitalized patients deserve special attention: a) patients with NTI without known thyroid dysfunction who have a high or low T4 status; b) patients with primary hypothyroidism and concurrent NTI and, c) patients with hyperthyroidism and concurrent NTI (13). Because the diagnostic reliability of FT4 testing is still questionable in sick hospitalized patients, a combination of both T4 (FT4 or TT4) and TSH may be needed to assess thyroid status in this setting (10,13).

In most clinical situations where FT4 and TSH results are discordant, the TSH test is the most diagnostically reliable, provided that the patient does not have pituitary failure or receiving medications such as glucocorticoids or dopamine that directly inhibit TSH secretion (110,142,181). Repetitive TSH testing may be helpful in resolving the cause of an abnormal FT4, because the TSH abnormalities of NTI are typically transient (Figure 6b) whereas the TSH abnormality will persist if due to underlying thyroid dysfunction (184-187). In some cases, it may be useful to test for TPOAb as a marker for underlying thyroid autoimmunity.

**A diagram of recovery and recovery

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**Figure 6. Effects of Nonthyroidal Illness (NTI) on Thyroid Tests. Figure 6a shows the magnitude and direction of changes in total (TT4 and TT3) and free (FT4 and FT3) thyroid hormone IMA tests versus FT4 measured by the RMP (ED-ID-LC-MS/MS), as the severity of illness increases, followed by recovery. Figure 6b shows the magnitude and direction of TSH changes as the severity of illness increases, followed by recovery. Data redrawn from reference 188 with permission.**

*Pediatric Patients*

The determination of normal reference limits for pediatric age groups is especially challenging, given the limited number of studies involving large numbers of healthy children (175-177). Most studies report that serum TSH peaks after birth and steadily declines throughout childhood to reach adult levels at puberty. Likewise, FT3 declines across the pediatric age groups during childhood and approaches the adult range at puberty, whereas FT4 levels for infants less than a year old are higher than for children 1 to 18 years old who have FT4 comparable to adults (175-180).

**Interferences with Thyroid Hormone Tests**

Only the ordering physician can suspect interference with a test result and request that the laboratory perform interference checks. This is because the hallmark of interference is discordance between the test result and the clinical presentation of the patient, and most specimens are sent to the laboratory with no clinical information. Failure to recognize interferences can have adverse clinical consequences (91,146,189-197).

Laboratory checks for interferences include, a) showing a discordance between different manufacturers methods (196,198-200), b) re-measurement of the analyte after adding a blocker of Heterophile antibodies (HAb) (196,200,201), c) performing linearity studies or d) precipitating interfering immunoglobulins with polyethylene glycol (PEG) (196,198). A change in the analyte concentration in response to any one of these maneuvers suggests interference, but a lack of an effect does not rule out interference.

Interferences can be classified as either (a) non-analyte-specific, or (b) analyte-specific (191,195,199).

NON-ANALYTE SPECIFIC INTERFERENCES

*Protein Interferences*

Either paraproteins or abnormal immunoglobulins can interfere with immunoassays (90,202-205).

Congenital TBG excess or deficiency:Free hormone immunoassays and free T4 index tests may be susceptible to interference from grossly abnormal TBG concentrations, such as seen in congenital TBG excess or deficiency states (26,62,103,148,149).

Pregnancy:Estrogen stimulation increases TBG, and consequently both TT4 and TT3, concentrations progressively rise to plateau at 2.5-fold pre-pregnancy values by mid-gestation (73). Despite the rise in total hormone, both FT4 and FT3 decline during gestation, in accordance with the law of mass action (73,157,158,206,207). However, the degree of FT4 decline during pregnancy is variable and method-dependent (Figure 5). The declining albumin concentrations typical of late gestation also affect some methods (208).

Familial Dysalbuminemias and Transthyretin Hyperthyroxinemias***:***Autosomal dominant mutations in the albumin or transthyretin (prealbumin) gene (209) can result in altered protein structures with enhanced affinity for thyroxine and/or triiodothyronine. These abnormal proteins can interfere with FT4 and/or FT3 measurements and result in inappropriately high FT4 and/or FT3 immunoassay values (105,151,210-212). Familial Dysalbuminemic Hyperthyroxinemia (FDH) is a rare condition with a prevalence of ~1.8 percent in the Hispanic population (119,213). It arises from a few genetic variants in the albumin gene, with the R218H being the most common. Some variants result in extremely high TT4, whereas other mutations (i.e. L66P) affect mainly TT3 (150). Affected individuals are euthyroid and have normal TSH and FT4 when measured by direct techniques such as equilibrium dialysis (105). Unfortunately, most FT4 estimate tests (immunoassays and indexes) report falsely high values for FDH patients that may prompt inappropriate treatment for presumed hyperthyroidism if the condition is not recognized (105,119).

*Heterophile Antibodies (HAb)*

It is well recognized that heterophile antibodies (HAb) - human poly-specific antibodies targeting animal antigens, can interfere with immunometric assays causing falsely high/positive or falsely low/negative test results (214,215). The most common interferant is human anti-mouse antibodies (HAMA) (199,215-220). Rheumatoid factor (RF), an immunoglobulin commonly associated with autoimmune conditions, is also considered a heterophile antibody that can interfere by targeting human antigens (199,217,221,222). Although HAb usually causes false positive tests, false-negative tests have also been reported (214). HAb has been shown to interfere with multiple endocrine tests that use IMA principles, including free and total thyroid hormones, TSH, Tg, and TgAb (138,193,200,214,221,223-225). The prevalence of HAbs is variable but has been reported as high as eleven percent (223,226,227). In recent years assay manufacturers have increased the immunoglobulin blocker reagents added to their tests and this has reduced HAb interference somewhat (223,225-227). However, interference is still seen in some patients with a high enough HAb to overcome the assay blocker (198,223,228). HAb interference mostly affects non-competitive immunometric assays (IMA) that employ monoclonal antibodies of murine origin (216). Assays based on the competitive format that employ high affinity polyclonal antibody reagents, are rarely affected (216). The test marketed by one manufacturer can be severely affected, whereas the test from a different manufacturer may appear unaffected (200). This is why the first step for investigating interference is re-measurement of the analyte by a different method. It should be noted that patients receiving recent vaccines, blood transfusions, or monoclonal antibodies (given for treatment or scintigraphy), as well as veterinarians and those in contact with animals, are especially prone to test interferences caused by induced HAb and human anti-mouse antibodies (HAMA)(198,229).

*Anti-Reagent Antibodies*

Interference can be caused by antibodies targeting assay reagents. For example, a number of reports have found that anti-rhuthenium antibodies can interfere with TSH, FT4, and FT3 tests (200,230). In addition, antibodies targeting either streptavidin (231,232) or Biotin (233,234) can interfere with assays employing streptavidin or biotin reagents.

*High Dose Dietary Biotin*

Some IMA tests have employed a biotin-streptavidin separation system (232). Patients who take a high dose of dietary biotin risk having test interferences with such methods (232). Depending on the specific test formulation, biotin interference can cause falsely high- or low- test results (234-236). Manufacturers are now prioritizing replacing their biotin-streptavidin separation systems to eliminate this problem (237).

ANALYTE-SPECIFIC INTERENCES

Analyte-specific interferences typically result from autoantibodies targeting the analyte (238). Autoantibodies targeting both TSH (macro-TSH) (238-241) and both thyroid hormones (T4 and/or T3) (154,155) have been reported. Autoantibody interferences may be more prevalent in patients with non-thyroid autoimmune conditions (242,243). Depending on the analyte and test formulation, thyroid hormone and TSH autoantibodies typically cause falsely high tests (239,244). It should be noted that transplacental passage of either HAb or anti-analyte autoantibodies (i.e. TSHAb or T4Ab) have the potential to interfere with neonatal screening tests (245-247). Specifically, maternal TSH autoantibodies can cross the placenta and cause a falsely high TSH screening test in the newborn mimicking congenital hypothyroidism (247), whereas maternal T4 autoantibodies could cause a falsely high neonatal T4 test and mask the presence of congenital hypothyroidism (246).

*Thyroid Hormone Autoantibodies (T4Ab/T3Ab)*

T4 and T3 autoantibodies can falsely elevate total hormone, free hormone, or THBR measurements depending on the method employed (153,155,210). The prevalence of thyroid hormone autoantibodies occurs in approximately 2 percent of the general population but may be present in over 30 percent of patients with autoimmune thyroid disease or other autoimmune conditions (242,243,248). However, despite their high prevalence, significant interference caused by thyroid autoantibodies is not common and depends on the qualitative characteristics of the autoantibody present (i.e. its affinity for the test reagents). Furthermore, different methods exhibit such interferences to a greater or lesser extent (120,154). Because autoantibody interference is difficult for the laboratory to detect proactively, it is the physician who should first suspect interference from an unexpected discordance between the clinical presentation of the patient and the test result(s) (249,250).

**TSH (THYROID STIMULATING HORMONE) MEASUREMENT**

Over the last five decades the dramatic improvements in TSH assay sensitivity and specificity have revolutionized thyroid testing and firmly established TSH as the first-line test for ambulatory patients who are not receiving drugs known to alter TSH secretion (10,13,251). Serum TSH has become the therapeutic target for levothyroxine (L-T4) replacement therapy for hypothyroidism (52) and suppression therapy for differentiated thyroid cancer (DTC) (57,252,253). The diagnostic superiority of TSH versus FT4 measurement arises from the inverse, predominantly log/linear, TSH/FT4 relationship, that is modified to some extent by factors such as age, sex, active smoking, and TPOAb status (8,10,13,143,144,254,255).

**TSH Assays**

TSH assay "quality" has historically been defined by clinical sensitivity – the ability to discriminate between hyperthyroid and euthyroid TSH values (8,10,13,256). The first generation of RIA methods had a detection limit approximating 1.0 mIU/L (1,3,257) that limited their clinical utility to diagnosing primary hyperthyroidism (258) and necessitated the use of TRH stimulation to diagnose hyperthyroidism, characterized by an absent TRH-stimulated TSH response (259-261). With the advent of immunometric assay (IMA) methodology that uses a combination of poly- and/or monoclonal antibodies targeting different TSH epitopes in a "sandwich" format (262-264), a ten-fold improvement in TSH assay sensitivity (~ 0.1 mIU/L) was achieved when using isotopic (I125) signals (265). This level of sensitivity facilitated the determination of the lower TSH reference limit (0.3-0.4 mIU/L) and the detection of overt hyperthyroidism without the need for TRH stimulation (266,267) but was still insufficient for distinguishing between differing degrees of hyperthyroidism (i.e. subclinical versus overt) (268). Assay sensitization continued until a third generation of TSH IMAs was developed by employing non-isotopic signals that could achieve a sensitivity of 0.01 mIU/L (8,251,267). Initially different non-isotopic signals were used that gave rise to a lexicon of terminology to distinguish between assays: immunoenzymometric assays (IEMA) used enzyme signals; immunofluorometric assays (IFMA) used fluorophors as signals, immunochemiluminometric assays (ICMA) used chemiluminescent molecules as signals, and immunobioluminometric assays (IBMA) used bioluminescent signal molecules (112,267). Current TSH methods are mostly automated ICMAs that achieve third generation functional sensitivity (FS = ≤0.01 mIU/L) - a FS level that has now become the standard of care (10,13,269).

FUNCTIONAL SENSITIVITY (FS) = THE LOWEST REPORTABLE ASSAY LIMIT

During the period of active TSH assay sensitization, different non-isotopic IMAs made competing claims for sensitivity. Methods were described as: "sensitive", "highly sensitive", "ultrasensitive", or "supersensitive" - marketing terms that had no scientific definition. This confusion led to a debate concerning what was the most clinically relevant parameter to use to determine the lowest reliable reportable TSH value for clinical practice (10,251,267). Functional sensitivity (FS) became defined as the lowest analyte concentration measured with 20 percent coefficient of variation (10) established over a clinically relevant timespan (6-8 weeks for TSH). FS is now recognized as the parameter that best represents the between-run precision for measuring low analyte concentrations in clinical practice (10,270,271). FS is used to define the lower reportable limit for not only TSH but also Tg and TgAb, as well as other non-thyroid assays for which analytic sensitivity is critical (10). FS protocols recognize that immunoassays tend to be matrix-sensitive and specify that precision be determined in human sera rather than a quality control material that uses an artificial protein matrix (71,72,272). The timespan used for determining precision is also analyte-specific and should reflect the frequency of testing employed in clinical practice - 6 to 8 weeks for TSH, but 6 to 12 months for the Tg and TgAb - assays that are used as tumor markers for monitoring DTC. An optimal timescale is important, because low-end precision erodes over time due to a myriad of variables including reagent lot-to-lot variability (71). Note that the FS parameter is more stringent than other biochemical sensitivity parameters such as limit of detection (LOD - a within-run parameter) and limit of quantitation (LOQ - a between-run parameter without stipulations regarding the matrix and the timespan used for determining precision (72,271,273). A ten-fold difference in FS has been used to define each generation of increasingly more sensitive methods (17,251,271,274). Thus, TSH RIA methods with FS approximating 1.0 mIU/L were designated "first generation", the TSH immunoradiometric (IRMA) methods that had a functional sensitivity approximating 0.1 mIU/L were designated " second generation", and current TSH ICMAs with FS approximating 0.01 mIU/L are now designated "third generation" assays (267,270,275).

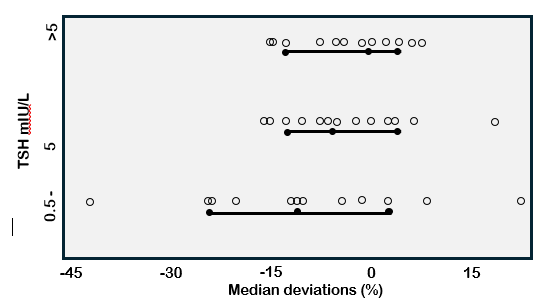
TSH BIOLOGIC VARIABILITY

TSH is a heterogeneous glycoprotein (276-278), and TRH-mediated changes in TSH glycosylation and thus detection by IMA methodology (279,280) have the potential to influence immunoactivity (277,281). Alterations in TSH glycosylation can occur in a number of pathophysiologic circumstances (278,282). Seasonal variability in TSH has been shown with 10% higher TSH levels in the winter than in the summer months (283). However, FT4 and FT3 levels show no such seasonal variability (283). The demonstration that harmonization of TSH methods successfully minimizes between-method differences (69), suggesting that under normal conditions current TSH IMAs appear to be "glycosylation blind" and detect different TSH glycoforms in an equimolar fashion (277,278). However, future studies need to include sera from conditions where TRH dysregulation may lead to abnormal TSH glycosylation and bioactivity, such as pituitary dysfunction, NTI, and aging (278,280,281,284-286).

TSH intra-individual variability is relatively narrow (20-25 percent) in both non-pregnant and pregnant subjects, as compared with between-person variability (13,287,288). In fact, the serum TSH of euthyroid volunteers was found to vary only ~0.5 mIU/L when tested every month over a span of one year (287). Twin studies suggest that there are genetic factors that determine hypothalamic-pituitary-thyroid setpoints (289-291). These studies report that the inheritable contribution to the serum TSH level approximates 65 percent (290,291). This genetic influence appears in part to involve single nucleotide polymorphisms in thyroid hormone pathway genes such as the phosphodiesterase gene (PDE8B) (292), polymorphisms causing gain (293) or loss of function TSH receptors (294), and the type II deiodinase enzyme polymorphisms (293). Undoubtedly, such polymorphisms account for some of the euthyroid outliers that skew TSH reference range calculations (295). The narrow TSH within-person variability and low (< 0.6) index of individuality (IoI) (287,288) limits the clinical utility of using the TSH population-based reference range to detect thyroid dysfunction in an individual patient (288,296-298). When evaluating patients with marginally (confirmed) low (0.1–0.4 mIU/L) or high (4–10 mIU/L) TSH abnormalities, it is more important to consider the degree of TSH abnormality relative to patient-specific risk factors for cardiovascular disease rather than the degree of the abnormality relative to the TSH reference range (13,52,299).

TSH REFERENCE RANGES

As with the thyroid hormone tests, the significant biases between different TSH methods (Figure 7) prevent establishing universal population or trimester-specific reference ranges that would apply across methods (13,170). These method biases also impact the detection of subclinical hypothyroidism (299,300). Since TSH is a complex glycoprotein, no reference measurement procedure (RMP) is available, or will likely be feasible in the future (13), given the current lack of commutability between the pituitary TSH reference preparations and patient specimens (33). A harmonization approach (31,301), whereby methods are recalibrated to the "all method mean", has been shown to have the potential to effectively eliminate current between-method TSH differences that are most pronounced at pathophysiologic levels (29,302). Better harmonization may also be possible using a reference panel of serum specimens (33). The IFCC is actively working with the IVD industry to encourage manufacturers to harmonize their methods. A reduction of between-method variability could eliminate the need to establish method-specific TSH reference ranges - a practice that is costly and inconvenient given the large numbers of rigorously screened participants that are necessary to establish reliable 2.5th to 97.5th percentiles for a population (87,303). However, even after harmonization minimizes inter-method differences, it remains to be determined to what extent universal ranges would be impacted by other factors such as age (254,304), ethnicity (254), and iodine intake (305). It may be that a reference range established in one geographic location may not be representative of a different locale or population. The harmonization of TSH methods would be advantageous for consolidating data from different studies and establishing universal reference limits (13).

****

**Figure 7. TSH Between-Method Variability. Figure shows deviations in TSH measurements made in the low (<0.5), medium (0.5-5.0), and high (>5) mIU/L range using 14 different immunoassays. Data is expressed as deviations from the trimmed all method mean (88).**

TSH POPULATION REFERENCE RANGE

The log/linear TSH/FT4 relationship (8,10,143,144,255) dictates that TSH will be the first abnormality to appear as mild (subclinical) as hypo- or hyperthyroidism develops. It follows that the setting of the TSH reference limits critically influences the frequency of diagnosing subclinical thyroid disease (50,53). It is recommended that “TSH reference intervals should be established from the 95 percent confidence limits of the log-transformed values of at least 120 rigorously screened normal euthyroid volunteers who have: (a) no detectable thyroid autoantibodies, TPOAb or TgAb; (b) no personal or family history of thyroid dysfunction; (c) no visible or palpable goiter and, (d) who are taking no medications (except estrogen)” (10,303).

Multiple factors influence population TSH reference limits, especially the upper (97.5th percentile) limit. Different methods report different ranges for the same population resulting from between-methods biases (Figure 7) (13). A key factor affecting the upper limit is the stringency used for eliminating individuals with thyroid autoimmunity from the population (306-308). Other factors relate to population demographics such as sex (254), ethnicity (254,309,310), iodine intake (311,312), BMI (313,314), and smoking status (311,315). The relationship between TSH and age is complex with most studies in iodine sufficient populations reporting an increase in the TSH upper limit with age (143,254,308,309,316). This has led to the suggestion that age-and sex-specific TSH reference limits be used (50,316). Conflicting data on this issue could merely represent population differences with an increasing prevalence of thyroid autoimmunity in iodine-sufficient populations (254,317). Whereas in iodine deficient populations, increasing autonomy of nodular goiter can result in decreased TSH with aging (318). Some studies have reported that a mild TSH elevation in elderly individuals may convey a survival benefit (319), whereas other studies dispute this (320). However, TSH is a labile hormone, and studies cannot assume that a TSH abnormality found in a single determination is representative of thyroid status in the long-term (321).

PEDIATRIC TSH REFERENCE RANGES

The adult TSH population reference range does not apply to neonates or children. Serum TSH values are generally higher in neonates and then gradually decline until the adult range is reached after puberty (178,179,322,323). This necessitates using age-specific TSH reference ranges for diagnosing thyroid dysfunction in different pediatric age groups.

SUBCLINICAL THYROID DYSFUNCTION

Subclinical Hyperthyroidism (SCHY) is defined as a low (<2.5th percentile) but detectable TSH (0.01 - 0.3 mIU/L range) without a FT4 abnormality. SCHY seems relatively independent of the method used (324-326). Endogenous SCHY prevalence is low (0.7 %) in iodine-sufficient populations (254) but may increase as an iatrogenic consequence of L-T4 replacement therapy (327-330). SCHY is a risk factor for osteoporosis and increased fracture risk (331) as well as atrial fibrillation and cardiovascular disease (325,332-334), especially in older patients.

Subclinical Hypothyroidism (SCHO)is defined as a TSH above the upper (>97.5th percentile) TSH reference limit without a FT4 abnormality (50,300,308,335). However, the setting of the TSH upper limit remains controversial, thus the prevalence of SCHO is highly variable - 4 to 8.5 percent rising to 15 percent in older populations (254,299,307,335). In most cases, SCHO is associated with TPOAb positivity, indicative of an autoimmune etiology (307). The clinical consequences of SCHO relate to the degree of TSH elevation (336,337). Most guidelines recommend L-T4 treatment of SCHO when TSH is above 10 mIU/L (49,50), but below 10 mIU/L L-T4 treatment is usually based on patient-specific risk factors (50). There is active debate concerning the efficacy of treating SCHO to prevent progression (338-340), or improve renal (341), cardiovascular (333,336,342-346), or lipid (347,348) abnormalities that can be associated with SCHO.

THYROID DYSFUNCTION IN PREGNANCY

Overt hypo- or hyperthyroidism is associated with both maternal and fetal complications (349-352). However, the impact of maternal subclinical thyroid dysfunction remains controversial (51), although no maternal or fetal complications appear associated with subclinical hyperthyroidism during pregnancy (349,353). First trimester "gestational hyperthyroidism" is typically transient and hCG-related (354). In contrast, short-term and long-term outcome studies of maternal subclinical hypothyroidism (51,355) are complicated by heterogeneity among studies arising from a myriad of factors influencing TSH cutoffs, such as gestational stage, TSH method used, maternal TPOAb status, and current and pre-pregnancy iodine intake (160,172). Using gestational age-specific reference intervals the frequency of SCHO in first trimester pregnancy approximates 2-5 percent (355,356). Studies have found that subclinical hypothyroidism is associated with increased frequency of maternal and fetal complications, especially when TPOAb is positive (51,160,349,357-362). Maternal complications have included miscarriage (358), preeclampsia (363,364), placental abruption (350), preterm delivery (349,358,365,366), and post-partum thyroiditis (359). Fetal complications have included intrauterine growth retardation and low birth weight (350,353) and possible impaired neuropsychological development (367,368). It remains controversial whether L-T4 treatment of SCHO in early gestation decreases the risk of complications (358,362,369).

Trimester-Specific TSH Reference Ranges. As with non-pregnant patients, TSH is the first-line test used for assessing thyroid status during pregnancy when gestation-related TSH changes occur (47,51,76,158,355). Currently, method specific TSH reference ranges are needed for each trimester because of between-method variability (Figure 8). In the first trimester, there is a transient rise in FT4 caused by high hCG concentrations stimulating the TSH receptor - because hCG shares some homology with TSH (370-372). The degree of TSH suppression is inversely related to the hCG concentration and can be quite profound in patients with hyperemesis who have an especially high hCG (165,370,372-374). As gestation progresses, TSH tends to return towards pre-pregnancy levels (165). Recent studies from different geographic areas with diverse iodine intakes using different TSH methods have reported higher trimester-specific TSH upper limits than recommended by previous guidelines (51,159,164,165, 355, 375). In response, the American Thyroid Association have revised their pregnancy guidelines (47,48) to replace trimester-specific reference limits by a universal upper TSH limit of 4.0 mIU/L, when TPOAb is negative and no local reference range data is available (376). However, at this time between-method biases (Figure 7) clearly preclude proposing universal TSH cut offs that would apply to all methods and all populations including pregnant patients (69,87,164,165). IVD manufacturers are being encouraged to harmonize their TSH methods so that universal reference limits can be established for pregnancy (69,87). Requiring each institution to establish their own trimester-specific reference ranges is impractical, given the costs, logistics and ethical considerations involved in recruiting the more than 400 disease-free pregnant women that would be needed to represent each trimester (158). Even after methods are re-standardized (FT4) or harmonized (TSH), trimester-specific reference ranges would still be influenced by differences in ethnicity and iodine intake, especially the pre-pregnancy iodine intake that influences thyroidal iodine stores (172). In addition, since the TSH upper limit is skewed by the inclusion of individuals with thyroid autoimmunity, reliable method-specific TPOAb cutoffs need to be established (165,372,377).

*A diagram of a method

AI-generated content may be incorrect.*

**Figure 8. Between-Method TSH Variability Impacts Thyroid Testing in Pregnancy. The figure is a summary of 43 published studies showing the upper and lower TSH reference limits (2.5–97.5 %) measured in each trimester of pregnancy by four different methods – Abbott (1), Beckman (2), Roche (3), and Siemens (4). The data shows the expected trend for a lower TSH in the first trimester, resulting from thyroidal human chorionic gonadotropin (HCG) stimulation of thyroxine, which is maximal in the first trimester. The data is re- drawn with permission from reference 156.**

**Clinical Utility of TSH Measurement**

AMBULATORY PATIENTS

In the outpatient setting the reliability of TSH testing is not usually influenced by the time of day of the blood draw, because the diurnal TSH peak occurs between midnight and 0400 (378-381). However, seasonal changes in TSH have been shown, with TSH approximately 10 % higher in winter than in summer (283). Third generation TSH assays (FS ~0.01 mIU/L) have now become the standard of care because they can reliably detect the full spectrum of thyroid dysfunction from overt hyperthyroidism to overt hypothyroidism, provided that hypothalamic-pituitary function is intact, and thyroid status stable (10,49,382,383). TSH is also used for optimizing L-T4 therapy - a drug with a narrow therapeutic range (49,384). Because TSH secretion is slow to respond to changes in thyroxine status there is no need to withhold the L-T4 dose on the day of the blood test (10,384). In addition, in differentiated thyroid cancer (DTC) patients, targeting the degree of TSH suppression relative to recurrence risk plays a critical role in management (57,385,386).

HOSPITALIZED PATIENTS WITH NONTHYROIDAL ILLNESSES (NTI)

Non-thyroidal illness, sometimes called the "sick euthyroid syndrome" is associated with alterations in hypothalamic/pituitary function and thyroid hormone peripheral metabolism, often exacerbated by drug influences (104,181,186,387-389). Routine thyroid testing in the hospital setting is not recommended because thyroid test abnormalities are frequently seen in sick euthyroid patients (Figure 6) (388-391). TSH also usually remains within normal limits or may become somewhat depressed in the early phase, especially in response to drug therapies such as dopamine or glucocorticoid (104,110,181). During the recovery phase, TSH frequently rebounds above the reference range (184). High TSH may also be seen associated with psychiatric illness (392). It is important to distinguish the generally mild, transient TSH alterations typical of NTI from the more profound and persistent TSH changes associated with hyper- or hypothyroidism (10,183,185,390).

**Causes of Misleading TSH Measurements**

A diagnostically misleading TSH can result from biological factors or interferants in the serum such as drugs, heterophile antibodies (i.e. HAMA), or endogenous TSH autoantibodies (91,195,197,378,393). In most cases such interferences cause a falsely high TSH.

BIOLOGIC FACTORS CAUSING MISLEADING TSH

*Unstable Thyroid Function*

TSH can be misleading when there is unstable thyroid status - such as in the early phase of treating hypo- or hyperthyroidism or non-compliance with L-T4 therapy - when there is a lag in the resetting of pituitary TSH to reflect a new thyroid status (394). During such periods of instability TSH will be misleading and FT4 will be the more diagnostically reliable test.

*Pituitary/Hypothalamic Dysfunction*

Pituitary dysfunction is rare in ambulatory patients (395). TSH measurement is unreliable in cases of both central hypothyroidism and central hyperthyroidism (285,395-397).

Central hypothyroidism (CH) is rare, 1/1000 less prevalent than primary hypothyroidism, 1/160,000 detected by neonatal screening) (395). CH can arise from disease at either the pituitary or hypothalamic level, or both (395). A major limitation of using a TSH-centered screening strategy is that current TSH tests will miss a diagnosis of CH because TSH IMAs are “glycosylation blind” and detect the abnormally glycosylated biologically inactive TSH as “normal” TSH, despite clinical hypothyroidism (276,285,396). This limitation necessitates that the clinical diagnosis of CH be confirmed biochemically as a low FT4/normal-low TSH discordance. and that L-T4 replacement therapy for CH be optimized using the serum FT4 not TSH. It should be noted that in the absence of clinical suspicion, investigations for pituitary dysfunction should only be initiated after ruling-out technical interference.

TSH secreting pituitary adenomas are characterized by a non-suppressed TSH associated with high thyroid hormone levels and clinical hyperthyroidism (397,398). Since this is a rare type of pituitary adenoma (0.7 %), technical interferences such as heterophile antibody (HAb) or TSH autoantibodies (macro TSH) should be excluded before initiating inconvenient and unnecessary pituitary imaging or dynamic diagnostic testing such as T3 suppression or TRH stimulation. This clinical/biochemical discordance reflects the TSH isoforms with enhanced biologic activity secreted by the adenoma. As with CH, current TSH IMA methods cannot distinguish these abnormal isoforms from normal TSH. Failure to diagnose the pituitary as the cause of the hyperthyroidism can lead to inappropriate thyroid ablation. The treatment of choice is surgery but in cases of surgical failure somatostatin analog treatment has been found effective (398). Note that the biochemical profile (high thyroid hormones and non-suppressed TSH) resembles that seen with thyroid hormone resistance syndromes (399,400) or interference from thyroid autoantibodies (120).

*Resistance to Thyroid Hormone (RTH)*

Resistance to thyroid hormone is caused by mutations in the *THRB* gene encoding the thyroid hormone receptor B and is biochemically characterized by high thyroid hormone (FT4 +/- T3) levels and a non-suppressed, sometimes slightly elevated TSH (402,403). Tissues expressing primarily the thyroid hormone receptor B are hypothyroid (e.g. the liver), whereas organs with a predominant expression of thyroid receptor A (e.g. the heart) display alterations consistent with thyroid hormone excess (400,401). Early cases of thyroid hormone resistance were shown to result from mutations in the thyroid hormone receptor *B* (400). More recently the syndromes with decreased sensitivity to thyroid hormones have been broadened to include mutations in thyroid hormone transporters (e.g. MCT8), the metabolism of thyroid hormone (e.g. SBP2), and resistance mediated by mutations in thyroid receptor A (401) (for detailed discussion see the Endotext chapter entitled “Impaired Sensitivity to Thyroid Hormone: Defects of Transport, Metabolism and Action”). These insensitivity and resistance syndromes display a spectrum of clinical and biochemical profiles and can now be identified by genetic testing.

*Activating or Inactivating TSH Receptor Mutations*

Non-autoimmune hyperthyroidism resulting from an activating mutation of the TSH receptor (TSHR) is rare (293,402). A spectrum of loss-of-function TSHR mutations (TSH resistance) causing clinical and subclinical hypothyroidism despite high thyroid hormone levels, have also been described (295,400,403,404). Because TSHR mutations are a rare cause of TSH/FT4 discordances, technical interferences should first be excluded before considering a TSHR mutation as the cause of these discordant biochemical profiles.

TECHNICAL FACTORS CAUSING MISLEADING TSH

*Non-Analyte Specific Interferences*

Heterophile Antibodies (HAbs) such as Human Anti Mouse Antibody (HAMA) can cause falsely high TSH IMA tests (200,220,241,405,406) and interfere with neonatal TSH screening (407). Since the HAb in some patient's sera interfere strongly with some manufacturers tests but appear inert in others (200), re-measurement using a different manufacturers assay should be the first test to identify interference. A fall in TSH in response to a blocker-tube treatment (43) is typically used to confirm HAb interference.

Anti-Reagent Antibody Interferences. As discussed for free hormone tests, some patients have antibodies that target test reagents such as rhuthenium and cause interference with TSH tests. (408). It should be noted that the anti-rhuthenium antibodies of different patients may affect different analytes to differing degrees (230,409,410).

Biotin Interferences. Tests employing streptavidin or biotin reagents are prone to interferences from antibodies targeting either streptavidin (231) or biotin (233). Alternatively, high dose biotin ingestion has been known to produce interference in an analyte-specific, platform-specific manner (241,411). The popularity of biotin therapy is now prompting assay manufacturers to reformulate their tests to remove biotin interference (237,412).

*Analyte-Specific Interferences*

Analyte-specific interferences typically result from autoantibodies targeting the analyte. Depending on the analyte and test formulation, autoantibody interferences most commonly cause falsely high test results. It should be noted that transplacental passage of both heterophile antibodies or anti-analyte autoantibodies (i.e. TSHAb or T4Ab) have the potential to interfere with neonatal screening tests (245-247,413). Patients with autoantibodies targeting both TSH and prolactin (PRL) have been described (414).

TSH Autoantibodies (Macro TSH*).* Analytically suspicious TSH measurements are not uncommon (205,238,239,244,415) and have been reported in up to five percent of specimens subjected to rigorous screening (405). There have been many reports of TSHAb, often referred to as "macro TSH” causing spuriously high TSH results in a range of different methods used for both adult (238,416) and neonatal screening (244,415). The prevalence of TSHAb approximates 0.8 percent but can be as high as 1.6 percent in patients with subclinical hypothyroidism (238). The most convenient test for TSHAb is to show a lowering of TSH in response to a polyethylene glycol (PEG) precipitation of immunoglobulins (415-417). Alternatively, column chromatography can show TSH immunoactivity in a high molecular weight peak representing a bioinactive TSH-immunglobulin complex (415,416).

TSH Variants.TSH variants are a rare cause of interference (403). Nine different TSH beta variants have been identified to date (286). These mutant TSH molecules may have altered immunoactivity and be detected by some TSH IMA methods but not others (403). The bioactivity of these TSH mutants is variable and can range from normal to bio-inert (286,403), resulting in discordances between the TSH concentration and clinical status (403) and/or a discordant TSH/FT4 relationship (286). These TSH genetic variants are one of the causes of central congenital hypothyroidism (418,419).

**THYROID SPECIFIC AUTOANTIBODIES (TRAb, TPOAb and TgAb)**

Tests for antibodies targeting thyroid-specific antigens such as thyroid peroxidase (TPO), thyroglobulin (Tg) and TSH receptors (TSHR) are used as markers for autoimmune thyroid conditions (420-422). Over the last four decades, thyroid antibody test methodologies have evolved from semi-quantitative agglutination, complement fixation techniques and whole animal bioassays to specific ligand assays using recombinant antigens or cell culture systems transfected with the human TSH receptor (20,420). Unfortunately, the diagnostic and prognostic value of these tests has been hampered by methodologic differences as well as difficulties with assay standardization (423,424). Although most thyroid autoantibody testing is currently made on automated immunoassay platforms, methods vary in sensitivity, specificity, and the numeric values they report because of standardization issues (45,377,425). Thyroid autoantibody testing can be useful for diagnosing or monitoring treatment for several clinical conditions, although these tests should be selectively employed as adjunctive tests to other diagnostic testing procedures.

**TSH Receptor Autoantibodies (TRAb)**

The TSH receptor (TSHR) serves as a major autoantigen (19,422,426-428). Thyroid gland stimulation occurs when TSH binds to the TSHR on thyrocyte plasma membranes and activates the cAMP and phospholipase C signaling pathways (427). The TSH receptor belongs to the G protein-coupled class of transmembrane receptors. It undergoes complex posttranslational processing in which the ectodomain of the receptor is cleaved to release a subunit into the circulation (426). The TSH-like thyroid stimulator found uniquely in the serum of Graves’ disease patients was first described using a guinea pig bioassay system in 1956 (429). Later, a mouse thyroid bioassay system was used to show this serum factor displayed a prolonged stimulatory effect as compared to TSH and hence was termed to be a “long-acting thyroid stimulator” or LATS (430,431). Much later, the LATS factor was recognized not to be a TSH-like protein but an antibody capable of stimulating the TSH receptor that was the cause of Graves’ hyperthyroidism (432). TSH receptor antibodies (TRAb) have also become implicated in the pathogenesis of Graves’ ophthalmopathy (432-436). TRAbs are heterogeneous (polyclonal) and fall into two general classes both of which can be associated with autoimmune thyroid disorders – (a) thyroid stimulating autoantibodies (TSAb) that mimic the actions of TSH and cause Graves’ hyperthyroidism and (b), blocking antibodies (TBAb) that block TSH binding to its receptor and can cause hypothyroidism (19,20,420,427,432,437-440). TSH, TSAb and TBAb appear to bind to different sites on the TSH receptor ectoderm with similar affinities and often overlapping epitope specificities (441). In some cases of Graves’ hyperthyroidism, TBAb have been detected in association with TSAb (442,443) and the dominance of one over the other can change over time in response to treatment (444,445). Because both TSAb and TBAb can be present in the same patient, the relative concentrations and receptor binding characteristics of these two classes of TRAb can influence the severity of Graves’ hyperthyroidism and the response to antithyroid drug therapy or pregnancy (426,442,446-448). For completeness, it should also be mentioned that a third class of “neutral” TRAb has also been described, of which the functional significance has yet to be determined (432,438,448,449).

Two different methodologic approaches have been used to quantify TSH receptor antibodies (425,437,450,451): (a) TSH receptor antibody tests (TRAb assays) also called TSH Binding Inhibition Immunoglobulin (TBII) assays, and (b) Bioassays that use whole cells transfected with human or chimeric TSH receptors that produce a biologic response (cAMP or bioreporter gene) when TSAb or TBAb are present in a serum specimen. In recent years automated immunometric assays using recombinant human TSHR constructs have been shown to have high sensitivity for reporting positive results in Graves' disease sera (18,425). However, assay sensitivity varies among current receptor versus bioassay methods (452).

TSH RECEPTOR (TRAb)/TSH BINDING INHIBITORY IMMUNOGLOBULIN (TBII)

TRAb methods detect serum immunoglobulins that bind TSHR but do not functionally discriminate stimulating from blocking antibodies (453). TRAb methods are based on standard competitive or noncompetitive principles. The first generation of methods were liquid-based whereby immunoglobulins in the serum inhibited the binding of 125I-labeled TSH or enzyme-labeled TSH to a TSH receptor preparation (451). These methods used TSH receptors of human, guinea pig, or porcine origin (454). After 1990, a second generation of both isotopic and non-isotopic methods were developed that used and immobilized porcine or recombinant human TSH receptors (451,455). These second-generation methods were shown to have significantly more sensitivity for detecting Graves' thyroid stimulating immunoglobulins than first generation tests (425). In 2003 a third generation of non-isotopic methods were developed that were based on serum immunoglobulins competing for immobilized TSHR preparation (recombinant human or porcine TSHR) with a monoclonal antibody (M22) (420,425,451,455-457). Third generation assays have also shown a good correlation and comparable overall diagnostic sensitivity with bioassay methods (425,442,458). Current third generation TRAb tests have now been automated on several immunoassay platforms (425). However, between-method variability remains high and between assay precision is often suboptimal (CVs > 10 %) despite calibration using the same International Reference Preparation (08/204) (423,459). This fact makes it difficult to compare values using different methods and indicates that further efforts focused on additional assay improvements are needed (420,423,455).

Over the last ten years automated IMA methods have dramatically lowered the cost and increased the availability of TRAb testing (18,428,452). Automated TRAb IMAs are not functional tests and do not distinguish between stimulating and blocking TRAbs (455), however, this distinction is usually unnecessary, since it is evident from clinical evidence of hyper- or hypothyroid features. Also, both TSHR stimulating and blocking antibodies may be detected simultaneously in the same patient and cause diagnostic confusion (460). Because the sensitivity and specificity of current third generation TRAb tests is over 98 percent, TRAb testing can be useful for determining the etiology of hyperthyroidism (425,428), as an independent risk factor for Graves’ ophthalmopathy (435,436,440), and may be useful for monitoring responses to therapy (76,425). TRAb measured prior to radioiodine therapy for Graves' hyperthyroidism can also help predict the risk for exacerbating ophthalmopathy (433,436,461). There is conflicting data concerning the value of using TRAb to predict the response to antithyroid drug treatment or the risk of relapse (443,458,462,463). An important application of TRAb testing is to detect high TRAb concentrations in pregnant patients with a history of autoimmune thyroid disease or active or previously treated Graves’ hyperthyroidism, in whom transplacental passage of stimulating or blocking TRAb can cause neonatal hyper- or hypothyroidism, respectively (76,352,425,437,451,464-466). Because the expression of thyroid dysfunction may be different in the mother and infant, automated IMA methods have the advantage of being able to detect both stimulating and blocking antibodies (467). It is currently recommended that TRAb be measured in the first trimester in all pregnant patients with active Graves’ hyperthyroidism or who have received prior ablative (radioiodine or surgery) therapy for Graves’ disease in whom TRAb can remain high even after patients have been rendered hypothyroid and are being maintained on L-T4 replacement therapy (47,48). When TRAb is high in the first trimester additional TRAb testing is recommended at 18-22 and 30-34 weeks (47,48,76,420,442,468).

BIOASSAY METHODS (TSAb/TBAb)

The first TSH receptor assays used surgical human thyroid specimens, mouse, or guinea pig thyroid cells, or rat FRTL-5 cell lines to detect TSH receptor antibodies. These methods typically required pre-extraction of immunoglobulins from the serum specimen (429,437,439,469,470). Later, TRAb bioassays used cells with endogenously expressed or stably transfected human TSH receptors and unextracted serum specimens (471-473). Current TRAb bioassays are functional assays that use intact (typically CHO) cells transfected with human or chimeric TSH receptors, which when exposed to serum containing TSH receptor antibodies use cAMP or a reporter gene (luciferase) as a biological marker for any stimulating or blocking activity in a serum (425,451,463). Bioassays are more technically demanding than the more commonly used receptor assays because they use viable cells. However, these functional assays can be modified to detect TBAb that may coexist with TSAb in the same sera and make interpretation difficult (451). The most recent development is for second generation assays to use a chimeric human/rat LH TSHR to effectively eliminate the influence of blocking antibodies. This new approach has shown excellent sensitivity and specificity for diagnosing Graves' hyperthyroidism and clinical utility for monitoring the effects of anti-thyroid drug therapy (463).

**Thyroid Peroxidase Autoantibodies (TPOAb)**

TPO is a large, dimeric, membrane-associated, globular glycoprotein that is expressed on the apical surface of thyrocytes. TPO autoantibodies (TPOAb) found in sera typically have high affinities for an immunodominant region of the intact TPO molecule. When present, these autoantibodies vary in titer and IgG subclass and display complement-fixing properties (474). Studies have shown that epitope fingerprints are genetically conserved suggesting a possible functional importance (475). However, it is still unclear whether the TPOAb epitope profile correlates with the presence of, or potential for, the development of thyroid dysfunction (474-477). TPOAb antibodies were initially detected as antibodies against thyroid microsomes (antimicrosomal antibodies, AMA) using semi-quantitative complement fixation and tanned erythrocyte hemaagglutination techniques (478). Studies have identified the principal antigen in AMA tests as the thyroid peroxidase (TPO) enzyme, a 100 kD glycosylated protein present in thyroid microsomes. Manual agglutination tests have now been replaced by more specific, automated TPOAb immunoassay or immunometric assay methods that use purified or recombinant TPO (10,420,479-482). There is considerable inter-method variability of current TPOAb assays (correlation coefficients 0.65 and 0.87), despite calibration against the same International Reference Preparation (MRC 66/387) (420,479,480,482). It appears that both the methodologic principles of the test and the purity of the TPO reagent used may influence the sensitivity, specificity, and reference range of the method (420,479). The variability in sensitivity limits and the reference ranges of different methods has led to different interpretations regarding the normalcy of having a detectable TPOAb (377,420,424,482).

TPOAb CLINICAL SIGNIFICANCE

Estimates of TPOAb prevalence depend on the sensitivity and specificity of the method employed (377,424,482). In addition, ethnic and/or geographic factors (such as iodine intake) influence the TPOAb prevalence in population studies (317). For example, TPOAb prevalence is significantly higher (~11 percent) in countries like the United States and Japan where dietary iodine is sufficient, as compared with iodine deficient areas in Europe (~ 6 percent) (254,483). The prevalence of TPOAb is higher in women of all age groups and ethnicities, presumably reflecting the higher propensity for autoimmunity as compared with men (254,483). Approximately 70-80 percent of patients with Graves' disease and virtually all patients with Hashimoto’s or post-partum thyroiditis have detected TPOAb (479,484). TPOAb has, in fact, been implicated as a cytotoxic agent in the destructive thyroiditic process (477,485,486).

TPOAb prevalence is also significantly higher in various non-thyroidal autoimmune disorders in which no apparent thyroid dysfunction is evident (487). Aging is associated with an increasing prevalence of TPOAb that parallels the increasing prevalence of both subclinical and clinical hypothyroidism (254). In fact, the NHANES III survey reported that TPOAb prevalence increases with age and approaches 15-20 percent in elderly females even in the iodine-sufficient United States (254). This same study found that the odds ratio for hypothyroidism was strongly associated with the presence of TPOAb but not TgAb, suggesting that primarily TPOAb contribute to the autoimmune etiology of hypothyroidism (254). Although the presence of TgAb alone did not appear to be associated with hypothyroidism or TSH elevations, the combination of TPOAb and TgAb versus TPOAb alone may be more pathologically significant (Figure 9), however further studies would be needed to confirm this (254,307,477). It is now apparent that the presence of TPOAb in apparently euthyroid individuals (TSH within reference range) appears to be a risk factor for future development of overt hypothyroidism that subsequently becomes evident at the rate of approximately two percent per year in such populations (474,488,489). Furthermore, TPO-positivity in pregnant women is a risk factor for preterm birth (490).

**A graph of a number of women and men

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**Figure 9. Prevalence of thyroid antibodies in women (A) and men (B). Abscissa TSH values correspond to the upper and lower limits of the intervals spanning each set of bars. Asterisks denote a significant difference in prevalence from the TSH range with lowest antibody prevalence, 0.1 and 1.5 mIU/liter for women and 0.1 and 2.0 mIU/liter for men from reference 307.**

TPOAb measurement can serve as a useful prognostic indicator for future thyroid dysfunction (489,491). However, a hypoechoic ultrasound pattern can often be seen before the biochemical TPOAb abnormality appears (492). Further, some individuals with unequivocal TSH elevations, presumably resulting from autoimmune destructive disease of the thyroid, do not have TPOAb detected (307). Presumably, this paradoxical absence of TPOAb in some patients with elevated TSH likely reflects the suboptimal sensitivity and/or specificity of current TPOAb tests or a non-autoimmune cause of thyroid failure (i.e. atrophic thyroiditis) (254,307,482,493).

Although changes in autoantibody concentrations often occur with treatment, or reflect a change in disease activity, serial TPOAb measurements are not recommended for monitoring treatment for autoimmune thyroid diseases (49,479,494). This is not surprising since treatment of these disorders addresses the consequence (thyroid dysfunction) and not the cause (autoimmunity) of the disease. However, where it may have an important clinical application is to use the presence of serum TPOAb as a risk factor for developing thyroid dysfunction in patients receiving amiodarone, interferon-alpha, interleukin-2, or lithium therapies which all appear to act as triggers for initiating autoimmune thyroid dysfunction in susceptible (especially TPOAb-positive) individuals (10,110,495-500).

During pregnancy the presence of TPOAb has been linked to reproductive complications such as miscarriage, infertility, IVF failure, fetal death, pre-eclampsia, pre-term delivery, post-partum thyroiditis, and depression (47,76,484,501-508). However, whether this association represents cause or effect remains unresolved.

**Thyroglobulin Autoantibodies (TgAb)**

Thyroglobulin autoantibodies belong predominantly to the immunoglobulin G (IgG) class, are not complement fixing and are generally conformational (509). Tg autoantibodies were the first thyroid antibody to be detected in the serum of patients with autoimmune thyroid disorders using tanned red cell hemagglutination techniques (478). Subsequently, methodologies for detecting TgAb have evolved in parallel with those for TPOAb measurement, from semi-quantitative techniques to more sensitive ELISA and RIA methods and now to non-isotopic competitive or non-competitive immunoassays (45,420,482,510,511). Unfortunately, the between-method variability of TgAb assays is even greater than that of the TPOAb tests (Figure 10) (45,420,510-512). Additionally, high levels of thyroglobulin in the serum have the potential to influence TgAb measurements (511). Between-method variability is influenced by the purity and the epitope specificity of the Tg reagent, as well as the patient-specific epitope specificity of the TgAb secreted (513). As with TPOAb methods, TgAb tests have highly variable sensitivity limits and manufacturer-recommended cut-off values for "positivity", despite the use of the same International Reference Preparation (MRC 65/93) (Figure 10) (45,510-512,514). Whereas the FS limit is the recommended cutoff to define TgAb-positivity for DTC monitoring, the FS is typically much lower than the manufacturer-recommended cut-off for “positivity” (Figure 10) (10,45). This is because manufacturer-recommended cutoffs (MCO) are set for diagnosing thyroid autoimmunity and are too high to detect the low TgAb levels that can interfere with Tg measurements (515,516). Although there are reports that low levels of TgAb may be present in normal euthyroid individuals, it is unclear whether this represents assay noise due to matrix effects or "natural" antibodies (21). Further complicating this question are studies suggesting that there may be qualitative differences in TgAb epitope specificities expressed by normal individuals versus patients with either differentiated thyroid cancers (DTC) or autoimmune thyroid disorders (517). These differences in test sensitivity and specificity negatively impact the reliability of determining the TgAb status (positive versus negative) of specimens prior to Tg testing of DTC patients.

**A graph with lines and dots

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**Figure 10. TgAb Measurements Made by Different Methods. Figure shows the relative TgAb concentrations reported for 143 DTC patient sera with evidence of TgAb interference with serum Tg measurements. Each serum was measured by four different methods each with a different manufacturer-recommended cutoff value for “TgAb positivity” (open bars) and with different experimentally determined (10) functional sensitivity limits (closed bars). From reference 45.**

CLINICAL UTILITY OF TgAb TESTING

Tg autoantibodies (TgAb) are encountered in autoimmune thyroid conditions, usually in association with TPOAb (254,489,490). However, the NHANES III survey found that only three percent of subjects with no risk factors for thyroid disease had serum TgAb present without detectable TPOAb (Figure 9) (254,307). Furthermore, there was no association between the isolated presence of TgAb and TSH abnormalities in these subjects (254,307). This suggests that it is unnecessary to measure both TPOAb and TgAb for a routine evaluation for thyroid autoimmunity (307,420,489). However, when autoimmune thyroid disease is present, there is some evidence that assessing the combination of TPOAb and TgAb has greater diagnostic utility than the TPOAb measurement alone (Figure 9) (307,489,490,518). In pregnant women, both TPOAb and TgAb-positivity have been shown to be risk factors for preterm birth (490).

The role of TgAb for monitoring patients with DTC is two-fold: 1) to authenticate that a Tg measurement is not compromised by TgAb interference, and 2) as an independent surrogate tumor-marker (519,520). Immunoassay methods detect TgAb in approximately 25 percent of patients presenting with DTC, double the TgAb prevalence of the general population (45,254,521,522). In patients with thyroid nodules the presence of TgAb is a risk factor for lymph node metastases (520,523,524) and may be a useful marker for papillary thyroid cancer in cases of indeterminate cytology (523,525,526). The prevalence of TgAb is typically higher in patients with papillary versus follicular tumors (22,510,519,527-529). After TgAb-positive patients are rendered disease-free by surgery, TgAb concentrations typically progressively decline during the first few post-operative years and typically become undetectable after a median of three years of follow-up (22,530,531). In contrast, a rise in, or de novo appearance of, TgAb is often the first indication of tumor recurrence (14,22,531,532). In patients with persistent disease, serially determined TgAb concentrations may serve as an independent surrogate tumor marker for changes in tumor mass (Figure 11) (14,17,22,520,530,531,533-536). However, the use of the TgAb trend as a surrogate tumor marker necessitates that TgAb be measured by the same method in preferably the same laboratory, because of the large differences in the sensitivities and cut off values for “positivity” between different methods (Figure 10) (9,45,511,512,514,519,521).

**THYROGLOBULIN (Tg)**

Thyroglobulin plays a central role in a variety of pathophysiologic thyroid conditions, including acting as an autoantigen for thyroid autoimmunity (421,509,537). Serum Tg levels can serve as a marker for iodine status of a population (538-540) and genetic defects in Tg biosynthesis causing dyshormonogenesis can result in congenital hypothyroidism (10,541,542). Because Tg has a thyroid-tissue specific origin, a serum Tg measurement can be used to investigate the etiology of congenital hypothyroidism (athyreosis versus dyshormonogenesis) (543,544). Likewise, a paradoxically low serum Tg can be used to distinguish factitious hyperthyroidism from the high Tg expected with endogenous hyperthyroidism (14,545-547). However, the primary clinical use of Tg measurement is as a post-operative tumor-marker test used to monitor patients with follicular-derived (differentiated) thyroid cancer (DTC) (14,17,57,271,274,548-550).

Most Tg testing is currently by rapid, automated immunometric assays (IMA), most of which now have second generation functional sensitivity (FS≤ 0.1 µg/L) - a sensitivity level that obviates the need for recombinant human TSH (rhTSH) stimulation (57,274,551-554). TgAb interference, causes falsely low/undetectable serum Tg IMA tests and this is the major limitation of using IMA methodology since this direction of interference can mask disease (14,17,23,512,521,555,556). Currently, most laboratories first establish the TgAb status of the specimen (negative or positive) and restrict Tg-IMA testing to TgAb-negative sera, while reflexing TgAb-positive specimens to other methodologies believed less prone to TgAb interference from TgAb - RIA (14,274,512,521) or LC-MS/MS (14,24,43,555,557,558).

**Technical Limitations of Tg Methods**

Thyroglobulin measurement remains technically challenging. Five methodologic problems impair the clinical utility of this test: (a) suboptimal functional sensitivity; (b) between-method biases; (c) "hook" problems (some IMA methods) and interferences caused by (e) Heterophile antibodies (HAb) and/or (f) Tg autoantibodies (TgAb).

Tg ASSAY SENSITIVITY

As with TSH, assay functional sensitivity (FS) represents the lowest analyte concentration that can be measured in human serum with 20 percent CV, calculated from runs made over a clinically relevant timespan (6 -12 months for Tg) and using at least two different lots of reagents (10). These stipulations are necessary because assay precision erodes over time, especially during the long clinical interval (6-12 month) typically used when monitoring Tg as a tumor marker for DTC, during which time assay reagents and conditions can change (9,71,559). The use of FS as the assay sensitivity limit is more relevant than either a limit of detection (LOD) or limit of quantitation (LOQ) calculation - parameters that do not stipulate using a clinically relevant time span for assessing precision (10,560). The FS protocol (10) also stipulates that precision be determined in human sera rather than a commercial QC preparation, because instruments and methods are matrix-sensitive (72,560). Tg IMA methods should have precision determined in TgAb-negative human sera (560) and TgAb-positive human serum pools should be used to determine the precision of Tg methodologies used to measure TgAb-positive specimens - most commonly RIA or LC-MS/MS.

In accord with TSH a generational approach to Tg assay nomenclature has been adopted (1,17). Early Tg RIAs (5) had FS approximating 1 μg/L and were designated "first generation" assays. Currently, some RIAs, IMAs and LC-MS/MS methods still only have first generation functional sensitivity (FS = 0.5-1.0 µg/L) (17,271,274,512,561,562). However, in recent years 2nd generation assays (FS 0.05-0.10 µg/L) have become the standard of care (57,271,274,549,550,561,563). These second-generation tests obviate the need for recombinant human TSH (rhTSH) stimulation, because basal Tg correlates with rhTSH-stimulated Tg (17,57,561). However, the use of a second-generation assay does not eliminate the need for periodic ultrasound examinations, because many histologically confirmed lymph nodes metastases may not secrete enough Tg to be detected (14,563,564).

SERUM Tg REFERENCE RANGES

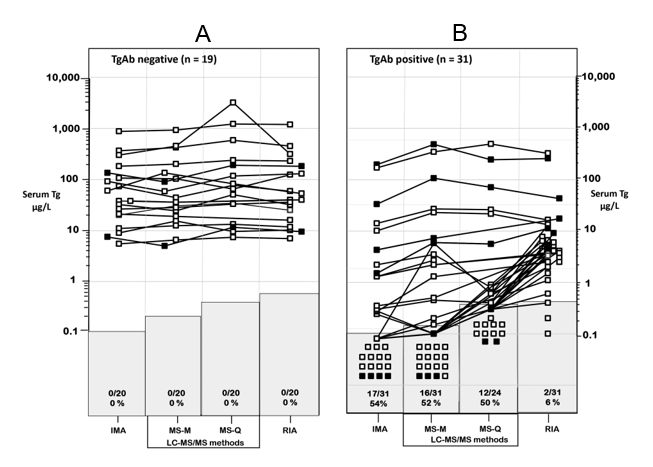
The adult serum Tg reference range approximates 2-40 µg/L (10,565). Newborn infants have a higher serum Tg that falls to the adult range after two years of age (566). However, most Tg testing is made following surgery (thyroidectomy or lobectomy) for DTC, the Tg reference range is only relevant in the preoperative period (567-570). Different Tg methods may report two-fold differences in numeric values for the same serum specimen (14,274,571). This between-method variability reflects differences in assay standardization as well as the assay specificity for detecting different Tg isoforms in the serum (512,572-576). When evaluating a thyroidectomized patient, the assay reference range should be adjusted for thyroid mass (thyroidectomy versus lobectomy) as well as the TSH status of the patient (10,570).

BETWEEN METHOD Tg BIASES

Thyroglobulin in frozen sera is remarkably stable. The between-run precision for repetitive serum Tg measurements made over 6-12 months (the typical DTC monitoring interval), approximates 10 percent. In contrast, between-method variability can exceed 30 percent (14,274,516,571) despite CRM-457 standardization (584,585). In fact, in some cases different methods can report more than a two-fold difference in Tg for the same serum specimen (14,274,571). This between-method variability significantly exceeds the biologic variability of Tg in normal euthyroid subjects (~16 %) (559,577). This between-method variability reflects matrix differences between methods as well as specificity differences for detecting different Tg isoforms in the serum (45,512,572-574,576).

Some Tg should be detected in all TgAb-negative normal euthyroid subjects when using a second-generation IMA method standardized against the International Reference Preparation CRM-457. Although the intra-individual serum Tg variability is relatively narrow (CV ~15 %) (577), the Tg population reference range is quite broad (2-40 µg/L) (512,565,575,578)*.* It follows that 1 gram of normal thyroid tissue gives rise to ~1.0 µg/L Tg in the circulation, unless TSH is elevated (10,579). Following a lobectomy, euthyroid patients should be evaluated using a mass-adjusted reference range (1.5 - 20 µg/L). The range should be lowered a further 50 percent (0.75 - 10 µg/L) during TSH-suppression (10,570). After thyroidectomy, the typical 1-to-2-gram thyroid remnant (580) would be expected to produce a serum Tg below 2 µg/L (at low-normal TSH) (581,582). By this same reasoning, truly athyreotic patients would be expected to have no Tg detected irrespective of their TSH status (10). However, a rising Tg trend after lobectomy in the absence of recurrent disease is not unexpected due to a compensatory increase in normal remnant tissue (583).

Since TgAb interferes with different methods to differing extents (14,45,586), a false negative TgAb test could also lead to significant between-method differences with the potential to disrupt serial Tg monitoring and negatively impact clinical management (516). Between method variability is the reason current guidelines stress the necessity of using the same Tg method (and preferably the same laboratory) for monitoring Tg trends and the need to re-baseline the Tg level if a change in method becomes necessary (57,587).

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**Figure 11. Between-Method Serum Tg Variability in DTC Patients +/- TgAb. Serum Tg measured by different methodologies in patients with distant metastatic DTC who were either TgAb-negative (panel A) or TgAb-positive (panel B). Three Tg methodologies were compared: IMA, LC-MS/MS (MS-M = Mayo; MS-Q = Quest), and RIA. Tg measurements below the assay FS limit are indicated in the shaded areas and expressed as a percentage relative to the total number of tests performed with that method. Patients who died of DTC-related complications are shown by solid symbols. From reference 14.**

HIGH-DOSE HOOK EFFECT

Tumor marker tests employing IMA methodology can be prone to so-called "high-dose hook effects", whereby very high antigen concentrations can overwhelm the binding capacity of the monoclonal antibody reagents leading to a falsely normal/low value (9,588-591). Manufacturers have largely overcome hook problems by adopting a two-step procedure, whereby a wash step is used to remove unbound antigen after the first incubation of specimen with the capture monoclonal antibody before introducing the labeled monoclonal during which the signal binds the captured antigen during a second incubation (580). When using IMA methodology, it is the laboratory’s responsibility to determine whether a hook effect is likely to generate falsely normal or low values.

There are two approaches for detecting and overcoming hook effects with Tg IMA methods when an unexpectedly low serum Tg value is encountered for a patient with known metastatic disease: 1) Measure the Tg in the specimen at two dilutions. For example, a hook effect is likely present when the value of the test serum measured at a 1/5 or 1/10 dilution is higher than that obtained with the undiluted specimen. 2) Assess the recovery of added Tg antigen. If a hook effect is present, the Tg result will be inappropriately low.

INTERFERENCES WITH Tg MEASUREMENT

*Heterophile Antibody (HAb) Interferences*

HAb interferes with Tg IMAs, but not RIA or Tg-LC-MS/MS methodologies (43, 214, 221, 223, 228, 592-595). HAb interferences are thought to reflect the binding of HAb to the monoclonal antibody IMA reagents (murine origin). RIA methods are not prone to HAb interference because their polyclonal antibody reagents (rabbit origin) do not bind human IgG. In most cases HAb interferences are characterized by a false-positive Tg-IMA result (223,228,592), although falsely low Tg IMA results have also been reported (214,596). Recent reports find that Tg LC-MS/MS methodology appears free from HAb interferences (43,595). A presumptive test for HAb interference is a lowering of the analyte value in the presence of a blocking agent (43,201,597). The laboratory cannot proactively test for HAb because specimens are typically sent to the laboratory without clinical information. Physicians should request the laboratory test for HAb interference when an apparently disease-free patient has an unexpectedly high Tg result.

*Tg Autoantibody (TgAb) Interference*

TgAb interference with Tg measurement remains the major limitation for using Tg as a DTC tumor marker. TgAb has the potential to interfere with Tg measured by each of the current methodologies: IMA, RIA and LC-MS/MS. The prevalence of TgAb in DTC patients approximates 25 percent - twice that of the general population (9,254,522). There appears to be no threshold TgAb concentration that precludes TgAb interference (45,57,386,510,512,521). TgAb is thought to interfere by both in vitro (epitope masking) (45,512,521,598) and/or in vivo mechanisms such as enhanced TgAb-mediated Tg clearance (599-603). High TgAb concentrations do not necessarily interfere, whereas low TgAb may profoundly interfere (9,22,45,521,555,598,604,605). Unfortunately, the recovery approach appears to be unreliable for detecting TgAb interference (512,521,598).

TgAb Interference - In-Vivo Mechanisms. Studies over past decades have suggested that the presence of TgAb enhances Tg metabolic clearance. In 1967 Weigle showed enhanced clearance of endogenously I25-labeled Tg in rabbits, after inducing TgAb by immunizing the animals with an immunogenic Tg preparation (599,603). In humans, Tg and TgAb acute responses to sub-total thyroidectomy have also suggested that TgAb may increase Tg metabolic clearance (603,606). Changes (a rise or fall) in TgAb versus Tg-RIA concentrations have typically been concordant and appropriate for clinical status, whereas the direction of change in Tg-IMA is typically discordant with Tg-RIA and clinical status (45,274,521,556). In general, the change in TgAb concentrations tends to be steeper than for Tg-RIA (521), as would be consistent with TgAb-mediated Tg clearance, perhaps because some TgAbs act as "sweeper" antibodies that facilitate clearance of antigen (602,603,607).

TgAb Interference - In-Vitro Mechanisms. TgAb interferes with Tg measurement in a qualitative, quantitative, and method-dependent manner (22,45,521,608,609). The potential for in vitro interference is multifactorial and depends not only on the assay methodology (IMA, RIA or LC-MS/MS) (39), but also the concentration and epitope specificity of the TgAb secreted by the patient (22,512,610). RIA methodology appears to quantify total Tg (free Tg + TgAb-bound Tg) whereas IMA primarily detects only the free Tg moiety, i.e. Tg molecules with epitopes not masked by TgAb complexing. Steric masking of Tg epitopes is the reason why TgAb interference with IMA methodology is always unidirectional (underestimation) and why a low Tg-IMA/Tg-RIA ratio has been used to indicate TgAb interference (45,521,555,611,612). The recently developed Tg-LC-MS/MS methodology uses trypsin digestion of Tg-TgAb complexes to liberate a proteotypic Tg peptide. This conceptually attractive approach was primarily developed to overcome TgAb interference with IMA methods thereby eliminating falsely low/undetectable Tg-IMA results that can mask disease. However, recent studies report that a high percentage (>40 %) of TgAb-positive DTC patients with structural disease have paradoxically undetectable Tg-LC-MS/MS tests (14,24,43,555,557,558). More studies are needed to determine why LC-MS/MS fails to detect Tg in TgAb-positive DTC patients with disease. Possibilities to investigate include tumor Tg polymorphisms that prevent the production of the Tg-specific tryptic peptide (38), suboptimal trypsinization of Tg-TgAb complexes, or Tg levels that are truly below detection because of increased clearance of Tg-TgAb complexes by the hepatic asialoglycoprotein receptor (599-602).

TgAb interference with Tg-RIA Methodology. Radioimmunoassay (RIA) was the earliest methodology used to measure Tg (5). Thyroglobulin antigen (from serum or added 125I-Tg tracer) competes for a low concentration of polyclonal (PAb) (usually rabbit) Tg antibody. After incubation, the Tg-PAb complex is precipitated by an anti-rabbit second antibody and the serum Tg concentration is quantified from the 125I-Tg in the precipitate. The first Tg-RIAs developed in the 1970s were insensitive (~2 µg/L) (5,613). Over subsequent decades some Tg-RIAs have achieved first generation functional sensitivity (FS = 0.5 µg/L) by using a long (48-hour) pre-incubation before adding a high specific activity 125I-Tg tracer (614,615). The use of a high affinity polyclonal antibody (616) coupled with a species-specific second antibody appears to minimize TgAb interference. Resistance to TgAb interference is evidenced by appropriately normal Tg-RIA values for TgAb-positive euthyroid controls (512) and detectable Tg-RIA in TgAb-positive DTC patients with structural disease (14,555). The clinical performance of this Tg-RIA contrasts with IMA methods that fail to detect Tg in some TgAb-positive normal euthyroid subjects (512), some TgAb-positive Graves' hyperthyroid patients (14,617), or TgAb-positive patients with structural disease (14,512,618). It should be noted that the propensity of TgAb to interfere with Tg-RIA determinations and cause under- or overestimation (546,608) depends on the patient-specific interactions between Tg and TgAb in the specimen and the RIA reagents (609).

TgAb interference with Tg-IMA Methodology. Most Tg testing is currently made by automated IMAs, whereby antigen is captured by two monoclonal antibodies (MAb) that target different epitopes on the Tg protein (619). TgAb interferes with IMA methodology by steric inhibition – i.e. by blocking the epitope(s) necessary for Tg to bind the MAb(s), so that the MAb-Tg-MAb reaction cannot take place and Tg is reported as falsely low or undetectable. This mechanism of epitope masking is supported by timed recovery studies. Clinically, TgAb interference is evident from the paradoxically low/undetectable Tg-IMA seen for TgAb-positive normal controls (512), patients with Graves' hyperthyroidism (14,617), and DTC patients with active disease (Figures 10 and 11) (14,43,555). High Tg concentrations can overwhelm the TgAb binding capacity rendering Tg-IMA concentrations detectable and lessening the degree of interference (45,555). It follows that as Tg concentrations rise, more Tg is free, the influence of TgAb lessens and the discordance between Tg-IMA and Tg-RIA lessens (Figure 11B) (45,555). Although some IMA methods have claimed to overcome TgAb interference by using monoclonal antibodies directed against specific epitopes not involved in thyroid autoimmunity (580), this approach has not overcome TgAb interferences in clinical practice, possibly because less restricted TgAb epitopes are associated with thyroid carcinomas than with autoimmune thyroid conditions (510,517,620).

TgAb Interference with Tg LC-MS/MS.Liquid Chromatography, Tandem Mass Spectrometry (LC-MS/MS) is the newest methodology used to measure Tg. This methodology measures Tg by trypsinizing the Tg-TgAb complexes in the serum to generate a Tg-specific peptide(s) that can be measured by LC-MS/MS (37-39,41,580,621). Most Tg LC-MS/MS methods only have first generation functional sensitivity (FS ~ 0.5 µg/L) (24,39,40) although more sensitive methods are being developed (621). Tg-LC-MS/MS methodology has been shown free from HAb interferences (43,595) and has been promoted as being free from TgAb interference (24,39,40). However, these claims are not supported by clinical studies in which paradoxically undetectable LC-MS/MS Tg tests are seen for many TgAb-positive DTC patients with structural disease (14,24,43,555,557,558). The higher the TgAb, the more likely that no Tg would be detected by LC-MS/MS in patients with disease (558). It currently appears that when TgAb is present LC-MS/MS methodology offers no diagnostic advantage over IMA.

**Clinical Utility of TGAb Used as a Surrogate DTC Tumor Marker**

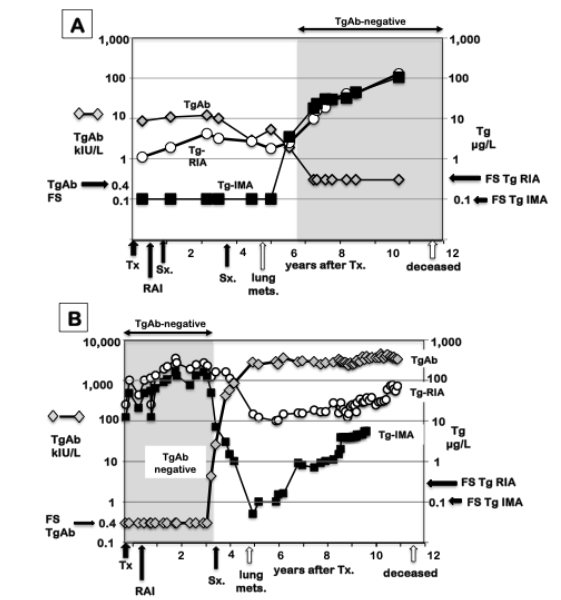
The serum TgAb trend has become recognized as a postoperative surrogate DTC tumor-marker. A declining TgAb trend is a good prognostic sign, whereas a stable or rising TgAb may indicate persistent/recurrent disease (23,57,509,519,521,530,531,533,536,612,622-624). The TgAb half-life in blood approximates 10 weeks (522). Following successful surgery (± radioiodine treatment), TgAb typically falls more than 50 percent in first post-operative year and often decreases to <10 percent after 3-4 years eventually becoming undetectable with reduced stimulation of the immune system by lower Tg antigen levels (45,57,274,522,524,530,531,625). The time needed for a TgAb-positive patient to become TgAb-negative in response to successful treatment is inversely related the initial TgAb concentration, perhaps representing the long-lived memory of plasma cells (274,626). Patients exhibiting a TgAb decline of more than 50 percent by the end of the first post-operative year have been shown to have a low recurrence risk (515,531,534,612,627). However, a significant percentage (~5 %) of TgAb-negative patients may develop transient de novo TgAb-positivity in the early post-operative period, presumably in response to Tg antigen released by surgical trauma (532,628,629). A rise in TgAb can also be seen soon after fine needle aspiration (FNA) biopsy (630-632) or more chronically (months) in response to radiolytic damage following radioiodine treatment (22,633,634). However, the 5 percent of DTC patients that display a sustained de novo TgAb appearance are likely to have recurrent disease (Figure 11B) (532,635). These TgAb-negative to TgAb-positive conversions are the reason why guidelines mandate that TgAb be measured with every Tg test (23,57,635). Patients with persistent disease may exhibit only a marginal TgAb decline or have stable, rising or a de novo TgAb appearance (511,521,531-533,612,622). If serum Tg remains detectable after TgAb becomes negative (~3 % of cases), the risk for disease remains (Figure 11A). Since TgAb tests differ in sensitivity and specificity (Figure 10) (23,45,513,514,636) it is essential to measure the serum TgAb trend by the same method, preferably in the same laboratory (23,45,57,482,511,512,514,535,636).

**Serum Tg Monitoring of Patients with DTC**

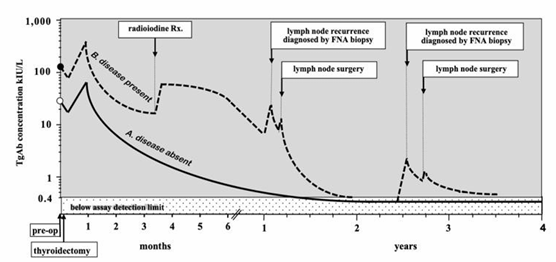
Over the past decade, the incidence of DTC has substantially risen with the detection of small thyroid nodules and micropapillary cancers by ultrasound and other anatomic imaging modalities (57,637-640). Although most DTC patients are rendered disease-free by their initial surgery, approximately 15 percent of patients experience recurrences and approximately 5 percent die from disease-related complications (580,641-644). A risk-stratified approach to diagnosis and treatment is now recommended by current guidelines (57).

In most cases, persistent/recurrent disease is detected within the first five post-operative years, although recurrences can occur decades after initial surgery necessitating life-long monitoring for recurrence (642,643). Since most patients have a low pre-test probability for disease, protocols for follow-up need a high negative predictive value (NPV) to eliminate unnecessary testing, as well as a high positive predictive value (PPV) for identifying patients with persistent/recurrent disease. Because Tg testing is generally recognized as being more sensitive for detecting disease than diagnostic 131I whole body scanning (645), biochemical testing (serum Tg. + TgAb) is used in conjunction with periodic ultrasound (57,645). The persistent technical limitations of Tg and TgAb measurements necessitate close physician-laboratory cooperation.

The majority (~75 %) of DTC patients have no Tg antibodies detected (521). In the absence of TgAb, four factors influence the interpretation of serum Tg concentrations: (1) the mass of thyroid tissue present (normal tissue + tumor); (2) The intrinsic ability of the tumor to secrete Tg; (3) the presence of any inflammation of, or injury to, thyroid tissue following fine needle aspiration biopsy, surgery, RAI therapy, or thyroiditis; and (4) the degree of TSH receptor stimulation by TSH, hCG, or TSAb (10). The presence of TgAb necessitates a shift in focus from monitoring serum Tg as the primary tumor-marker, to monitoring the serum TgAb trend as a surrogate tumor-marker (519).

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**Figure 12. TgAb Effects on Serial Tg IMA and Tg RIA Measurements. Serial TgAb, Tg-RIA and Tg-IMA measurements made in two DTC patients who underwent a change in TgAb status (panel A, positive to negative) or (panel B negative to positive) before death from structural DTC. These cases illustrate why a Tg measurement cannot be interpreted without knowing the TgAb status of the patient (57). The de novo appearance of TgAb (Patient B) either reflects a change in tumor-derived Tg heterogeneity (secretion of a more immunogenic Tg molecule), or recognition of tumor-derived Tg by the immune system. In contrast, TgAb can become undetectable despite the exacerbation of disease (Patient A).**

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**Figure 13. TgAb Trends in Response to Treatment. Typical trends in TgAb following thyroidectomy in patients rendered disease-free by thyroidectomy (pattern A) versus patents with persistent/recurrent disease (pattern B). TgAb levels may rise or become detectable de novo in response to an increase in Tg antigen following surgical injury, lymph node recurrence(s), lymph node resection(s), FNA biopsy of metastatic lymph nodes or radioiodine therapy.**

PRE-OPERATIVE Tg MEASUREMENT

An elevated Tg is merely a non-specific indicator of thyroid pathology and cannot be used to diagnose malignancy (568). However, studies have reported that a Tg elevation detected decades before a DTC diagnosis, is a risk factor for thyroid malignancy (567-569,646-648). This suggests that most thyroid cancers secrete Tg protein to an equal or greater degree than normal thyroid tissue, underscoring the importance of using Tg as a DTC tumor marker. Approximately 50 percent of DTC patients have an elevated preoperative serum Tg the highest being seen in follicular > oncocytic (formerly “Hurthle cell cancer”) > papillary thyroid carcinoma (567-569). Up to one-third of tumors may be poor Tg secretors relative to tumor mass, especially BRAF-positive tumors that are associated with reduced expression of Tg protein (649). Although current guidelines do not recommend routine pre-operative serum Tg measurement (57,549,650), some believe that a preoperative serum Tg (drawn before or more than two weeks after FNA) can provide information regarding the tumor’s intrinsic ability to secrete Tg and thus aid with the interpretation of postoperative Tg changes (567-569,648,650). For example, knowing that a tumor is an inefficient Tg secretor could prompt a physician to focus more on anatomic imaging and less on postoperative Tg monitoring (649,651,652).

POST-OPERATIVE Tg MEASUREMENT

Because TSH exerts such a strong influence on serum Tg concentrations it is important to promptly initiate thyroid hormone therapy after surgery to establish a stable post-operative Tg baseline to begin biochemical monitoring. When surgery is followed by RAI treatment it may take time (months) to establish a stable Tg baseline because the Tg rises in response to TSH-stimulation and may be augmented by Tg release from radiolytic damage of the thyroid remnant. Short-term rhTSH stimulation is expected to produce an approximate 10-fold serum Tg elevation (561), whereas chronic endogenous TSH stimulation following thyroid hormone withdrawal results in an approximate 20-fold serum Tg rise (653). Serum Tg measurements performed as early as 6 to 8 weeks after thyroidectomy have been shown to have prognostic value - the higher the serum Tg the greater the risk of persistent/recurrent disease (526, 654, 655). Since the half-life of Tg in the circulation approximates 3 days (656), the acute Tg release resulting from the surgical trauma and healing of surgical margins should largely resolve within the first six months, provided that post-operative thyroid hormone therapy prevents TSH from rising. Patients who receive RAI for remnant ablation may exhibit a slow Tg decline over subsequent years, presumably reflecting the long-term radiolytic destruction of remnant tissue (657,658).

The Tg secretion expected from the ~1 gram of normal remnant tissue left after thyroidectomy (580) is expected to produce a serum Tg concentration ~1.0 µg/L, provided TSH is not elevated (10). A recent study found that in the first six months following thyroidectomy (without RAI treatment) disease-free PTC patients had a serum Tg nadir < 0.5 µg/L when TSH was maintained below 0.5 mIU/L (274,581,582). This is consistent with earlier studies using receiver operator curve (ROC) analysis that found a 6-week serum Tg of <1.0 µg/L, when measured during TSH suppression, had a 98 percent negative predictive value (NPV) for disease (although positive predictive value (PPV) was only 43 percent) (654).

LONG-TERM Tg MONITORING (WITHOUT TSH STIMULATION)

The higher the post-operative serum Tg measured without TSH stimulation, the greater the risk for persistent/recurrent disease (654). If a stable TSH is maintained (≤0.5 mIU/L) (274,582) changes in serum Tg will reflect changes in tumor mass. Under these conditions a rising Tg would be suspicious for tumor recurrence whereas declining Tg levels suggests the absence or regression of disease. When using a sensitive Tg-IMA method, the trend in serum Tg (measured without TSH stimulation) is a more reliable indicator of disease status than using a fixed Tg cutoff value for disease (57,274,548,562,587,654,659-661). It is the degree of Tg elevation, not merely a "detectable" Tg that is the risk factor for disease, since Tg “detectability” varies according to the method used (563,575,578,582). As with other tumor-markers, such as calcitonin, the Tg doubling time (measured without TSH stimulation) is a useful prognostic marker that has an inverse relationship to mortality (252,581,660,662-666). However, between-method variability necessitates that the serum Tg trend be established using the same method, and preferably the same laboratory (Figure 11) (57,587). One approach used to mitigate between-run imprecision and improve the reliability of assessing the Tg trend has been to measure the current specimen concurrently (in the same run) with the patient’s previous archived specimen, thereby eliminating run-to-run variability and increasing the confidence to detect small Tg changes (9,10,587).

SERUM Tg RESPONSES TO TSH STIMULATION

The degree of tumor differentiation determines the presence and density of TSH receptors that in large part determines the magnitude of the serum Tg response to TSH stimulation (667,668). The serum Tg rise in response to endogenous TSH (thyroid hormone withdrawal) is twice that seen with short-term rhTSH stimulation (~20-fold versus ~10-fold, respectively) (386,653,669). Recombinant human TSH (rhTSH) administration was adopted as a standardized approach for stimulating serum Tg into the measurable range of the insensitive first-generation tests (386,549,561,653,669,670). A rhTSH-stimulated serum Tg cut-off of ≥2.0 µg/L, measured 72 hours after the second dose of rhTSH, was found to be a risk factor for disease (653,669). A "positive" rhTSH response had a higher NPV (>95 percent) than the basal Tg measured by an insensitive first-generation test, (553,564,654,671). However, a negative rhTSH test did not guarantee the absence of tumor (653,671). Furthermore, the reliability of adopting a fixed numeric rhTSH-Tg cut-off value for a positive response is problematic, given that different methods can report different numeric Tg values for the same specimen (Figure 11) (14, 512, 575). Other variables include differences in the dose of rhTSH delivered relative to absorption from the injection site as well as the surface area and age of the patient (672,673). One critical variable is the TSH sensitivity of tumor tissues, with poorly differentiated tumors having blunted TSH-mediated Tg responses (649,651,652,668). When using a sensitivesecond-generation Tg-IMA, an undetectable basal Tg (<0.10 µg/L) had a comparable NPV to rhTSH stimulation and was rarely associated with a "positive" rhTSH-stimulated response (>2.0 µg/L) (561, 563, 575, 674, 675). This would be expected given the strong relationship between basal Tg and rhTSH-stimulated Tg values (553,561,578,676). Once sensitive Tg-IMA methods had become the standard of care, it became apparent that rhTSH-stimulation provided no additional information over and above a basal Tg measured by second generation assay (57, 553, 561, 563, 575, 578, 674-676).

One potential use of rhTSH-stimulated Tg would be to test for HAb interferences. Specifically, when a Tg-IMA value appears clinically inappropriate (usually high), an absent rhTSH-stimulated Tg response would suggest interference that could be confirmed by a blocker tube test (561). An alternative reason for an absent/blunted rhTSH-stimulated response would be the presence of TgAb (578), with TgAb-enhanced clearance of Tg-TgAb complexes (599,602,606).

Tg MEASUREMENT IN FNA NEEDLE WASHOUTS (FNA-Tg)

Because the Tg protein is tissue-specific, the detection of Tg in non-thyroidal tissues or fluids (such as pleural fluid) indicates the presence of metastatic thyroid cancer (677). Struma ovarii is the only (rare) condition in which the Tg in the circulation does not originate from the thyroid (678,679). Cystic thyroid nodules are commonly encountered in clinical practice, the large majority arising from follicular epithelium and the minority from parathyroid epithelium. A high concentration of Tg or parathyroid hormone (PTH) measured in the cyst fluid provides a reliable indicator of the tissue origin of the cyst (thyroid versus parathyroid, respectively), information critical for surgical decision-making (677,680). Lymph node metastases are found in up to 50 percent of patients with papillary cancers but only 20 percent of follicular cancers (681,682). High-resolution ultrasound has now become an important component of postoperative surveillance for recurrence (57,386,669). Although ultrasound characteristics are helpful for distinguishing benign reactive lymph nodes from those suspicious for malignancy, the finding of Tg in the needle washout of a lymph node biopsy has higher diagnostic accuracy than the ultrasound appearance (632,683-691). An FNA needle washout is now widely accepted as a useful adjunctive test that improves the diagnostic sensitivity of a cytological evaluation of a suspicious lymph node or thyroid mass, even in the presence of TgAb (683-687). The current protocol for obtaining FNA-Tg samples recommends rinsing the biopsy needle in 1.0 mL of saline and sending this specimen to the laboratory for Tg analysis. In thyroidectomized patients a common cutoff value for a "positive" FNA-Tg result is 1.0 µg/L, however this cutoff can vary by method and institution (685,686,690-692). For investigations of suspicious lymph nodes in patients with an intact thyroid, a higher FNA-Tg cutoff value (~35-40 µg/L) is recommended (683). There is still controversy whether TgAb interferes with FNA-Tg analyses (528,684). It should be noted that when the serum TgAb concentration is high there can be TgAb contamination of the FNA wash fluid. Although a ~40-fold dilution of TgAb in the wash fluid would be expected, this could still be insufficient to lower TgAb below detection and eliminate the possibility of TgAb interference with the FNA-Tg IMA test producing a falsely low result. The FNA needle wash-out procedure can also be used to detect calcitonin in neck masses of patients with primary and metastatic medullary thyroid cancer (680,693,694). In addition, FNA-PTH determinations may be useful for identifying lymph nodes arising from parathyroid tissue (680).

**THYROID SPECIFIC mRNAs USED AS THYROID TUMOR MARKERS**

Reverse transcription-polymerase chain reaction (RT-PCR) has been used to detect thyroid-specific mRNAs (Tg, TSHR, TPO and NIS) in the peripheral blood of patients with DTC (579,695-697). Initial studies suggested that circulating Tg mRNA might be employed as a useful tumor marker for thyroid cancer, especially in TgAb-positive patients in whom Tg measurements were subject to TgAb interference (695,698,699). More recently, this approach has been applied to the detection of NIS, TPO, and TSH receptor (TSHR) mRNAs (699,700). Although some studies have suggested that thyroid specific mRNA measurements could be useful for cancer diagnosis and detecting recurrent disease, most studies have concluded that they offer no advantages over sensitive serum Tg measurements (579,699,701). Further, the recent report of false positive Tg mRNA results in patients with congenital athyreosis (702) suggests that Tg mRNA can arise as an assay artifact originating from non-thyroid tissues, or illegitimate transcription (703,704). Conversely, false negative Tg mRNA results have also been observed in patients with documented metastatic disease (705,706). Although Tg, TSHR, NIS and TPO are generally considered “thyroid specific” proteins, mRNAs for these antigens have been detected in non-thyroidal tissues such as lymphocytes, leukocytes, kidney, hepatocytes, brown fat and skin (427,707,708)). Additional sources of variability in mRNA analyses relate to the use of primers that detect splice variants, sample-handling techniques that introduce variability, and difficulties in quantifying the mRNA detected (701,705). The general consensus is that thyroid specific mRNA measurements lack the optimal specificity and practicality to be useful tumor markers (579,699,701). MicroRNA (miRNA) has recently been proposed as an alternate candidate biomarker when Tg measurement is unreliable (709). The growing number of reports of functional TSH receptors and Tg mRNA present in non-thyroidal tissues further suggests that these mRNA measurements will have limited clinical utility in the management of DTC in the future (427,707,708). Further studies in thyroid cancer genomics may yield additional DTC tumor markers with optimal sensitivity and specificity to monitor DTC (710).

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