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THYROID FUNCTION TESTS: ASSAY OF THYROID HORMONES AND RELATED SUBSTANCES

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ABSTRACT

This chapter reviews how improvements in sensitivity and specificity of thyroid function tests [total and free thyroid hormones, TSH, thyroid autoantibodies (TRAb, TPOAb and TgAb) and thyroglobulin (Tg)] have dramatically improved clinical strategies for detecting and treating thyroid disorders. The review discusses the strengths and limitations of the different methodologies currently used (RIA, IMA and LC-MS/MS) and their propensity for analyte-specific interferences caused by heterogeneity (TSH, TgAb and Tg) or analyte-specific autoantibodies (T4Ab, T3Ab, TSHAb and TgAb). In addition, non-analyte related interferences from heterophile antibodies, including human anti-mouse antibodies (HAMA) and Rheumatoid Factor (RF), and interferences related to the use of Biotin and Streptavidin reagents, are discussed. The review provides an update on collaborations between the International Federation of Clinical Chemistry (IFCC) committee for the standardization of thyroid function tests (C-STFT) and the in-vitro diagnostic (IVD) industry- the goal being to eliminate between-method biases. Although re-standardization of thyroid hormone tests against established reference measurement procedures, and harmonization of TSH tests to the all-method mean has proved effective, recalibration has yet to be implemented by the IVD. Until between-method biases are eliminated, it is not feasible to propose universal reference ranges that would apply across methods. The review contains a comprehensive discussion of the clinical utility of Tg methodology (RIA, IMA or LC-MS/MS), used to monitor patients with differentiated thyroid cancer (DTC). Mechanisms for in-vitro and possible in-vivo TgAb interference with Tg testing are proposed. The methodologic and clinical strengths and weakness of each test are discussed relative to current guidelines. For complete coverage of this and related areas in Endocrinology, visit our free web-books, www.endotext.org and www.thyroidmanager.org.

INTRODUCTION

Over the past forty years, improvements in the sensitivity and specificity of thyroid testing

methodologies have dramatically impacted clinical strategies for detecting and treating thyroid disorders. In the 1950s, only one thyroid test was available - an indirect estimate of the serum total (free + protein-bound) thyroxine (T4) concentration, using the protein bound iodine (PBI) technique (1). Since 1970, technological advances in radioimmunoassay (RIA) (2-6), immunometric assay (IMA) (7-11) and most recently liquid chromatography-tandem mass spectrometry (LC-MS/MS) (12-23) have progressively improved the specificity, reproducibility and sensitivity of thyroid tests (24,25). Currently, serum-based immunoassays and LC-MS/MS techniques are available for measuring total and free thyroid hormones, [Thyroxine (T4) and Triiodothyronine (T3)] (23,26-28), as well as the pituitary thyroid stimulator, Thyrotropin (Thyroid Stimulating Hormone, TSH) (8,29) and the thyroid hormone precursor protein, Thyroglobulin (Tg) (9,16,21,30-33). In addition, measurements can be made of the thyroid hormone binding proteins, Thyroxine Binding Globulin (TBG), Transthyretin (TTR)/Prealbumin (TBPA) and Albumin (34-36). Methods to detect the thyroid autoantibodies (24,37): TSH receptor antibodies (TRAb) (38-43), thyroid peroxidase antibodies (TPOAb) and thyroglobulin antibodies (TgAb) (31,44,45) have been developed in response to the recognition that autoimmunity is a major cause of thyroid dysfunction (46-48). Currently, most thyroid testing is performed on serum specimens using manual or automated immunoassays employing specific antibody reagents targeting these ligands (22,24).

Over the last ten years the International Federation of Clinical Chemistry (IFCC) committee for the standardization of thyroid function tests (C-STFT)* has been working with manufacturers to identify and reduce between-method variability for total and free thyroid hormones as well as TSH (49). Reference measurement procedures (RMP) for TT4 and TT3 using primary calibrators have been developed (12,13,26) and used to establish isotope-dilution liquid chromatography/tandem mass spectrometry (ID-LC-MS/MS) as the RMP for FT4 and FT3 after isolating free hormone by equilibrium dialysis (26,27,50-53) or ultrafiltration (14,23,27,28,54,55). Thyroglobulin has also been detected by LC-MS/MS after trypsinization (16,19-21). Despite technical improvements, sensitivity, specificity and standardization issues still result in substantial between-method variability for many thyroid analytes (8,9,16,32,44,49,53,56-58). The C-STFT studies have shown that recalibrating thyroid hormone methods to their RMPs (50-53) and harmonizing TSH methods to the all-method mean, derived by a robust factor analysis model, significantly reduces between-method biases (29,52,53,59,60). It is hoped that the industry will shortly recalibrate their thyroid tests to remove current biases thereby allowing establishment of universal reference ranges that could apply to all methods and improve the clinical utility of thyroid testing. This chapter is designed to give an overview of the current status and limitations of the thyroid testing methods most commonly used in clinical practice, as recommended by current guidelines (24,61-74). Table 1

Table 1-Reference ranges for Thyroid Function Tests Used in USC Clinical Laboratory

Test	Method	USC Reference Ranges *
Total Thyroxine (TT4)	Roche Cobas	57-159 nmol/L (4.5-12.5 ug/dL)
Total Triiodothyronine (T3)	Roche Cobas	1.2-2.8 nmol/L (80-180 ng/dL)
Thyroid Hormone Binding Ratio (THBR)	Roche Cobas	0.72 - 1.24 (unitless)
Thyrotropin (TSH)	Roche Cobas	0.3 - 4.0 mIU/L
Thyroxine Binding Globulin (TBG)	Siemens Immulite	14.0-31.0 mg/L (14.0-31.0 µg/mL)

Thyroid Peroxidase Antibody (TPOAb)	Kronus/RSR	<1.0 kIU/L
Thyroglobulin (2G-Tg-IMA)	Beckman Access	3-40 µg/L (3-40 ng/mL) #
Thyroglobulin RIA (Tg-RIA)	USC LDM [31]	3-40 µg/L (3-40 ng/mL) #
Tg Autoantibody (TgAb)	Kronus/RSR	<0.4 kIU/L

***These ranges are only applicable to the method listed. They were established for a non-pregnant <60 year-old euthyroid cohort recruited by USC.**

Tg range should be adjusted for thyroid mass and TSH status [see below].

** My sincere thanks to the C-STFT committee chair Professor Linda Thienpont for informative discussions and for providing some of the data contained in this chapter.*

TOTAL THYROID HORMONE MEASUREMENTS (TT4 AND TT3)

Thyroxine (T4) circulates 99.97% bound to the plasma proteins, primarily TBG (60-75%) but also Transthyretin TTR/TBPA (15-30%) and Albumin (~10%)(Table 2) . In contrast, approximately 99.7% of Triiodothyronine (T3) is protein-bound, primarily to TBG [34,35,75]. Total (free + protein-bound) concentrations of thyroid hormones (TT4 and TT3) circulate at nanomolar concentrations and are considerably easier to measure than the free hormone moieties (FT4 and FT3) that circulate in the picomolar range. Serum TT4 measurement has evolved over the past four decades from the protein-bound iodine and competitive protein binding tests [1,76] to non-isotopic immunometric assays [77] and LC-MS/MS methods [13,78-80].

Table 2: Conditions that Influence Thyroid Hormone Binding Proteins

	Increased TBG	Decreased TBG	Albumin Transthyretin (TTR) Abnormalities
Drugs	Estrogens Tamoxifen 5-Fluorouracil Heroin/Methadone Clofibrate Nicotinic Acid Perphenazine	Thyroid hormones Androgens Anabolic steroids Glucocorticoids L-asparaginase Interleukin-6	
Pathophysiologic conditions	Pregnancy Hypothyroidism Acute/chronic hepatitis HCC/PBC Adrenal insufficiency AIDS Angioneurotic edema Acute intermittent porphyria Oat cell carcinoma	Hyperthyroidism Critical illness Sepsis Hepatic failure Nephrotic syndrome Diabetic ketoacidosis Chronic alcoholism Malnutrition Acromegaly Cushing's syndrome Extreme prematurity	Nonthyroidal illness Malnutrition Inflammation Pregnancy
Congenital conditions	TBG excess	TBG deficiency	Familial Dysalbuminemic Hyperthyroxinemia, FDH Transthyretin-Associated Hyperthyroxinemia, TTR-AH

From: references 35 and 36

Serum TT4 measurement has evolved over the past four decades from the protein-bound iodine and competitive protein binding tests [1,76] to non-isotopic immunometric assays [77] and LC-MS/MS methods [13,78-80]. Total hormone methods require the inclusion of inhibitors, such as 8-anilino-1-naphthalene-sulphonic acid, to block hormone binding to serum proteins in order to facilitate binding to the antibody reagent [81]. Methodology for TT4 measurement has changed over the decades and been paralleled by changes in TT3 methodology. However TT3 measurement presents a greater sensitivity and precision challenge, because TT3 concentrations are ten-fold lower than TT4 [13,82-86]. Most laboratories currently measure TT4 and TT3 concentrations by non-competitive immunometric assays performed on automated platforms using enzymes, fluorescence or chemiluminescent molecules as signals [25,75,87]. A recent IFCC C-STFT study compared eleven TT4 and twelve TT3 immunoassays marketed by eight diagnostic companies [80]. TT4 and TT3 measurements were made in sera from healthy individuals using the various immunoassays and compared with values reported by isotope dilution tandem mass spectrometry (ID-LC-MS/MS) - the reference measurement procedure (RMP) based on using primary T4 and T3 standards for calibration [80,88]. 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 [80, 88] (Figure 1). Thus, as would be expected, TT4 assays are more reliable than TT3 although assay variability persists, likely as a result of matrix differences between calibrators and patient sera, the efficiency of the blocking agent employed by different manufacturers and lot-to-lot variability [53,56,89,90].

Figure 1- Between-method TT4 and TT3 Variability

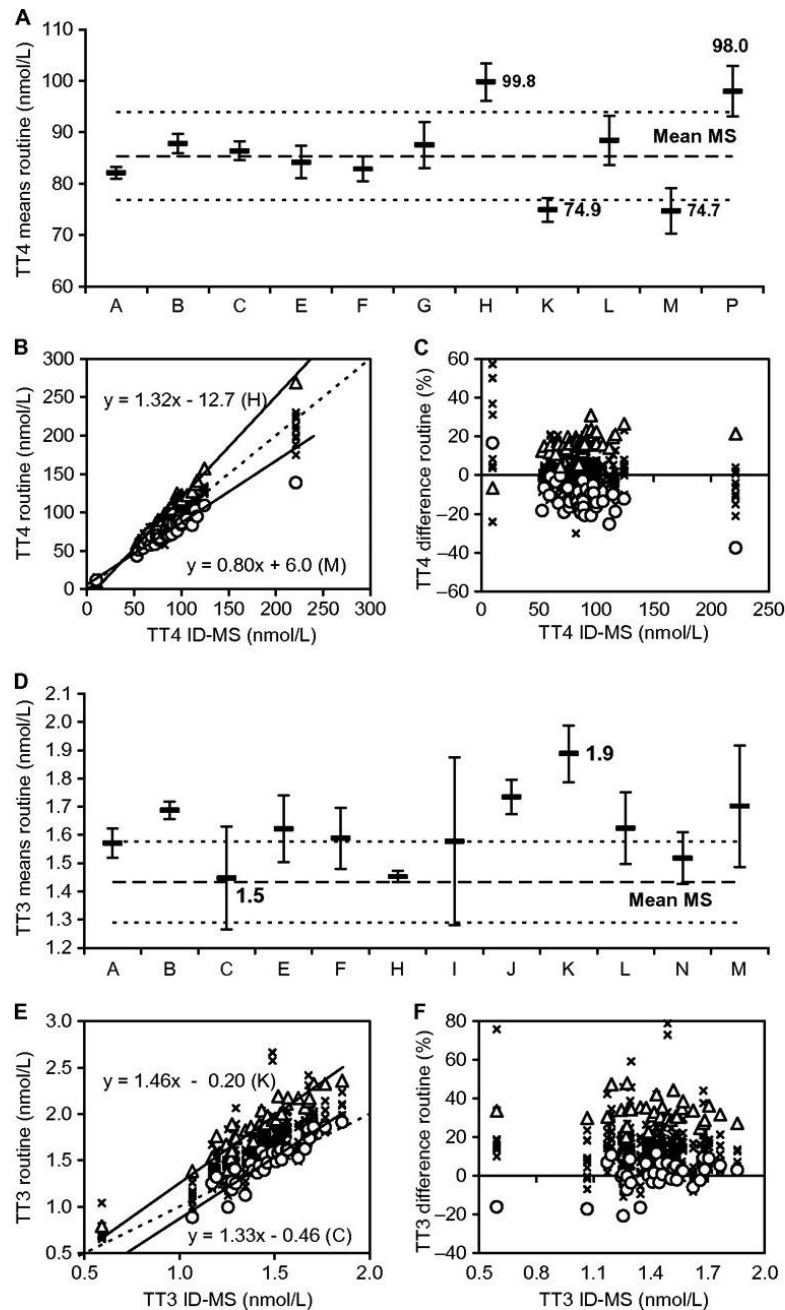


Figure 1. (A), (TT4); (D) (TT3): assay means (1-sided 95% CIs) vs the mean by the RMPs. The x axis gives the codes of the different assays, the dotted lines represent the mean of the RMP \pm 10%. For the assays differing $>10\%$ from the mean of the RMP, the numerical value of the mean is listed. (B), (TT4); (E), (TT3): scatter plot (x = mean of the RMP, y = mean of singlicate results per assay) with indication of the line of equality (dotted) and the most extreme Deming regression lines/equations. The results for the most deviating assays are indicated by circles and triangles; all other assays are indicated with the same symbol, X. (C), (TT4); (F), (TT3): percent-difference plot with indication of the strongest negatively (circles) and positively (triangles) biased assays. Note that (B), (C), (E), and (F) are extended to show the complete range (10–221 nmol/L for TT4, 0.6–1.9 nmol/L for TT3) [80].

Clinical Utility of TT4 and TT3 Measurements

The diagnostic accuracy of total hormone measurements would be equivalent to that of free hormone tests if all patients had similar binding protein concentrations [35,75]. In fact, a recent study has reported that a screening cord blood TT4 < 7.6 µg/dL (< 98 nmol/L) can be used as a screening test for congenital hypothyroidism [91]. Unfortunately, many conditions are associated with TBG abnormalities that distort the relationship between total and free thyroid hormones (Table 1). Additionally, some patients have abnormal thyroid hormone binding albumins (dysalbuminemias) [92-94], thyroid hormone autoantibodies [95-98], or are taking drugs [25,99-101] that render total hormone measurements diagnostically unreliable [Table 1]. Consequently, TT4 and TT3 measurements are rarely used as stand-alone tests, but are typically employed in conjunction with a direct TBG measurement or an estimate of binding proteins [i.e. a thyroid hormone binding ratio test, THBR, that can be used to calculate a free hormone index (FT4I or FT3I)]. This index approach effectively corrects for the most common thyroid hormone binding protein abnormalities that distort total hormone measurements [102-104]. Because free hormone immunoassays are more technically challenging than total hormone measurements [49,86] total hormone tests can be useful confirmatory when a free hormone immunoassay result appears questionable, especially in pregnancy and critical illness where changes in binding protein concentrations and affinity for thyroid hormones can occur [22,104-106]. Suboptimal FT3 assay sensitivity limits reliable FT3 measurements to the high (hyperthyroid) range [86]. However, since T3 is typically only a 3rd-line test of thyroid status used for diagnosing unusual cases of hyperthyroidism, TT3 measurement can usually suffice in preference to FT3, especially when TT3 is used as a ratio with TT4 to eliminate binding protein effects [107]. In fact, in Graves' hyperthyroidism preferential thyroidal T3 secretion resulting from increased deiodinase activity secondary to thyroidal stimulation by TSH receptor antibodies (TRAb) [108] such that a high serum TT3/TT4 or FT3/FT4 ratio that can be used to differentiate Graves' from other causes of hyperthyroidism [107,109,110].

TT4 and TT3 Reference Ranges

Total T4 reference ranges have approximated 58 to 160 nmol/L (4.5-12.5 µg/dL) for more than four decades, although some between-method differences and sample-related variability remains [80, 104]. The IFCC C-STFT found that most TT4 methods report values within 10 percent of the ID-LC-MS/MS RMP (Figure 1) [80]. In euthyroid pregnant subjects the major influence on TT4 is the TBG concentration that rises approximately two-fold by mid-gestation. As a consequence, TT4 steadily increases from the first trimester to plateau at approximately 1.5-fold pre-pregnancy levels by mid-gestation [104,106,111-114]. Thus the non-pregnant TT4 reference range, adjusted by a factor of 1.5 can be used to assess thyroid status in the latter half of gestation [66,67,104,106,115,116].

TT3 reference ranges generally approximate 1.2 - 2.7 nmol/L (80 –180 ng/dL) [84]. However, TT3 methods display far more between-method variability than TT4, and most display more than a 10 percent bias relative to the reference method [79,80,86]. The IFCC C-STFT continues to work with manufacturers to reduce variability and improve the calibration of TT3 methods against the RMP.

Free Thyroid Hormone Tests (FT4 and FT3)

In accord with the free hormone hypothesis, it is the free fraction of the thyroid hormones

(0.02% of TT4 and 0.2% of TT3) that exerts biologic activity at the cellular level [117], whereas protein-bound hormone is considered as biologically inactive. Since binding-protein abnormalities are highly prevalent (Table 1) [35], free hormone measurement is considered preferable to total hormone testing [22,118]. However, free hormone measurement that is independent of thyroid hormone binding proteins remains challenging [22,118-120]. Free hormone methods fall into two categories – **direct** methods, that employ a physical separation of the free from protein-bound hormone, and **estimate** tests, that either calculate a free hormone “index” from a measurement of total hormone corrected for binding proteins with either a TBG measurement or a binding-protein estimate, or immunoassays that employing an antibody to sequester a small amount of the total hormone that is purportedly proportional to the free hormone concentration [22,75,118]. All free hormone tests are subject to limitations. 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 abnormal [52,92,118-128]. 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 [22,128-133]. The IFCC C-STFT has now established a reference measurement procedure (RMP) for free thyroid hormones that is based on equilibrium dialysis-dilution-mass spectrometry (ED-ID-MS) and primary calibrators [15,51,54,134]. An evaluation of current FT4 immunoassays has revealed major between-method variability and significant biases relative to the RMP that are far in excess of FT4 biological variation [50,53]. Recalibrating methods against the RMP was shown to significantly reduce biases that currently preclude implementing universal reference intervals that would apply across methods. The C-STFT is actively working with the in vitro diagnostic industry to re-standardize free hormone methods against the RMP to reduce current biases.

Direct FT4 and FT3 Methods

Direct free hormone methods have employed equilibrium dialysis [51,54,135-137], ultrafiltration [14,17,18,23,131,138-142] or gel filtration [143] to separate free hormone from the dominant protein-bound moiety. These separation techniques can be prone to inaccuracies causing under- or overestimate of free hormone due factors relating to dilution, adsorption, membrane defects, temperature, pH, the influence of endogenous binding protein inhibitors, fatty acid formation and sample-related effects [22,118,128,130-133,141,142,144-146]. The IFCC C-STFT has now established the RMP for FT4 as ED ID-LC-MS/MS. Specifically, equilibrium dialysis of serum is performed under defined conditions before FT4 is measured in the dialysate by isotope-dilution-liquid chromatography/tandem mass spectrometry [15,51,54]. Manufacturers are recommended to use this RMP to recalibrate their FT4 immunoassay tests [52-54,134]. Because direct free hormone methods are technically demanding, inconvenient and expensive, they are typically only readily available in reference laboratories. Most FT4 and FT3 testing is made using estimate tests - either the two-test “index” approach or an immunoassay “sequestration” method [118]. However, all current FT4 and FT3 estimate tests are binding-protein dependent to some extent [118,147-150], and 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 [22].

Equilibrium Dialysis

Early equilibrium dialysis methods used I⁻¹³¹ and later I⁻¹²⁵ 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 [135]. Subsequently, symmetric dialysis in which serum was dialyzed without dilution (or employing a near-physiologic medium) was used to overcome dilution effects [132]. By the early 1970s higher affinity T4 antibodies ($>1 \times 10^{11}$ L/mol) and high specific activity T4- I^{125} tracers were used to develop sensitive RIA methods that could to directly measure FT4 and FT3 in dialyzates and ultrafiltrates [82,136-138,142,151-154]. Subsequent improvements have involved employing more physiologic buffer diluents and improving the dialysis cell design [132,137]. More recently, isotope-dilution liquid chromatography/tandem mass spectrometry (ID-LC-MS/MS) [155] has been used to measure FT4 in ultrafiltrates [14,156,157] and dialyzates [27,50,51,134]. The FT4 RMP recently established by the IFCC C-STFT is based on ED followed by ID-LC-MS/MS [15,51].

Figure 2. FT4 and FT3 Immunoassay Method Comparison

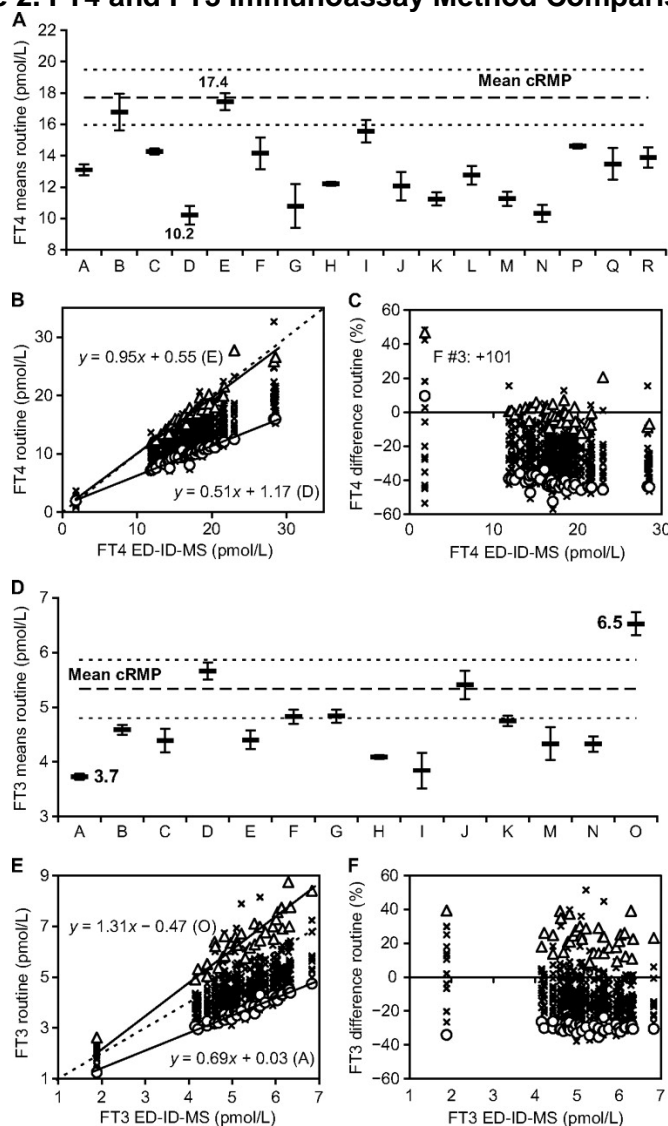


Figure 2. Between Assay Comparison of FT4 and FT3 Measurements in Healthy Euthyroid Subjects. A=FT4 and D=FT3: assay means versus the mean by the RMPs. Different assays

are coded A-O on the x axis, manufacturer codes used to designate assays were different for FT4 and FT3 assays. The dotted lines represent mean \pm 10% of the RMP ED-ID-MS). B=FT4 and E=FT3: scatter plot (x =mean of the RMP vs. y = mean of 6 singlicate results per assay. Line of equality indicated by dotted line. The results for the most deviating assays are indicated by circles and triangles; all other assays are indicated with the same symbol, X. C=FT4 and F=FT3: percent-difference plot indicating the strongest negatively (circles) and positively (triangles) biased assays [50].

Ultrafiltration Methods

A number of studies have used ultrafiltration to remove protein-bound T4 prior to LC-MS/MS measurement of FT4 in the ultrafiltrate [14,17,18,23,55,131,138-142]. Direct FT4 measurements employing ultrafiltration are sometimes higher than those made by equilibrium dialysis, because ultrafiltration avoids dilution effects [140]. Furthermore, ultrafiltration is not influenced by dialyzable inhibitors of T4-protein binding that can be present in conditions such as non-thyroidal illness (NTI) [130]. 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 [127]. In addition, ultrafiltration is temperature sensitive and ultrafiltration performed at ambient temperature (25°C) will report FT4 results that are 67 percent lower than ultrafiltration performed at 37°C [133,158]. However, FT4 concentrations measured by ID-LC-MS/MS following either ultrafiltration at 37°C or equilibrium dialysis usually correlate [159].

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 [75].

Indirect FT4 and FT3 Estimate Tests

Two-Test Index Methods (FT4I and FT3I)

Free hormone indexes (FT4I and FT3I) are unitless mathematical calculations made by correcting the total hormone test result for the binding protein, primarily TBG, concentration. These indexes require two separate tests and have been used to estimate free hormone concentrations for more than 40 years [118]. The first test involves the measurement of total hormone (TT4 or TT3), whereas the second test assesses the binding protein concentration using either (i) a direct TBG immunoassay, (ii) a Thyroid Hormone Binding Ratio (THBR) or "Uptake" test or (iii) an isotopic determination of the free hormone fraction [118,160].

TBG Immunoassays

There is conflicting data concerning whether indexes employing THBR in preference to direct TBG are diagnostically superior [161]. Free hormone indexes calculated using direct 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 [101,162-165]. Regardless, the TT4/TBG index is not totally independent of the TBG concentration, nor does it correct for Albumin or Transthyretin binding protein abnormalities (Table 1) [120].

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) [119,166,167]. The "uptake" of T3 tracer onto the scavenger provided an indirect, reciprocal estimate of the TBG concentration of the specimen. Initially, T3 uptake tests were reported as percent uptakes (free/total tracer). Typically, sera with normal TBG concentrations 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, calculating uptakes based on the ratio between absorbent and total minus absorbent counts, and expressing results expressed as a ratio with normal sera having an assigned value of 1.00 [160,167]. Historically, the use of T3 as opposed to T4 tracer was made for practical reasons relating to the ten-fold lower the affinity of TBG for T3 versus T4, facilitating a higher percentage of T3 tracer being taken up by the scavenger and allowing lower 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 [168]. 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) [104,122,169-171], although they may fail to correct for grossly abnormal binding proteins [94] in euthyroid patients with congenital TBG extremes [120,122,172], Familial Dysalbuminemic Hyperthyroxinemia (FDH) [75,92,173-176], thyroid hormone autoantibodies [95,97,177,178], non-thyroidal illness (NTI) [120,128,179,180] or medications that directly or indirectly influence thyroid hormone binding to plasma proteins [75,99,120,164,181,182].

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 [135,183,184]. These early isotopic detection systems were technically demanding and included paper chromatography, electrophoresis, magnesium chloride precipitation and column chromatography [135,153,185-187]. The free fraction index approach was later extended to ultrafiltration and symmetric dialysis, the latter measuring the rate of transfer of isotopically-labeled hormone across a membrane separating two chambers containing the same undiluted specimen [92,138,140,184,188-190]. Ultrafiltration and symmetric dialysis had the advantage of eliminating dilution effects that influenced tracer dialysis values [129,191]. However, free hormone indexes calculated using an isotopic free fraction were not completely independent of the TBG concentration and furthermore were influenced by tracer purity and the buffer matrix employed [137,192].

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

Some favored the two-test FT4I approach for evaluating the thyroid status of patients with abnormal binding protein states like pregnancy and NTI [104,193]. Continued use of the FT4I remains controversial [194]. However, until FT4 immunoassays are re-standardized to remove biases [50,52,53], FT4I remains a useful confirmatory test when binding proteins are abnormal and when diagnosing central hypothyroidism [195].

Free Thyroid Hormone Immunoassay Methods (FT4 and FT3)

Most free hormone testing is made using FT4 and FT3 immunoassays [87]. These immunoassays are based on "one-step", "labeled antibody" or "two-step" principles, as described below [75,118,196]. 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 [22,104], or due to polymorphisms, drug interactions, high free fatty acid (FFA) levels or thyroid binding inhibitors such as those present in NTI [25,53,75,92,119,120, 126-128,130,147,150,196-200]. Studies showing correlations between FT4 immunoassay values and both TBG and albumin concentrations, as well as weak inverse FT4/TSH log/linear relationships [17,18,23,126], have emphasized the need to evaluate each method with clinical specimens containing abnormal binding proteins. Currently, most FT4 and FT3 immunoassays display significant negative or positive biases that exceed the intra-individual biological variability (Figure 2) [50,52,53]. The IFCC C-STFT is actively working with the IVD industry to recalibrate their free hormone immunoassays against the RMP [15,50,53,60]. However, although recalibration to the RMP has been shown to greatly reduce between-method biases [50,52,53], implementation of a global re-calibration effort has been delayed by 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 [22,75,118,119,201,202]. 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 critically dependent on the degree that the analog is "inert" with respect to binding protein abnormalities [17,18,23,118,119,147,180,200,203-208].

Labeled Antibody FT4 and FT3 Methods

Labeled antibody methods are "one-step" methods that use 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 [22,75,118,120,202,209]. The labeled antibody approach is used as the basis for a number of automated immunoassay platforms because it is easy to automate and considered less binding-protein dependent than the labeled analog approach, because the solid phase hormone does not compete with endogenous free hormone for hormone binding proteins [22,87,118,210,211].

Two-Step, Back-Titration FT4 and FT3 Methods

The two-step approach was first developed by Ekins and colleagues in the late 1970s [75,119,128,202]. 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 [75,118]. After removing unbound serum constituents by washing, a labeled probe (^{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" [118].

Clinical Utility of FT4 and FT3 Measurements

Most FT4 methods give diagnostically reliable results when binding proteins are near-normal, provided that a method-specific reference range is employed [53]. However, both TT3 and FT3 immunoassay methods tend to be inaccurate in the low range [86,212] and have no value for diagnosing or monitoring treatment for hypothyroidism [70,213], although T3 measurement can be useful for diagnosing or confirming unusual cases of hyperthyroidism.

Ambulatory Patients

Free hormone tests (FT4 or FT3) are used in preference to total hormone (TT4 or TT3) measurements in order to improve diagnostic accuracy for detecting hypo- and hyperthyroidism in patients with abnormal thyroid hormone binding proteins (Table 1). FT4 is typically employed 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 [24,100,101,165,214-219].

Mild "subclinical" thyroid dysfunction is characterized by TSH/FT4 discordances (abnormal TSH/normal FT4). This reflects the intrinsic complex nature of the inverse log/linear TSH/FT4 relationship [24,220,226] - a relationship that is modified by age and gender [227,228]. 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 (for subclinical hyperthyroidism) and 5 and 10 mIU/L (for subclinical hypothyroidism). An unexpected TSH/FT4 discordance, if confirmed, should prompt an investigation for interference with FT4, TSH or both tests [229,230]. FT4 interference can result from severe binding protein abnormalities such as congenital TBG excess or deficiency [75,94,122,159,231,232], dysalbuminemias [92,233-236], thyroid hormone autoantibodies [95,97,98,177,178,230,237] or drug interferences [75,99,120].

Hospitalized Patients with Nonthyroidal Illnesses (NTI)

The diagnostic performance of current FT4 methods has not been evaluated in hospitalized patients with NTI where binding protein inhibitors and drug therapies can negatively impact the reliability of both thyroid hormone and TSH testing [24,75,126,130,180,218,238,239]. 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 [238,240,241]. 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 [24,53,180,242]. 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 is receiving medications such as glucocorticoids and dopamine that directly inhibit TSH secretion [101,165,218]. Repetitive TSH testing may be helpful in resolving the cause of an abnormal FT4, because the TSH abnormalities of NTI are typically transient whereas the TSH abnormality will persist if due to

underlying thyroid dysfunction [243-246]. It may be useful to test for TPOAb as a marker for underlying thyroid autoimmunity

FT4 and FT3 reference ranges

Current reference ranges for FT4 and FT3 immunoassays are method-dependent because of calibration biases [50,52,53] (Figure 2). This calibration problem negatively impacts the clinical utility of FT3 and FT4 tests because it precludes establishing universal reference ranges that would apply across methods.

Pediatric FT4 and FT3 Reference Ranges

The determination of normal reference limits for pediatric age-groups is especially challenging, given the limited number of studies involving sufficient numbers of healthy children [247-249]. 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 similar to that observed for adults [247-252].

Pregnancy FT4 Reference Ranges

As with non-pregnant patients, TSH is the first-line test to use for assessing thyroid status during pregnancy [253]. However, FT4 measurement is needed for monitoring anti-thyroid drug treatment of hyperthyroid pregnant patients who have undetectable TSH. The question whether an isolated low FT4 during pregnancy is a maternal or fetal risk factor, remains controversial [254-259]. However, a number of studies suggest that low FT4 may be a risk factor for gestational diabetes and fetal complications [260-264]. Non-pregnant FT4 reference ranges do not apply to pregnancy since FT4 progressively declines as gestation progresses, necessitating the use of trimester-specific reference ranges [104,113,265-271]. Currently it is not possible to propose universal trimester-specific FT4 reference ranges given current between-method differences [50,53,271] (Figure 2) compounded by differences related to the ethnicity [193,270,272-275], iodine intake [276-278], smoking [279] and BMI [269,270,280-283] between study cohorts. Establishing institution-specific trimester-specific reference ranges from the 2.5 to 97.5 percentiles of least 400 pregnant patients from each trimester [270] is not practical for most institutions. The feasibility of establishing universal trimester-specific reference ranges will improve after the proposed re-standardization of FT4 methods against the RMP [53]. However, binding protein effects will remain and population-specific factors will still have to be considered.

Interferences with Total and Free Thyroid Hormone Tests

Only the 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. Failure to recognize interferences can have adverse clinical consequences [229,284-289].

Laboratory checks for interference include showing discordance between different manufacturers methods [290-293], re-measurement of analyte after adding blocking agents [293-297] and performing linearity studies or precipitating immunoglobulin with polyethylene

glycol (PEG) [229,290,291,293,294,298-300]. A change in analyte concentration in response to one of these maneuvers suggests interference, but a lack of effect does not rule out interference. Interferences can be classified as either (a) non-analyte-specific or (b) analyte-specific [301,302].

Non-Analyte-Specific Interferences

Protein Interferences

Immunoassays can be affected by interferences from both paraproteins [303-305] and abnormal immunoglobulins [306,307].

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 those seen in congenital TBG excess or deficiency states [75,94,122,159,231,232].

Pregnancy.

Estrogen stimulation causes TBG concentrations to progressively rise to plateau 2.5-fold higher than pre-pregnancy values by mid-gestation [193,308,309]. As a consequence, both TT4 and TT3 increase to approximately 1.5-fold of pre-pregnancy values by mid-gestation [113,310]. Despite the rise in total hormone, both FT4 and FT3 decline to a method-related degree during gestation [104,265-269]. It should be noted that lower FT4 levels would be expected during pregnancy from a consideration of the law of mass action as applied to T4-binding protein interactions [310]. However, the degree of FT4 decline during pregnancy is variable and method-dependent due to standardization differences (Figure 2) and in some cases method sensitivity to the declining albumin concentrations typical of late gestation [18,193,311].

Familial Dysalbuminemic and Transthyretin-Associated Hyperthyroxinemias.

Autosomal dominant mutations in the Albumin or Transthyretin (prealbumin) [312] gene 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 [92,173,237,312]. Familial Dysalbuminemic Hyperthyroxinemia (FDH) is a rare condition with a prevalence of ~1.8 % in the Hispanic population [313]. It arises from a number of genetic variants, with the R218H being the most common, some variants result in extremely high TT4, whereas other mutations (i.e. L66P) affect mainly T3 [233]. Affected individuals are euthyroid and have normal TSH and FT4 when measured by direct techniques such as equilibrium dialysis [92]. 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 [92].

Heterophile Antibodies (HABs)

Heterophile antibodies (HAb) are human poly-specific antibodies targeted against animal antigens, the most common being human anti-mouse antibodies (HAMA) [293,302,314,315]. Alternatively, HAb can target human antigens [302] such as rheumatoid factor (RF), an immunoglobulin commonly associated with autoimmune conditions that is widely considered a heterophile antibody [316]. RF has been shown to interfere with free and total thyroid hormone tests [87] as well as TSH [317] and Tg [318]. HABs have a prevalence of 30-40 percent [319-321] and have the potential to interfere with a broad range of methods that use IMA principles [290,300,306,322]. In

recent years assay manufacturers have increased the immunoglobulin blocker reagents added to their tests and this has reduced interference from 2 to 5 percent [290,297,323]. However, interference is still seen in approximately one percent of patients who have high enough HAb concentrations to overcome the assay blocker [296,298,322,324]. HAMA interference mostly affects non-competitive immunometric assays (IMA) that employ monoclonal antibodies of murine origin [325]. Assays based on the competitive format that employ high affinity anti-antigen polyclonal antibody reagents, are rarely affected [296,319]. HAb has the potential to interfere with both free [178,321,326-328] and total [178,326,327] thyroid hormone tests, as well as THBR [327], TSH [289,294,300,328-330] and Thyroglobulin (Tg) [295,296,323,324,331,332], TgAb [333] and calcitonin (CT) [300,334-337] methods. Interference from HAb or HAMA typically causes falsely high results for one or more analytes. Less commonly falsely low test results may be seen [332]. The test marketed by one manufacturer can be severely affected, whereas the test from a different manufacturer may appear unaffected. This is why the first step for investigating for interference is re-measurement of the analyte in a different manufacturers 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 coming into contact with animals, are especially prone to test interferences caused by induced HAb and HAMA [298,338].

Anti-Reagent Antibodies

Interference can be caused by antibodies against assay reagents. For example, there are a number of reports of anti-Rhuthenium antibodies interfering with TSH, FT4 and FT3 by [339-343]. In Streptavidin-Biotin based assays interference can result from antibodies targeting either Streptavidin [344] or biotin reagents [345]. Alternatively, high dose biotin ingestion has been known to produce interference with thyroid and other tests in an analyte-specific, platform-specific manner [346-350].

Analyte-Specific Interferences

Analyte-specific interferences typically result from autoantibodies targeting the analyte. Depending on the analyte and test formulation, autoantibody interferences typically cause falsely-high test results, but can cause falsely-low test results, as in the case of Tg autoantibodies . It should be noted that transplacental passage both heterophile antibodies or anti-analyte autoantibodies (i.e. TSHAb or T4Ab) have the potential to interfere with neonatal screening tests [351-354]. Specifically, maternal TSH autoantibodies can cross the placenta and may cause a falsely high TSH screening test in the newborn mimicking congenital hypothyroidism, whereas maternal T4 autoantibodies could cause falsely high neonatal T4 masking the presence of congenital hypothyroidism [230,353].

T4 and T3 Autoantibodies (T4Ab/T3Ab)

T4 and T3 autoantibodies can falsely elevate total hormone, free hormone or THBR measurements depending on the method employed [95,97,98,177,178,230,237]. The prevalence of thyroid hormone autoantibodies approximates 2 percent in the general population but may be as much as 30 percent in patients with autoimmune thyroid disease or other autoimmune conditions [316,355-358]. 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). Further, different methods exhibit such interferences to a greater or lesser degree [95,97]. Because autoantibody interference is difficult for the laboratory to detect proactively, it is the physician who should first suspect interference characterized

by unexpected discordance between the clinical presentation of the patient and the test result(s) [96, 178].

SERUM TSH (THYROID STIMULATING HORMONE/THYROTROPIN) MEASUREMENT

Over the last four 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 not receiving drugs known to alter TSH secretion [24,70,71,120,216,218,359]. Serum TSH has become the therapeutic target for levothyroxine (L-T4) replacement therapy for hypothyroidism and suppression therapy for differentiated thyroid cancer [72]. 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 [7,24,221-228].

TSH Assays

TSH assay "quality" has historically been defined by clinical sensitivity – the ability to discriminate between hyperthyroid and euthyroid TSH values [24,360-364]. The first generation of RIA methods had a detection limit approximating 1.0 mIU/L [365-367] that limited their clinical utility to diagnosing primary hypothyroidism [368-370] and necessitated the use of TRH stimulation to diagnose hyperthyroidism that was characterized by an absent TRH-stimulated TSH response [371-376]. With the advent of immunometric assay (IMA) methodology that uses a combination of poly- and/or monoclonal antibodies targeting different TSH epitope(s) in a "sandwich" format [377-379], a ten-fold improvement in TSH assay sensitivity (~ 0.1 mIU/L) was achieved when using isotopic (I^{125}) signals [380]. This level of sensitivity facilitated the determination of the lower TSH reference limit (as 0.3-0.4 mIU/L), and the detection of overt hyperthyroidism without the need for TRH stimulation [7,374-376,380-386], but was still insufficient for distinguishing between differing degrees of hyperthyroidism (i.e. subclinical versus overt). Sensitization continued until a third-generation of TSH IMAs, using non-isotopic signals, were developed that could achieve a sensitivity of 0.01 mIU/L [7,8,374,387-389]. Initially different non-isotopic signals were used that gave rise to a lexicon of terminology to distinguish between assays: immunoassayometric 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 [8,390]. Current TSH methods are automated ICMA [87] that all achieve third-generation functional sensitivity (FS = ≤ 0.01 mIU/L) - a sensitivity the FS level that has subsequently become the standard of care [7,8,52,53,388,391-396].

Functional Sensitivity (FS) - determines the lowest reportable assay limit

During the period of active TSH assay improvement, 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 [8,397-403]. Functional sensitivity (FS), defined as the lowest analyte concentration measured with 20 percent coefficient of variation [24] is now recognized as the parameter that best represents the between-run precision for measuring low analyte concentrations in clinical practice [24,395,404]. FS is used to define the lower clinical reporting limit for not only for TSH

assays, but also Tg and TgAb measurements, for which assay sensitivity is critical [8,24,397,404,405]. Protocols used for establishing FS specify that precision be determined in human serum, not quality control materials based on artificial protein matrices, since immunoassays tend to be matrix-sensitive [406,407]. The time-span 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 when used as tumor markers for monitoring differentiated thyroid cancer (DTC). This time-span is important because low-end, between-run assay precision erodes over time as a result of a myriad of variables, reagent lot-to-lot variability being a key variable [9,408-410]. 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 matrix and time-span for determining precision) [404,411]. A ten-fold difference in FS has been used to define each more sensitive "generation" of TSH [397] or Tg [32,404,412,413] method. Thus, TSH RIA methods with FS approximating 1.0 mIU/L were designated "first generation", TSH IMA methods with functional sensitivity approximating 0.1 mIU/L were designated "second generation", and TSH IMAs with FS approximating 0.01 mIU/L are designated "third generation" assays [8,57,395,397,405,414]. Analogous to TSH, Tg assays [Section 6A] with FS approximating 1 µg/L are designated "first generation", whereas Tg IMAs with FS approximating 0.10 µg/L meet the criteria for a "second generation" method [32,58,296,395,404,413,415,416].

TSH Biologic Variability

As compared with between-person variability, TSH intra-individual variability is relatively narrow (20-25 percent) in both non-pregnant and pregnant subjects, as compared with between-person variability [29,222,417,418]. 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 [417]. Twin studies suggest that there are genetic factors that determine hypothalamic-pituitary-thyroid setpoints [419-421]. These studies report that the inheritable contribution to the serum TSH level approximates 65 percent [420,422]. This genetic influence appears, in part, to involve single nucleotide polymorphisms in thyroid hormone pathway genes such as the phosphodiesterase gene (PDE8B) [423-425], polymorphisms causing gain [426-433] or loss [434-436] of function TSH receptors [423,437,438] and the type II deiodinase enzyme polymorphisms [423,439]. Undoubtedly, such polymorphisms account for some of the euthyroid outliers that skew TSH reference range calculations [423,434,440].

Figure 3. TSH Between-Method Variability

Figure 3. A. Geometric mean of the TSH results for the range 0.5– 6.6 mIU/L, (x axis, different assays; dotted lines, overall mean and 10% error). In the plots, the 1-sided 95% CIs of the means are shown (note: the wide interval of assay O is due to results from only 2 runs with a high between-run variation and $df = 1$ by the Satterthwaite approximation). For the assays outside the 10% limit, the mean value is listed. **B.** Plot showing the %-difference between TSH methods. The most discrepant assays are shown by triangles and circles. Other assays are shown with the same symbol (x) [29,52].

The narrow TSH within-person variability and low (< 0.6) index of individuality (Iol) [222,417,418,441-443] limits the clinical utility of using the TSH population-based reference range to detect thyroid dysfunction in an individual patient [222,418,443,444]. 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 [69,445,446].

TSH Reference Ranges - General Considerations

IFCC C-STFT comparison studies (Figure 3) report significant biases between different TSH methods. Currently this prevents establishing universal population or trimester-specific TSH reference ranges that would apply across methods [52,447]. These method biases also impact the frequency of detecting subclinical hypothyroidism [61,448]. Since TSH is a complex glycoprotein, no reference measurement procedure (RMP) is available, or will likely be feasible in the future. However, a harmonization approach [59,60], where 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 [29,449]. The IFCC C-STFT 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 population and trimester-specific TSH reference ranges for each method - 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 [450]. 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 [451], ethnicity [396,452] and iodine intake [453]. It may be that a reference range established in one geographic location may not be representative of a different locale or population. After harmonization of TSH methods the advantages of consolidating data from different studies and establishing universal reference limits is clearly apparent.

The TSH Population Reference Range

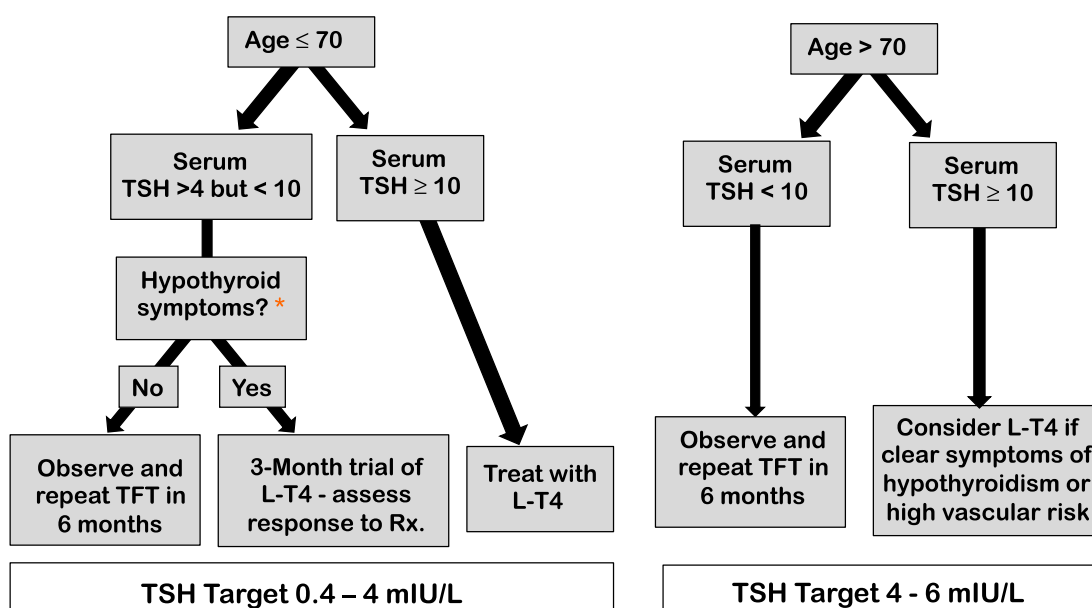
The complex log/linear TSH/FT4 relationship [7,24,221-228] dictates that TSH will be the first abnormality to appear with the development of mild (subclinical) hypo- or hyperthyroidism. It follows that the setting of the TSH reference limits critically influences the frequency of diagnosing subclinical thyroid disease [69,445,448,454].

Guidelines recommend 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 (measured by sensitive immunoassay); (b) no personal or family history of thyroid dysfunction; (c) no visible or palpable goiter and, (c) who are taking no medications except estrogen" [24,450].

Multiple factors influence population TSH reference limits, especially the upper (97.5th percentile) limit. Different methods report different ranges for the same population as a result of between-methods biases (Figure 3) [396,448,451,455]. A key factor affecting the upper limit is the stringency used for eliminating individuals with thyroid autoimmunity (thyroid autoantibody positive [456]) from the population [452,456-461]. Other factors relate to population demographics such as sex [452], ethnicity [452,462-464], iodine intake [465], BMI [466-477] and smoking status [462,478,479]. Age is a major factor the influences the TSH upper limit [460,463,480-482] leading to the suggestion that age-specific TSH reference limits should be used (Figure 4) [69,451,480]. However, the relationship between TSH and age is complex. Most studies in iodine sufficient populations have shown an increase in TSH with age [440,452,460,483], whereas other studies have reported no change or a decreased TSH with aging [457,484,485]. This conflicting data could merely represent population differences - with a rising TSH with

age reflecting an increasing prevalence of thyroid autoimmunity in iodine-sufficient populations [452], whereas in iodine deficient populations, increasing autonomy of nodular goiter can result in decreased TSH with aging [486-488]. Some studies have reported that a mild TSH elevation in elderly individuals may convey a survival benefit [481,489-492], whereas other studies dispute this [493,494]. 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 [495,496].

Figure 4. Guidelines for Diagnosing and Managing Subclinical Hypothyroidism



- Depending on patient-specific circumstances: Consider treating individuals with goiter, dyslipidaemia, diabetes, TPOAb-positivity and preconception planning.

*Figure 4. Suggested management algorithm from reference # 69 Initial management of persistent subclinical hypothyroidism in non-pregnant adults: persistent subclinical hypothyroidism describes patients with elevated serum TSH and within reference range serum FT 4 on two occasions separated by at least 3 months. This algorithm is meant as a guide and clinicians are expected to use their discretion and judgment in interpreting the age threshold around 70 years. * Depending on circumstances, individuals with goiter, dyslipidaemia, and diabetes may also be considered for treatment, along with those with planning pregnancy in the near future.*

TSH is a heterogeneous glycoprotein [497,498], and TRH-mediated changes in TSH glycosylation [499] have the potential to influence immunoactivity [500,501]. A number of pathophysiologic circumstances are known to alter TSH glycosylation [498,500,502-504]. The demonstration that harmonization of TSH methods successfully minimizes between-method differences [52,53] suggests that under normal conditions current TSH IMAs appear to be "glycosylation blind", and detect different TSH glycoforms in an equimolar fashion [52,53,501]. 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 [215,239,246,498,505-509].

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 [250-252, 485, 510-514]. This necessitates using age-specific TSH reference ranges for diagnosing thyroid dysfunction in these different pediatric age groups.

Subclinical Thyroid Dysfunction

Subclinical Hyperthyroidism (SCHY)

The lower (2.5th percentile) TSH reference limit approximates 0.3-0.4 mIU/L, and is fairly independent of the method used [445,452,484,485,515-520]. Subclinical hyperthyroidism (SCHY), is defined as a low but detectable TSH (0.01 –0.3 mIU/L range) without a FT4 abnormality. The prevalence of endogenous SCHY is low (0.7%) in iodine-sufficient populations [452], but is higher in patients reporting thyroid disease as an iatrogenic consequence of L-T4 replacement therapy [521-523]. SCHY is a risk factor for osteoporosis and increased fracture risk [474,524-526] as well as atrial fibrillation and cardiovascular disease [445,474,527], especially in older patient patients.

Subclinical Hypothyroidism (SCHO).

Subclinical hypothyroidism is defined as a TSH above the upper (97.5th percentile) TSH reference limit without a FT4 abnormality [69,448,454,460,516,528-530]. However, since the setting of the TSH upper limit remains controversial, the prevalence of SCHO is highly variable - 4 to 8.5 % [452,521], rising to 15% in older populations [446,456]. In most cases, SCHO is associated with TPOAb positivity, indicative of an autoimmune etiology [452,456]. The clinical consequences of SCHO relate to the degree of TSH elevation [531]. Most guidelines recommend L-T4 treatment of SCHO when TSH is above 10 mIU/L [68,69] but below 10 mIU/L recommend L-T4 treatment based on patient-specific risk factors (Figure 4) [69]. There is active debate concerning the efficacy of treating SCHO to prevent progression [532-535], or improve renal [536,537], cardiovascular [474,524,531,538-543], or lipid [544-546] abnormalities that can be associated with SCHO [69,547].

Thyroid Dysfunction and Pregnancy

It is well documented that overt hypo- or hyperthyroidism is associated with both maternal and fetal complications [548-550]. However, the impact of maternal subclinical thyroid dysfunction remains controversial [253]. No maternal or fetal complications appear associated with subclinical hyperthyroidism during pregnancy [258,551]. First trimester "gestational hyperthyroidism" is typically transient and hCG-related, as described above. In contrast, short-term and long-term outcome studies of maternal subclinical hypothyroidism [550] 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 [277,454]. Using gestational age-specific reference intervals the frequency of SCHO in first trimester pregnancy approximates 2-5 percent [552-556]. A number of studies have

reported that subclinical hypothyroidism is associated with increased frequency of maternal and fetal complications, especially when TPOAb is positive [557-559]. Maternal complications have included miscarriage [474,548,560-562], preeclampsia [548,563], placental abruption [552], preterm delivery [552,562,564] and post-partum thyroiditis [565]. Fetal complications have included intrauterine growth retardation and low birth weight [258,548,566-568] and possible impaired neuropsychological development [550,569,570]. It remains controversial whether L-T4 treatment of SCHO in early gestation decreases risk of complications [559,562,564,571].

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 [66,67,253,254,555,556,572]. 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 [254,308,309,573,574]. The degree of TSH suppression is inversely related to the hCG concentration and can be quite profound in patients with hyperemesis who have especially high hCG [271,575-577]. As gestation progresses, TSH tends to return towards pre-pregnancy levels [271]. Recent studies from different geographic areas with diverse iodine intakes have using different TSH methods have reported higher trimester-specific TSH upper limits than recommended by previous guidelines [253,269,271,454,556,578-580]. In response, the American Thyroid Association has recently revised their pregnancy guidelines [66,74] to replace trimester-specific reference limits by a universal upper TSH limit of 4.0 mIU/L, when TPOAb is negative and local reference range data is not available. However, at this time between-method biases (Figure 3) clearly preclude proposing universal TSH or FT4 reference ranges that would apply to all methods and all populations [52,53,267,271,447]. It is critical that the IVD manufacturers respond to the urging of the IFCC C-STFT and harmonize their TSH methods to increase the feasibility of establishing TSH universal reference limits for pregnancy [52,53]. Requiring each institution to establish their own trimester-specific reference ranges for thyroid tests is impractical, given the costs, logistics and ethical considerations involved in recruiting the more than 400 disease-free pregnant women needed to establish reliable ranges for each trimester [270]. Only after methods are re-standardized (FT4) or harmonized (TSH), will it be feasible to propose trimester-specific reference ranges that would apply across methods. However, such ranges would still be influenced by differences in ethnicity [280] and iodine intake, especially pre-pregnancy iodine intake that influences thyroidal iodine stores [277]. There is also a current need to reevaluate optimal TPOAb cutoffs needed to exclude those individuals with thyroid autoimmunity whose inclusion skews TSH upper limits [271,280,454,574,581,582].

Clinical Utility of TSH Measurement

Ambulatory Patients

In the outpatient setting the reliability of TSH testing is not influenced by the time of day of the blood draw, because the diurnal TSH peak occurs between midnight and 0400 [583-586]. 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 is stable [24,57,216,242,359,414,587,588]. TSH is also used for optimizing L-T4 therapy - a drug with a very narrow therapeutic index [359,589,590]. 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 [24]. In addition, targeting the degree of TSH suppression relative to recurrence risk plays a critical role in the management of thyroid cancer [72,591-593].

Hospitalized Patients with Nonthyroidal Illnesses (NTI)

Routine thyroid testing in the hospital setting is not recommended because thyroid test abnormalities are frequently seen in euthyroid sick patients [238,594]. 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 [100,218,239,245,595]. T3 levels typically fall early in the illness followed by a fall in T4 as the severity of illness increases. [24,244,595-597]. As thyroid hormone levels fall TSH typically remains unchanged, or may be low early in the illness, especially in response to drug therapies such as dopamine or glucocorticoid [100,101,218]. During the recovery phase, TSH frequently rebounds above the reference range [243]. However, high TSH may also be seen associated with psychiatric illness [598]. 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 [24,238,244].

Misleading TSH Measurements

TSH can be diagnostically misleading either because of (a) biological or (b) technical factors. From heterophile antibodies (HABs) or endogenous TSH autoantibodies are the most common causes of a falsely high TSH [299,329,599].

Biologic factors causing TSH diagnostic dilemmas

Unstable thyroid function

TSH can be misleading when there is unstable thyroid status - such as in the early phase of treating hyper- 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 [600]. 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 [509]. TSH measurement is unreliable in cases of both central hypothyroidism and central hyperthyroidism caused by TSH-secreting adenomas [215,217,219,508].

Central Hypothyroidism (CH)

Central hypothyroidism (CH) is rare (1/1000 as prevalent as primary hypothyroidism, 1/160,000 detected by neonatal screening) [509, 601]. CH can arise from disease at either the pituitary or hypothalamic level, or both [509]. A major limitation of using a TSH-centered screening strategy is that this strategy will miss a diagnosis of CH, because the TSH isoforms secreted in CH are abnormally glycosylated and bio-inactive, yet will be detected as paradoxically normal TSH by current IMA methods

despite the presence of clinical hypothyroidism [215, 217, 602]. The clinical diagnosis of CH can be confirmed biochemically as a low FT4/normal-low TSH discordance. Serum FT4 should be used to optimize L-T4 replacement therapy. In the absence of clinical suspicion, investigations for pituitary dysfunction should only be initiated after ruling-out technical interference.

TSH-secreting pituitary adenomas

TSHomas are characterized by near-normal TSH despite clinical hyperthyroidism [603]. Since this is a rare (0.7%) type of pituitary adenoma, technical interference causing paradoxically high TSH, such as a TSH autoantibody should be excluded before initiating inconvenient and unnecessary pituitary imaging or dynamic (T3 suppression or TRH stimulation) diagnostic testing. TSHomas are characterized by discordance between the clinical presentation and a paradoxically non-suppressed TSH despite high thyroid hormone levels and clinical hyperthyroidism [604]. This clinical/biochemical discordance reflects adenoma secretion of TSH isoforms with enhanced biologic activity that cannot be distinguished from bioactive TSH by IMA methods. 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 [604]. Note that the biochemical profile (high thyroid hormones and non-suppressed TSH) is similar to that seen with thyroid hormone resistance syndromes [605]. When pituitary imaging is equivocal, genetic testing may be necessary to distinguish between these two conditions.

Resistance to Thyroid Hormone (RTH)

Resistance to thyroid hormone is biochemically characterized by high thyroid hormone (FT4 +/- T3) levels and a non-suppressed, sometimes slightly elevated TSH without signs and symptoms of thyroid hormone excess [606]. Early cases of resistance to thyroid hormone were shown to result from mutations in the thyroid hormone receptor *B* [607]. More recently the definition of RTH has been broadened to include other causes of thyroid hormone resistance - mutations in the thyroid hormone cell membrane transporter MCT8, and a range of genetic thyroid hormone metabolism defects (SBP2) [608]. These resistance syndromes display a spectrum of clinical and biochemical profiles may need to be identified by specialized genetic testing.

Activating or Inactivating TSH Receptor Mutations

Non-autoimmune hyperthyroidism resulting from an activating mutation of the TSH receptor (TSHR) is rare [426-433]. A spectrum of loss-of-function TSHR mutations (TSH resistance) causing clinical and subclinical hypothyroidism despite high thyroid hormone levels, have also been described [434-436]. 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 TSH Diagnostic Dilemmas

Causes of technical interferences with TSH measurement are similar to those discussed for thyroid hormone tests.

Non Analyte -Specific Interferences

Heterophile Antibodies (HABs) can cause falsely high TSH IMA tests [289,294,300,328-

330, 609]. The HAb in some patient's sera interfere strongly with some manufacturers tests but appear inert in others [609]. This is why re-measurement in a different manufacturers assay should be the first test for interference. A fall in TSH in response to blocker-tube treatment 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 Rhuthenium) that cause interference with TSH and/or free hormone tests. It should be noted that the anti-Rhuthenium antibodies of different patients may affect different analytes to different degrees [339-342].

Tests employing Streptavidin-Biotin

reagents are prone to interferences from antibodies targeting either Streptavidin [344] or biotin reagents [345]. Alternatively, high dose biotin ingestion has been known to produce interference with thyroid and other tests in an analyte-specific, platform-specific manner [346-350].

Analyte-specific interferences typically result from autoantibodies targeting the analyte. Depending on the analyte and test formulation, autoantibody interferences typically cause falsely-high test results, but can cause falsely-low test results, as in the case of Tg autoantibodies. It should be noted that transplacental passage both heterophile antibodies or anti-analyte autoantibodies (i.e. TSHAb or T4Ab) have the potential to interfere with neonatal screening tests [351-354]. Specifically, maternal TSH autoantibodies can cross the placenta and may cause a falsely high TSH screening test in the newborn mimicking congenital hypothyroidism, whereas maternal T4 autoantibodies could cause falsely high neonatal T4 masking the presence of congenital hypothyroidism [230, 353].

TSH Autoantibodies (TSHAb)/"Macro TSH".

Analytically suspicious TSH measurements are not uncommon [290] and have been reported in up to five percent of specimens subjected to rigorous screening [294]. In recent years there have been a number of reports of TSHAb, often referred to as "macro" TSH, causing spuriously high TSH results in a range of different methods [610,611]. The prevalence of TSHAb approximates 0.8 percent, but can as high as ~1.6 percent in patients with subclinical hypothyroidism. Showing a lowering of TSH in response to a polyethylene glycol (PEG) precipitation of immunoglobulins is the most convenient test for TSHAb [599,611]. Alternatively, column chromatography can show TSH immunoactivity in a high molecular weight peak representing a bioinactive TSH-immunglobulin complex [599,611].

TSH Variants.

TSH variants are a rare cause of interference [612]. Nine different TSH beta variants have been identified to date [613]. These mutant TSH molecules may have altered immunoactivity and be detected by some TSH IMA methods but not others [612]. The bioactivity of these TSH mutants is variable and can range from normal to bio-inert [613], resulting in discordances between the TSH concentration and clinical status [612] and/or discordant TSH/FT4 relationships [613]. These TSH genetic variants are one of the causes of congenital hypothyroidism [614-616].

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 [37,617]. 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 [37,618,621]. Unfortunately, the diagnostic and prognostic value of these tests has been hampered by methodologic differences as well as difficulties with assay standardization [622]. 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 [44,582,620,623]. Thyroid autoantibody testing can be useful for diagnosing or monitoring treatment for a number of clinical conditions, however 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 [624,625]. Thyroid gland stimulation occurs when TSH binds to TSHR on thyrocyte plasma membranes and activates the cAMP and phospholipase C signaling pathways [625]. 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 [624]. A TSH-like thyroid stimulator found uniquely in the serum of Graves' disease patients was first described using a guinea pig bioassay system in 1956 [626]. Later, using a mouse thyroid bioassay system 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 [627,628]. Much later, the LATS factor was recognized not to be a TSH-like protein but an antibody that was capable of stimulating the TSH receptor causing Graves' hyperthyroidism [629]. TSH receptor antibodies have also become implicated in the pathogenesis of Graves' ophthalmopathy [629-632]. 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 that the actions of TSH and cause Graves' hyperthyroidism and (b), blocking antibodies (TBAb) that block TSH binding to its receptor and can cause hypothyroidism [37,48,621,625,629,633,634]. Although TSH, TSAb and TBAb appear to bind to different sites on the TSH receptor ectoderm, TSAb and TBAb have similar affinities and often overlapping epitope specificities [635]. In some cases of Graves' hyperthyroidism, TBAb have been detected in association with TSAb [636,637] and the dominance of one over the other can change over time in response to treatment [638]. 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 may influence the severity of Graves' hyperthyroidism and the response to antithyroid drug therapy or pregnancy [624,636,639-643]. 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 [641,644].

Two different methodologic approaches have been used to quantify TSH receptor antibodies [40,620,633,645]: (i) TSH receptor tests (TRAb assays) also called TBII or TSH Binding Inhibition Immunoglobulin assays, and (ii) 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 [620,646]. However, assay sensitivity varies among current receptor versus bioassay methods [43]

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 [626,633,647-652]. Later, TRAb bioassays used cells with endogenously expressed or stably transfected human TSH receptors and could use unextracted serum specimens [653-655]. 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 [40,42,620,648,651,653,656]. 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 [40,657]. The most recent development is for 2nd 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 [42].

TSH Receptor (TRAb)/TSH Binding Inhibitory Immunoglobulin (TBII) Methods

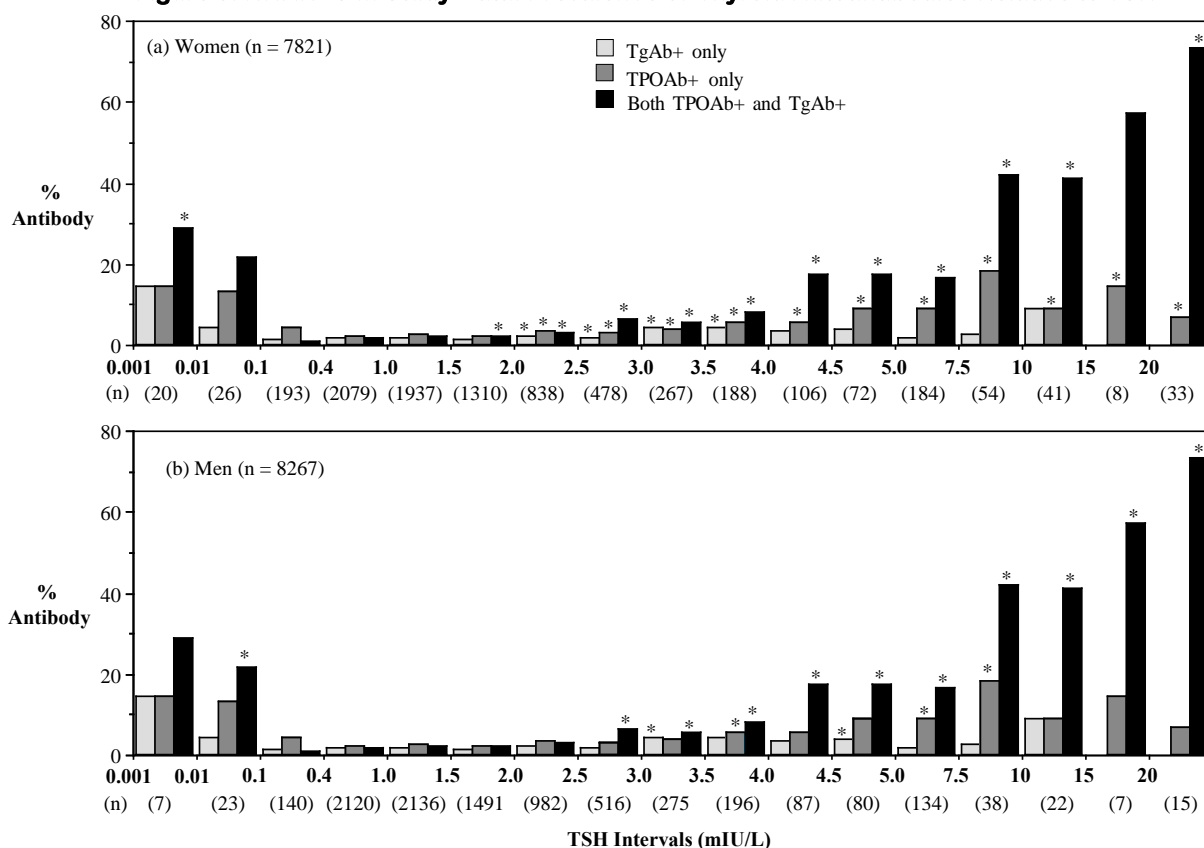
TRAb methods detect serum immunoglobulins that bind TSHR but do not functionally discriminate stimulating from blocking antibodies. TRAb methods are based on standard competitive or noncompetitive principles. First generation methods were liquid-based whereby immunoglobulins in the serum inhibited the binding of ¹²⁵I-labeled TSH or enzyme-labeled TSH to a TSH receptor preparation [40,658]. These methods used TSH receptors of human, guinea-pig or porcine origin [658]. After 1990, a second-generation of both isotopic and non-isotopic methods were developed that used and immobilized porcine or recombinant human TSH receptors [40,659-661]. These second-generation methods were shown to have significantly more sensitivity for detecting Graves' thyroid stimulating immunoglobulins than first-generation tests [620]. 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) [37,40,42,620,648,656,660,662-666]. 3rd generation assays have also shown a good correlation and comparable overall diagnostic sensitivity with bioassay methods [620,636,648,667,669]. Current third-generation tests have now been automated on several immunoassay platforms [620]. However, between-method variability remains high and interassay precision often suboptimal (CVs > 10 %) despite the use of the same international reference preparation for calibration [622,670]. This fact makes it difficult to compare values using different methods and indicates that further efforts focused on additional assay improvements are needed [37,622,671].

Clinical Use of TRAb Tests

Over the last ten years automated IMA methods have dramatically lowered the cost and increased the availability of TRAb testing [43,646,672]. Automated TRAb IMAs are not functional tests and do not distinguish between stimulating and blocking TRAbs. 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 [42,673]. 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 [620,672], as an independent risk factor for Graves' ophthalmopathy [632] and may be useful for monitor responses to therapy [620,674,675]. TRAb measured prior to radioiodine therapy for Graves' hyperthyroidism can also help predict the risk for exacerbating ophthalmopathy [630,676-680]. There is conflicting data concerning the value of using TRAb to predict the response to antithyroid drug treatment or risk of relapse [42,637,661,667,681-685]. 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 [40,67,620,645,686-689]. 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 [690]. It is currently recommended [74] 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. When TRAb is high in the first trimester additional TRAb testing is recommended at weeks 18-22 and 30-34 [24,37,67,74,636,687,691].

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 titre and IgG subclass and display complement-fixing properties [692]. Studies have shown that epitope fingerprints are genetically conserved suggesting a possible functional importance [693]. However, it is still unclear whether the epitope profile correlates with the presence of, or potential for, the development of thyroid dysfunction with which TPOAb presence is most commonly associated [692,694,697].

Figure 5. NHANES III Study Data: Prevalence of Thyroid Autoantibodies Relative to TSH

Prevalence of thyroid antibodies across TSH intervals in women (A) and men (B). The 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 [456].

TPOAb antibodies were initially detected as antibodies against thyroid microsomes (antimicrosomal antibody, AMA) using semi-quantitative complement fixation and tanned erythrocyte hemaagglutination techniques [698-700]. Recent studies have identified the principal antigen in the AMA tests as the thyroid peroxidase (TPO) enzyme, a 100 kD glycosylated protein present in thyroid microsomes [701, 702]. Manual agglutination tests have now been replaced by automated, more specific TPOAb immunoassay or immunometric assay methods that use purified or recombinant TPO [24,37,619,703-710]. Despite calibration against the same International Reference Preparation (MRC 66/387), there is considerable inter-method variability of current TPOAb assays (correlation coefficients 0.65 and 0.87) that precludes the numeric comparison of serum TPOAb values reported by different tests [37,618,619,706,709,710]. 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 [37,619]. 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 [37,582,710].

TPOAb Clinical Significance

Estimates of TPOAb prevalence depend on the sensitivity and specificity of the method employed [582,710,711]. In addition, ethnic and/or geographic factors (such as iodine intake) influence the TPOAb prevalence in population studies [487]. For example, TPOAb prevalence is significantly higher (~11 percent) in dietary iodine-sufficient countries like the United States and Japan as compared with iodine deficient areas in Europe (~ 6 percent) [452,515,712]. 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 [452,712]. Approximately 70-80 % of patients with Graves' disease and virtually all patients with Hashimoto's or post-partum thyroiditis have TPOAb detected [619,706,709,711,713]. TPOAb has, in fact, been implicated as a cytotoxic agent in the destructive thyroiditic process [697,714,717]. However, TPOAb prevalence is also significantly higher in various non-thyroidal autoimmune disorders in which no apparent thyroid dysfunction is evident [718-720]. Aging is associated with an increasing prevalence of TPOAb that parallels the increasing prevalence of both subclinical (mild) and clinical hypothyroidism [452]. In fact, the NHANES III survey reported that TPOAb prevalence increases with age and approaches 15-20 percent in elderly females in the iodine-sufficient United States [452]. This same study found that the odds ratio for hypothyroidism was strongly associated with the presence of TPOAb but not TgAb, suggesting that only TPOAb has an autoimmune etiology [452]. 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 5), although further studies would be needed to confirm this [452,456,459,697]. It is now apparent that the presence of TPOAb in the serum of 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 2 percent per year in such populations [46,532,692,693].

In this context, it is reasonable to assume that TPOAb measurement may serve as a useful prognostic indicator for future thyroid dysfunction [46,721]. However, it is noteworthy that the detection of TPOAb does not always precede the development of thyroid dysfunction. A recent study suggests that a hypoechoic ultrasound pattern can be seen before a biochemical TPOAb abnormality is detected [458,487]. Further, some individuals with unequivocal TSH elevations, presumably resulting from autoimmune destructive disease of the thyroid, do not have TPOAb detected [456]. 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) [452,456,710,722].

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 [359,619,723]. 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 employ 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 [24,101,724-730].

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 and

post-partum thyroiditis and depression [66,67,564,731-742]. However, if this association represents cause or effect has yet to be resolved.

Thyroglobulin Autoantibodies (TgAb)

Thyroglobulin autoantibodies predominantly belong to the immunoglobulin G (IgG) class, are not complement fixing and are generally conformational [743]. Serum TgAb were the first thyroid antibody to be detected in patients with autoimmune thyroid disorders using tanned red cell hemagglutination techniques [699]. 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 most recently non-isotopic competitive or non-competitive immunoassays [10,37,44,706,710,713,744-747]. Unfortunately, the inter-method variability of these TgAb assays is even greater than that of TPOAb tests discussed above [10,37,44,745-747]. Additionally, high levels of thyroglobulin in the serum have the potential to influence TgAb measurements [747-750]. 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 in the serum [751,752]. As with TPOAb methods, TgAb tests have highly variable sensitivity limits and cut-off values for "TgAb positivity", despite the use of the same International Reference Preparation (MRC 65/93) (Figure 6) [10, 44, 745-747, 753]. It should be noted that the manufacturer-recommended cutoffs are set for diagnosing thyroid autoimmunity and are too high for detecting levels of TgAb that interfere with Tg measurements - the much lower assay FS limit (Figure 6) is the recommended cutoff to define TgAb-positivity for DTC monitoring [24]. 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 [744,754]. 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 [744,752,755]. 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.

Clinical Utility of TgAb Tests

Autoantibodies against Tg are encountered in autoimmune thyroid conditions, usually in association with TPOAb [46,452,746, 756]. 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 5) [452,456]. Further, in these subjects there was no association observed between the isolated presence of TgAb and TSH abnormalities [452,456]. This suggests that it may be unnecessary to measure both TPOAb and TgAb for a routine evaluation of thyroid autoimmunity [37,46,456]. In fact, 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 5) [46,456,459,757].

TgAb measurement is primarily used as an adjunctive test to serum Tg measurement when monitoring patients with differentiated thyroid cancers (DTC) [72,593]. The role of TgAb testing is two-fold: 1) to authenticate that a Tg measurement is not compromised by TgAb interference, 2) as an independent surrogate tumor-marker in the ~20 percent of patients with circulating TgAb. Current guidelines recommend that all sera be prescreened for TgAb by a sensitive immunoassay method prior to serum Tg testing, because there appears to be no threshold TgAb concentration that precludes TgAb interference with Tg measurements

[9,10,24,44,593,713,746,758]. Immunoassay methods detect TgAb in approximately 25 percent of patients presenting with DTC [44,713,759-761]. The prevalence of TgAb is typically higher in patients with papillary versus follicular tumors and is frequently associated with the presence of lymph node metastases [746,759,761, 62]. Perhaps of even greater importance is the observation that serially determined TgAb concentrations may also serve as an independent parameter for detecting changes in tumor mass in patients with an established diagnosis of DTC [Figure 6Ad(ii)] [761-766]. For example, 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 [761,762,766]. In contrast, a rise in, or de novo appearance of, TgAb is often the first indication of tumor recurrence [713,761,762]. However, when using serial TgAb measurements as a surrogate marker for changes in tumor burden it is essential to use the same TgAb method, because of the large between-method differences observed with this assay (Figure 6) [9,10,44,713,745,747,753].

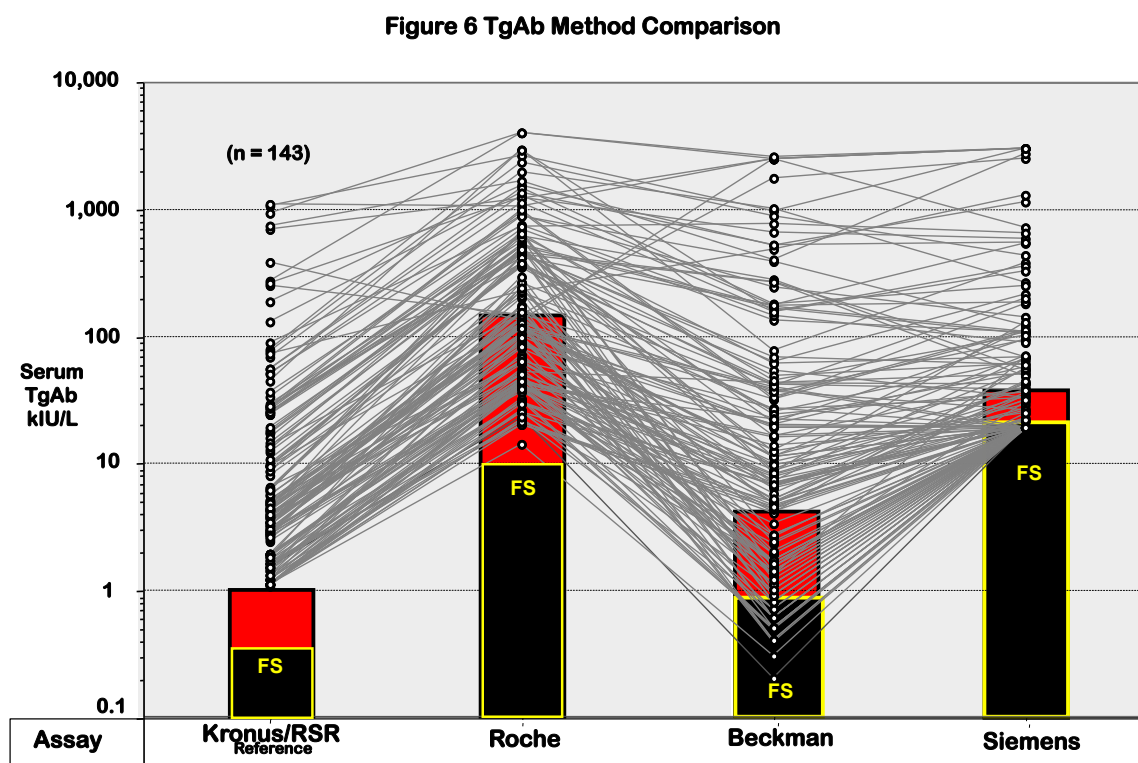


Figure 6. TgAb Method Comparison. 143 DTC sera with TgAb above the MCO for the reference method (Kronus/RSR) and evidence of TgAb interference with Tg measurement (Tg-IMA/Tg-RIA ratios < 80 percent) were measured by three different TgAb methods. The red bars show the manufacturer recommended cutoff for TgAb-positivity for each method. The black bar denotes the functional sensitivity (FS) of each method [44].

THYROGLOBULIN (TG)

Thyroglobulin plays a central role in a wide variety of pathophysiologic thyroid conditions, including acting as an autoantigen for thyroid autoimmunity [617,743,767]. Serum Tg levels can serve as a marker for iodine status of a population [768-771], whereas dyshormonogenesis resulting from genetic defects in Tg biosynthesis is a cause of congenital hypothyroidism [24,772-775]. Because Tg has a thyroid-tissue specific origin, a Tg measurement can aid in determining the etiology of congenital hypothyroidism (athyreosis versus dyshormonogenesis) [776,777]. Likewise, a paradoxically low serum Tg can be used to distinguish factitious hyperthyroidism from the high Tg expected with endogenous hyperthyroidism [778-780]. This chapter focuses on the primary clinical use of Tg measurement - a tumor-marker test for post-operative monitoring of patients with follicular-derived (differentiated) thyroid cancer (DTC) [32,72,404,781-788]. (Table 3)

Most Tg testing is currently made by rapid, automated immunometric assays (IMA) with second-generation functional sensitivity (2G -Tg-IMA, $FS \leq 0.1 \mu\text{g/L}$). Assays with this level of FS obviate the need for recombinant human TSH (rhTSH) stimulation [11,32,72,416,784,789-793]. The major limitation of IMA methodology is its propensity for TgAb interference causing falsely low/undetectable serum Tg-IMA that can mask disease [10,31,45,58,760,790,794-798]. Currently, most laboratories in the United States first establish the TgAb status of the specimen (negative or positive) in order to restrict Tg-IMA testing to TgAb-negative sera, whereas TgAb-positive specimens are reflexed for testing by Tg methodologies believed to be less prone to interferences, such as RIA [30,32,796] or LC-MS/MS [31,799-801].

Technical Limitations of Tg Methods

Thyroglobulin measurement remains technically challenging [788]. Five methodologic problems impair the clinical utility of this test: (a) between-method biases; (b) suboptimal functional sensitivity; (c) suboptimal between-run precision over the typical clinical interval used to monitor DTC patients (6-12 months); (d) "hook" problems (some IMA methods), and interferences caused by both (e) Heterophile antibodies (HAb) and (f) Tg autoantibodies (TgAb).

Tg Assay Functional Sensitivity

As discussed for TSH, assay functional sensitivity (FS) represents the lowest analyte value that can be reliably detected under clinical practice conditions. For Tg assays FS is defined by the lowest Tg concentration that can be measured in human serum with 20 percent coefficient of variation (CV) in runs made over a 6-12 month period using at least two different lots of reagents and two instrument calibrations [24,58,72,404,802]. These stipulations are needed because assay precision erodes over time and the clinical interval for serum Tg monitoring of DTC patients is typically long (6-12 months) [9,408,803]. For Tg assays it is critical to use FS as the lowest reporting limit in preference to a LOQ calculation (20 percent CV), because LOQ does not stipulate a relevant time-span for assessing precision [24,405,407,804,805]. Another stipulation of the FS protocol [24] is to assess precision using the appropriate test matrix (human serum) in preference to a commercial QC preparation, because instruments and methods are matrix-sensitive [407]. Since Tg-IMA testing is typically restricted to TgAb-negative sera, precision estimates should be made in TgAb-negative human serum pools [407]. In contrast, Tg-RIA and Tg-LC-MS/MS testing is typically reserved for sera containing TgAb, necessitating precision estimation in TgAb-positive human serum pools.

As with TSH [220,397], there has been a progressive improvement in the FS of Tg methods

that has led to the adoption of a generational approach to Tg assay nomenclature. Currently, some Tg-IMAs, all Tg-RIAs and all Tg-LC-MS/MS methodologies still only have first-generation functional sensitivity (FS = 0.5-1.0 µg/L) [4,10,32,33,58]. Over the last ten years second-generation immunometric assays (^{2G}-Tg-IMA), characterized by an order of magnitude greater functional sensitivity (FS 0.05-0.10 µg/L), have become available. ^{2G}-Tg-IMA testing is now considered the standard of care in the absence of TgAb [31-33,58,72,296,783,806-808]. When disease is absent the basal serum ^{2G}-Tg-IMA is typically below 0.5 µg/L, even without RAI treatment [809,810]. It follows that the inferior FS (~1 µg/L) of first-generation assays can barely distinguish subnormal values from the Tg levels seen when an intact thyroid gland is present (~2-40 µg/L), and are clearly too insensitive to detect recurrences in thyroidectomized patients unless recombinant human TSH (rhTSH) stimulation is employed [296,593,758,782,811,812]. Now that ^{2G}-IMA-Tg testing has become the standard of care [72], there is no longer a need for routine rhTSH stimulation to boost the Tg level to values detectable by first-generation tests, because basal (TSH suppressed) Tg correlates with rhTSH-stimulated Tg measured by ^{2G}-Tg-IMA [10,11,32,58,72,296,413, 416,789,791-793,806,807,813-816]. Studies have shown that a basal ^{2G}-Tg-IMA below 0.1 µg/L predicts a negative rhTSH test (rhTSH-stimulated Tg <2.0 µg/L) with a high degree of confidence [72,296,791,792,817]. Even so, the use of a 2nd generation Tg assay does not eliminate the need for periodic ultrasound examinations, because many histologically confirmed lymph nodes metastases are inefficient Tg secretors and may be associated with an undetectable serum Tg, even when measured by ^{2G}-Tg-IMA [807,818-821].

Figure 7. Serum Tg Measurements in DTC Patients with Structural Disease

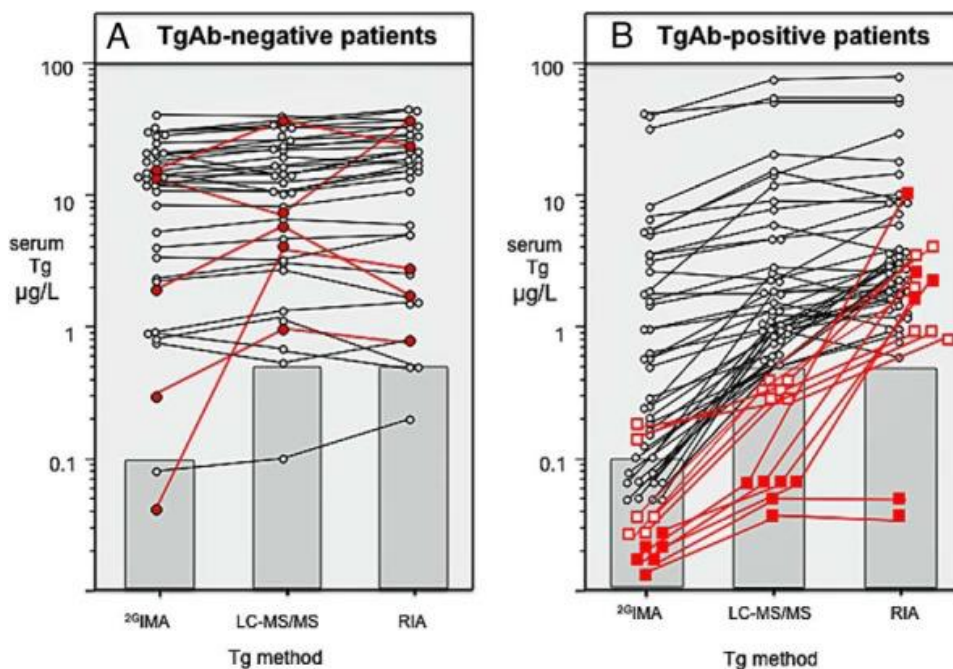


Figure 7. Panel A shows the comparison of serum Tg values reported for 37 TgAb-negative DTC patients with persistent/recurrent DTC measured by a ^{2G}-Tg-IMA (Beckman), Tg-LC-MS/MS (Mayo Medical Labs) and the USC Tg-RIA method. Sera with Tg values below the FS limit of the method are shown in the shaded areas, Although each method was standardized against CRM-457, the sera marked in red displayed > 30% difference in Tg

values that reflected different method specificities for detecting tumor-derived Tg molecules - differences with the potential to disrupt clinical management following a change in Tg method. Panel B shows the method comparison for 52 TgAb-positive DTC patients with structural disease. Sera with unequivocally undetectable Tg-LC-MS/MS values (no peak) are shown by solid red squares, whereas sera with marginally detectable Tg-LC-MS/MS values in the 0.3 to 0.5 µg/L range are shown by open red squares [31].

Between-Method Biases

Although most Tg methods claim to be standardized against the Certified Reference Preparation CRM-457 [9,822,823] there can be significant differences between the Tg values reported for the same serum measured by different methods, even in the absence of TgAb (Figure 7A) [10,19,24,32,58,799,824]. Between-method Tg variability is higher than the biologic variability (~16 percent) in euthyroid subjects [442, 803]. In fact, studies have shown that there can be a two-fold difference in Tg values reported for the same serum measured by different methods [32]. Although this reflects standardization and matrix differences to some extent [299,797], for the most part this between-method variability reflects differences in method specificities for detecting heterogeneous serum Tg isoforms [10,825-827]. It should be noted that because IMA methodology uses monoclonal antibody reagents, IMAs have narrower specificities for detecting Tg heterogeneity than RIA methods that use polyclonal antibodies [9,10,826-829]. Because Tg-IMAs differ in their sensitivity to TgAb interference, between-method Tg variability can also result from using an insensitive TgAb test that reports false-negative TgAb values (Figure 6) [19,44,830].

When TgAb is absent and a ²⁶G-Tg-IMA method is used consistently, the between-run precision across a 6-12 month timespan (the typical interval for monitoring DTC patients) is less than 10%, yet the between-method variability seen for some TgAb-negative patients (shown in Figure 7A by red lines) can be greater than 30 percent [31]. These differences likely reflect different method specificities for detecting heterogeneous serum Tg isoforms. Clearly this magnitude of between-method difference has the potential to disrupt serial Tg monitoring and could negatively impact clinical management should a change in Tg method be made without re-baselining the Tg level [10,24,58,72,805]. In recognition of the differences between Tg methods, current guidelines stress the critical importance of using the same Tg method (and preferably the same laboratory) to monitor the serum Tg trend in DTC patients [72].

High-Dose Hook Effect

Tumor marker tests employing IMA methods 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 [9,831-834]. This phenomenon reduces the ability of the endogenous analyte to form a bridge between the capture and signal monoclonals resulting in an inappropriately low signal [9,831,835,836]. 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 followed by a second incubation when signal binds captured antigen [790,832]. When using any particular IMA method, it is primarily the laboratory's responsibility to determine whether a hook effect is likely to generate falsely normal or low values.

Approaches for detecting and overcoming hook effects occurring with IMA methods are:

- Routinely run each specimen at two dilutions. For example, the value obtained with a 1/5 or 1/10 dilution of the test serum would, if a hook effect were present, be higher than that obtained with an undiluted sample.
- To carry out appropriate dilution studies to rule out a possible hook effect when an unexpectedly low serum Tg value is encountered for a patient with known metastatic disease. In such cases, consultation with the physician may provide valuable information regarding this issue.
- To perform a Tg recovery test. If there is a hook effect present, the recovery of added antigen (Tg) will produce an inappropriately low result.

Interferences with Tg Measurement

Heterophile Antibody (HAb) Interferences

As discussed for FT4 and TSH, HAb, including human anti-mouse antibodies (HAMA) and Rheumatoid Factor (RF), interferes selectively with IMA but not RIA or Tg-LC-MS/MS methodologies [295,296,318,323,324,331,332,761,837]. HAb interferences are thought to reflect the binding of human immunoglobulins in the serum specimen to the murine-derived monoclonal antibody IMA reagents. The rabbit polyclonal antibodies (PAb) used for Tg-RIA methods are not susceptible to this problem. In most cases HAb interferences are characterized by a false-positive Tg-IMA result [323,324,331,784], although falsely-low Tg-IMA results have also been reported [332].

Tg Autoantibody (TgAb) Interferences

TgAb interference with Tg measurement remains the major problem that limits the clinical utility of Tg testing. TgAb has the potential to undermine the clinical reliability of Tg measurements by both in-vitro mechanisms (epitope masking/low recoveries) [10,760,796,838, 839] and/or in-vivo mechanisms (enhanced TgAb-mediated Tg clearance) [677,840-842], irrespective of the Tg methodology used. There appears to be no threshold TgAb concentration that precludes TgAb interference [9,10,24,31,44,72,746,796,830]. High TgAb concentrations do not necessarily interfere, whereas low TgAb may profoundly interfere [9,31,44,761,795,796,830,839,843-846]. The Tg recovery approach is not reliable for detecting TgAb interference [10,752,839].

In-vitro Mechanisms of TgAb Interference

TgAb interferes with Tg testing in a qualitative, quantitative and method-dependent manner [44,761,796,838,847,848]. The potential for in vitro interference is multifactorial and depends not only on the assay methodology (IMA, RIA or LC-MS/MS), but also the concentration and epitope specificity of the patient's TgAb [10,761,844]. RIA methodology appears to quantify total Tg (free Tg + TgAb-bound Tg) whereas IMA primarily detects only the free Tg moiety - Tg molecules whose epitopes are 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 [31,44,713,797,849,850]. The new Tg-LC-MS/MS methodology uses trypsin digestion of Tg-TgAb complexes to liberate a Tg proteotypic peptide. This conceptually attractive approach was primarily developed to overcome TgAb interference with IMA and thereby eliminate falsely low/undetectable Tg-IMA results that can mask disease. However, recent studies have reported a high percentage (>40%) of TgAb-positive DTC patients with structural disease who have paradoxically undetectable Tg-LC-MS/MS [31,799-801]. The reason

why LC-MS/MS fails to detect Tg despite disease when TgAb is present needs further study. Possibilities to investigate include, tumor Tg polymorphisms that prevent the production of the Tg-specific tryptic peptide [21], 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 [677,840-842].

In-vivo Mechanisms of TgAb Interference.

A number of studies over past decades have suggested that the presence of TgAb enhances Tg metabolic clearance. In the 1967 Weigle et al showed enhanced clearance of endogenously ¹³¹I-labeled Tg in rabbits, after inducing TgAb by immunizing the animals with an immunogenic Tg preparation (840). Human studies of Tg and TgAb acute responses to sub-total thyroidectomy have also suggested that TgAb may increase Tg metabolic clearance (851). Changes (rise or fall) in TgAb versus Tg-RIA concentrations are typically concordant and appropriate for clinical status, whereas the direction of change of Tg-IMA is typically discordant with not only TgAb but also Tg-RIA and clinical status (31,32,44,713,798). In general, the change in TgAb concentrations tends to be steeper than for Tg-RIA (713), as would be consistent with TgAb-mediated Tg clearance. It may be that some TgAbs act as "sweeper" antibodies that facilitate clearance of antigen (842,852-854).

Figure 9. Influence of Changing TgAb status on Tg-IMA & Tg-RIA)

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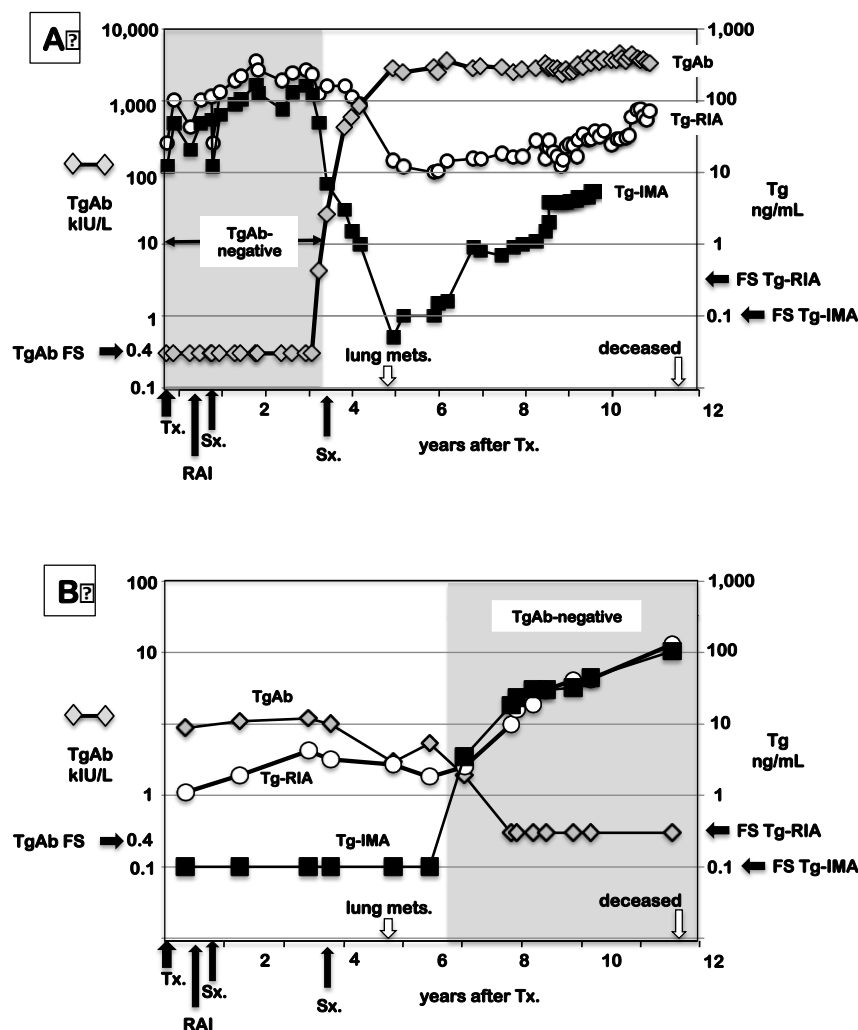


Figure 9 Serial TgAb, Tg-RIA and Tg-IMA concentrations in two DTC patients who underwent a change in TgAb status (panel A, negative to positive) or (panel B, positive to negative) before death from structural DTC. Panel A: When TgAb appeared de novo 2.5 years after initial treatment (thyroidectomy, Tx + RAI) for PTC a progressive fall in Tg-IMA to undetectable levels occurred together with an approximate 90 percent fall in Tg-RIA. Thereafter as disease exacerbated, TgAb remained elevated and Tg-IMA rose to parallel Tg-RIA but at an 80 percent lower concentration. Panel B. This patient was TgAb-positive at the time of initial Tx+RAI treatment at which time Tg-RIA was detectable and Tg-IMA was undetectable. Despite extensive disease, TgAb became undetectable 5 years after initial treatment. This change in TgAb status was associated with a rapid rise in Tg-IMA to parallel a steep increase in Tg-RIA with a doubling time <1 year before demise.

Figure 9 provides insights on the influence of TgAb on Tg measurements. These two

DTC patients who eventually died of structural disease, illustrate how changes in TgAb status (Panel A-TgAb-negative to TgAb-positive versus Panel B- TgAb-positive to TgAb-negative) can produce Tg method discordances. These patients also serve to illustrate how disparate TgAb versus Tg responses can be associated with a poor prognosis and emphasize why a Tg measurement cannot be interpreted without knowing the TgAb status of the specimen (72). The de novo appearance of TgAb in the patient shown in Figure 9A either reflects a change in tumor-derived heterogeneity (secretion of a more immunogenic Tg molecule), or immune system recognition of tumor-derived Tg. In the patient shown in Figure 9B, TgAb was lost despite exacerbation of disease. This TgAb loss could be a response to the decrease in normally iodinated Tg antigen as normal remnant tissue was destroyed by RAI, at the same time as poorly iodinated (less immunogenic) tumor-derived Tg was rising with exacerbation of disease.

TgAb interference with Tg-RIA.

Tg-RIA methodology is based on Tg antigen (from serum or added ^{125}I -Tg tracer) competing for a low concentration of polyclonal (rabbit) Tg antibody (PAb). After incubation, the Tg-PAb complex is precipitated and the serum antigen concentration quantified as an inverse relationship to the ^{125}I -Tg in the precipitate. The first Tg-RIAs developed in the 1970s were very insensitive ($\sim 2 \mu\text{g/L}$) (4,855). Over subsequent decades some Tg-RIAs have achieved first-generation functional sensitivity ($\text{FS} = 0.5 \mu\text{g/L}$) by using a 48-hour pre-incubation before adding a high specific activity ^{125}I -Tg tracer (856,857). The use of a high affinity PAb (858) coupled with a species-specific second antibody minimizes TgAb interference. Resistance to TgAb interference is evidenced by appropriately normal Tg-RIA values for TgAb-positive euthyroid controls (10) and detectable Tg-RIA for TgAb-positive DTC patients with structural disease (Figures 7B and 8) (31). The clinical performance of this Tg-RIA contrasts with IMA methods that report paradoxically undetectable serum Tg for some TgAb-positive normal euthyroid subjects (10) as well as TgAb-positive Graves' hyperthyroid patients (794) TgAb-positive patients with structural disease (Figures 7B and 8) (10). It should be noted that the propensity of TgAb to interfere with Tg-RIA determinations and cause underestimation (859) or overestimation (847,860) depends on not only the assay formulation but also patient-specific interactions between the endogenous Tg and TgAb in the specimen and the exogenous RIA reagents (848).

TgAb interference with Tg-IMA.

Non-competitive IMA methodology is based on a two-site reaction that involves antigen capture by a solid-phase monoclonal antibody (MAb) followed by addition of a labeled MAb that targets different epitopes of the captured antigen (377). TgAb interferes with IMA methodology by steric inhibition. Specifically, when the Tg epitope(s) necessary for binding to the IMA monoclonals are blocked by TgAb complexing, the 2-site reaction cannot take place and the test antigen is reported as falsely low or undetectable. This mechanism involving epitope masking is supported by recovery studies (data not shown). Clinically, the Tg-IMA underestimation caused by TgAb interference is evident from paradoxically low/undetectable Tg-IMA seen for TgAb-positive normal controls (10), patients with Graves' hyperthyroidism (794) and DTC patients with active disease (Figures 7B and 8) (9,10,44,45,752,755,846,861-863). High Tg concentrations can overwhelm the TgAb binding capacity rendering Tg-IMA concentrations detectable and lessening the degree of interference (31,44). 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 disappears (Figure 9) (31,44). Although some IMA methods have claimed to

overcome TgAb interference by using monoclonal antibodies directed against specific epitopes not involved in thyroid autoimmunity (790,864), this approach does not overcome TgAb interferences in clinical practice, possibly because less restricted TgAb epitopes are more often associated with thyroid carcinoma than with autoimmune thyroid conditions (746,752,755,862,865).

TgAb Interference with Tg LC-MS/MS.

The new LC-MS/MS methods measure Tg as a Tg-specific peptide(s) generated after trypsinization of serum containing Tg-TgAb complexes (16,21,790,866). Currently LC-MS/MS methods only have first-generation functional sensitivity (FS ~ 0.5 µg/L) (19,20,799). Tg-LC-MS/MS methodology has been shown free from HAb/HAMA interferences (837), and is being promoted as being free from TgAb interference (19,20,799). However, the reliability of using LC-MS/MS to detect Tg in the presence of TgAb is currently questionable. A number of studies have reported that over 40 percent of TgAb-positive patients with structural disease have paradoxically undetectable Tg-LC-MS/MS values (31,799-801). The most recent study concluded that Tg-LC-MS/MS offers no diagnostic advantage over ²⁶Tg-IMA when TgAb is present (801). This study also confirmed earlier observations (867) that the higher the TgAb concentration, the more likely that Tg-LC-MS/MS would be undetectable despite disease (801). An inverse relationship between TgAb concentration and Tg-LC-MS/MS detectability would be expected if the presence of TgAb enhanced Tg clearance in vivo (see above).

Use of the TgAb Trend as a Surrogate DTC Tumor-Marker (Table 2)

It is now generally recognized that the serum TgAb concentration can be used as a surrogate tumor-marker for TgAb-positive DTC patients in whom the reliability of Tg testing is compromised by TgAb interference [Figures 9 and 10] (24,32,45,72,743,761-764,766,796,868-874). Following successful surgery (± RAI treatment), TgAb concentrations typically decline progressively over subsequent months, and may become undetectable during the first few post-operative years as a result of reduced Tg antigen stimulation of the immune system (32,44,72,762-766,870,875). The time needed for TgAb to become undetectable is inversely related to TgAb concentration around the time of initial treatment (32). It should be noted that in the early post-operative period a significant percentage (~5%) of TgAb-negative patients develop transient de novo TgAb-positivity, presumably a response to Tg antigen released by surgical trauma (876). Such TgAb-negative to TgAb-positive conversions is one reason why Guidelines mandate that TgAb be measured with every Tg test (45,72). Transient rises in TgAb may be seen in response to the acute release of Tg following thyroid surgery (877,878), fine needle aspiration biopsy (879,880) or more chronically (months) in response to radiolytic damage following RAI treatment (759,761,881-884). 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 (762,874,876,885,886). In contrast, patients with persistent/recurrent disease may exhibit only a marginal TgAb decline, or have stable or rising TgAb (760,762,764,796,868,874). In fact, a rise, or de novo appearance of, TgAb, is an indication of persistent/recurrent disease (Figure 9A) (9,10,32,44,745,747,753,762-764,796,850,873,887). Because TgAb tests differ in sensitivity and specificity (44,45,745,753,888,889) (Figure 6), it is essential that serum TgAb concentrations be measured using the same manufacturers method and preferably the same laboratory (10,44,45,72,710,745,747,753,796,888,890,891).

Table 3--Clinical Significance of Changes in TgAb Concentrations:

1. Approximately 25 percent of DTC patients have TgAb detected before or within three months of surgery [713,760]. TgAb prevalence in DTC patients is double that of the general population [452,713].
2. Pre-operative TgAb-positivity is a risk factor for PTC in nodules with indeterminate cytology [892-895].
3. The post-operative trend in TgAb (measured with the same method and preferably by the same laboratory) can be a useful surrogate tumor marker. A declining TgAb trend is a good prognostic sign, whereas a stable or rising TgAb may indicate persistent/recurrent disease [24,32,45,72,743,762-764,766,796,868-870,872-874].
4. After successful treatment for DTC, TgAb (and Tg-RIA) concentrations typically fall more than 50% in first post-operative year and continue to fall in subsequent months-years, often becoming undetectable within a median time of four years [32,760,761,876].
5. With successful treatment of disease, serum Tg-RIA typically becomes undetectable (< 0.5 µg/L) before TgAb [32,896].
6. The time needed for a TgAb-positive patient to become TgAb-negative in response to successful treatment is proportional to the initial TgAb concentration, perhaps
7. Approximately 10 percent of TgAb-negative DTC patients develop TgAb-positivity during post-operative monitoring [850], necessitating TgAb measurement with every Tg test [45,72].
8. Most (75 %) TgAb-negative to TgAb-positive conversions are transient (months) and occur in response to the release of Tg antigen by surgical trauma [677,877], fine-needle biopsy [880] or RAI treatment [759,761,881-884].
9. Approximately 3 percent of TgAb-negative DTC patients exhibit a de novo TgAb appearance more than one year following thyroidectomy without an initiating factor (surgery, biopsy or RAI treatment). Such TgAb-negative to TgAb-positive conversions are often associated with the presence representing the long-lived memory of plasma cells [32,896,897]. of metastatic disease, such as illustrated in Figure 9A [763,887].
10. The de novo appearance of TgAb is typically associated with a rapid fall in Tg-IMA, often to undetectability, as a result of TgAb interference (Figure 9A). TgAb interference is less apparent when Tg-IMA is high before a TgAb appearance, because a high Tg concentration can saturate TgAb binding sites and reduce interference [31,44].
11. When serum Tg (RIA or IMA) persists after TgAb disappearance (~3% of cases) risk for disease remains (Figure 9B).

The Use of Serum Tg for Monitoring Patients with DTC

Over the past decade, the incidence of DTC has substantially risen partly as a result of detecting small thyroid nodules and micropapillary cancers (72,898-900) by ultrasound and other anatomic imaging modalities used for nonthyroidal purposes (901-904). Although most DTC patients are rendered disease-free by their initial surgery, overall approximately 15 percent of patients experience recurrences and approximately 5 percent die from disease-related complications (790,905-908). A risk-stratified approach to diagnosis and treatment is now recommended by current guidelines (72,785,787,908). 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 (906,907). 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. Serum Tg testing is generally recognized more sensitive

for detecting disease than diagnostic ^{131}I whole body scanning (909-912). It is recommended that biochemical testing (serum Tg+TgAb) be used in conjunction with periodic ultrasound (72,787,912,913). 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 (713). In the absence of TgAb, four factors primarily influence the interpretation of serum Tg concentrations: (1) the mass of thyroid tissue present (normal tissue + tumor); (2) The intrinsic ability the tumor to secrete Tg; (3) the presence of any inflammation of, or injury to, thyroid tissue, such as following fine needle aspiration biopsy, surgery, RAI therapy or thyroiditis; and (4) the degree of TSH receptor stimulation by TSH, hCG or TSAAb (24). In the presence of TgAb, interference with Tg measurement remains a problem necessitating a shift in focus from monitoring serum Tg as the primary tumor-marker, to monitoring the serum TgAb concentration as a surrogate tumor-marker.

Serum Tg Reference Ranges

The serum Tg reference range in adults approximates 3-40 $\mu\text{g/L}$ (24,914). Serum Tg is higher in newborn infants but falls to the adult range after two years of age (915,916). However, because most Tg testing is made following surgery (thyroidectomy or lobectomy) for DTC, the Tg reference range is only relevant in the preoperative period. Tg methods can report up to 2-fold differences in numeric values for the same serum specimen (32). Between-method variability reflects differences in assay standardization and specificity for recognizing different serum Tg isoforms (10,58,825-827). When evaluating a thyroidectomized patient, the reference range of the assay should be adjusted for thyroid mass (thyroidectomy versus lobectomy) as well as the TSH status of the patient (24,882).

When using a ^{26}Tg -IMA method standardized directly against the International Reference Preparation CRM-457, Tg should be detectable in all sera from TgAb-negative normal euthyroid subjects. Although the intra-individual serum Tg variability is relatively narrow (CV ~15%) (442,825), the Tg population reference range for TgAb-negative euthyroid subjects is broad, (~ 3 to 40 $\mu\text{g/L}$) (30,58,817,914). It follows that 1 gram of normal thyroid tissue results in ~1.0 $\mu\text{g/L}$ Tg in the circulation under euthyroid TSH conditions (24,917,918). Following a lobectomy, euthyroid patients should be evaluated using a mass-adjusted reference range (1.5 - 20 $\mu\text{g/L}$). The range should be lowered a further 50 percent (0.75 - 10 $\mu\text{g/L}$) during TSH-suppression (24,882). After thyroidectomy, the typical 1-2 gram thyroid remnant (790,919) would be expected to produce a serum Tg below 2 $\mu\text{g/L}$ (with a non-elevated TSH) (809,810). By this same reasoning, truly athyreotic patients would be expected to have no Tg detected irrespective of their TSH status (24).

Pre-operative Tg Measurement

An elevated Tg is a non-specific indicator of thyroid pathology and cannot be used to diagnose malignancy. However, a number of studies have reported that a Tg elevation, detected decades before a DTC diagnosis, is a risk factor for thyroid malignancy (920-926). This suggests that most thyroid cancers secrete Tg protein to an equal or greater degree than normal thyroid tissue, underscoring the importance of Tg as a DTC tumor marker (927). Approximately 50 percent of DTC patients have an elevated preoperative serum Tg, the highest serum Tg concentrations are seen in Follicular > Hurthle > Papillary (927). Up to one-third of tumors may be poor Tg secretors relative to tumor mass, especially tumors containing the BRAF mutation associated with reduced expression of Tg protein (928).

Although current guidelines do not recommend routine pre-operative serum Tg measurement (72,782), 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 (929,930). 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 (928,931).

Post-operative Tg measurement - First Post-Operative Year

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 (882). 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 may be augmented by Tg release from radiolytic damage. Short-term rhTSH stimulation is expected to produce an approximate 10-fold serum Tg elevation (412), whereas chronic endogenous TSH stimulation following thyroid hormone withdrawal results in an approximate 20-fold serum Tg rise (811). 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 (813,895,932-940). Since the half-life of Tg in the circulation approximates 3 days (941), the acute Tg release resulting from the surgical injury 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 (942,943).

The Tg secretion expected from the ~1 gram of normal remnant tissue left after thyroidectomy (790,919), is expected to result in a serum Tg concentration ~1.0 µg/L under non-elevated TSH conditions (24). It should be noted that many thyroidectomized patients have a low serum Tg (0.10 – 0.99 µg/L) detected by ²⁵¹I-Tg-IMA. 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 (32,809,810). 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) (although the positive predictive value (PPV) was only 43 percent) (940).

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 (813,932-940). If a stable TSH is maintained (≤0.5 mIU/L) (32,810), changes in the serum Tg will reflect changes in tumor mass. Under these conditions a rising Tg would be suspicious for tumor recurrence whereas a declining Tg levels suggests the absence or regression of disease. Now that sensitive ²⁵¹I-Tg-IMA methods have become the standard of care, the *trend* in serum Tg, measured without TSH stimulation, is a more reliable indicator for disease status than using a fixed Tg cutoff value to assess disease (32,72,413,785,806,911,940,944-949). It is the degree of Tg elevation, not merely a "detectable" Tg, that is the risk factor for disease, since Tg "detectability" is merely determined by the assay FS (58,783,807,810,816). As with other tumor-markers such as Calcitonin, the Tg doubling time, measured without TSH stimulation, can be used as a prognostic marker that has an inverse relationship to mortality (809,949-956). However,

between-method variability necessitates that the serum Tg trend be established using the same method, and preferably the same laboratory. One approach used to mitigate between-run imprecision and improve the reliability of establishing the Tg trend has been to measure the current specimen concurrently (in the same run) with an archived specimen from the patient, thereby eliminating run-to-run variability and increasing the confidence for detecting small changes in serum Tg (9,804).

Serum Tg responses to TSH Stimulation

The degree of tumor differentiation determines the presence and density of TSH receptors that in large part determine the magnitude of the serum Tg response to TSH stimulation (928,931,957,958). 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) (593,758,811,819,959). RhTSH administration was adopted as a standardized approach for stimulating serum Tg into the measureable range of the insensitive first-generation tests (296,593,758,782,811,812). A consensus rhTSH-stimulated serum Tg cut-off of ≥ 2.0 $\mu\text{g/L}$, measured 72 hours after the second dose of rhTSH, was found to be a risk factor for disease (758,811). A "positive" rhTSH response had a higher NPV (>95 percent) than basal (unstimulated) Tg measured by an insensitive first-generation test (813,818,819,911,937,940,945,946,948,960,961). However, a negative rhTSH test did not guarantee the absence of tumor (811,819,960). 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 7) (10,58). 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 (962-965). One critical variable is the TSH sensitivity of tumor tissues, with poorly differentiated tumors having blunted TSH-mediated Tg responses (928,958,966). When using a more sensitive ^{251}I -Tg-IMA, an undetectable basal Tg (<0.10 $\mu\text{g/L}$) had a comparable NPV to rhTSH stimulation, and was rarely associated with a "positive" rhTSH-stimulated response (>2.0 $\mu\text{g/L}$) (58,296,416,792,806,807,814,816,967,968). This relationship would be expected, given the strong relationship between basal Tg and rhTSH-stimulated Tg values (296,816). As ^{251}I -Tg-IMA methods have become the standard of care, it became apparent the rhTSH-stimulated Tg value provides no additional information over and above a basal Tg measured by 2nd generation assay (58,72,296,416,792,807,814,816,967).

One important use of rhTSH-stimulated Tg testing remains - that as a test for HAb/HAMA/RF interferences. Specifically when the Tg-IMA value appears clinically inappropriate (usually high), an absent rhTSH-stimulated Tg response suggests interference, and a blocker tube test is indicated (296). An alternative reason for an absent/blunted rhTSH-stimulated response is the presence of TgAb (816). A blunted rhTSH-stimulated Tg response might be expected if TgAb enhanced the clearance of Tg-TgAb complexes (794,840,842,851).

Tg Measurement in FNA Needle Washouts (FNA-Tg)

Because 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 (779). Struma ovarii is the only (rare) condition in which the Tg in the circulation does not originate from the thyroid (969,970). 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 (779,971). Lymph node metastases are found in up to 50 percent of patients with papillary cancers but only 20 percent of follicular cancers (972-975). High-resolution ultrasound has now become an important component of the protocols used for postoperative surveillance for recurrence (72,593,758). 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 (976-988). An FNA needle washout is now widely accepted as a useful adjunctive test for improving the diagnostic sensitivity of the cytological evaluation of a suspicious lymph node or thyroid mass (976-981,983,986,987,989). 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 (980,987,990), however this cutoff can vary by assay and Institution (986,991). For investigations of suspicious lymph nodes in patients with an intact thyroid, a higher FNA-Tg cutoff value (~35-40 µg/L) is recommended (978,982). There is still controversy whether TgAb interferes with FNA-Tg analyses (979,992,993). It should be noted that when the serum TgAb concentration is very high and there is serum contamination of the FNA wash, the expected ~40-fold dilution in the wash fluid may be insufficient to lower TgAb below detection, and there is potential for TgAb to interfere with the FNA-Tg IMA test causing a falsely low/undetectable FNA-Tg 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 (971,994-996), and FNA-PTH determinations may be useful for identifying lymph nodes arising from parathyroid tissue (971).

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 (918,997-999). 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 assay interference (1000,1001). More recently, this approach has been applied to the detection of NIS, TPO and TSH receptor (TSHR) mRNA (1001-1005). 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 (918,1001,1006,1007). Further, the recent report of false positive Tg mRNA results in patients with congenital athyreosis (1008) suggests that Tg mRNA can arise as an assay artifact originating from non-thyroid tissues, or illegitimate transcription (1009,1010). Conversely, false negative Tg mRNA results have also been observed in patients with documented metastatic disease (1011-1013). Although Tg, TSHR, NIS and TPO are generally considered "thyroid specific" proteins, mRNAs for these antigens have been detected in a number of non-thyroidal tissues such as lymphocytes, leukocytes, kidney, hepatocytes, brown fat and skin (625,1014-1019). 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 (1006,1011). There is now a general consensus is that thyroid specific mRNA measurements presently lack the optimal specificity and practicality to be useful tumor markers (918,1001,1006). Finally, 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 (625,1017-1019).

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