THYROID
Volume 24, Number 3, 2014

DOI: 10.1089/thy.2013.0119

Thyrotropin Isoforms: Implications for Thyrotropin Analysis and Clinical Practice

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Serum thyrotropin (TSH) is considered the single most sensitive and specific measure of thyroid function in the general population owing to its negative logarithmic association with free triiodothyronine and free thyroxine concentrations. It is therefore often the test of choice for screening, diagnosis, and monitoring of primary hypothyroidism. Serum TSH concentrations can be analyzed quantitatively using third-generation immunoassays, whereas its bioactivity can be measured by TSH activity assays in cell culture. Theoretically, if serum TSH concentrations are directly related to TSH activity, the two tests should yield comparable results. However, on occasion, the results are discordant, with serum concentrations being higher than TSH biological activity. This review focuses on the dissociation between the clinical state and serum TSH concentrations and addresses clinically important aspects of TSH analysis.

Thyrotropin Synthesis

Thyrotropin (TSH) is a heterodimeric 28-kDa-glycoprotein hormone released from the anteromedial pituitary and is a regulator of thyroid function. Its synthesis is controlled by the hypothalamic neuropeptide TSH-releasing hormone (TRH). The two peptide subunits of TSH are noncovalently linked and cotranslationally glycosylated with mannose-rich oligosaccharides (1). Posttranslationally, the two subunits are combined and the attached oligosaccharides are further processed. Synthesis of a mature TSH molecule requires the excision of signal peptides from both TSH α- and β-subunits, followed by trimming of mannose and further addition of fucose, galactose, and sialic acids (2). Thus, mature TSH molecules are asparagine-(N)-linked [Asp(N)-linked] complex carbohydrate structures capped with sulfate and/or sialic acid molecules (3,4) (Fig. 1). TSH oligosaccharide structures vary according to the source of TSH: human pituitary–derived TSH comprises fucosylated biantennary glycans with terminal *N*-acetylgalactosamine sulfate and low sialic acid content (5–7), while recombinant human TSH (rhTSH), synthesized in Chinese hamster ovary cells, specifically terminates in α2,3-linked sialic acid (8–11) and rhTSH formed in yeast lacks sialic acid residues altogether (12).

Glycosylation Patterns and TSH Bioactivity

Appropriate glycosylation is necessary to maintain normal TSH bioactivity (13). Indeed, both α- and β-subunits of TSH have functionally important domains associated with TSH receptor (TSHR) binding and activation. The α-subunit has two Asp(N)-linked oligosaccharide chains with a typical biantennary structure, while TSH β-subunit has only one chain (Fig. 2). The transcriptional and posttranscriptional mechanisms involved in TSH glycosylation result in folding of these subunits, leading to their heterodimerization. These mechanisms also qualitatively regulate TSH secretion, prevent intracellular degradation, and influence TSH clearance rate from the circulation (14,15). A three-dimensional TSH structure has been proposed in the 1990s based on crystallographic studies and comparisons with other glycoprotein hormones (16), but the schemes have not been fully confirmed by more recent studies, including structures of the follicle stimulating hormone extracellular domain and the TSH extracellular domain (17–19).

Human pituitary–derived TSH carbohydrate chains are subject variations in glycan structures, effecting TSH bioactivity (6,7). Such variations in glycosylation can be normal and have been observed in healthy subjects during the nocturnal TSH surge, normal fetuses during the last trimester of pregnancy, primary hypothyroidism, nonthyroidal illnesses, and in TSH-secreting pituitary adenomas (6,7).

TSH can be modified by changes in its terminal mannose, *N*-acetylgalactosamine sulfate, GlcNAc galactose, and/or core fucose. Specific hepatic receptors rapidly capture these modified TSH forms (20–23), resulting in blood enriched with highly branched and sialylated TSH glycoforms. Thus,

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*The authors are saddened to note that our friend and colleague, Dr. Offie Soldin, died shortly before the final publication of this article. She was a leader in the field and was always excited to explore new frontiers clinically and scientifically. She is greatly missed.

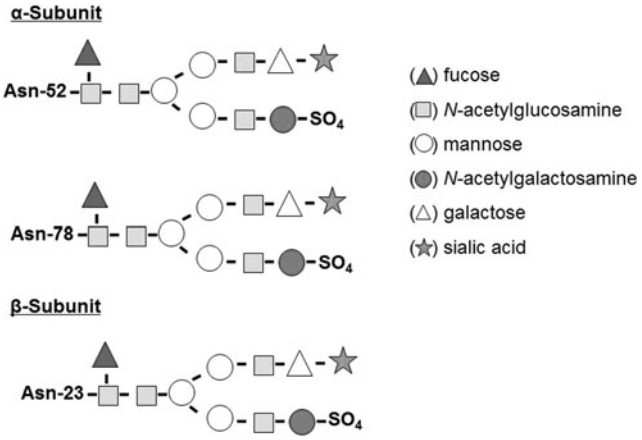

FIG. 1. TSH α- and β-subunits. A normal human TSH molecule contains an α-subunit with two oligosaccharide chains (located at Asn-52 and Asn-78) and a β-subunit with one oligosaccharide chain (located at Asn-23). Each subunit contains a terminal sialic acid and a sulfate residue that confer TSH binding and biological activity. Asn, asparagine; TSH, thyrotropin.

patients with primary hypothyroidism exhibit TSH molecules with increased sialylation (6,7,24,25) and decreased inner fucosylation (26). Despite the loss of *N*-linked oligosaccharides, TSH isoforms maintain effective binding to the cognate receptor. However, loss of glycosylation leads to the loss of TSH biological activity (27). Moreover, deglycosylated isoforms may disrupt the biological activity of normal TSH and human thyroid-stimulating immunoglobulins (28), possibly by preventing the binding of other isoforms (29,30).

On the other hand, a higher glycosylation rate lowers the TSH clearance rate from the circulation; therefore, modulations of the TSH molecule result in changes in hepatic and renal clearance. Because of the nonhepatocyte cellular location of various liver lectin receptors, even in cases of hepatic insufficiency, such as chronic liver disease, fatty liver and cirrhosis serum TSH concentrations, as assessed by immunoassay, remain normal (31–33). In cases of chronic kidney disease patients, impaired glycosylation is associated with a change in TSH clearance rate, resulting in increased TSH biological half-life, decreased pulsatility, and a response to hypothalamic TRH feedback. Yet, in these patients as well, normal TSH levels are maintained (34–36).

TSH Measurements

As a single hormone determination, serum TSH immunoassays provide the most sensitive index to reliably detect thyroid function abnormalities (37–39). The current standards of care call for the use of "third-generation" TSH assays with a functional sensitivity of <0.02 mIU/L (37,40–42) in order to detect differing degrees of TSH suppression. Of note, not all immunoassays perform consistently in measuring very low levels of TSH, and the sensitivity of TSH assays used in clinical laboratories (functional sensitivity) may differ significantly from that stated by the manufacturer (analytical sensitivity) (38).

The usefulness of population-based TSH reference intervals to detect thyroid dysfunction in individuals may be somewhat limited (43,44). This is because it has been demonstrated that for TSH the between-person variability is more variable than within-person variability (45–47). Several epidemiological studies indicate that within-person (intraindividual) TSH variability is relatively narrow, and varies by only about 0.5 mIU/L when tested every month over a span of 1 year. Theoretically, it may be important to evaluate individuals with marginally (yet confirmed) low (e.g., 0.3–0.4 mIU/L) or high (3.0–4.5 mIU/L) TSH levels relative to patient-specific risk factors for cardiovascular disease, rather than relative to the normal TSH reference interval (48). However, it has not been demonstrated that serum TSH levels outside a person's individual range (but still within the population reference interval) can result in increased morbidity and mortality.

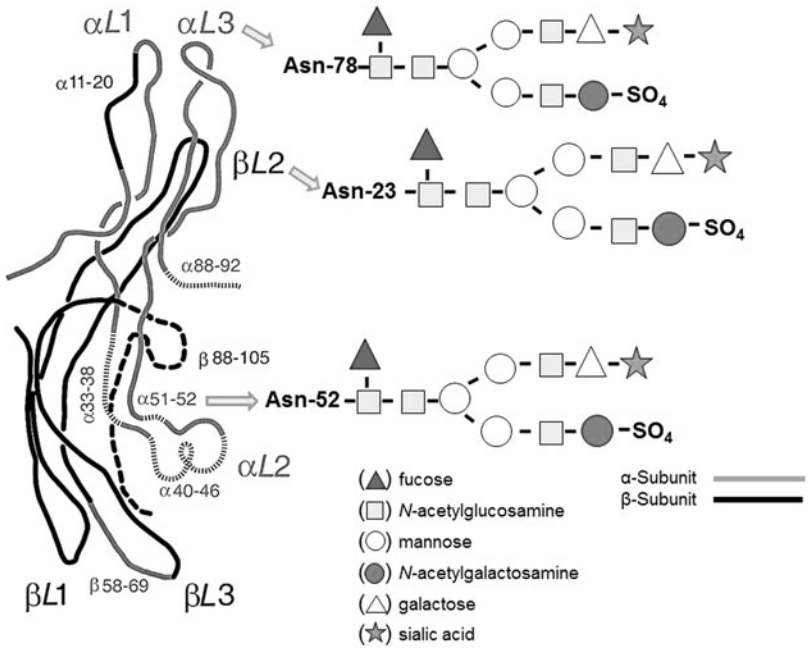

FIG. 2. Suggested structure of the human TSH molecule with two major subunits that confer biological and immunological activity. Each subunit contains glycan branches (designated by the arrows) whose terminal ends can alter TSH binding to its receptors and prevent proper renal clearance. [Structure is modified from (88).]

TSH is used in some U.S. states and regions of the world to screen newborns for underactive thyroid. It is also used for monitoring thyroid replacement therapy, in the workup of female infertility, and to screen adults for thyroid disorders. An abnormal serum TSH concentration indicates an excess or deficiency in thyroid hormone levels but does not provide information about the underlying etiology.

Baseline serum TSH concentrations are important when thyroid abnormalities are first suspected, and then again when equilibrium has been reached or the clinical status has changed (Table 1). Close monitoring of TSH and free thyroxine (FT4) is recommended for pregnant women with thyroid disease, for severely ill patients with nonthyroidal illness (49), and for severely ill hypothyroid patients with malabsorption of oral levothyroxine (LT4).

Nonthyroidal illness can frequently alter thyroid hormone peripheral metabolism and hypothalamic–pituitary–thyroidal function, resulting in thyroid test abnormalities, including decreased or increased serum TSH levels (50). It is important to distinguish the generally mild, transient TSH alterations typical of nonthyroidal illness from the more profound and persistent TSH changes associated with hyper- or hypothyroidism (51).

Primary hypothyroidism is the most common cause of elevated serum TSH. In such patients, serum FT4 is low-normal or reduced, and serum free triiodothyronine (FT3) concentrations usually remain normal until thyroid function level has markedly declined (52). In an iodine-sufficient patient, a transient elevation in TSH occurs during the recovery phase after a severe illness (15), when the hypothalamic–pituitary–thyroid axis is temporarily overactive. In general, such patients do not have an underlying thyroid dysfunction.

Hypothalamic–pituitary dysfunction may be associated with normal or modest increases in TSH, resulting from abnormal TSH glycosylation in the TRH-deficient patient. TSH resistance is associated with molecular defects that hinder adequate transmission of TSH stimulatory signals into thyrocytes. The defect may affect steps along the cascade after TSH binding to its receptor. Resistance to TSH because of either TSHR or G_s alpha mutations (53) and the resulting TSH elevations does not necessarily indicate hypothyroidism (54). Compound heterozygotes or homozygotes for partially inactivating mutations of the TSHR may have an elevated TSH, but with normal peripheral hormones, and be euthyroid, while monoallelic mutations in the G_s alpha subunit are associated with pseudohypoparathyroidism. If the mutation is on the maternal allele, then this can be associated with mild TSH elevations. Because of silencing of the paternal allele caused by imprinting, there is no thyroid phenotype if the mutation is on the paternal allele (55).

Isolated TSH elevations (with normal serum FT4) may be observed in a variety of conditions, including subclinical (mild) hypothyroidism, recovery from hypothyroxinemia of nonthyroidal illnesses, partial TSH resistance, hypothalamic hypothyroidism, and the presence of medications (such as amiodarone), which can inhibit thyroid hormone synthesis and metabolism and may cause transient reversible elevation of serum TSH (56).

In older adults, serum TSH may be higher than the normal reference interval for healthy adults (57–60). Epidemiological studies indicate elevated TSH levels with aging (61,62). Theoretically, this may be because of the presence of TSH isoforms with low bioactivity, especially when there are no accompanying clinical symptoms of hypothyroidism. The treatment of subclinical hypothyroidism in the elderly (48) may be resolved once the issue of declining TSH bioactivity in aging is resolved, by creating appropriate reference intervals for TSH for older adults, or by new technologies for TSH testing that can differentiate and quantify only TSH isoforms with normal bioactivity (63).

Fine-tuning the degree of TSH suppression plays a critical role in the management of thyroid cancer (64,65). It is well recognized that in patients with advanced-stage differentiated thyroid cancer, maintaining serum TSH below the normal range ensures greater progression-free disease and longevity (64). Suppression may be persistent for a prolonged period. In such cases, it is important to note that TSH concentrations ranging between 0.01 and 0.1 mIU/L can indicate a significant risk for atrial fibrillation and bone loss in older patients (66,67).

In the case of hyperthyroidism, including cases of Graves' disease, toxic adenoma and nodular goiter, subacute and lymphocytic (silent, postpartum) thyroiditis, iodine-induced hyperthyroidism, and exogenous thyroid hormone excess, serum FT4 and FT3 measurements are indicated when TSH levels are less than 0.1 mIU/L and also when TSH is in the range of 0.1–0.4 mIU/L. Further, in cases of Graves' disease, TSH should not be used to monitor therapy, as it may not respond to alterations in circulating thyroid hormones in an

Table 1. Clinical Situations in Which Measurements of Serum Thyrotropin Alone May Yield Misleading Results

Condition	*Serum TSH*	*Consequences of clinical action based on serum TSH value alone*	*Serum FT4*
Heterophile antibodies	Normal	Failure to diagnose thyrotoxicosis	High
Central hypothyroidism	Low, normal, or elevated	Failure to diagnose hypothyroidism and investigate hypothalamic–pituitary structure function	Low
TSH-secreting pituitary adenoma	Normal or elevated	Failure to diagnose thyrotoxicosis and investigate pituitary structure and function	High
Thyroid hormone resistance	Normal or elevated	Failure to recognize the condition	High
Poor compliance with T4 therapy	High	Inappropriate increase in dose of T4	High
Delayed recovery of TSH secretion	Normal or low	Failure to diagnose impending hypothyroidism	Low

FT4, free thyroxine; TSH, thyrotropin.

appropriate and rapid fashion. Patients with overt hyperthyroidism are expected to have serum TSH concentrations of less than 0.01 mIU/L except in TSH-induced thyrotoxicosis and T4/T3 resistance. In the case of resistance to thyroid hormones, the patients may have a mixed phenotype, with signs of hypothyroidism in some tissues and thyrotoxicosis in others. Because of the impaired feedback at the level of the thyrotrophs, the TSH levels are normal or mildly elevated serum. Isolated TSH suppression may be seen in hyperthyroidism resulting from causes such as subclinical hyperthyroidism, recovery from overt hyperthyroidism, nonthyroidal illnesses, during the first trimester of pregnancy, and in patients using medications such as dopamine and high-dose glucocorticoids.

Methods for Serum TSH Analysis

TSH immunoassays

The immunometric assay methodology ("sandwich" or "noncompetitive" methodology) is based on the excess antibody approach, and provides functional sensitivity of 0.1 mIU/L to reliably detect the low serum TSH values characteristic of hyperthyroidism (68,69). TSH assay sensitivity and specificity have been further enhanced by the adoption of nonisotopic (chemiluminescent and fluorescent) signals, resulting in narrower TSH reference intervals because of reduced cross-reactivity and improved precision, and offer the additional advantage of being easier to automate (70,71). Automated third-generation TSH assays have become the current standard of care. Fourth-generation assays, which have a sensitivity of 0.001–0.002 mIU/L, have been developed for research purposes (72).

TSH testing provides better sensitivity for detecting thyroid dysfunction than does FT4 testing (37,42,73,74). Because serum T4 has a half-life of approximately 7 days, it does not change sufficiently in 1 day to raise TSH secretion, and therefore there is no need to withhold LT4 therapy on the day of blood testing for TSH (37). TSH normally exhibits a diurnal variation peaking between midnight and 04:00 am, with the lowest levels between 10:00 and 16:00 hours (75). This variation should not usually influence the diagnostic interpretation of test results since most clinical TSH measurements are performed on ambulatory patients during similar hours. Similarly, a diurnal variation exists in T3 levels (76).

Laboratory-Related Variability in TSH Measurements

Although assay antibodies recognize peptide determinants of TSH, studies indicate that the presence or absence of specific glycans modify the immunoreactivity of the hormone, particularly when these changes occur in the β-subunit—the subunit specific to TSH (77–79). Furthermore, although pituitary and recombinant TSH usually share high cross-reactivity, clearly, they do not behave in an identical manner (10). Differences between the glycosylation patterns of a pituitary TSH calibrator and serum samples have been shown to be responsible for differences in epitope conformation and for introducing discrepancies in TSH measurements (10).

TSH is the primary test for thyroid function and one of the most common tests performed in the clinical chemistry laboratory (80). Inter- and intralaboratory variations in TSH measurements have been observed depending on the different methods used for analysis (41,81–85). Assays typically measure a nondetermined, highly heterogeneous mixture of glycoforms in addition to degradation products of TSH against a distinct internal standard or reference material (86). Although both preparations may share common glycoforms, they should not be assumed to behave identically (87). There are indications that circulating forms of TSH differ between euthyroid and hypothyroid persons (6,7,24–26). Under pathophysiologic conditions, TSH, similar to many other circulating glycoproteins, exhibits large structural variations (88). For instance, TSH glycosylations related to hypothyroidism may alter epitope expression between serum samples and the calibrator resulting in discordances in TSH measurements (79,89–91).

Monoclonal anti-TSH antibodies can be divided into two groups depending on their capacity to bind different forms of TSH (92). Some antibodies are unable to bind sialylated forms of TSH, whereas others fail to detect nonfucosylated forms of TSH (92). Both forms are seen in patients developing common thyroid disorders, such as hypothyroidism.

TSH sialylation and sulfation

Sialylation of the terminal end of the *N*-linked oligosaccharides is subject to variation that affects biological activity (93). Highly sialylated terminal ends lead to reduced intrinsic biological activity *in vitro* since the negatively charged sialic acid residues tend to repel the negatively charged TSHRs, while the addition of sialic acids increases the circulation half-life of TSH *in vivo* by the reduction of TSH binding to hepatic asialoglycoprotein receptors. The net effect of reduced intrinsic *in vitro* activity, yet longer *in vivo* half-life, is the increased bioactivity of sialylated TSH *in vivo* (14). Sulfation effectively increases TSH biological activity and reverses the effects of highly sialylated carbohydrate chains (94). Sialylation and sulfation of termini are two separate processes; sulfation does not necessarily follow desialylation, and immunoassay analysis does not differentiate between the sialylated and sulfated forms.

Alterations in TSH bioactivity relative to immunoactivity ratio (B/I) have physiological implications that are important in explaining unexpected laboratory results when clinical findings seem discordant. The sulfated TSH isoforms have higher affinity for TSHRs and increased *in vitro* bioactivity, and therefore a higher B/I ratio when measured by *in vitro* cyclic adenosine monophosphate (cAMP) production (although the *in vivo* bioactivity might be reduced by desialylation because of a faster clearance of TSH molecules by the liver). For example, even the normal circadian rhythm influences sialylation, reflected in higher levels of sialic acid–rich TSH isoforms at night compared with daytime (14). The sialic acid–rich TSH forms have lower biological activity *in vitro* than the normal sulfated forms. Examples are adults and fetuses with resistance to thyroid hormone who have an inappropriately normal or elevated TSH because of the resistance at the pituitary level and demonstrate upregulated α-2,6 sialyltransferase activity, which greatly increases the sialic acid content of secreted TSH molecules, resulting in lower bioactivity *in vitro* (14).

Fucosylation

TSH contains a fucose group attached to the GlcNAc residue, leading to increased activation of the IP3 pathway,

stimulating cAMP (88,95). In addition, fucosylation is known to increase TSH antibody recognition and immunoreactivity (92). In primary hypothyroidism, fucose residues are decreased, forming a TSH isoform with reduced immunological and biological activity (92).

Terminal truncation

Human TSH has been shown to be heterogeneous at the amino-terminus of each subunit because of terminal truncation of both unit polypeptide chains. Shortened isoforms may affect antibody-binding interactions at the carboxy terminus while conducting immunoassay analysis, resulting in lower immunoreactivity.

Macro-TSH

Case reports suggest an alternative form of TSH, namely, macro-TSH, a form that leads to falsely elevated levels of TSH (96,97). Macro-TSH is a rare macromolecule composed of a bond between TSH and anti-TSH IgG molecules (96). These rare macromolecules have reduced biological activity and similar binding efficiency to immunoassay antibodies. In such patients, FT4 levels appear to be normal and the clinical presentation suggests the absence of thyroid dysfunction despite elevated serum TSH because of macro-TSH presence.

Antibody Recognition of TSH

Most existing antibodies target three antigenic regions in the TSH molecule. Each cluster comprises at least two close epitopes (92), with a main immunogenic region that has been characterized as dependent on changes in glycosylation (92). Some antibodies display a preference for specific glycoforms and thereby induce discordances among assays, particularly when these assays have been calibrated against the normal pituitary TSH standard.

Both the presence and the nature of specific linkages of the sialic acid residues are of high importance for optimizing antibody recognition. Moreover, fucosylation adds a further level of complexity as evidenced by the preference of some antibodies for nonfucosylated forms of TSH. This indicates that discordance in measurements can arise when an internal calibrator lacks fucose, such as in rhTSH. Thus, the identity of the TSH calibrator is of eminent importance to ensure that serum TSH can be measured with higher accuracy and possibly on a molar basis. Most of the formats tested were quite divergent when the calibrator was missing sialic acid, as in pituitary TSH, or a core fucose, as in nonfucosylated rhTSH fractions (98). The use of a pituitary international reference preparation is likely to mask part, if not all, of the influence of sialic acid and may explain the variable measurements encountered with rhTSH in 1999 and noted in most third-generation assays (41,99).

The use of monoclonal antibodies in TSH immunoassays has virtually eliminated cross-reactivity with other glycoprotein hormones (such as luteinizing hormone or human chorionic gonadotropin [hCG]). However, because monoclonal antibodies differ in their specificity for recognizing various circulating TSH isoforms, these antibody differences can result in the reporting of TSH values that may differ by as much as 1.0 mIU/L between different laboratories (who use different TSH assays) for the same serum specimen (100).

Endogenous antibodies

Some patients may develop autoantibodies against the TSH molecule. Endogenous antibody interferences are most frequently characterized by falsely high TSH values, depending on the type and composition of the antibody assay employed.

Heterophile antibodies

Heterophile antibodies are a group of relatively weak, multispecific, polyreactive antibodies with specificity for poorly defined antigens that react with immunoassays derived from two or more species (101,102). Most frequently, heterophile antibody interferences result from IgM rheumatoid factor or human antimouse antibodies (HAMA).

Human antimouse antibodies

Immunometric assays based on monoclonal antibodies of murine origin are more prone to HAMA interference than competitive immunoassays because HAMA are able to form a bridge between the capture and signal antibody reagents and create a signal that is reported as a falsely high value (15,103). Of concern are the instances when such HAMA interferences produce inappropriately normal values in patients who eventually prove to have clinical disease (104). Conducting serial dilutions of the suspect serum, and finding nonlinear results upon comparison with that of patients with a suppressed TSH can usually identify such assay-related artifacts. Despite the measures used by manufacturers to neutralize interferences, both the clinician and the laboratory must be aware of this possibility when an apparently inappropriate test result is encountered.

TSH Bioactivity Assays

Serum TSH concentration measurement alone may fail to detect the presence of pituitary and/or hypothalamic disease such as central hypothyroidism or TSH-secreting pituitary tumors, because TSH levels are often within the normal reference interval in these circumstances (105,106). Moreover, current TSH immunoassays cannot distinguish between normal and biologically altered TSH isoforms. TSH isoforms with impaired biologic activity are typically secreted in central hypothyroidism, whereas TSH isoforms with enhanced biologic activity are often secreted by TSH-secreting pituitary tumors (105,106). These abnormal TSH isoforms can result in paradoxically low, normal, or high reported TSH in the face of clinical and biochemical hypo- or hyperthyroidism (106). Therefore, in cases of suspected pituitary or hypothalamic disease, it is important to obtain serum FT4 and TSH concentrations concomitantly.

TSH bioactivity assays indicate the total amount of the biologic potencies of the various circulating TSH isoforms (107). Two methodologies are used to detect TSH antibodies, namely, bioassays and TSH-binding or receptor assays. Bioassays assess the functionality of the antibodies, and as such are able to distinguish between thyroid-stimulating immunoglobulins and thyroid-blocking antibodies (TBAb) (108). On the other hand, TSH-binding inhibiting immunoglobulin (TBII) assays are able to detect antibodies at the TSHR on membranes of the thyroid follicular cells, but they do not measure their functionality, and are thus unable to distinguish between stimulating and blocking antibodies (109).

TSH bioactivity measured *in vitro* provides a different parameter than the bioactivity of TSH measured *in vivo*. For example, *in vitro*, sialic acid (a negatively charged residue) tends to lower the affinity of TSH for the negatively charged TSHR. In comparison, *in vivo*, sialic acid binds and covers a galactose residue in the oligosaccharides, which then "hides" that galactose from hepatic galactose receptors, prolonging the half-life of circulating TSH. Thus, adding sialic acid to TSH tends to decrease intrinsic *in vitro* bioactivity slightly, but tends to increase *in vivo* bioactivity dramatically. This may be the reason why organisms evolved a mechanism to increase the sialylation of TSH when hypothyroid, a biochemical effect demonstrated in hypothyroid patients (25).

Bioassay methods (thyroid-stimulating antibody/TBAb)

Bioactivity assays detect cAMP released to indicate serum antibody action (110). Increased cAMP production indicates stimulating antibody activity at the receptor level, while inhibition of cAMP production indicates a blocking action (TBAb). As such, the bioassay methods are generally able to differentiate between the presence of thyroid-stimulating antibody (TSAb) and TBAb. However, the interpretation of these tests can prove to be difficult, because both TSAb and TBAb can be present in the same patient, and because TBAb may demonstrate stimulating activity in some cases.

TSAb bioassays measure biological activity by using a cAMP-inducible luciferase gene stably transfected in cell lines to measure cAMP levels through light production (111). Alternatively, TSH bioactivity and TSAb activity can be measured by determining cAMP in the extracellular fluid of Chinese hamster ovary cells transfected with recombinant human TSHR (CHO-R cells-JP26) (111–114). CHO-R testing has proven more reliable in determining autoantibody action and possesses better sensitivity, specificity, and reproducibility than its immediate precursor, the FRTL-5 bioassay (112). Currently, assays for TBAb are not available for routine clinical use.

Receptor methods (TBII)

TSHR binding assays, as noted above, are unable to distinguish between TSAb and TBAb. However, in a clinical setting, a physical examination together with biochemical testing can be used to interpret the binding assay results and determine the difference between these effects for a patient. TBII assays have evolved over time (115–118). The current third-generation assays do not use labeled TSH, but rather human monoclonal thyroid-stimulating immunoglobulins binding to recombinant TSHR. The third-generation assay has 100% specificity (119) and a detection limit of about 0.01 mIU/L, and can therefore reliably distinguish between normal and hyperthyroid patients. However, these assays have restrictive clinical application and are not generally widely utilized because the distinction between normal and hyperthyroid patients is usually not a clinical problem. These assays are more frequently used when the distinction between Graves' disease and other causes of hyperthyroidism is difficult, and in pregnant women with Graves' disease, when appropriate, to assess the likelihood of the fetus developing thyrotoxicosis *in utero* or after delivery. The assay can also be employed to help determine if a patient with Graves' disease will develop or has developed a remission.

Bioassays Versus Immunometric Assays of TSH and B/I Ratio

TSH bioassays reflect the sum of the biopotencies of the various circulating TSH isoforms, whereas immunometric assays quantify serum TSH concentrations. Analyzed simultaneously by immunometric and biological assays, the ratio between the bioactivity and immunoactivity serves as an index of the overall potency of circulating TSH molecules (120). Thus, variations in the B/I ratio of immunopurified TSH samples result from changes in the amount of biological activity per unit of immunological activity. This serves as an estimate of the circulating TSH molecules' overall biological potency, or intrinsic bioactivity. Multiplying the B/I ratio by the concentration of TSH in serum produces the bioactive TSH concentration or intrinsic TSH bioactivity (107).

Alterations to the TSH structure affect its binding capacity and clearance. Since TSH undergoes modifications before its release from the anterior pituitary, it is subject to a variety of glycosylations that would affect its binding action. In a case of subclinical hypothyroidism in a young adult with Down's syndrome, antibody recognition of TSH significantly increased with sialylation and fucosylation (121). The modified TSH demonstrated a much stronger and more efficient binding to the assay antibodies compared with the normal pituitary TSH. This competitive binding preference for the modified TSH suggests that the normal active TSH was not as accurately detected in serum, at least in this one patient. This sialylation modification is known to prevent liver uptake and metabolic clearance, and therefore prolongs the half-life of serum TSH (121).

TSH isoform bioactivity is similarly subject to modifications that affect bioassay measurements. TSH glycosylation affects antibody binding activity and its ability to induce cAMP release. Altered glycan groups on TSH affect its bioactivity; deglycosylation may increase receptor binding and decrease signal transduction. Sialylation increases receptor binding action on TSH proteins (88). Thus, to reach accurate diagnoses, both bioactivity and biochemical analysis should be taken into account, especially in cases such as TSH-secreting pituitary tumors and hypothalamic hypothyroidism. This potential discordance should also be considered when routine TSH immunoassay values are not congruent to FT4 and T3 and/or the clinical circumstance (14,122).

Of note, in some clinical conditions (Table 1), the measurements of serum TSH alone as a first-line test may yield misleading information; it is difficult for the laboratory to proactively detect interference from a single measurement such as an isolated TSH test. A more common occurrence is for the physician to suspect assay interference when a reported value is inconsistent with the clinical status of a patient. The most practical way to investigate possible interference is to test the specimen by a different manufacturer's method and check for discordance between the test results. This approach is effective because methods vary in their susceptibility to interfering substances. Occasionally, a biological check can be made using TRH stimulation (which is not presently clinically available in the United States) or thyroid hormone suppression to validate a suspected inappropriate serum TSH level. Interferences producing a falsely elevated TSH value will usually be associated with a blunted (<2-fold increase) response to stimulation.

Disease states associated with apparently higher serum TSH

Extremely rare conditions associated with elevated TSH include inherited autosomal recessive forms of partial (euthyroid hyperthyrotropinemia) or complete (congenital hypothyroidism) TSH resistance that are associated with biallelic inactivating point mutations of the *TSHR* gene (15). Inherited dominant forms of partial TSH resistance have also been described in the absence of *TSHR* gene mutations (15). More frequently, in patients who have elevated serum FT4, normal or elevated TSH requires further investigation for resistance to thyroid hormone or a thyrotroph tumor (123,124).

Psychiatric illness may be associated with either elevated or suppressed TSH, but abnormal levels are not usually in the range typically associated with symptomatic thyroid dysfunction. In patients with Addison's disease (with slightly elevated TSH in the absence of primary thyroid disease) corticosteroid results in the normalization of TSH levels.

Central hypothyroidism

Idiopathic central hypothyroidism (seen in various hypothalamic–pituitary conditions) may result from the secretion of biologically inactive TSH. In these cases, serum TSH levels are normal or slightly elevated with decreased bioactivity (112,125–127). TRH regulates not only the secretion of TSH but also its specific molecular and conformational characteristics required for TSH hormone action (126). In such cases, chronic TRH administration has been shown to increase TSH bioactivity and restore thyroid function (126). In patients with primary hypothyroidism and TSH-secreting pituitary adenomas, normal, reduced, and increased TSH bioactivities have all been reported (112,122,128–131).

Resistance to thyroid hormone

As previously mentioned, TSH bioactivity is increased in patients with thyroid hormone resistance (122,132). These patients demonstrate greatly increased sialic acid content of secreted TSH molecules (14).

Sheehan's syndrome (hypothyroidism caused by postpartum panhypopituitarism)

Sheehan's syndrome is a peripartum condition that follows massive necrosis of the anterior pituitary gland (133,134). Many patients with Sheehan's syndrome have low TSH levels (135). The majority of cases are classified as chronic since patients can remain asymptomatic for several months to years after massive postpartum uterine bleeding, which leads to amenorrhea and loss of lactation. An acute state is diagnosed when symptoms of headaches and hormonal deficiencies appear within days after a traumatic delivery (136). Despite the differences in time and diagnosis, these patients present with similar TSH characteristics and serum levels because of a necrotic anterior pituitary gland. While TSH secretion is elevated because of increased tonic (not pulsatile) TSH secretion, the TSH circadian rhythm is severely blunted (135,137). Patients with Sheehan's syndrome demonstrated mannose content of TSH isoforms similar to that of normal controls, but the degree of TSH sialylation is higher (135,137). Moreover, although intrinsic TSH bioactivity is decreased, serum TSH concentrations were higher than those in controls, so the resultant bioactive serum TSH concentration (the product $B/I \times I$) in patients with Sheehan's syndrome did not differ from that of healthy controls. The observation that serum FT4 levels correlated significantly with bioactive TSH concentrations, but not with immunoreactive TSH levels or intrinsic TSH bioactivity in Sheehan's patients, reflects the relevant role of bioactive TSH in residual T4 secretion in Sheehan's patients. Thus, the paradox of the presence of hypothyroidism with increased serum immunoreactive TSH levels in patients with Sheehan's syndrome cannot be solved simply by demonstrating that serum TSH has decreased intrinsic bioactivity. Another paradox is posed by the observation that in Sheehan's patients bioactive serum TSH, although normal, fails to sustain normal T4 levels.

TSH and Aging

TSH immunoactivity and reference intervals in older adults

Several epidemiological studies indicate that the normal reference interval for serum TSH increases with age (61,62). The NHANES III survey of the U.S. population demonstrates that the 2.5th, 50th, and 97.5th percentiles of serum TSH rise with age, and the most significant effects are seen at the 97.5th percentile, which increases by 0.3 mIU/L with each 10-year increase in age (138). It was demonstrated that in individuals who are TPO antibody or thyroglobulin antibody negative, serum TSH concentrations >3.0 mIU/L were observed with increasing frequency with aging. Moreover, in elderly subjects (>80 years), 23.9% had serum TSH concentrations between 2.5 and 4.5 mIU/L, and 12% had concentrations greater than 4.5 mIU/L (60). The mild rise may indicate that slightly elevated TSH in the elderly may not necessary reflect subclinical thyroid dysfunction, but instead be a normal manifestation of aging. This observation has called into the question the applicability of adult TSH reference intervals to the elderly population, which may need to be adjusted with increasing age. Surks *et al.* acknowledge that serum TSH levels between 3.0 and 4.5 may indicate early signs for subclinical hypothyroidism (138,139). Nonetheless, if the upper limit is set for 2.5–3.0 mIU/L, in the United States alone, there would be a 300–400% increase in individuals diagnosed with hypothyroidism (22–28 million additional individuals). Therefore, Surks *et al.* suggest the use of age-specific reference intervals in order to avoid the misclassification of healthy patients as hypothyroid (60). Consequently, the upper limit will be set higher for older individuals than for younger individuals, averting unwarranted hypothyroidism diagnoses and, in turn, unnecessary thyroid hormone therapies for healthy, aging individuals (63).

TSH biological activity and aging

Although immunoassay discrepancy may be the underlying reason for the elevated serum TSH, the increase in serum TSH has also been associated with relatively higher concentrations of biologically inactive isoforms of TSH, as described above. When higher TSH is not accompanied by the normal thyroidal response of a rise in FT4/FT3, it suggests the presence of bioinactive TSH. Sera with bioinactive TSH would show elevated TSH concentrations (immunoactivity) although bioactivity may be normal.

The current standard of care calls for the use of third-generation TSH assays that have a functional sensitivity of <0.02 mIU/L (37,40–42). This level of sensitivity is important for determining the degree of TSH suppression. In older patients, serum TSH concentrations between 0.01–0.1 mIU/L (implying an increase in FT4/T3 for this individual patient) represents a significant risk for atrial fibrillation and are often an iatrogenic consequence of LT4 suppression or an unanticipated side effect of replacement therapy (66,67). Further, since T4 (and LT4) has a narrow therapeutic index, monitoring TSH levels provides an improved sensitivity than does FT4 testing.

TSH Assessment in Pregnancy

Thyroid disorders are relatively frequent in women of childbearing age (140,141); consequently, TSH measurements are useful in detecting subtle thyroid dysfunction associated with reproductive issues and poor pregnancy outcome. During pregnancy, the placenta secretes high levels of hCG, a glycoprotein hormone sharing a common α-subunit with TSH (142). As discussed above, currently used immunometric TSH assays have overcome the problems posed by hCG cross reactivity (141). Moreover, enhanced sensitivity of third-generation assays has established the lower TSH limit for nonpregnant women as ~0.3 mIU/L and affords the accurate detection of the low serum TSH concentrations often detected in early pregnancy (143). During the first trimester, elevated hCG secretion results in higher T4 and T3 and suppressed serum TSH concentrations. Since hCG concentrations are higher in multiple pregnancies than in singleton pregnancies, a greater downward shift is observed in twin pregnancies (144). However, in the case of maternal hypothyroxinemia (low FT4 accompanied by normal TSH), which is increasingly recognized for its association with neurodevelopmental deficits, TSH is within the normal range and is therefore not a useful assay for detecting this problem (145). FT4 immunoassays are notoriously unreliable during pregnancy (146), but relying on TSH is not sufficient in this case.

Trimester-specific reference intervals of TSH

During pregnancy, increased hCG can lead to transient hCG-induced thyrotoxicosis. This can be reflected in lower limits of TSH during the first trimester. The lower and upper reference limits for serum TSH are decreased by about 0.1–0.2 mIU/L and 1.0 mIU/L, respectively, compared with the TSH reference interval of 0.4–4.0 mIU/L of nonpregnant women (142,147). Accordingly, the Endocrine Society and the recent American Thyroid Association guidelines recommend using a TSH upper limit of 2.5 mIU/L for preconception and first trimester, and 3.0 mIU/L for the second and third trimesters (74,148). Of note, in 1.7% of pregnancies, TSH can be greatly suppressed (<0.01 mIU/L) and yet still represent a euthyroid pregnant patient (149). The differences in normal TSH intervals in the various ethnic groups persist during pregnancy (150); therefore, providing specific normal ranges for the local population is of particular advantage. Taken together, and in accordance with the new American Thyroid Association guidelines for women in pregnancy, trimester-specific reference intervals for TSH, as defined in populations with optimal iodine intake, should be applied, although they are not generally provided (74). If trimester-specific reference intervals for TSH are not available in the laboratory, the following reference intervals are recommended by the American Thyroid Association: 1st trimester 0.1–2.5 mIU/L; 2nd trimester 0.2–3.0 mIU/L; 3rd trimester 0.3–3.0 mIU/L (74,151).

Finally, immunoassay methods for TSH analysis using nonisotopic, primarily chemiluminescent signals have become available on a variety of high-throughput immunoassay analyzer platforms that employ bar-coding, multiple-analyte random-access, primary tube sampling, auto dilution, STAT testing, and computerized data output (152). Since most thyroid disease is treated on an outpatient basis, point-of-care thyroid tests are unlikely to replace centralized automated thyroid testing. Liquid chromatography–tandem mass spectrometry testing of thyroid hormones requires equilibrium dialysis or ultrafiltration before analysis. Currently, there is no available methodology to measure TSH, since it is a larger molecule with variable secondary modifications. However, TSH analysis may change when liquid chromatography–tandem mass spectrometry for TSH is made available, by finding smaller fragments of the molecule that can adequately represent TSH activity.

Acknowledgments

Dr. O.P. Soldin was supported in part by NIH R01AG033867 and by the Flight Attendants Medical Research Institute. The project was also supported in part by award number P30CA051008 from the National Cancer Institute. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health. We thank Chen Fei for help with constructing the figures.

Author Disclosure Statement

The authors declare that no competing financial interests exist.

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