

A Novel Thyroid Stimulating Immunoglobulin Bioassay Is a Functional Indicator of Activity and Severity of Graves' Orbitopathy

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Context: Immunoglobulins stimulating the TSH receptor (TSI) influence thyroid function and likely mediate extrathyroidal manifestations of Graves' disease (GD).

Objectives: The aim of this study was to assess the clinical relevance of TSI in GD patients with or without Graves' orbitopathy (GO), to correlate the TSI levels with activity/severity of GO, and to compare the sensitivity/specificity of a novel TSI bioassay with TSH receptor (TSH-R) binding methods (TRAb).

Design: TSI were tested in two reporter cell lines designed to measure Igs binding the TSH-R and transmitting signals for cAMP/CREB/cAMP regulatory element complex-dependent activation of luciferase gene expression. Responsiveness to TSI of the novel chimeric (Mc4) TSH-R (amino acid residues 262–335 of human TSH-R replaced by rat LH-R) was compared with the wild-type (wt) TSH-R.

Results: All hyperthyroid GD/GO patients were TSI-positive. TSI were detected in 150 of 155 (97%, Mc4) and 148 of 155 (95%, wt) GO patients, in six of 45 (13%, Mc4) and 20 of 45 (44%, wt) mostly treated GD subjects, and in 0 of 40 (Mc4) and one of 40 (wt) controls. Serum TSI titers were 3- and 8-fold higher in GO vs. GD and control, respectively. All patients with diplopia and optic neuropathy and smokers were TSI-positive. TSI strongly correlated with GO activity ($r = 0.87$ and $r = 0.7$; both $P < 0.001$) and severity ($r = 0.87$ and $r = 0.72$; both $P < 0.001$) in the Mc4 and wt bioassays, respectively. Clinical sensitivity (97 vs. 77%; $P < 0.001$) and specificity (89 vs. 43%; $P < 0.001$) of the Mc4/TSI were greater than TRAb in GO. All 11 of 200 (5.5%) TSI-positive/TRAb-negative patients had GO, whereas all seven of 200 (3.5%) TSI-negative/TRAb-positive subjects had GD only.

Conclusion: The novel Mc4/TSI is a functional indicator of GO activity and severity. (*J Clin Endocrinol Metab* 95: 2123–2131, 2010)

The autoantibodies of clinical relevance in Graves' disease (GD) stimulate the TSH receptor (TSH-R) and directly influence the metabolic activity of the thyroid gland leading to hyperthyroidism (1–4). The TSH-R-stimulating Igs (TSIs) with inflammatory cytokines mediate metabolic changes in TSH-R-positive fibroblasts, target cells of orbital tissues, and supposedly lead to Graves' orbitopathy (GO) (5–9). Recent evidence for the coex-

pression of TSH and IGF-I receptors on lymphocytes (10, 11) and specialized subsets of fibroblasts (12, 13) indicates that other self-antigens and TSI not linked to thyroid hormone production *per se* may contribute to extrathyroid inflammation in GD.

Identifying TSIs and differentiating them from non-functional TSH-R remain important goals (14, 15). So far, the tests for detection of TSH-R autoantibodies in GD

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

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doi: 10.1210/jc.2009-2470 Received November 18, 2009. Accepted February 12, 2010.
First Published Online March 17, 2010

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Abbreviations: CAS, Clinical activity score; CHO, Chinese hamster ovary; CSS, clinical severity score; CV, coefficient of variation; fT3, free T₃; fT4, free T₄; GD, Graves' disease; GO, Graves' orbitopathy; RLU, relative light unit; ROC, receiver operating characteristic; SRR%, percent specimen-to-reference ratio; Tg, thyroglobulin; TPO, thyroid peroxidase; TRAb, TSH-R antibody; TSH-R, TSH receptor; TSI, TSH-R stimulating Ig; wt, wild-type.

patients do not necessarily identify the pathogenic TSH-R antibodies that determine the clinical outcome of thyroid autoimmunity (14, 16, 17). Methods that measure the binding of antibodies in patient sera to TSH-R immobilized on the surface of plastic-coated tubes, plates, or beads display high analytical sensitivity and specificity. Unfortunately, these methods do not measure the functional activity of Igs, nor do they discriminate between the Igs with stimulating, blocking, or neutral activity (16–20). Over the past 30 yr, biological assays to detect TSI in the sera of GD patients have been developed in individual laboratories. These experimental cell-based systems assess TSI activity on human thyroid cells, FRTL-5 primary rat thyrocytes, or Chinese hamster ovary (CHO) cells transfected with recombinant human TSH-R (7, 8, 21–28). The protocols involve several days of cell culture with cell lines that are not quality controlled and require the measurement of radioactive cAMP released into the supernatant of cell lysates. Despite these limitations, the monitoring of TSI in GD patients undergoing treatment with anti-thyroid medication and B-cell depletion indicate a promising role for TSI bioassays in the evaluation of responses to therapy (16, 29).

Thus, to assess the relevance of TSI in the pathogenesis of GD and its extrathyroid manifestations, we performed a controlled cross-sectional trial in a large cohort of GD subjects. Two commercial TSI bioassays with quality-controlled cell lines that employ a luciferase reporter readout were compared with anti-TSH-R binding assays for their clinical sensitivity and specificity in GD and GO.

Patients and Methods

This cross-sectional study consisted of 200 Graves' patients, most of whom had been treated, and 40 euthyroid healthy controls (Table 1). The patients had either clinically overt orbitopathy (GO patients) or no orbital involvement (GD patients). All patients provided informed consent, and the study had the approval of the State Ethical Committee and the Institutional Review Board at the Gutenberg University Medical Center. All patients had blood samples drawn after complete endocrine and ophthalmic assessment at the university joint thyroid and eye clinic.

The activity of the eye disease was assessed by one author (K.A.P.) who was unaware of the laboratory data. The clinical activity score (CAS), based on the classical signs of inflammation, consists of seven items: spontaneous pain behind the globe, pain on attempted upgaze, redness of the conjunctiva, redness of the eyelid, chemosis, swelling of the lacrimal caruncle, and eyelid swelling. One point is added for each item present. The score ranges from 0 to 7 (30–32). The overall severity of the disease was assessed using the clinical severity score (CSS) based on the NOSPECS classification (33). For all NOSPECS categories, at least one assessment was selected. The lid aperture (in millimeters) was measured at the midline in primary gaze (class 1). Class

TABLE 1. Demographic, clinical, and serological data of patients and controls

GD patients	n = 200
Gender	162 females, 38 males
Age (yr)	48 (13–81)
Smokers	70/200 (35%)
Other autoimmune diseases	30/200 (15%)
Graves' thyroidal disease, only	45/200 (22.5%)
GO	155/200 (77.5%)
Graves' dermopathy/acropachy	10/200 (5%)
Duration of GD (months)	8 (1–96)
Therapy of Graves' hyperthyroidism	
Methimazole (2.5–10 mg/d)	123/200 (61.5%)
Thyroidectomy	18/200 (9%)
Radioiodine therapy	11/200 (5.5%)
Patients in remission	48/200 (24%)
GD patients treated for hyperthyroidism	33/45 (73.3%)
GO patients treated for hyperthyroidism	114/155 (73.5%)
Peripheral thyroid function	
GD euthyroid	37/45 (82%)
GD hyperthyroid	8/45 (18%)
GO euthyroid	92/155 (59%)
GO hyperthyroid	63/155 (41%)
Duration of GO (months)	4 (1–83)
CAS of GO	3 (0–7)
CSS of GO	5 (1–12)
Active GO	99/155 (64%)
Mild GO	46/155 (29.7%)
Moderately severe GO	104/155 (67%)
Sight-threatening GO	5/155 (3.2%)
Diplopia	89/155 (57.4%)
Previous therapy of GO	
Steroids	61/155 (39%)
Retrolbulbar irradiation	24/155 (15.5%)
Thyroid-related hormones	
TSH (mU/liter)	0.13 (0.01–22)
fT3 (pg/ml)	3.3 (1.5–30)
fT4 (ng/dl)	1.3 (0.5–5.9)
Thyroid autoantibodies	
Anti-TSH-R (IU/ml)	43 (0.5–574)
Anti-TPO (IU/ml)	245 (0–1000)
Anti-Tg (IU/ml)	20 (0 to >3000)
Euthyroid controls	n = 40
Gender	21 females, 19 males
Age (yr)	21 (7–68)
TSH (mU/liter)	1.5 (0.4–2.9)
fT3 (pg/ml)	3.3 (2.2–4.1)
fT4 (ng/dl)	1.1 (0.6–1.5)

Data are expressed as number of patients/total subjects (%) or as median (range).

2 (soft tissue involvement) was assessed using a color atlas. Class 3 (proptosis) was assessed using a Hertel exophthalmometer. Eye muscle involvement (class 4) was classified according to the Gorman diplopia score. Class 5 (corneal involvement) and class 6 (optic nerve involvement) were ophthalmologically assessed (slit lamp microscopy, funduscopy, vision field testing, color vision tests, and visual acuity test). Visual acuity was measured using the decimal system, and optic nerve involvement was defined to be present if there was disc swelling or pallor, a visual field defect, or if visual acuity was less than 0.5 in the absence of other reasons for sight loss. The severity score is calculated as the sum of each class present and ranges from 1 to 18. Each NOSPECS class A is being substituted by 1, B by 2, and C by 3, and the sum of the scores constitutes the CSS.

Cell-based TSI bioassay

All materials to perform the novel TSI bioassay were supplied by the manufacturer (Diagnostic Hybrids, Inc., Athens, OH). The cells used in the assay are CHO-K1 cells (ATCC no. CCL-61; American Type Culture Collection, Manassas, VA) that stably express a fully intact wild-type (wt) human TSH-R (wtCHO) or the chimeric human TSH-R, which has amino acids 262 to 335 substituted with 73 amino acids from the rat LH receptor (Mc4CHO) under control of the simian virus 40 promoter. Stable cell lines were generated by transfection with linearized (Xmn1-digested) pMC 1-GPH/Luciferase containing the wt TSH-R insert or the pMC4-GPH/Luciferase plasmid using Hy-Fect (Denville Scientific, Metuchen, NJ) and selected for growth in medium containing antibiotic neomycin (34). Both Mc4CHO and wtCHO cells were also transfected with the luciferase reporter gene, the expression of which is under control of a 236-nucleotide sequence (GenBank sequence AF401991) derived from the glycoprotein α -subunit promoter, which contains a cAMP regulatory element (CRE). Mc4CHOLuc and wtCHOLuc were obtained as quality controlled fresh frozen cells (FreshFrozenCells; Diagnostic Hybrids, Inc.) shipped on dry ice.

On d 1, the inner 48 wells of each Corning 96-well clear flat-bottom black plate were treated with Cell Attachment Solution and seeded with thawed Mc4CHOLuc or wtCHOLuc cells diluted in the prescribed planting medium. Plates were then incubated for 15 to 18 h in a humidified, 5% CO₂, 35 to 37 C incubator. The wtCHOLuc were incubated an additional 16 h in serum-free starvation medium before contact with patient sera to enhance the specific TSI-induced cAMP signal. The extra preparation time of wtCHOLuc cells resulted in a 3-d bioassay. In contrast, the plates of Mc4CHOLuc are ready for use in a 1-d assay. After all the wells of each plate were inspected to ensure that the monolayer was confluent and appeared healthy, the wells were rinsed with prewarmed (37 C) reaction buffer, and 100 μ l reaction buffer was then added to each well. Specimens of patients and controls were then prepared by adding one part serum to 10 parts of reaction buffer. Each diluted serum specimen was added in 100 μ l to the appropriate wells in triplicate, and the plate was placed in a humidified, 5% CO₂, 35 to 37 C incubator for 3 h. After the incubation period, all liquid was decanted from the plate, and 75 μ l of luciferase substrate and lysis reagent was added to each well. After 10 min at room temperature, the plate was read on a multiwell plate luminometer (Veritas Microplate Luminometer; Turner BioSystems, Sunnyvale, CA; or Tecan Infinite M200; Tecan, Crailsheim, Germany).

Protein G adsorption of sera

To confirm that IgGs mediate the stimulating activity, the TSI-positive sera were preadsorbed with Immunopure immobilized Protein G cross-linked to fast flow Sepharose 4B (catalog no. 17-0618-01; GE Healthcare Life Sciences, Munich, Germany). Sera samples diluted 1:1 vol/vol with PBS were added in 750- μ l aliquots to 2 ml microfuge tubes containing 250 μ l of Protein G Sepharose gel and incubated by gentle mixing for 1 h at room temperature. After centrifugation for 2–3 min in a microcentrifuge, the unbound material was carefully removed and centrifuged a second time to eliminate residue of beaded Sepharose, and the supernatant was transferred to fresh tubes. The TSI activity of nonadsorbed *vs.* preadsorbed sera samples was then assayed.

Assays for TSH-R antibody (TRAb) detection

The anti-TSH-R binding activity was measured with the human TRAK RIA (Brahms AG, Hennigsdorf, Germany) interassay precision coefficient of variation (CV) between 3.9 and 7.5% for TRAb 13.2–27 IU/liter and 14.1% for TRAb 1.1 IU/liter, evaluation limit of 1 IU/liter, and TRAb positivity greater than 1.5 IU/liter. Sera tested with the TRAK RIA that gave discordant results with the TSI-reporter bioassay were retested with the automated Cobas electrochemiluminescence ECLIA Elecsys, an anti-TSH-R immunoassay that uses a porcine TSH-R and human anti-TSH-R autoantibody M22 (Roche Diagnostics GmbH, Penzberg, Germany) with interassay precision, CV 3.3% for TRAb greater than 2 IU/liter and 14.9% for TRAb levels 0.4–1 IU/liter, and TRAb positivity greater than 1.5 IU/liter (18). The sera with discordant test results were tested a third time with TRAb Coated Tube Kit (KRONUS, Boise, ID), interassay precision CV 7% for TRAb 11.4 IU/liter, 10% for TRAb 3.7 IU/liter, and 16% for TRAb 1 IU/liter. TRAb positivity was reported for values greater than 1.1 IU/liter.

Thyroid-related hormones and autoantibody immunoassays

The serum levels of TSH, free T₄ (fT₄), and free T₃ (fT₃) were measured with chemiluminescent microparticle immunoassays that have analytical sensitivities of less than 0.02 mIU/liter, 0.4 ng/dl, and 1 pg/ml, respectively (Architect analytical system third generation kits; Abbott Diagnostic Division, Abbott Park, IL). The IgG classes of thyroglobulin (Tg) autoantibodies and thyroid peroxidase (TPO) autoantibodies were measured with chemiluminescent microparticle immunoassays having precision of less than 10% CV for samples of at least 4 IU/ml and at least 5.61 IU/ml, respectively.

Data analysis and statistics

The acquisition file templates and the parameters of data analysis were defined using template software (Veritas Microplate Luminometer Software, version 1.7.1) or the Tecan instrument control and data analysis software (Magellan Tracker, version 2.4). All correlations were performed using Spearman's correlation coefficient. The percent specimen-to-reference ratio (SRR%) for each result was calculated according to the formula: $SRR\% = (\text{mean } TSI_{\text{specimen}} / \text{mean } TSI_{\text{reference}}) * 100$, whereby mean TSI_{specimen} is the mean of triplicate relative light unit (RLU) measurements in the wells containing cells in contact with the specimen of diluted serum of a patient or control, and mean $TSI_{\text{reference}}$ is the mean of triplicate RLU measurements in the wells containing cells in contact with the reference 0.1 IU/ml of bovine TSH. Patient or control specimen was considered positive for the presence of TSI if the resultant SRR% measured at least 140 over the reference control. This cutoff was established by the U.S. Food and Drug Administration cleared protocol and was specified in the manufacturer's product insert for Thyretain TSI Reporter BioAssay (reference 40-25000.v2/50000.v2; Diagnostic Hybrids Inc.). To compare the prognostic evaluation of the wt and the Mc4 TSI bioassays, receiver operating characteristic (ROC) analysis was performed with the MedCalc Software version 11.1.1.0. For each result, the CV% was calculated according to the formula: $CV\% = (SD_{RLU \text{ specimen}} / \text{mean}_{RLU \text{ specimen}}) * 100$. The interassay precision of Mc4CHO TSI bioassay was previously established by testing serum containing TSI of normal (SRR% = 61), low (SRR% = 183), mild (SRR% = 313), and high

(SRR% = 509) levels and reported in the manufacturer's product insert: intraassay CVs of 5.9, 4.7, 1.9, and 1.8%, respectively; intraday CVs of 5, 4.2, 2.6, and 3.6%, respectively; and interday CVs of 15.7, 14.5, 12.8, and 11.5%, respectively. Any specimen having a CV greater than 15% was excluded from the data set and retested. The records of all acquired data were stored in the format of the original acquisition source files and as data calculations in exported Excel spreadsheets (Microsoft, Redmond, WA).

Results

Analytical sensitivity and specificity

All 200 GD patients and 40 normal donors of the trial site were tested twice in both TSI bioassays by two independent investigators using the same reagents and materials. The control panels were comprised of positive sera, reference bovine TSH, and TSI-negative patient sera and were repeatedly tested alongside the patient sera. No test results were disqualified due to poor confluence or turbidity of the culture medium. Agreement of 100% was attained between the two users at the same TSI bioassay facility. To confirm that the IgG fraction in the sera of the GD patients is responsible for TSI activity, the sera were incubated with Protein G Sepharose. The TSI activity in nonadsorbed sera, 300–400 SRR%, was reduced to less than 50 SRR% after adsorption on Protein G Sepharose (results not shown). When comparing the two bioassays, the ROC analyses (Fig. 1A) revealed small differences in sensitivity (80 vs. 85%) and specificity (100 vs. 95%). Intraassay precision was higher in Mc4/TSI than the wt/TSI as indicated by lower CVs at all levels of TSI SRR% above 140. The total intraassay precision of Mc4/TSI (mean, 5.8%; range, 0.4–25%) was significantly lower than that of wt/TSI (7.3%, 0.9–21%; $P < 0.001$) (Fig. 1B). The interassay precisions of the Mc4/TSI and wt/TSI reveal similar CV% for the negative control (mean SRR%, 85; CV, 5%; vs. SRR%, 88; CV, 7.7%), but the CV of wt/TSI was significantly higher than Mc4/TSI for the positive control (SRR%, 377; CV, 23%; vs. SRR%, 371; CV, 5%; $P < 0.001$) (Fig. 1C).

Clinical diagnosis and TSI levels

Demographic, clinical, and serological data of the 200 Graves' patients, most of whom had been treated, are shown in Table 1. All hyperthyroid patients were TSI-positive; however, at the time of blood sampling the vast majority of treated patients were euthyroid. The TSI-positive results for GO, GD, and controls were 150 of 155 (97%, Mc4/TSI) or 148 of 155 (95%, wt/TSI), six of 45 (13%, Mc4/TSI) or 20 of 45 (44%, wt/TSI), and 0 of 40 (Mc4/TSI) or one of 40 (2.5%, wt/TSI), respectively. All five TSI-negative GO patients already had radioiodine

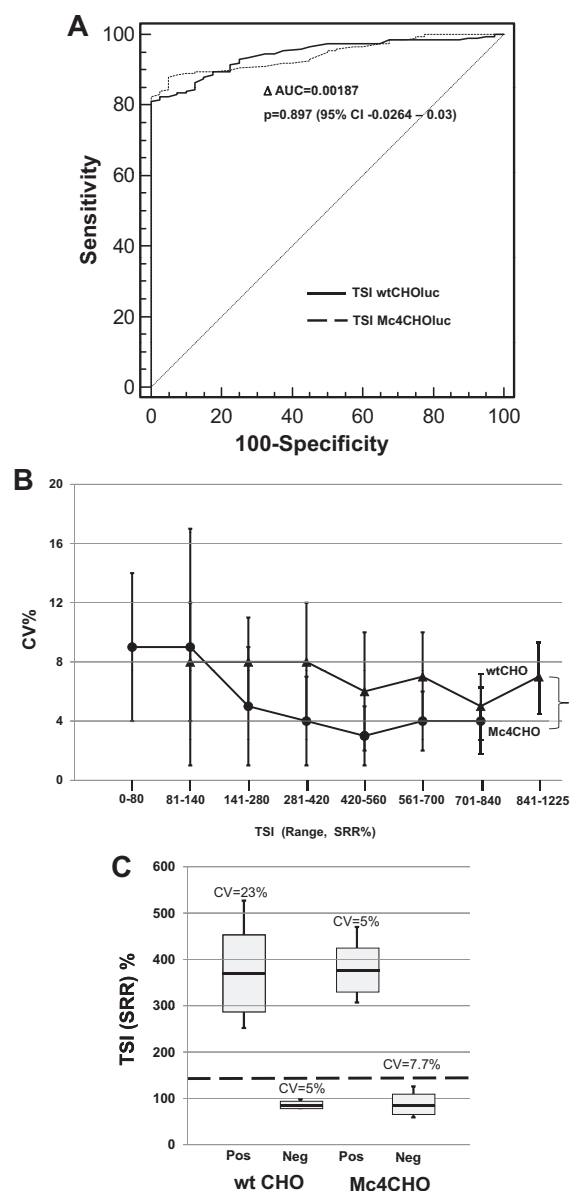


FIG. 1. Comparison of the wtCHO TSH-R and the chimeric TSH-R Mc4CHO TSI bioassays. ROC analysis of the data of 200 patients with GD and 40 healthy individuals. A, Δ AUC is the difference in the area under the curve between the two bioassays. B, Intraassay precision. The mean CV% mean and sd for each TSI SRR% range obtained from all 240 serum samples run on wt/TSI bioassay (solid triangles) and Mc4/TSI bioassay (solid circles). The CVs were calculated from the mean of triplicate RLU values as described in *Patients and Methods*. The number of specimens in each TSI SRR% range (n), the mean value of CV, and the range of CV (in parentheses) were as follows: Mc4—SRR% 0 to 79, n = 58 and CV = 9% (2–25%); SRR% 80–140, n = 31 and CV = 9% (1–26%); SRR% 141–280, n = 35 and CV = 5% (1–21%); SRR% 281–420, n = 30 and CV = 4% (0–10%); SRR% 421–560, n = 69 and CV = 3% (1–11%); SRR% 561–700, n = 7 and CV = 4% (1–5%); SRR% 701–840, n = 10 and CV = 4% (1–11%); SRR% 841–1225, n = 0. wt—SRR% 0 to 79, n = 0; SRR% 80–140, n = 69 and CV = 8% (1–21%); SRR% 141–280, n = 45 and CV = 8% (2–20%); SRR% 281–420, n = 16 and CV = 8% (3–16%); SRR% 421–560, n = 33 and CV = 6% (0–13%); SRR% 561–700, n = 25 and CV = 7% (2–19%); SRR% 701–840, n = 20 and CV = 5% (1–10%); SRR% 841–1225, n = 32 and CV = 7% (1–21%). *, Paired sample *t* test of the % CVs of wtCHO vs. Mc4CHO ($P < 0.001$). C, Interassay precision CV calculated from 19 repeated independent plate runs of the positive (Pos) and negative (Neg) control set. The horizontal dotted line represents the manufacturer's cutoff limit calculated at TSI SRR% of 140.

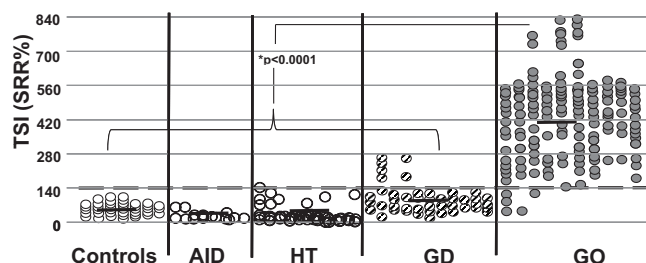


FIG. 2. Clinical diagnosis and TSI levels. Scatterplot of the TSI values (Mc4/TSI) of controls ($n = 40$, open symbols) and patients ($n = 200$, solid symbols). Additionally shown are TSI levels of patients with confirmed Hashimoto's thyroiditis (HT) ($n = 54$) and various autoimmune diseases (AID; i.e. systemic lupus, rheumatoid arthritis) ($n = 16$). *, Unpaired two-tailed t test comparison of TSI of GO ($n = 155$) vs. the TSI of GD ($n = 45$). It should be noted that the majority of the patients with GD had undergone antithyroid treatment and were rendered euthyroid at the time of blood sampling.

treatment or thyroid surgery, and three of five previously received steroids. Distribution of SRR% frequency reveals a distinct separation of the TSI levels of GO patients (412 ± 170) that are greater ($P < 0.001$) than that of GD patients (95 ± 54) and of controls (53 ± 21) (Fig. 2).

TSI levels strongly correlate with the clinical activity and clinical severity of GO

The TSI or the TRAb values of all patients were sorted with respect to the CAS or CSS, and then the data points were plotted. Strong positive correlations of TSI with both CAS (Fig. 3, A and B) and CSS (Fig. 4, A and B) as well as weaker correlations of two independent TRAb assays *vs.* CAS (Fig. 3, C and D) or *vs.* CSS (Fig. 4, C and D) were

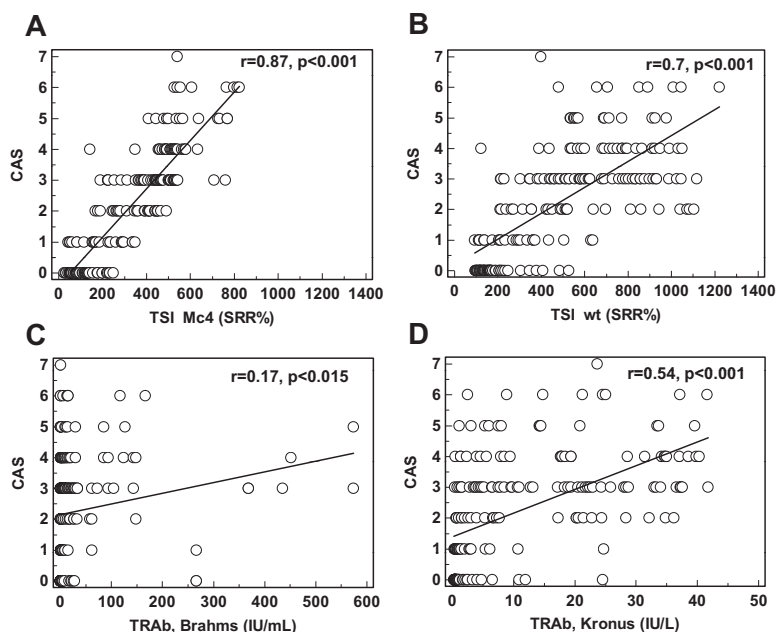


FIG. 3. Correlation of the thyroid-stimulating Igs in GO with the clinical disease activity. CAS and both the TSI (Mc4 bioassay *vs.* wt bioassay) represented by value of SRR% (A and B), as well as the anti-TSH-R binding activity, TRAb (C and D, Brahms and Kronus).

noted. The coefficients of correlation for Mc4/TSI were consistently higher compared with the coefficients of correlation of wt/TSI for both the CAS and CSS. All subjects with optic neuropathy and diplopia and smokers were TSI-positive. TSI levels were highest in GO patients with diplopia. In contrast, TPO and/or Tg autoantibodies correlated with neither activity/severity of GO nor TSI levels (results not shown).

TSI levels were also plotted *vs.* fT3 ($r = 0.253$, $P < 0.001$, Mc4/TSI; $r = 0.173$, $P = 0.014$, wt/TSI; $r = 0.194$, $P = 0.006$, TRAb) and fT4 ($r = 0.197$, $P = 0.005$, Mc4/TSI; $r = 0.148$, $P = 0.038$, wt/TSI; $r = 0.147$, $P = 0.039$, TRAb). The free thyroid hormone levels positively correlated with both bioassays and the Brahms binding assay, respectively. To eliminate the bias introduced in patients undergoing treatment with L-T₄, patients on hormone replacement therapy were not included in the plotted analysis.

TSI bioassay compared with anti-TSH-R binding assays

For all subsequent comparisons of the TSI *vs.* TRAb, only the Mc4/TSI was used. Both the sensitivity and the specificity of TSI were markedly greater (both $P < 0.001$) than that of the TRAb in GO (Table 2). The performance of the TSI bioassay was compared with anti-TSH-R binding assays by first testing all of the sera with TRAb RIA and tallying the percent positive and percent negative agreement with TSI. The discordant test results [TSI positive/TRAb RIA negative, 31/200 (15.5%); and TSI negative/TRAb RIA positive, 18/200 (9%)] (Table 2) were then confirmed by retesting with a second binding assay, TRAb ECLIA and Kronus RIA. Retesting resulted in a significant reduction in the number of discordants: Mc4/TSI positive/TRAb ECLIA negative, 11/200 (5.5%); and TSI negative/TRAb ECLIA positive, 7/200 (3.5%). The 11 patients whose sera tested TRAb-negative in at least two independent anti-TSH-R binding methods were scored as confirmed discordant findings. All patients whose sera were Mc4/TSI positive/TRAb negative had GO with severe disease, and most of these individuals were smokers (Table 3). Ten of these 11 were hyperthyroid and received antithyroid medications. In contrast, the seven GD patients that tested Mc4/TSI negative/TRAb positive presented with neither GO nor systemic involvement, and all were nonsmokers. All seven patients had a milder course of GD and became rapidly euthyroid during treatment with methimazole.

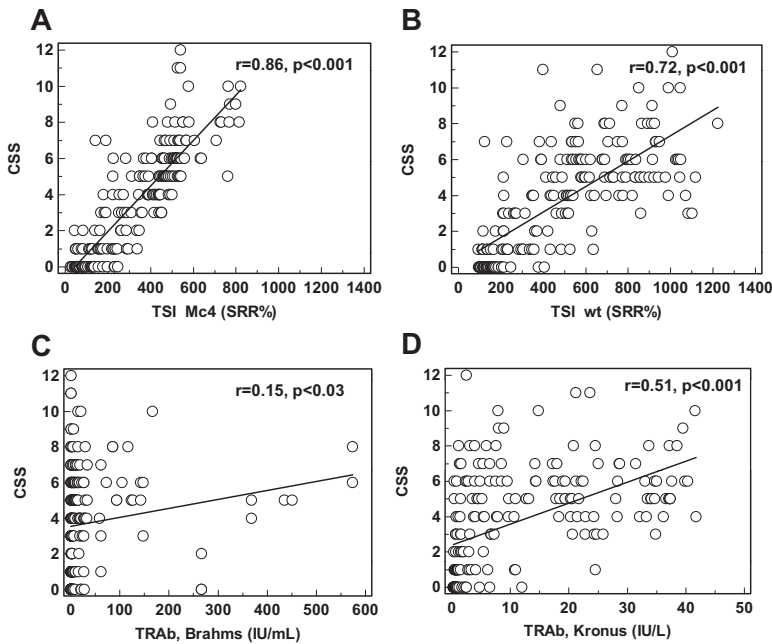


FIG. 4. Thyroid-stimulating Igs in GO correlate with the clinical disease severity. The CSS vs. TSI (both the Mc4 TSI and wt TSI, A and B) as well as the TRAb (C and D, Brahms and Kronus). Coefficient of correlations, TSI vs. CAS and of TSI vs. CSS are from data obtained from all 200 GD patients.

Discussion

In this controlled trial, all hyperthyroid GD/GO patients and the vast majority of the GO patients, independent of their thyroid function, were TSI-positive. Interestingly, all TSI-positive/TRAb-negative subjects had GO, whereas all TRAb-positive/TSI-negative subjects had GD only. Thus, the TSI levels in this large cohort of GD patients reveal striking positive correlation with the clinical activity and severity of GO. The present findings reinforce previous

TABLE 2. Comparison of the Mc4 TSI bioassay vs. the anti TSH-R binding assays (TRAb)

	GD	GO	Total	Controls
Mc4/TSI bioassay				
Positive	6	150	156	0
Negative	39	5	44	40
Total	45	155	200	40
TRAb RIA				
Positive	19	120	139	0
Negative	26	35	61	40
Total	45	155	200	40
Sensitivity				
TSI		97%		
TRAb		77%		
TSI vs. TRAb	$P < 0.001$			
Specificity				
TSI		89%		
TRAb		43%		
TSI vs. TRAb	$P < 0.001$			

The Mc4/TSI levels in all 200 GD patient sera were compared with the human TRAb RIA Brahms. Sensitivity and specificity of TSI vs. TRAb in the patients with GO are shown (an χ^2 test).

reports of the association between functional TSH-R autoantibodies with the clinical features of GO (9, 35) and support the conclusion that TSI levels are a strong indicator of disease activity and severity. Furthermore, these findings suggest that the TSI measured in the TSI/Mc4 assay are likely to represent the TSI that persistently provoke GO pathogenesis after antithyroid therapy. Although the TSI were ostensibly present in patients having extrathyroidal manifestations, it cannot be definitively concluded that the TSI cause stimulation of TSH-R expressing target cells in the periphery. It is not unexpected that the GD patients without GO showed a low prevalence of TSI positivity, given that the majority of these patients had undergone prolonged antithyroid treatment before time of sampling. Because a TSI test should have the best sensitivity and specificity in untreated GD, a prospective trial including untreated GD/GO patients is currently under way at our institution.

The novel Mc4/TSI bioassay described in this report can be performed in 1 d, requires limited technical skill, and displays a sensitivity and specificity similar to that of the wt/TSI assay. Although the Mc4/TSI as well as the TRAb assays show 100% specificity on control sera, the Mc4/TSI demonstrates greater sensitivity and specificity for detection of TSH-R autoantibodies in GO. In contrast to the weak correlation between TRAb and the clinical scores of GO, the Mc4/TSI shows a strong correlation with both CAS and CSS. The stimulating activity of several patient sera in Mc4/TSI was abolished by adsorption of serum on Protein G coupled beads. This finding is consistent with evidence that the TSH-R stimulation in TSI bioassays is attributed to IgG. Furthermore, it confirms the results of a previous study showing that the IgG purified from sera of GO patients retains TSI activity in a radioactive bioassay and associates with the clinical activity of GO (9). Unlike conventional TSI bioassays that require several days of cell culture and/or special sample preparation, the 1-d Mc4/TSI directly assesses TSI in unfractionated patient serum and performs with a precision and reproducibility favorable for routine reporting of TSI. The weak correlations of TRAb with CAS and CSS suggest that the Igs detected by binding assays engage the receptor’s extracellular domain (*e.g.* blocking or neutral binding) but do not necessarily activate the intact TSH-R and initiate pathogenic effects in target cells.

Previous evaluations of TSH-R binding inhibitory Igs in GO report increased levels of TSH-R autoantibodies

TABLE 3. Patients with discordant TSH-R autoantibody test results

Patient sera	CAS	CSS	Smoker	TPO-Abs	Tg-Abs	Mc4/TSI		Anti-TSH-R binding (IU/liter)		
						POS	SRR%	Brahms RIA	Elecsys LIA	Kronus RIA
All GO										
5	5	8	Yes	<10	<20	Yes	479%	0.5	6.67	7.5
11	2	3	No	21	<20	Yes	168%	<0.5	<1	6.6
12	3	4	Yes	<10	<20	Yes	224%	<0.5	<1	7.86
20	3	5	Yes	<20	<20	Yes	224%	<0.05	<1	0.6
54	3	4	Yes	>1000	<20	Yes	236%	1.3	<1	0.83
61	3	5	Yes	14.7	<20	Yes	248%	<0.05	<1	0.8
79	3	6	Yes	<10	<20	Yes	284%	<0.5	<1	28
87	1	2	Yes	92	<20	Yes	348%	1.5	<1	0.63
103	2	3	No	>1000	177	Yes	191%	<0.05	<1	1
104	7	11	Yes	<10	<20	Yes	541%	0.5	25.45	26
119	6	12	Yes	3	<20	Yes	541%	<0.05	<1	2.2
121	5	8	No	611	165	Yes	409%	1.2	<1	1.2
195	1	1	Yes	0	<20	Yes	276%	1.1	<1	0.63
All GD, no GO										
3			No	245	524	No	25%	267	34.2	0.6
73			No	98	<20	No	96%	4.6	5.98	0.6
93			No	976	782	No	131%	2.5	4.2	2.6
186			No	14	<20	No	125%	2.5	10.5	1
189			No	31	<20	No	133%	1.1	4.2	1.23
190			No	245	524	No	131%	1.8	2.2	42
191			No	629	<20	No	51%	1.9	2	0.9

The patient sera samples that gave discordant test results between the TSI and the RIA Brahms were all retested using a second anti-TSH-R binding method, LIA Elecsys Roche. Samples were also retested using a third binding method, RIA Kronus. Tg-Abs, Tg autoantibodies (normal values, <20 IU/ml); TPO-Abs, TPO autoantibodies (normal values, <10 IU/ml); POS, positive. The cutoff of TSI-positive is SRR% = 140. Normal values of anti-TSH-R binding assays are <1.5 IU/liter.

among cases of severe disease, independent of the patient's age and smoking habits. This trend was observed both at disease onset and at early time points after antithyroid medication (35, 36). In a clinical trial of anti-B-cell therapy in GD, TSI was assessed by a cAMP RIA in JP09 CHO cells (16). GD patients were first rendered euthyroid and then randomized to treatment with methimazole alone or methimazole plus anti-CD20 (Rituximab) therapy. Both treatment regimens demonstrably reduced the TRAb levels, but only the patients receiving Rituximab showed significant diminution of TSI. These results may indicate that TSI, in contrast to TRAb, are derived from B-cell subsets that persist after antithyroid drugs, and more aggressive therapy involving B-cell depletion may be required to eliminate TSI from the circulation. A controlled trial comparing the effects of Rituximab *vs.* iv steroids in GO patients found no significant reduction in the level of TRAb during B-cell depletion (29). Although these studies emphasize the importance of monitoring TSI during disease follow-up and therapy, unlike the present work, they do not differentiate between the levels of TSI and TRAb among patients with GD *vs.* GO.

The present evaluation of the Mc4/TSI bioassay revealed two unique discordant data sets. The first set was sera that tested positive for TSI and negative for TRAb; all sera from this set were from GD patients with severe GO and/or systemic involvement. The second set was sera that

tested negative for TSI and positive for TRAb; all sera from this set were from GD patients without extrathyroidal manifestations. The former discordant group was hyperthyroid and required antithyroid medications and close clinical management of their orbitopathy and systemic inflammation. The latter discordant group consisted of euthyroid GD patients who were in remission or had undergone radioiodine treatment or thyroidectomy and were mostly on L-T₄ therapy. In this regard, the high TRAb values and conspicuously low SRR% in the latter group are likely to be Igs with either blocking or neutral activity.

In conclusion, further insight into the clinical performance of this novel bioassay is forthcoming by prospective monitoring of TSI in untreated GD/GO at onset of diagnosis, at the time of relapse, and during treatment follow-up. A functional prognostic tool in GD has promising implications for the prediction of disease progression, relapse, and responses to antithyroid medications, immune suppressive drugs, and other therapeutic modalities.

Acknowledgments

The authors gratefully acknowledge the technical support of Lauren Grippa and Jeffrey Houtz [Diagnostic Hybrids, Inc. (DHI)]. They also thank Dr. Paul D. Olivo (DHI) so much for critically reviewing the manuscript and Dr. David R. Scholl (DHI) for helpful discussions.

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This work was supported by a grant from Diagnostic Hybrids, Inc. (Athens, OH).

Disclosure Summary: S.D.L., K.A.P., M.K., and N.M. have nothing to declare. L.D.K. has previously consulted for DHI, and G.J.K. consults for DHI.

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