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 coexpression 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 nonfunctional TSH-R remain important goals (14, 15). So far, the tests for detection of TSH-R autoantibodies in GD

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Abbreviations: CAS, Clinical activity score; CHO, Chinese hamster ovary; CSS, clinical severity score; CV, coefficient of variation; fT3, free T_3; fT4, free T_4; 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 antithyroid 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%)
Retrobulbar 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 (Fresh*Frozen*Cells; 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% CO_2, 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 μl 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 μl to the appropriate wells in triplicate, and the plate was placed in a humidified, 5% CO_2, 35 to 37 C incubator for 3 h. After the incubation period, all liquid was decanted from the plate, and 75 μl 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-μl aliquots to 2 ml microfuge tubes containing 250 μl 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 T_4 (fT4), and free T_3 (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% = (mean $TSI_{specimen}$/mean $TSI_{reference}$)*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 $TSI_{reference}$ 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% = ($SD_{RLU\,specimen}$/$mean_{RLU\,specimen}$)*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).
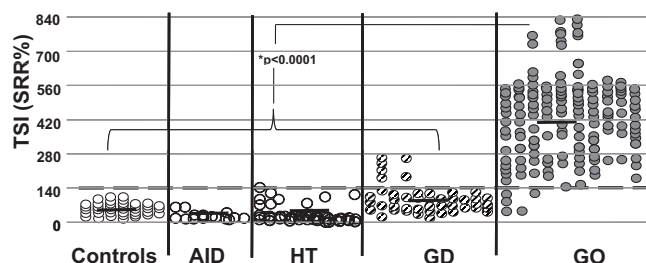
Clinical diagnosis and TSI levels

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
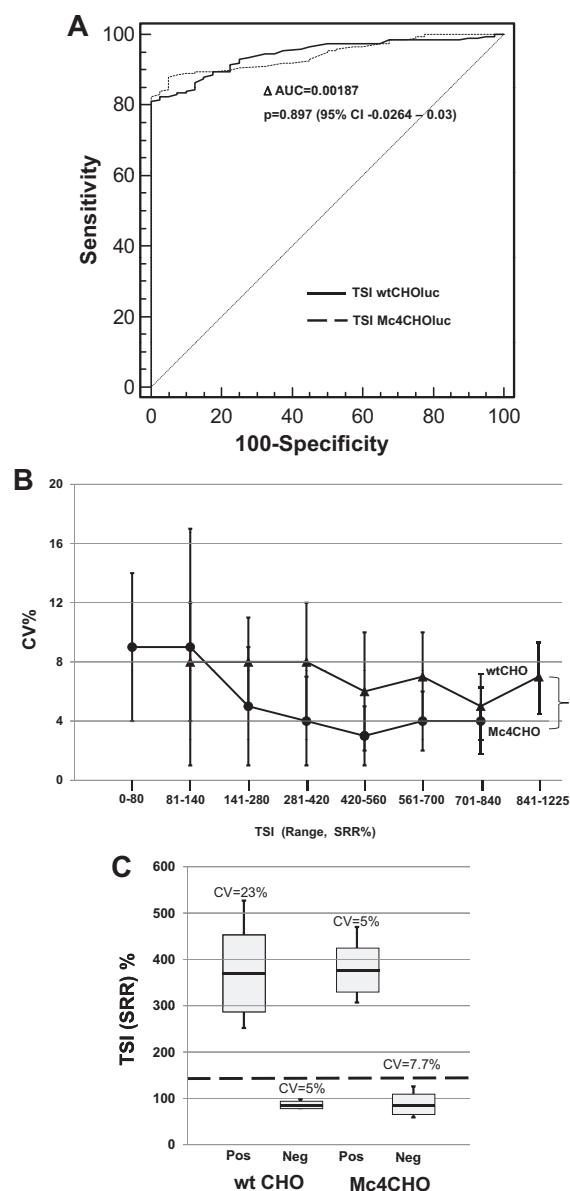
FIG. 1. Comparison of the wtCHO TSH-R and the chimeric TSH-R Mc4CHO TSI bioassays. ROC analysis of the data of 200 patients with GD and 40 healthy individuals. A, Δ AUC is the difference in the area under the curve between the two bioassays. B, Intraassay precision. The mean CV% mean and SD for each 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

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-T_4, 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/TS 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.
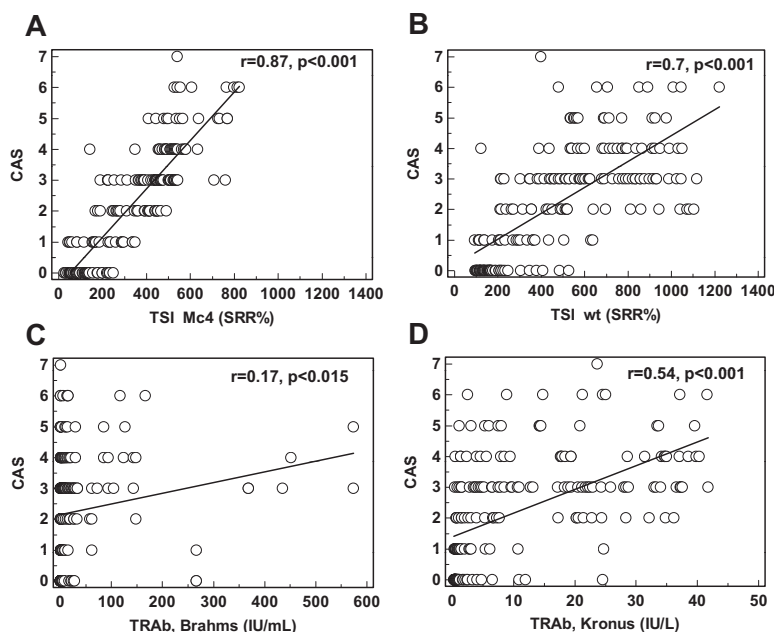
FIG. 3. Correlation of the thyroid-stimulating Igs in GO with the clinical disease activity. CAS and both the TSI (Mc4 bioassay *vs.* wt bioassay) represented by value of SRR% (A and B), as well as the anti-TSH-R binding activity, TRAb (C and D, Brahms and Kronus).
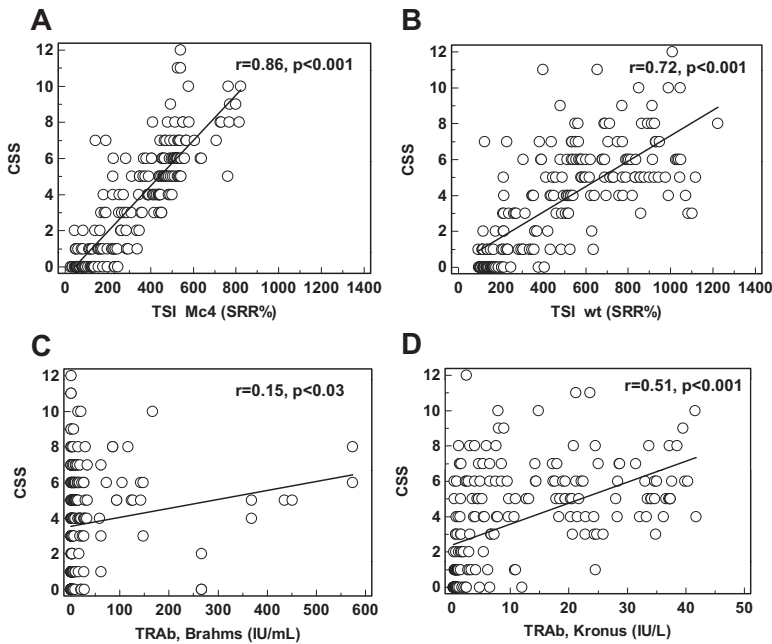
FIG. 4. Thyroid-stimulating Igs in GO correlate with the clinical disease severity. The CSS *vs.* TSI (both the Mc4 TSI and wt TSI, A and B) as well as the TRAb (C and D, Brahms and Kronus). Coefficient of correlations, TSI *vs.* CAS and of TSI *vs.* CSS are from data obtained from all 200 GD patients.

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 POS	Mc4/TSI SRR%	Anti-TSH-R binding (IU/liter) Brahms RIA	Anti-TSH-R binding (IU/liter) Elecsys LIA	Anti-TSH-R binding (IU/liter) 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-T$_4$ 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

References

1. Smith BR, Hall R 1974 Thyroid-stimulating immunoglobulins in Graves' disease. Lancet 304:427–431
2. McKenzie JM, Zakarija M 1976 A reconsideration of a thyroid-stimulating immunoglobulin as cause of hyperthyroidism in Graves' disease. J Clin Endocrinol Metab 42:778–781
3. Weetman AP, McGregor AM 1994 Autoimmune thyroid disease: future developments in our understanding. Endocr Rev 15:788–830
4. Michalek K, Morshed SA, Latif R, Davies TF 2009 TSH receptor autoantibodies. Autoimmun Rev 9:113–116
5. Orgiazzi J 2007 Pathogenesis. In: Wiersinga WM, Kahaly GJ, eds. Graves' orbitopathy: a multidisciplinary approach. Bale, Switzerland: Karger; 41–55
6. Lazarus JH, Marino M 2007 Orbit-thyroid relationship. In: Wiersinga WM, Kahaly GJ, eds. Graves' orbitopathy: a multidisciplinary approach. Bale, Switzerland: Karger; 27–33
7. Kahaly G, Yuan JP, Krause U, Hülbusch K, Beyer J 1989 Ciclosporin and thyroid-stimulating immunoglobulins in endocrine orbitopathy. Res Exp Med (Berl) 189:355–362
8. Kahaly G, Moncayo R, Stover C, Beyer J 1991 Relationship of eye muscle antibodies with HLA phenotypes and thyroid-stimulating immunoglobulins in endocrine orbitopathy. Res Exp Med (Berl) 191:137–144
9. Gerding MN, van der Meer JW, Broenink M, Bakker O, Wiersinga WM, Prummel MF 2000 Association of thyrotrophin receptor antibodies with the clinical features of Graves' ophthalmopathy. Clin Endocrinol (Oxf) 52:267–271
10. Douglas RS, Naik V, Hwang CJ, Afifiyan NF, Gianoukakis AG, Sand D, Kamat S, Smith TJ 2008 B cells from patients with Graves' disease aberrantly express the IGF-1 receptor: implications for disease pathogenesis. J Immunol 181:5768–5774
11. Tsui S, Naik V, Hoa N, Hwang CJ, Afifiyan NF, Sinha Hikim A, Gianoukakis AG, Douglas RS, Smith TJ 2008 Evidence for an association between thyroid-stimulating hormone and insulin-like growth factor 1 receptors: a tale of two antigens implicated in Graves' disease. J Immunol 181:4397–4405
12. Douglas RS, Afifiyan NF, Hwang CJ, Chong K, Haider U, Richards P, Gianoukakis AG, Smith TJ 2010 Increased generation of fibrocytes in thyroid-associated ophthalmopathy. J Clin Endocrinol Metab 95:430–438
13. Kahaly GJ 2010 The thyrocyte-fibrocyte link: closing the loop in the pathogenesis of Graves' disease? J Clin Endocrinol Metab 95:62–65
14. Massart C, Sapin R, Gibassier J, Agin A, d'Herbomez M 2009 Intermethod variability in TSH-receptor antibody measurement: implication for the diagnosis of Graves' disease and for the follow-up of Graves' ophthalmopathy. Clin Chem 55:183–186
15. Mizutori Y, Chen CR, Latrofa F, McLachlan SM, Rapoport B 2009 Evidence that shed thyrotropin receptor A subunits drive affinity maturation of autoantibodies causing Graves' disease. J Clin Endocrinol Metab 94:927–935
16. El Fassi D, Banga JP, Gilbert JA, Padoa C, Hegedüs L, Nielsen CH 2009 Treatment of Graves' disease with rituximab specifically reduces the production of thyroid stimulating autoantibodies. Clin Immunol 130:252–258
17. Morris 3rd JC, Hay ID, Nelson RE, Jiang NS 1988 Clinical utility of thyrotropin-receptor antibody assays: comparison of radioreceptor and bioassay methods. Mayo Clin Proc 63:707–717
18. Morgenthaler NG, Ho SC, Minich WB 2007 Stimulating and blocking thyroid-stimulating hormone (TSH) receptor autoantibodies from patients with Graves' disease and autoimmune hypothyroidism have very similar concentration, TSH receptor Affinity, and binding sites. J Clin Endocrinol Metab 92:1058–1065
19. Costagliola S, Morgenthaler NG, Hoermann R, Badenhoop K, Struck J, Freitag D, Poertl S, Weglöhner W, Hollidt JM, Quadbeck B, Dumont JE, Schumm-Draeger PM, Bergmann A, Mann K, Vassart G, Usadel KH 1999 Second generation assay for thyrotropin receptor antibodies has superior diagnostic sensitivity for Graves' disease. J Clin Endocrinol Metab 84:90–97
20. Schott M, Hermsen D, Broecker-Preuss M, Casati M, Mas JC, Eckstein A, Gassner D, Golla R, Graeber C, van Helden J, Inomata K, Jarausch J, Kratzsch J, Miyazaki N, Moreno MA, Murakami T, Roth HJ, Stock W, Noh JY, Scherbaum WA, Mann K 2009 Clinical value of the first automated TSH receptor auto-antibody assay for the diagnosis of Graves' disease (GD): an international multicentre trial. Clin Endocrinol (Oxf) 71:566–573
21. Hermsen D, Broecker-Preuss M, Casati M, Mas JC, Eckstein A, Gassner D, van Helden J, Inomata K, Jarausch J, Kratzsch J, Mann K, Miyazaki N, Navarro Moreno MA, Murakami T, Roth HJ, Noh JY, Scherbaum WA, Schott M 2009 Technical evaluation of the first fully automated assay for the detection of TSH receptor autoantibodies. Clin Chim Acta 401:84–89
22. Zakarija M, McKenzie JM, Banovac K 1980 Clinical significance of assay of thyroid stimulating antibody in Graves' disease. Ann Intern Med 93:28–32
23. Kasagi K, Konishi J, Iida Y, Ikekubo K, Mori T, Kuma K, Torizuka K 1982 A new in vitro assay for human thyroid stimulation using cultured thyroid cells: effects of sodium chloride on adenosine 3'5' monophosphate increase. J Clin Endocrinol Metab 54:108–114
24. Rapoport B, Greenspan FS, Filetti S, Pepitone M 1984 Clinical experience with a human thyroid cell bioassay for thyroid stimulating immunoglobulin. J Clin Endocrinol Metab 58:332–338
25. Kim TY, Park YJ, Park DJ, Chung HK, Kim WB, Kohn LD, Cho BY 2003 Epitope heterogeneity of thyroid-stimulating antibodies predicts long-term outcome in Graves' patients treated with antithyroid drugs. J Clin Endocrinol Metab 88:117–124
26. Kung AW, Lau KS, Kohn LD 2001 Epitope mapping of TSH receptor-blocking antibodies in Graves' disease that appear during pregnancy. J Clin Endocrinol Metab 86:3647–3653
27. Na CH, Lee MH, Cho BY, Chae CB 2003 A method for identification of the peptides that bind to a clone of thyroid-stimulating antibodies in the serum of Graves' disease patients. J Clin Endocrinol Metab 88:1570–1576
28. Tahara K, Ishikawa N, Yamamoto K, Hirai A, Ito K, Tamura Y, Yoshida S, Saito Y, Kohn LD 1997 Epitopes for thyroid stimulating and blocking autoantibodies on the extracellular domain of the human thyrotropin receptor. Thyroid 7:867–877
29. Salvi M, Vannucchi G, Campi I, Currò N, Dazzi D, Simonetta S, Bonara P, Rossi S, Sina C, Guastella C, Ratiglia R, Beck-Peccoz P 2007 Treatment of Graves' disease and associated ophthalmopathy with the anti-CD20 monoclonal antibody rituximab: an open study. Eur J Endocrinol 156:33–40
30. Wiersinga WM, Perros P, Kahaly GJ, Mourits MP, Baldeschi L, Boboridis K, Boschi A, Dickinson AJ, Kendall-Taylor P, Krassas GE, Lane CM, Lazarus JH, Marcocci C, Marino M, Nardi M, Neoh C, Orgiazzi J, Pinchera A, Pitz S, Prummel MF, Sartini MS, Stahl M, von Arx G 2006 Clinical assessment of patients with Graves' orbitopathy: the European Group on Graves' Orbitopathy recommendations to generalists, specialists, and clinical researchers. Eur J Endocrinol 155:387–389
31. Bartalena L, Baldeschi L, Dickinson A, Eckstein A, Kendall-Taylor P, Marcocci C, Mourits M, Perros P, Boboridis K, Boschi A, Currò N, Daumerie C, Kahaly GJ, Krassas GE, Lane CM, Lazarus JH, Marinò M, Nardi M, Neoh C, Orgiazzi J, Pearce S, Pinchera A, Pitz

S, Salvi M, Sivelli P, Stahl M, von Arx G, Wiersinga WM 2008 Consensus statement of the European Group on Graves' Orbitopathy (EUGOGO) on management of GO. Eur J Endocrinol 158:273–285

32. Bartalena L, Baldeschi L, Dickinson AJ, Eckstein A, Kendall-Taylor P, Marcocci C, Mourits MP, Perros P, Boboridis K, Boschi A, Currò N, Daumerie C, Kahaly GJ, Krassas G, Lane CM, Lazarus JH, Marinò M, Nardi M, Neoh C, Orgiazzi J, Pearce S, Pinchera A, Pitz S, Salvi M, Sivelli P, Stahl M, von Arx G, Wiersinga WM 2008 Consensus statement of the European Group on Graves' Orbitopathy (EUGOGO) on management of Graves' orbitopathy. Thyroid 18:333–346

33. Werner SC 1977 Modification of the classification of the eye changes of Graves' disease: recommendations of the Ad Hoc Committee of the American Thyroid Association. J Clin Endocrinol Metab 44:203–204

34. Tahara K, Ban T, Minegishi T, Kohn LD 1991 Immunoglobulins from Graves' disease patients interact with different sites on TSH receptor/LH-CG receptor chimeras than either TSH or immunoglobulin from idiopathic myxedema patients. Biochem Biophys Res Commun 179:70–77

35. Eckstein AK, Plicht M, Lax H, Neuhäuser M, Mann K, Lederbogen S, Heckmann C, Esser J, Morgenthaler NG 2006 Thyrotropin receptor autoantibodies are independent risk factors for Graves' ophthalmopathy and help to predict severity and outcome of the disease. J Clin Endocrinol Metab 91:3464–3470

36. Marcocci C, Pinchera A 2007 Thyroid treatment. In: Wiersinga WM, Kahaly GJ, eds. Graves' orbitopathy: a multidisciplinary approach. Bale, Switzerland: Karger; 100–110