Bridge Technology with TSH Receptor Chimera for Sensitive Direct Detection of TSH Receptor Antibodies Causing Graves’ Disease: Analytical and Clinical Evaluation

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Abstract

Graves’ disease is caused by stimulating autoantibodies against the thyrotropin receptor inducing uncontrolled overproduction of thyroid hormones. A Bridge Assay is presented for direct detection of these thyroid-stimulating immunoglobulins using thyrotropin receptor chimeras. A capture receptor, formed by replacing aa residues 261–370 of the human thyrotropin receptor with residues 261–329 from rat lutropin/choriogonadotropin receptor and fixed to microtiter plates, binds one arm of the autoantibody. The second arm bridges to the signal receptor constructed from thyrotropin receptor (aa 21–261) and secretory alkaline phosphatase (aa 1–519) inducing chemiluminescence. The working range of the assay is from 0.3 IU/l to 50 IU/l with a cutoff of 0.54 IU/l and functional sensitivity of 0.3 IU/l. Sensitivity and specificity are 99.8 and 99.1 %, respectively, with a diagnostic accuracy of 0.998. The low grey zone is from 0.3–0.54 IU/l. The stimulatory character of the assayed antibodies is shown through a good correlation ($r = 0.7079$, $p < 10^{-7}$) to serum T4 levels of untreated patients. In Graves’ disease, titers are increased in associated eye disease. In 3 hypothyroid patients with sera positive in the thyrotropin receptor competition assay and in the blocking bioassay, antibodies are not detected by the Bridge Assay, while the monoclonal blocking antibody K1–70 was detected. In Hashimoto disease thyrotropin receptor autoantibodies are detected in some patients, but not in goiter. This Bridge Assay delivers good diagnostic accuracy for identification of Graves’ disease patients. Its high sensitivity may facilitate early detection of onset, remission, or recurrence of Graves’ disease enabling timely adaption of the treatment.

Nonstandard Abbreviations

- AITD  Auto immune thyroid disease
- CRE  Cyclic AMP response element
- ECD  Extracellular domain
- EO  Endocrine orbitopathy
- GD  Graves’ disease
- IQR  Interquartile range
- LHCG  Luteinizing hormone/choriogonadotropin
- SEAP  Secretory alkaline phosphatase
- TRAb  Thyroid hormone receptor autoantibody
- TRBAb  Thyroid hormone receptor-blocking autoantibody
- TSH  Thyrotropin, thyroid-stimulating hormone
- TSHR  Thyrotropin receptor, thyroid-stimulating hormone receptor
- TSI  Thyroid-stimulating immunoglobulin

Introduction

Graves’ disease (GD) is an autoimmune disease caused by autoantibodies, which bind to the thyrotropin (TSH) receptor (TSHR) on the surface of thyrocytes, resulting in uncontrolled overproduction of thyroid hormones [1–3]. GD continues to pose a major health risk, with a prevalence of 2–3 % and an incidence of 0.2–0.5 % in iodine sufficient countries, females being affected 10 times more often than males [4–6]. For quantification of TSHR autoantibodies (TRAb) and confirmation of the clinical diagnosis, 2 different types of assay technology are commonly used in laboratory medicine. The most widely practiced assays measure the competition between binding of
TRAb and TSH [7,8] or a TSHR directed human monoclonal autoantibody [9], respectively, at the TSHR. In contrast to these in vitro TSH competition assays, bioassays measure increased production of cyclic AMP in cellular systems [10–14]. These assays exhibit high specificity but are delicate and laborious. The recently commercialized Thyretain bioassay, which detects the stimulatory activity of TRAb by a chimeric TSHR and a cyclic AMP response element (CRE)-reporter gene and luciferase signaling [12], has been approved by the FDA for use in the clinical laboratory. During revision of this manuscript, the standardization of the Thyretain bioassay [13] as well as a standardized rapid bioassay with detection of thyroid stimulation using cyclic AMP-gated calcium channel and aequorin [14] were published. Most direct assays of TRAb measure the amount of immunoglobulin bound to the TSHR based on immunoprecipitation [15–18], but they do not achieve high sensitivity and are therefore not used in the clinical laboratory. We have finally developed an in vitro assay, which directly detects the concentration of TRAb in sera by applying Bridge technology, which was preliminarily reported by us [19] and used by others for TSHR autoantibody studies [20]. By this technology the autoantibody is detected by binding with one arm to a capture receptor on the solid phase and bridging with the other arm to a detection receptor giving a signal. The assay uses chimeric TSHRs detecting thyroid stimulating immunoglobulins (TSI) based on an understanding of the structure of the extracellular domain (ECD) of the TSHR and its interactions with anti-TSHR antibodies [21,22]. Studies with mutant receptors indicate that the epitopes for TSI binding are fairly near the N-terminus of the ECD [12,23]. Therefore, in our in vitro assay, a chimeric human TSHR, in which aa residues 261–370 were replaced by an equivalent section of the rat LHCG receptor [23,24], was used as capture receptor, and the N-terminus of the TSHR (aa 21–261) was used as signal receptor for determination of the autoantibodies. The main aims of this communication are first to describe the technology of the novel assay and second to demonstrate its effectiveness in diagnosing GD with high sensitivity. In addition, the stimulatory character of the autoantibodies detected by the method had to be examined, all in comparison to a standard competition assay (TRAK human assay).

Materials and Methods

Principle of the Bridge Assay
The assay is based on the principle that the 2 antigen binding sites of the antibody form a bridge between 2 different TSHR molecules. One arm of the antibody binds to a capture receptor fixed to the surface of a microtiter plate via a coating antibody. The density of the capture receptor is such that its spacing allows only one arm of an antibody molecule to interact with a capture receptor. Once the TRAb molecule has been attached to the capture receptor, a signal receptor with secretory alkaline phosphatase (SEAP) attached is introduced. The signal receptor being in free solution binds to the other arm of the antibody. The amount of signal receptor bound, and thus the amount of antibody bound, is determined by the intensity of enhanced chemiluminescence development by reaction of SEAP with luminescent substrate. The 2 forms of the TSHR and the sequence of events in the assay are illustrated in Fig. 1.

Plasmids, chemicals, and substances
The cDNA for human TSHR (pSVL-TSHR plasmid) was kindly provided by Dr. B. Rapoport (Cedars-Sinai Research Institute, Los Angeles, CA, USA). Plasmid pcDNA3-rLHR(B9) was a gift from Dr. D. L. Segaloff (University of Iowa, Iowa City, USA). Other reagents were from the following suppliers: plasmids pIRESneo and pSEAP2-Basic (Clontech, Palo Alto, CA, USA); FuGene transfection reagent (Promega, Madison, OR, USA); DMEM, FCS and G418 (Biochrom, Berlin, Germany); and chemiluminescence substrate (AP-Juice 1 × Low Background, PJK, Kleinblittersdorf, Germany). Interferences were tested with the following substances from BioRad Laboratories Quality Control Products (BioRad, München, Germany): Liquicheck ANA Control Centromere/Homogeneous/Nucleolar/Speckled Pattern; Lyphocheck Immunoassay Plus Control Trilevel; Liquid Assayed Multiqual Trilevel; Liquicheck Rheumatoid Factor Control Trilevel; and anti-Islet Cell Positive

Fig. 1 TSH receptor engineering for Bridge Assay demonstrated by linear structure and illustrating the principle of the assay. The capture receptor chimera is coated on the solid phase via a polyclonal or monoclonal antibody (*) directed against the cytosolic tail of the receptor. The signal receptor is constructed from the chimeric ECD of the receptor and N-terminally fused SEAP.
Control. Interference testing for anti-Tg and anti-TPO were performed with anti-Tg Plus Standard S6 and anti-TPO Standards (Thermo Fischer Scientific/Brahms GmbH, Henningsdorf, Germany). Testing for Thyroiditis deQuervain was done with serum from a patient clinically positive for Thyroiditis deQuervain and negative for Graves’ disease. Total T4 was measured with a VITROS TT4 assay (Ortho Clinical Diagnostics, Neckargemünd, Germany). TRAB competition assays were determined with the TRAK human assay (Thermo Fischer Scientific/Brahms GmbH, Henningsdorf, Germany). TSHR stimulating or blocking activity was measured with our in-house cAMP response element (CRE) reporter gene bioassay [25].

Construction of plasmids
The capture receptor was formed by replacing amino acid residues 261–370 of the human TSHR with residues 261–329 from rat LHCG receptor [24], as described previously [16]. For the signal receptor, the DNA sequence encoding amino acids (aa) 21–261 was amplified by PCR and cloned giving pIRESneo-Chimera B(ECD). DNA encoding aa 1–519 for SEAP (pSEAP2-Basic) was amplified by PCR to give the SEAP ampiclon, which was then inserted into pIRESneo-Chimera B(ECD) to give pIRESneo-SEAP-Chimera B(ECD) vector.

Generation of receptor-producing cells
HEK293 cells in DMEM supplemented with 10% fetal bovine serum were cultivated in a 5% CO₂ atmosphere at 37°C. Cells were transfected either with pIRESneo-Chimera B or pIRESneo-SEAP-Chimera B(ECD) vector using FuGene transfection reagent. Forty-eight hours after transfection, selection was started with 0.8 mg/ml G418. Stable high expressing clones were subcloned and 5% CO₂.

Capture receptor
Cell extract for the capture receptor was prepared by standard methods. Shortly, confluent cells were resuspended in cold PBS (2–8°C) and centrifuged (10 min/4°C/4000×g). The cell pellet was lysed in buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Triton X100, protease inhibitors) and centrifuged (20 min/4°C/20000×g). The supernatant was frozen in nitrogen. Total protein concentration was determined by a DC-Protein Assay (BioRad) with BSA as standard. A Western blot was carried out to confirm the correct product size. A Bridge Assay was performed with each lot to confirm correct functionality of the protein. To exclude cross-reactivity with other cellular components, cell extracts of nontransfected cells were tested with 20 different stimulating sera or pooled sera.

Signal receptor
Signal receptor was secreted into the cell culture supernatant. Shortly, 9×10⁶ cells were incubated for 24 h at 27°C with 5% CO₂. Supernatant was collected, pooled, and centrifuged for 10 min at 4000×g to remove cells and debris, and frozen at −196°C. A Western blot was carried out to confirm the correct product size. The deduced protein size is 85 kDa. SEAP activity and binding to TRAB was confirmed in a Bridge Assay for each lot. To exclude cross-reactivity between autoantibodies and SEAP, supernatant of cells transfected with SEAP, but without receptor chimera, was used instead of the signal receptor.

Coating antibody
An epitope consisting of aa 741–762 from the cytosolic tail of the human TSHR was used to immunize sheep at Labor Dr. Merk und Kollegen (Ochsenhausen, Germany). Antibodies were affinity purified and stored in 50% glycerol at −20°C. Dilution experiments were carried out to find the optimal coating antibody concentration.

Bioassay of stimulating or blocking TSH receptor autoantibodies
Stimulatory activity of autoantibodies was measured as presented at the International Thyroid Congress 2010 in Paris [25] (Fig. 1S). Blocking activity was measured by inhibition of TSHR stimulation with 0.5 U/l bovine TSH, added directly before addition of serum.

Patient samples
Patients were referred because of thyroid problems or suspected or verified GD according to usual clinical practice in 2 thyroid clinics from surrounding noniodine-deficient regions with up to 2 million inhabitants. For ROC analysis, inclusion criteria for the diagnosis of GD, following the guidelines of the ATA and AACC [26], were: clinical and biochemical hyperthyroidism, exclusion of toxic adenoma, toxic multinodular goiter or subacute thyroiditis, hypoechogenicity and hypervascularization in ultrasound. Waste serum of blood withdrawal from these patients was collected and frozen at −20°C. Ethical approval was obtained from the Regierungspräsidium Stuttgart. Altogether 599 samples, collected from 2 local clinics referred with suspected or proven GD, were used for ROC analysis, of which 274 turned out to be clinically GD positive and 325 GD negative. Among the GD positive samples, 248 were from patients in treatment at the time of blood withdrawal and 27 were from untreated patients; 24 samples were from patients with active endocrine orbitopathy (EO) classified according to the EUGOGO guidelines [27]. Samples from 48 euthyroid patients with goiter diagnosed by ultrasound and autoimmune thyreopathy excluded were collected from one local clinic. Additionally 57 patients with Hashimoto thyroiditis were collected by usual inclusion criteria (clinical and biochemical hypothyroidism, typical thyroid imaging by ultrasound, elevated anti-TPO). These patients were transferred to the thyroid clinic from a large region of southern Germany. A separate retrospective collection of 42 samples from untreated GD patients was used for comparison of Bridge Assay results with T4 values. Three patients with hypothyroidism and positive TRAB (TRAK human) were found among hundreds of patients with thyroid problems during referral to 2 thyroid clinics.

Assay procedure
A 96-well microtiter plate was coated with 100μl of coating antibody (10μg/ml in 100 mM carbonate buffer pH 9.6), followed by overnight incubation at 4°C and washing with assay buffer (0.1% v/v Triton X-100, 100 mM NaCl, 50 mM Tris–HCl pH 8.0). After blocking for 1 h at 37°C with 300μl blocking buffer (5% milk powder and 5% glucose in 100 mM carbonate buffer pH 9.6), the liquid was discarded and 100μl of the capture receptor cell extract were added (500μg/ml in assay buffer), followed by incubation at room temperature for 1 h with shaking (300 rpm) and a wash step with assay buffer. Calibrators, controls or samples (50μl) and assay buffer (50μl) were added, and the plate was incubated at room temperature for 90 min with shaking
the range of standards are shown in the working range. Details for the intra- and inter-assay CVs over is shown by a mean within-run CV of 5.1 % for values covering only values with inter-assay CVs lower than 20 %. High precision 0.1 IU/l to 50.0 IU/l were made using the WHO standard 90/672 for 0.0 IU/l (zero calibrator).

The effective working range was from 0.3 IU/l up to 50 IU/l, using between autoantibodies and SEAP alone (data not shown).

To exclude cross-reactivity with other cellular components, extracts of nontransfected cells were tested with 20 different GD patient sera or pooled sera. None of these sera gave a signal higher than background. No cross-reactivity could be detected between autoantibodies and SEAP alone (data not shown).

The effective working range was from 0.3 IU/l up to 50 IU/l, using only values with inter-assay CVs lower than 20 %. High precision is shown by a mean within-run CV of 5.1 % for values covering the working range. Details for the intra- and inter-assay CVs over the range of standards are shown in Fig. 2a; results are from 50 independent runs (except 0.6 IU/l, 35 runs) with duplicate samples. Multiple dilution experiments with patient sera or pooled sera, showing antibody titers exceeding the measuring range up to 178 IU/l, did not show any hook effect as shown in a typical example in Fig. 3A. Results of the same dilutions measured with TRAB competition assay also show no hook effect but with pronounced flattening of the curve.

Interference testing
No interference was found for nonthyroidal autoimmune disease, and for endogenous metabolites and exogenous compounds (pharmaceuticals) in the panel of BioRad Laboratories Quality Control Products and thyroid autoantibodies of Thermo Fischer Scientific/Brahms GmbH.

Clinical characteristics of the Bridge Assay and comparison with competition assay

Serum samples from 599 individuals, GD positive (n = 274) or GD negative (n = 325), were used to assess the clinical characteristics of the Bridge Assay. Fig. 3 illustrates results for GD negative (median 0.01 IU/l; range undetectable to 0.49 IU/l) and GD positive samples (median 4.42 IU/l; range 0.54 IU/l to 50 IU/l). There were no false positive and no false negative results.

For the same patient samples, a TRAB competition assay (TRAK human) was performed (Fig. 3B). The working range of the latter is from 0.1 IU/l to 40 IU/l. Median and range for the TRAB competition assay for GD negative samples were 0.01 IU/l and undetectable up to 18.8 IU/l, respectively, and for GD positive samples 6.3 IU/l and 0.01 IU/l to 40 IU/l, respectively. The criterion for positivity in the Bridge Assay, calculated by ROC analysis, was > 0.54 IU/l, with a grey zone of 0.3–0.54 IU/l, and in the TRAB competition assay > 1.3 IU/l, with a grey zone of 1.0–1.5 IU/l according to manufacturer’s data. The sensitivity and specificity of the Bridge Assay were 99.8 and 99.1 %, respectively; the corresponding results for the TRAB competition assay were 96.7 and 95.4 %. The diagnostic accuracy of the 2 assays were 0.998 (Bridge Assay) and 0.978 (TRAB competition assay). These
results are illustrated in the ROC plot (Fig. 2c). There were 10 false positive and 7 false negative results in the TRAb competition assay; there were no false positive and no false negative results in the Bridge Assay. In the Bridge Assay 26 sera were in the grey zone. All but one of these sera were diagnosed as GD negative, the only clinically GD-positive serum had a result of 0.54 IU/l in the Bridge Assay, whereas this serum had a value of 1.4 IU/l in the TRAb competition assay. In contrast, there were 19 sera in the grey zone of the TRAb competition assay, with 7 of them clinically diagnosed GD positive and 12 as GD negative.

Table 1

<table>
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<th>Sample</th>
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<th>Clinical status</th>
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<td>R0527</td>
<td>1.0</td>
<td>0.0</td>
<td>Clinically normal</td>
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<td>R4565</td>
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<td>0.34 *</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>B0187</td>
<td>1.5</td>
<td>1.65</td>
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</table>

Comparison of results of serum from patients with TSHR blocking antibodies

PL02 18.8 0.0 Clinically hypothyroid, treated with l-thyroxine
A001a 13.0 0.0
A002 7.0 0.63

Comparison of results using a commercial TSHR blocking mAb

K1–70 ~3.5 1.53 * Close to the lower limit of the Bridge assay grey zone
<2 0.24 * At 20 ng/ml in normal human serum
* At 4 ng/ml in normal human serum

Thyroid disease other than GD

TRAb in serum from patients with goiter (n = 48) was undetectable, resulting in a specificity of 100% for both the Bridge Assay and the competition assay, while in samples of Hashimoto patients (n = 57) specificity was 82.5% for Bridge Assay and 86.0% for competition assay (Fig. 3).

The presence of TSHR blocking antibodies

In sera of 3 patients exhibiting clinical and biochemical hypothyroidism, using the TRAb competition assay, the presence of TRAb was within the range seen in GD patients. In contrast, using the Bridge Assay only one patient had a level within the GD low range (Table 1). Sera of 2 of these patients, who were suspected of possessing TSHR blocking antibodies, did not exert any stimulation in our bioassay [25], but blocked TSH stimula-
tion by 37 and 22 %, respectively. This qualitative in house bioassay measures SEAP secreted after being cleaved from CRE-reporter gene and secreted into the cell culture medium. No serum was left from the third patient for assaying blocking activity.

It has to be noted that the commercially available monoclonal blocking antibody, K1–70, showed results of 1.53 and 0.24 IU/l in the Bridge Assay at a concentration of 20 and 4 ng/ml, respectively. These concentrations correlate with estimations of the range of total TRAb concentration in patients' sera [30, 31].

Correlation with serum T4
The correlation between TRAb levels results from 42 untreated patients and the corresponding T4 serum values is shown in Fig. 4. The linear correlation of results using the Bridge assay had values of $r^2 = 0.5011$; $r = 0.7079$ ($p < 10^{-7}$), as compared with $r^2 = 0.3526$; $r = 0.5938$ ($p < 10^{-4}$) in the competition assay.

Relation of Bridge Assay results and endocrine orbitopathy
Among the 274 GD positive sera, the Bridge Assay results of a subgroup of 24 GD patients with active EO were compared with the results of 151 GD patients without EO. A subgroup of 265 GD-negative sera without Hashimoto or other thyroid diseases served as control group. Table 2 shows a highly significant difference ($p < 0.001$), with a mean of 29.64 IU/l (median 22.77 IU/l) in sera from patients with active EO vs. a mean of 6.65 IU/l (median 2.96 IU/l) in sera from patients with GD but without eye disease. For the control group, the mean was 0.06 IU/l (median 0.01 IU/l).

Discussion

Technical data
The measurement of thyroid-stimulating autoantibodies (TSA) described herein is in agreement with former and recent studies, which show that TSA bind to the N-terminus of the extracellular domain (ECD) of the TSHR and induce thyroid stimulation [11, 12, 23]. Thus, the Bridge Assay uses 2 TSHR chimeras, one, the capture receptor, with an intact N-terminus and having aa residues 261–370 near the C-terminus of the ECD substituted with a corresponding section from the LHCG receptor, and second, the signal receptor consisting of aa 21–261, thus, of the N-terminus of the ECD of the TSHR fused with SEAP as chemiluminescence monitor. The novelty of our Bridge Assay is the technology of TRAb measurement enabling double direct epitope recognition of autoantibodies by using these genetically engineered chimeric human TSHRs (Fig. 1). The capture TSHR, extracted from stable transfected cell lines, is anchored by a coating antibody to a solid phase to bind only one arm of the antibody. This is guaranteed by spacing of the capture TSHRs achieved by experimentally adjusted dilution of coating antibody. The second chimeric signal receptor fused with SEAP binds to the free second arm of the antibody and gives a signal for the quantification of the antibodies. The signal receptor is secreted into the cell culture medium and needs no further purification and consequently allows accurate detection of TRAb achieved by enhanced chemiluminescence signaling.

Analytical data
As reported herein, by comparison with the widely accepted TRAB competition assay (TRAK human), this novel technology acting through double direct detection of TRAb confers increased sensitivity (99.8 vs. 96.7 %) and specificity (99.1 vs. 96.5 %).

Table 2 Bridge Assay and endocrine orbitopathy.

<table>
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<th>Healthy</th>
<th>GD</th>
<th>GD with active EO</th>
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<tr>
<td>Number</td>
<td>265</td>
<td>151</td>
<td>24</td>
</tr>
<tr>
<td>Mean (IU/l)</td>
<td>0.07</td>
<td>6.65</td>
<td>29.64</td>
</tr>
<tr>
<td>Median (IU/l)</td>
<td>0.01</td>
<td>2.96</td>
<td>22.77</td>
</tr>
<tr>
<td>t-Test Healthy – GD $p &lt; 10^{-13}$</td>
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<tr>
<td>Healthy – GD with active EO $p &lt; 10^{-6}$</td>
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<tr>
<td>GD – GD with active EO $p &lt; 10^{-8}$</td>
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</tbody>
</table>

$^a$Bridge Assay results of 3 distinct groups of patients recruited from the collection of subjects used for ROC analysis: patients with GD without active or inactive endocrine orbitopathy (EO, n = 151), patients with GD and active EO (n = 24), and healthy subjects without any thyroid disease (n = 265). Patients with GD and inactive EO (n = 78) were excluded. The t-test shows highly significant differences.

Fig. 4 Correlation of anti-TSHR titers with results of T4 serum concentrations representing the main thyroid secretion product; retrospective collection of 42 samples from untreated GD patients. a Correlation of Bridge Assay results with T4 values showing linear correlation between titers in the Bridge Assay and T4 with $r = 0.7079$ ($p < 10^{-7}$). b Correlation of competition assay with T4 values illustrating linear correlation between TRAb and T4 with $r = 0.5938$ ($p < 10^{-4}$).
95.4%) as well as a very low cut-off of 0.54 vs. 1.53 IU/l and a low and small grey zone (0.3–0.54 IU/l vs. 1.0–1.5 IU/l). This also explains the lower functional sensitivity of the Bridge Assay Fig. 2S. Furthermore, a broad working range from 0.54–50 IU/l is given and serial dilution curves show a slope of one and beyond this range higher concentration of TRAb are without any hook effect.

Clinical evaluation
The Bridge Assay differentiates low TRAb values in GD patients from individuals without GD (Fig. 3). The grey zone from the 2nd generation TRAb assay can be resolved. These characteristics will allow early diagnosis of onset and remission as well as relapse of disease. Early treatment of GD ameliorates the severity of the course of the disease [32] by avoiding an increase of the TSHR autoantibody titer and consequently worsening of hyperthyroidism. According to Eckstein et al. [33], there is an increasing risk of induction of “frightened face” endocrine orbitopathy (EO), when the titer of TRAb increases. Our studies clearly support these findings, demonstrating increased antibody titers in active EO (Table 2).

The Bridge Assay showed excellent variance data and high agreement with the diagnosis of GD (see ROC analysis). Furthermore, in untreated GD patients the antibody titers measured by the Bridge Assay correlated significantly with the main thyroid secretion product represented by serum T4 levels, whereas the 2nd generation TRAb assay showed a relatively low correlation (Fig. 4). Comparatively, in untreated GD patients the Thyretain assay using similar TSHR chimera as the presented Bridge Assay (with nearly identical aa exchange) was reported to exhibit a higher correlation to fT4 serum levels than did the bioassay using wild-type TSHR [12]. The Thyretain bioassay by its chimera binds TRAbs, which exhibited stimulation of cAMP production more effectively than its version using wildtype TSHR [12]. Altogether, these data strongly suggest that the thyroid antibodies measured by the Bridge Assay in fact are thyroid-stimulating antibodies.

Possible interferences with TSH receptor blocking antibodies (TRBAb)
We did not find binding activity in the sera of 3 patients, who were positive in the TRAK human assay and exhibited clinical and biochemical hypothyroidism. This discrepancy is explained by the existence of TRBAb, as shown by inhibition of TSH stimulation by our in house CRE reporter gene bioassay [25] in 2 of 3 of these patient sera. Nevertheless, the commercial K1-70 antibody [34,35], a human monoclonal blocking antibody, exhibited binding activity in our assay, but considerably less than in the TRAK human assay (Table 1). The Thyretain bioassay was reported as capable of measuring inhibition of TSH-stimulated cyclic AMP production for the detection of TRBAb in some patients withAITD [36]. On the other hand, serum samples of 22 TRAB (TRAK human) positive patients with hypothyroidism and blocking activity in the bioassay the majority (19) showed binding at the C-terminus of the TSHR ECD having the N-terminal part (aa8–165) replaced by LHCG receptor residues and being coated to tubes for measurement of TRBab by TSH competition [37]. There are several further reports on TRBAb binding at the C-terminus as well as at the N-terminus of the TSHR ECD, which findings may thus be explained by epitope heterogeneity of TRBAb binding [20,37–40]. None of the studies provided a recruitment regimen, which would allow to estimate the prevalence of TRBAb positive patients. The prevalence of patients with TRBAb seems to be very low. Our experience with 2 clinics is that less than one of such patients occurs among 100 of GD patients recruited by usual transferal practice. Concerning stimulating TRAb, there is unanimous opinion that they bind exclusively at the N-terminus of the TSHR ECD [12,20,40,41].

Other thyroid diseases
Patients with non-autoimmune euthyroid diffuse, uni- or multinodular goiter are not recognized by our Bridge Assay. This fact is of importance because goiter is a very frequent thyroid disease. However, in serum of 57 hypothyroid patients with Hashimoto disease high TPO Ab titers are accompanied by moderately elevated titers of TRAb measured by our Bridge Assay as well as by the TRAK human assay. These data are in agreement with the literature [42] reporting increased sensitivity accompanied by loss of specificity when detecting TRAb titers in hypothyroid Hashimoto disease.

Perspectives
Knowledge of the TRAb titers measured by the Bridge Assay at onset as well as during monitoring of the disease under treatment may contribute to the interesting observations on the prediction of the course and prognosis of the disease [43,44]. Furthermore, the Bridge Assay may assist for elucidating the character of elevated TRAb values during the course of methimazole treatment and in remission, which are suspected of having changed biological activity [45]. Concerning the binding site of blocking TRAb, extensive studies with many TRBAb harboring patients will be necessary to show by epidemiological planned recruitment the differentiation and relation of the different binding locations at the TSHR ECD.

Conclusion
The technology of the Bridge Assay presented here leads to good accuracy for detection of thyroid stimulating immunoglobulins. The high sensitivity allows early diagnosis and therefore timely treatment of GD, thus avoiding aggravation and complications of this disease. TRAb measured by the Bridge Assay using hybrid TSHR for capturing and quantifying TRBAb correlated closely with serum T4 levels (Fig. 4) and thus strongly suggests that this Bridge Assay measures stimulation of thyroidal secretion by TRAb very effectively. This is supported by the generally acknowledged opinion that thyroid stimulating immunoglobulins bind at the N-terminal part of the TSHR ECD, although it is not excluded that some of the rare blocking autoantibodies are binding at the N-terminus and are thus recognized by our Bridge Assay. Nevertheless, the Bridge Assay will permit new, detailed evaluation of GD patients both at presentation and throughout their management. Finally, the robustness of the Bridge Assay may enable high throughput by performance on automated platforms.

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Conflict of Interest

Ulrich Loos is co-owner of KreLo GmbH Medical Diagnostics.
Sigrid Brath is employee at KreLo GmbH Medical Diagnostics.
Claudia Frank was employee at KreLo GmbH Medical Diagnostics until 2013. KreLo GmbH Medical Diagnostics has a cooperation agreement with Siemens Healthcare Diagnostics.

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