Functional diagnostics for thyrotropin hormone receptor autoantibodies: bioassays prevail over binding assays

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1. ABSTRACT

Autoantibodies to the thyrotropin hormone receptor (TSH-R) are directly responsible for the hyperthyroidism in Graves’ disease and mediate orbital manifestations in Graves’ orbitopathy (otherwise known as thyroid eye disease). These autoantibodies are heterogeneous in their function and collectively referred to as TRAbs. Measurement of TRAbs is clinically important for diagnosis of a variety of conditions and different commercial assays with high sensitivity and specificity are available for diagnostic purposes. This review provides overwhelming evidence that the TRAbs detected in binding assays by mainly the automated electrochemical luminescence immunoassays (ECLIA) do not distinguish TRAbs that stimulate the TSH-R (called TSIs or TSAb) and TRAbs that just inhibit the binding of TSH without stimulating the TSH-R (called TBAbs). However, TSAbs and TBAbs have divergent pathogenic roles, and depending which fraction predominates cause different clinical symptoms and engender different therapeutic regimen. Therefore, diagnostic distinction of TSAbs and TBAbs is of paramount clinical importance. To date, only bioassays such as the Mc4 TSH-R bioassay (Thyretain, Quidel) and the Bridge assay (Immuleite 2000, Siemens) can measure TSAbs, with only the former being able to distinguish between TSAbs and TBAbs. On this note, it is strongly recommended to only use the term TSI or TSAb when reporting the results of bioassays, whereas the results of automated TRAb binding assays should be reported as TRAbs (of undetermined functional significance). This review aims to present a technical and analytical account of leading commercial diagnostic methods of anti-TSH-R antibodies, a metaanalysis of their clinical performance and a perspective for the use of cell based TSH-R bioassays in the clinical diagnostics of Graves’ disease.

2. INTRODUCTION

Formation of autoantibodies against the master endocrine G-protein coupled receptor; thyrotropin hormone receptor or thyroid stimulating hormone receptor (TSH-R), is the hallmark of thyroid autoimmunity and a powerful example of how autoantibodies themselves actually cause the illness
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via signaling either an overstimulation or inhibition of intracellular cAMP (1,2). The most remarkable feature of anti-TSH-R autoantibodies are their polar opposite function - they are grouped into two camps according to either stimulating or blocking activity which are linked to distinct clinical symptoms, hyper- and hypothyroid function respectively (3,4). A third type of 'neutral' antibodies may be involved in apoptosis via their interaction with the cleavage region of TSH-R ectodomain (5). The stimulating and blocking autoantibodies bind into a common concave pocket of the TSH-R extracellular domain which when occupied by the thyroid stimulating hormone (TSH) normally activates the receptor leading to thyroxine and triiodothyronine production (3,6,7). In hyperthyroid Graves’ Disease (GD) stimulating immunoglobulins (TSAb or TSI) prevail with positive TRAb, whereas in cases of autoimmune hypothyroidism, anti-TSH-R autoantibodies are produced which block TSH binding and inhibit the activation of TSH-R (TBAb) (2) and are termed TSH blocking antibodies (TBAbs). However, the pathogenic role of blocking TRAb has not been proven in clinical studies, since it is not known in which percentage of the patients with autoimmune thyroid disease the hypothyroidism is the result of destructive thyroiditis rather than the activity of blocking TRAb. Both TSAbs and TBAbs are readily measured by bioassays (8). Other assays are solely in vitro based and measure TSH binding inhibiting immunoglobulins which detect both TSAbs and TBAbs but cannot differentiate between them and are referred to as TBII (9). The TBII assays in current use are third generation assays with high sensitivity and specificity for diagnostic laboratories.

The urgent need to accurately diagnose autoimmune thyroid disease and to distinguish stimulating and blocking autoantibodies is driven by the high incidences of GD of 0.1.- 0.3.%, which includes extra thyroid complications notably Graves’ orbitopathy (GO) in 40% of cases (2, 10). Previous reviews of the thyrotropin hormone receptor (TSH-R) focus on its discovery, biochemistry and role in GD (11-13) and the update of functional anti-TSH-R autoantibody diagnostics and applications of assay methods in thyroid clinical practice (4, 14). The lessons learned are summarized as follows;

2.1. Tissue expression

TSH-R is present on the basal surface of thyroid follicular cells and is intimately involved in regulating thyrocyte cell growth and function (11). The TSH-R is also present in extra-thyroid tissues such as fibroblasts, heart, kidney, bone, astrocytes and human brain (15, 16). Moreover, the TSH-R also associates with other receptors such as insulin like growth factor receptors (IGF-R) by cross talk leading to pathogenicity in GO (17). Anti-IGF-1 receptor monoclonal antibody therapy shows efficacy in clinical trials (18), but the exact role of extrathyroidal TSH-R in mediating inflammatory responses, metabolic activity and cell differentiation in GO remain poorly understood.

2.2. TSH-R / anti-TSH-R autoantibody immunopathology axis

Dendritic cell processing and presentation of thyrotropin receptor antigen and subsequent events of T-cell dependent B cell clonal expansion involve multiple immunogenic response elements including defective regulatory T cell function (19, 20) and memory B-cell survival factors such as B-cell activating factor (BAFF) (5). The anti-TSH-R autoantibodies produced are directed primarily against the extracellular domain of TSH-R with some reports of neutral non-functional autoantibodies which bind the hinge region. Mouse models of GD induced by genetic immunizations of TSH-R extracellular region termed TSH-R A-subunit replicate closely the human clinical characteristics of GD and importantly GO (21).

2.3. TSH-R structures

High resolution x-ray diffraction of human TSH-R leucine rich repeat region in complex with Fab fragments of human monoclonal autoantibodies reveal that stimulator and blocker autoantibodies share striking similarity in the type of amino acid contacts they make with the concave surface of leucine rich repeat domain. The flexible extracellular TSH-R sequences and hinge region remain unresolved. Molecular models of TSH-R based on known G-protein coupled receptors (GPCR) structures are currently in use to predict how the extracellular region of the receptor communicates conformational changes in transmembrane (TM) domains (7, 12, 13).

2.4. Generations of anti-TSH-R autoantibody diagnostics

Two types of assay technology are available for the quantification of TRAb binding: competition assays and assays applying bridge technology. Competition (TBII) assays are the most widely used. The 2nd generation TRAb binding assays used TSH-Rs that are immobilized on to plastic surfaces by capture monoclonal antibodies specific for C-terminal region of TSH-R. The binding of immunoglobulins of patient serum to TSH-R is measured by the binding inhibitory activity of iodide-125 labeled TSH radioreceptor or biotinylated TSH detected enzymatically by a colorimeter or chemiluminescence reagent. The 3rd generation TRAb binding use the antigen binding fragment (Fab) of stimulatory anti-TSH-R human monoclonal antibody (M22 or KSAb1) (22) instead of TSH to quantify the signal produced after the displacement of the labeled tracer from porcine or
human TSH-R protein by immunoglobulins in patient serum. Unlike labelled TSH, the monoclonal Fab competition immunoassays are applied in automated platforms of electro-chemiluminescent immunoassays (ECLIA) such as Cobas Elecsys (Roche Diagnostics) (23-26) and Kryptor/Phadia (ThermoFischer) (26) which show greater sensitivity than 2nd generation TRAb binding assays. TRAb binding assays applying bridge technology copy the chimeric thyrotropin receptor developed in the commercial TSH-R bioassay, and use this construct as both the capture receptor and the reporter receptor. The bridge binding assay has been configured to run on automated Immulite XPi (Siemens Diagnostics) (27-31).

In contrast to TRAb binding, the TSH-R bioassays utilize live cells to directly assess functional autoantibodies having either stimulatory, increased cAMP signaling, or blocking activity, inhibition of the TSH CAMP signaling. The history of GD diagnostics began with in vivo bioassays of thyrotropin after injection of rodents with radio-iodide and the use of home grown in vitro cell cultures of porcine thyrocytes (1). A long acting thyrocyte stimulator (LATS) assay was developed in the late 1960s to assess capacity of patient serum immunoglobulins to stimulate in mice uptake of I-125 radiolabeled thyroxine (8, 32). In the 1980s a commercial cell bioassay using either rodent FRTL5 cells or thyrocytes freshly isolated from normal human thyroid reported TSI-induced 125I-cAMP uptake of I-125 radiolabeled thyroxine (8, 32). The assay required 2 days of cell culture and its use was limited to only the Japanese market (32). In 2009 the first FDA approved commercial bioassay of thyrotropin receptor stimulating immunoglobulins (TSI or TSAb) using the Mc4 chimeric receptor, formed by replacing residues 261–370 of the human thyrotropin receptor with residues 261–329 from rat lutropin/choriogonadotropin receptor stably expressed in CHO cells, was launched under the name Thyretain™ (34-36). The blocking inhibitory bioassay, TBAb (37-39) was subsequently developed and is currently undergoing FDA 510K review. The TSI bioassay enjoys routine reimbursement in the USA, German and Korean Healthcare systems, but with substantially lower testing volume numbers than the 3rd generation TBI assays based on automated platforms. In this review the principle technologies and performances of the commercialized TSH-R bioassay and the automated commercial anti-TSH-R binding immunoassays are presented and compared. Original data on performance of these systems offers new insights into the analytical sensitivity and functional specificities.

3. TSH-R STRUCTURES: THE LESSONS LEARNED

The molecular structures and biochemical insights into autoantibody interactions with TSH-R by the independent teams of Bernard Rees-Smith (3, 6, 7, 13) and Basil Rapoport (11, 40) were decisive to understanding the concept of functional stimulating and blocking autoantibodies in thyroid autoimmune disease. The details are beyond the scope of this review. To summarize, the stimulating monoclonal M22 and blocking monoclonal K1-70 enter into the horse shoe shaped concave surface of leucine rich domain (LRD) repeats in the extracellular domain (ECD) and make predominantly the same TSH-R amino acid contacts which the hormone ligand TSH normally engages in order to transmit signals for cAMP-induced stimulation of thyroxine (Figure 1). High affinity binding of M22 (6) and K1-70 (7) to TSH-R LRD are attributed to hydrogen bonds and salt bridges and fewer hydrophobic interaction than TSH.

The disulfide loop of the ECD and the flexible hinge region which links ECD to transmembrane domain cannot be determined due to the crystal disorder (6, 7). This fact and the uncertainty of models to predict the conformational changes of transmembrane helices (12, 13) are major challenges to understanding signaling in the TM domains and G-protein activation of cAMP upon binding interactions of ECD with TSAb and TBAbs.

4. AUTOANTIBODY BIOACTIVITY VERSUS TRAb BINDING

4.1. A tale of two principles

The nomenclature in the literature referring to autoantibodies produced against the TSH-R is confusing (41). For the sake of clarity and consistency this review defines autoantibodies in accordance with the methods used to detect them. In the event of patient immunoglobulins or monoclonal antibodies (MAbs) measured by TSH-R binding immunoassays, the autoantibodies detected are termed “TRAb”. In the event of patient immunoglobulins or MAbs measured by the TSH-R cell based cAMP reporter bioassays, the increased cAMP production is attributed to thyrotropin hormone stimulating immunoglobulins termed “TSI” or “TSAb” (8, 33-36) (Figure 2). Those patient immunoglobulins or MAbs which inhibit bTSH stimulation of cAMP in the TSH-R bioassay are termed blocking and/or inhibitory immunoglobulins “TBAb” (37-39). As mentioned earlier, TBI assays are determined by binding immunoassays (TRAb) and refer to either the radioreceptor assays or 3rd generation TBI assays using MAbs as tracer instead of radiolabeled bTSH (24-26) or Bridge immunoassay (27-29) (Figure 3).

The CE-marked, FDA 510K commercial Mc4 TSH-R bioassay Thyretain™ uses CHO cells of stable chimeric Mc4 TSH-R expression to assess the stimulating immunoglobulins in patient serum (TSAb) (33, 34) (Figure 2). The Mc4 CHO cells have been configured for measuring autoantibodies which block
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**Figure 1.** TSH-receptor structures reveal similar contacts with stimulator and blocker monoclonal autoantibodies. The x-ray crystal structure of TSH-R complexes with Fab of human stimulating human monoclonal autoantibody M22 protein data base (PDB) accession 2XWT (blue, left) and the thyroid blocking human monoclonal autoantibody K1-70 PDB 3G04 (blue, right) reveal CDR variable H and L chain contacts to the concave surface of leucine rich domain (LRD) of extracellular domain (ECD) of human TSH-R (yellow) that closely mimic the binding of hormone TSH. Conformational changes in transmembrane (TM) domain after autoantibody binding lead to either the activation or the inhibition of G-protein coupled intracellular cAMP signaling. Molecular models predict “active” (open cone) and “inactive” (closed cone) conformations of the TM helices. The critical motifs of ionic lock, proline kink and distortion and alpha-bulge are considered important in transmission of stimulating versus blocking signals (12,15).

the TSH-R by measuring the amount of cAMP produced relative to TSH stimulation (TBAb) (38, 39) (Figure 2). The CE-marked TBAb bioassay is undergoing FDA review for the reporting of TBAbs. After the thaw from frozen shell vials, Mc4 CHO cells are plated on microtiter wells coated with cell attachment solution and incubated overnight. The bioassay is then ready to use. Patient serum is added and within 3 hours the level of TSAb is determined in cell lysates containing chemiluminescent substrate. The measurements of relative light units (RLU) in luminometer are proportional to the cAMP induced luciferase activity. The percentage sample signal to reference ratio (SRR%) defines the TSAb level and the percentage sample inhibition of luciferase expression relative to the induction by bovine TSH alone defines the blocking autoantibodies TBAb level (Figure 2).

A non-commercial TSI bioassay, using CHO cells that express wild-type human TSH-R fused to G-alpha S subunit with a GTPase-inactivating mutation in 8 hour work flow for luminescence endpoint measurement after overnight culture, reported a sensitivity of 93%, specificity of 99% and efficiency of 98% in testing of 56 GD, 27 toxic thyroid nodule patients and 119 normal euthyroid subjects (42). The idea of a “rapid” bioassay to directly assess TSH-R stimulating immunoglobulins on thawed cells without overnight culture was investigated in order to optimize and streamline the work flow of TSH-R bioassays. Instead of quantifying the cAMP level by luciferase reporter gene expression, the TSH-R G-protein activation of cAMP gated calcium channel was detected by measuring the calcium influx with bioluminescent substrate aequorin (43). These bioassays, like that of the GTPase inactivating mutation are of interest for experimental research. To prove reliability for routine diagnostic application their clinical performance will need to be compared with theMc4 Bioassay predicate.
The demand for replacing manual methods with high throughput automated liquid handling systems, is driven by cost-savings in labour and need for faster sample turn over and reporting time in routine clinical laboratory. Manufacturers have developed new generation methods for measuring anti-TSH-R autoantibodies, which can be adapted to the automated ECLIA immunoassay platform. The commercial fully automated anti-TSH-R ECLIA immunoassay developed from ELISA methods are based on the principle of either the displacement or Bridge format (Figure 3). The essential design was achieved by stable immobilization of extracellular domain (ECD) of TSH-R on beads via anti-TSH-R capture antibody directed against porcine C-terminus which does not interfere with TRAb binding to ECD. In subsequent steps the patient sera are added. After washing cycles the specific immunoglobulins are detected by either the displacement of a pre-bound complex of a labeled anti-TSH-R human monoclonal antibody Fab fragment, quantification of the signal from released ruthenium labeled M22 Fab (Figure 3), or by the formation of oligomeric bridge; the patient autoantibody captured in the first step binds with its second arm to a second TSH-R molecule which is linked to the signal alkaline phosphatase, referred to as the Bridge assay (Figure 3). The successful formation and preparation of native TSH-R after extraction in detergent micelles was the greatest technical advance to facilitate development of the fully automated ECLIA TRAb immunoassays. In the case of Cobas Elecsys, stability of porcine TSH-R immobilized on streptavidin magnetic beads was achieved by pre-complex with human monoclonal M22 (6, 23, 24) and the signal recovery after M22 displacement was significantly enhanced by addition of chemical chaperones (23). Curiously the Immulite ECLIA TRAb immunoassay (26-29) uses capture and signal TSH-R molecules that contain the same extracellular domain of Mc4 chimeric TSH-R developed by Len Kohn (33-35). Upon loading and start of pipetting from the reagent packs, immunoassay times are approximately 19 minutes and 1 hour on the Cobas Elecsys™ and the Immulite™ respectively. Both systems show high sensitivities and specificities 97-100% and 98-99% respectively with similar technical specifications. Intra-assay CV% 1.4.-7.6 and inter-assay CV% 2.4.-11.4. of operational range 0.1. U/L – 40 U/L with clinical cut off 1.7.5. U/L Cobas Elecsys™ assay (30, 31). The intra-assay CV% 4-9% and inter-assay CV% 11-22% of operational
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Anti-TSH-R Autoantibody Binding-TRAb Immunoassay

A. Displacement of labeled monoclonal M22 Fab

B. Bridge of TSH-R Capture and TSH-R Signal

Figure 3. Principle of anti-TSH-R autoantibody binding immunoassays. The automated commercially available enhanced electro-chemiluminescent immunoassays (ECLIA) detect immunoglobulins in patient sera having direct binding interactions with the extracellular domain (ECD) of human TSH-R (TRAb) The TRAb binding activity is measured using two technologies: A) displacement of the ruthenium-labeled antigen binding fragment (Fab) of thyroid stimulating human monoclonal autoantibody M22 in Cobas elecsys ECLIA (27-30) or by B) the formation of an oligomeric binding “bridge” between two different TSH-R molecules; one arm of the TRAb binds a capture TSH-R sequence fixed to the surface of a polystyrene bead via monoclonal antibody directed against the TSH-R C terminus and the second arm of the TRAb binds to signal TSH-R with an attached secretory alkaline phosphatase for quantification of the ECLIA signal intensity in Siemens Immulite (B) (27-30).

range 0.1 – 40 U/L clinical cut-off 0.5. U/L bridge immunoassay technology (26-31).

4.2. Analytical sensitivity and specificity of TSH-R bioassay vs TRAb binding immunoassay

The analytical sensitivity and specificity of TSAb and TBAb in the Mc4 bioassay versus TRAb in ECLIA binding immunoassays is evaluated (Figure 4). Serial dilutions of known concentrations of stimulatory monoclonal autoantibodies KSAb1 and M22 and the blocking monoclonal autoantibody K170 were made into serum of a normal euthyroid individual and the dose-response curves of SRR%, U/L binding and the percent inhibition TBAb were assessed (Figure 4).

The anti-TSH-R monoclonal antibody KSAb1 produced by immunizations of BALB/C mice with recombinant adenovirus expressing TSH-R A-subunit was first evaluated on BRAHMS human TSH-R TRAK I-125 TSH binding inhibition assay and on CHO cells transfected with wild type human TSH-R bioassay. In these systems the reported EC50 of KSAb1 were 3-10 ng/mL and 10-30ng/mL in the BRAHMS assay and bioassay respectively (22). A second study reported EC50 of M22 and KSAb1 10-100 ng/mL in wild type TSH-R cAMP assay on CHO cells with evidence that the affinity maturation of TSAbs occurs during the B cell responses to TSH-R ectodomain which is shed into the blood circulation rather than to the holoreceptor which remains intact in the cell membrane (0). In the results presented here, KSAb1 shows EC50 of 500-1000 ng/mL on TRAb immunoassays and 270-320 ng/mL on Mc4 bioassay (Figure 4). The EC50 of stimulating human M22 Mab was 200-500 ng/mL on the TRAb immunoassays and 2-6 ng/mL on Mc4 bioassay (Figure 4). The 50-100 fold greater activity of M22 than the KSAb1 on Mc4 bioassay and only 1-2 fold higher activity of M22 than KSAb1 on the TRAb immunoassays underscores the higher sensitivity of the bioassay to detect stimulating autoantibodies and its capacity to discriminate TSAb agonistic potency over a greater range than that of the TRAb immunoassays.

Mc4 bioassay and TRAb immunoassays were further evaluated for their specificity to detect blocking Mab K1-70 (Figure 4). Strong binding of K1-70 was detected on both Cobas and Immulite TRAb immunoassays; EC50 <500 ng/mL. In contrast, complete absence-null TSAb activity of K1-70 was found on the Mc4 bioassay. This finding refutes the claim of Immulite (Bridge) assay to detect only TSIs. To further investigate the K1-70 bioactivity, the blocking or inhibitory function was measured on the blocking, TBAb Mc4 bioassay as percent inhibition of TSH...
stimulation (Figure 4). All three concentrations of K1-70 of 333 ng/mL, 1000 ng/mL and 3000 ng/mL gave 100% inhibition on the TBAb Mc4 bioassay. The KSAb1 Mab showed negative percent inhibition, as expected by its stimulatory property. This strikingly strong binding of K1-70 Mab in TRAb immunoassays, no K1-70 activity in TSAb bioassay and strong K1-70 inhibitory activity in TBAb assay shows that the bioassay as expected distinguishes between stimulatory and blocking TRAbs. Furthermore, the data confirm the greater sensitivity of the bioassay for stimulating TRAbs as compared to binding assays.

4.3. Clinical diagnostics of anti-TSH-R antibodies

Graves’ disease is unique among the autoimmune diseases in that the clinical manifestation of disease, hyperthyroidism (or thyrotoxicosis) is entirely dependent on the interaction of the autoantibody with its autoantigen, the TSH-R. Clinical attention and awareness of anti-TSH-R autoantibody diagnostics has increased over the past decade. Other than the autoimmune manifestation, there are a variety of other causes of thyrotoxicosis including constitutive activations of TSH-R in toxic nodular goiter. Maternal TSAb in pregnant Graves’ disease patient of the IgG type, which persists in 1-5% of GD pregnancies can cross the placenta and pose a risk of life-threatening thyrotoxicosis to the fetus or newborn child (neonatal thyrotoxicosis) (4, 44). Monitoring the reduction of TSAb or the presence of non-stimulating TRAb during early GD pregnancy gives early indication to treat the hyperthyroidism before potential harmful effects on the fetus (44) or the prediction of mothers at risk for the development of post partum GD (45).

The American Thyroid Association (ATA) Guidelines 2016 recommend measurements of TSH-R autoantibodies in addition to radio-iodine uptake and thyroid scanning to accurately assess the autoimmune nature of thyrotoxicosis and the early diagnosis of autoimmune Graves’ disease (46). Furthermore the ATA guidelines recommend the use of engineered CHO cells transfected with TSH-R for monitoring the levels of TSH-R stimulating autoantibodies during the management of Graves’ disease patients after treatment (46). Other conditions such as Hashimoto’s disease under thyroxine replacement often experience a swing in pendulum from hypo to hyperthyroidism which is associated with TSAb or a switch from TBAb to TSAb (47). These clinical conditions justify TSAb (4, 22) and TBAb (37-39, 41) testing to assess the likelihood of a transient or long-term complication.
The clinical mandate for testing of anti-TSH-R autoantibodies by TSAb bioassay or TRAb immunoassay is also manifest in the increasing number of Phase II/III clinical trials of GD biological therapies; monoclonal antibodies and peptides targeting B cells or TSH-R/IGF-1 receptor signaling, which rely on TSAb endpoint to assess the patient responses (18, 48-50).

Diagnostic clues and biomarkers are currently insufficient to assist clinicians in estimating the prognosis of extra-thyroid manifestations in GD and GO. It is very difficult to predict which patients will suffer from severe sequelae.

The pioneering studies on the role of TSH-R autoantibodies in the etiopathology of GO (51) and the prediction of severity and outcome of GO hyperthyroidism (52, 53) were decisive for raising clinical awareness of the importance of TSH-R autoantibodies in GO. The measurements of TRAb or TSab in bioassay have since become a cornerstone of GO clinical practice, as evident by its use in monitoring prognosis, dose adjustment of anti-thyroid medication and most recently for prediction of GO relapse (54, 55).

### 4.4. Meta-analysis on clinical performance of TSH-R bioassay and TRAb immunoassays

The review of literature has identified five studies of the TSAb on Mc4 bioassay (35, 36, 54-56) and five studies of TRAb on automated ECLIA (24, 26, 28, 29, 55) having complete performance data and reporting of sensitivities and specificities from the receiver to operator curve (ROC) analysis. The meta-analysis of these studies demonstrates high sensitivities 97% (95-100) and specificities 98% (96-100%) of both the TSAb Mc4 Bioassay and the TRAb immunoassays in untreated GD (Figure 5).

Both the TRAb immunoassay and TSAb bioassay distinguish GD from other causes of thyrotoxicosis such as subacute or chronic thyroid conditions. The meta-analysis presented here indicates reliable and accurate performance of TRAb immunoassays in untreated GD and strongly supports the conclusion that both the TRAb immunoassays and bioassay show equivalent performance in the screening and early diagnosis of GD.

Survey of the TSAb Mc4 bioassay and TRAb immunoassays reveal that the percentage of treated GD patients testing positive in TSAb Mc4 bioassay (56%, range 13-87%) was consistently less than that of the ThermoFischer BRAHMS TRAK assay (67%, range 42-91%) and the automated ECLIA immunoassays (98.5%, 98-99%) (Table 1). Two of these independent studies (24, 55) compare BRAHMS TRAK versus Mc4 Bioassay on the identical patient group and allow calculation of an odds ratio in favor of lower percent positivity on Mc4 Bioassay; OR=2.76. Similarly a trend of a lower percentage of Hashimotos thyroiditis (HT) patients test positive in the Mc4 bioassay (3%, range 2-4%) than the TRAb immunoassays (6.4%, range 2-11%) (Table 1).

### 4.5. Meta-analysis of TSI versus TRAb in Graves’ orbitopathy disease activity

The clinical evidence of anti-TSH-R autoantibodies in extra-thyroid conditions of GD, mainly the Graves’ orbitopathy (GO) indicate that TSAb associates with both the activity and severity of GO (57-59). The levels of TRab or TSab usually decrease during 6 months of anti-thyroid medications (50-53).

The immunopathogenic hallmarks of GO include Inflammatory infiltrate in muscle fibers, increased extraocular muscle and fat tissue and deposition of the glycosaminoglycans such as hyaluronan resulting in fibrosis in the back of the eye. The TSH-R positive fibroblast cells differentiate into adipocytes and explain the link between GD and GO. The most compelling evidence for the etiopathogenic role of TSH-R in GO is the modeling of GO in GD mice immunized with human TSH-R (21).

The level of TRab 12-24 months after diagnosis are used to predict mild, moderate or severe GO. Several studies have identified TRab levels as an important decision-point for the use of oral steroid and intravenous steroid infusions to avoid risk of vision threatening optic neuropathy (59-61). To compare the TRAb immunoassay and Mc4 bioassay for their relevance in predicting the course of GO, a single center prospective study at Ophthalmology clinic enrolled 112 newly diagnosed GO patients having neither steroids nor radiation treatment one year after initial diagnosis the patients were classified as mild inactive GO or active severe GO according to the NOSPECS criteria (59). Higher initial TRab values were associated with higher risk of severe disease in multiple regression analysis (p<0.01). The clinical cut offs of 10.7. IU/l and 555%, for M22 ELISA and Mc4 bioassay gave similar sensitivities of 67% and 69 respectively with a trend of higher specificity of the Mc4 bioassay; 85% versus 89% (Figure 4).

To evaluate the clinical utility of TRab versus TSAb in GO, the coefficient of correlations of the autoantibody levels SRR% TSAb Mc4 Bioassay (34, 58-62) or U/L TRAb immunoassay (34, 58-61) with the score of NOSPEC parameters; clinical activity score (CAS), proptosis, soft tissue inflammation, orbital eye muscle involvement, total eye score and optic neuropathy or of modified NOSPEC criteria, were
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The total random effects of meta-analysis show coefficient of correlation 0.4. Mc4 bioassay versus 0.2. TRAb immunoassay (Figure 6). The reported coefficient of correlations of CAS with TSAb of Mc4 Bioassay were highly significant (p<0.001) in 3 of the 4 studies and coefficient of correlations with TRAB levels in immunoassay were significant (p<0.05) in only 1 of 3 studies. Significant coefficient of correlations (p<0.05.) of proptosis, soft tissue, orbital muscle involvement and total eye score (TES) were found with all studies of TSAb levels in Mc4 Bioassay compared with only 1 study of TES and TRAb levels. Expert opinions on the role of TSAb and TRAb in Graves’ orbitopathy remain controversial with keen interest to identify new biomarkers of the orbitopathy and extra-thyroid inflammation mediated by TSH-R and other G-protein coupled co-receptors. In cases of euthyroid orbitopathy TSAb is not detected in the bioassay indicating that the TSH-R may not be relevant in mediating the illness (63, 64). Immune related adverse effects with the possible involvement of TSH-R autoantibodies has also emerged among compiled and presented (Figure 6). The clinical cut-offs of receiver to operator curve (ROC) of Mc4 TSI bioassay were 140% (34,57), 128% (25) and 123% (36). The clinical cut-offs of ROC of TRAb immunoassays; a=cobas elecsys 1.75 U/L, b=M22 Elisa 0.4. U/L and c= Siemens Bridge Immulite 0.55 U/L. N=number of subjects. HC=euthyroid healthy controls. Other= non-thyroid autoimmune diseases, thyroid cancer or goiter. B) Prediction of severe GO. Clinical cut-off of ROC for the Mc4 TSI Bioassay 555% and cobas elecsys 10.6. U/L (58). GO patients were newly diagnosed and untreated, N=112. One year treatment follow-up, ocular examinations assigned the patients to either mild GO; clinical activity score (CAS)<4 and NO SPEC <5, N=73 or severe GO; CAS≥4 and NO SPEC ≥5, N=39. All plots and confidence intervals were determined using the Medcalc statistical analysis software.

Figure 5. Meta-analysis of TSI bioassay and TRAb binding immunoassay clinical performance. Forest plot of the random effects of sensitivities and specificities of Mc4 TSI bioassay and TRAb binding immunoassays. A) Untreated or newly diagnosed GD. The clinical cut-offs of receiver to operator curve (ROC) of Mc4 TSI bioassay were 140% (34,57), 128% (25) and 123% (36). The clinical cut-offs of ROC of TRAb immunoassays; a=cobas elecsys 1.75 U/L, b=M22 Elisa 0.4. U/L and c= Siemens Bridge Immulite 0.55 U/L. N=number of subjects. HC=euthyroid healthy controls. Other= non-thyroid autoimmune diseases, thyroid cancer or goiter. B) Prediction of severe GO. Clinical cut-off of ROC for the Mc4 TSI Bioassay 555% and cobas elecsys 10.6. U/L (58). GO patients were newly diagnosed and untreated, N=112. One year treatment follow-up, ocular examinations assigned the patients to either mild GO; clinical activity score (CAS)<4 and NO SPEC <5, N=73 or severe GO; CAS≥4 and NO SPEC ≥5, N=39. All plots and confidence intervals were determined using the Medcalc statistical analysis software.
Table 1. Detection of Anti-TSH-R Autoantibodies in Mc4 Bioassay versus TRAb Immunoassays

<table>
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<tr>
<th>Ref</th>
<th>Assay Method</th>
<th>Clinical Cut off</th>
<th>Percent Positive</th>
<th>Untreated GD % (n)</th>
<th>Treated GD % (n)</th>
<th>GO % (n)</th>
<th>HT % (n)</th>
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<tr>
<td>34</td>
<td>Mc4 TSI Bioassay</td>
<td>140%</td>
<td>97±2</td>
<td>13 (45)</td>
<td>56±37</td>
<td>97 (155)</td>
<td>2 (54)</td>
</tr>
<tr>
<td>35</td>
<td>140%</td>
<td>96 (54)</td>
<td>36 (44)</td>
<td></td>
<td></td>
<td></td>
<td>2 (62)</td>
</tr>
<tr>
<td>36</td>
<td>128%</td>
<td>95 (103)</td>
<td>86 (130)</td>
<td></td>
<td></td>
<td></td>
<td>4 (99)</td>
</tr>
<tr>
<td>55</td>
<td>123%</td>
<td>97 (67)</td>
<td>87 (75)</td>
<td></td>
<td></td>
<td>100 (31)</td>
<td>2 (42)</td>
</tr>
<tr>
<td>56</td>
<td>140%</td>
<td>100 (82)</td>
<td>100 (31)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>Brahms TRAK</td>
<td>0.45 U/L</td>
<td>99 (67)</td>
<td>99 (130)</td>
<td>67±35</td>
<td>77</td>
<td>9.5 (42)</td>
</tr>
<tr>
<td>34</td>
<td>1.5 U/L</td>
<td>42 (45)</td>
<td>77 (155)</td>
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<td>9.5</td>
</tr>
<tr>
<td>55</td>
<td>M22 Cobas elecsys</td>
<td>1.75 U/L</td>
<td>94 (82)</td>
<td>96±2</td>
<td>77 (31)</td>
<td>73</td>
<td>7±6</td>
</tr>
<tr>
<td>54</td>
<td>96 (101)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>98 (82)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>29</td>
<td>97 (30)</td>
<td>99 (196)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11 (57)</td>
</tr>
<tr>
<td>28</td>
<td>Immulite TSI bridge</td>
<td>0.55 U/L</td>
<td>100 (30)</td>
<td>98±3</td>
<td>98</td>
<td>NA</td>
<td>5.4 (56)</td>
</tr>
<tr>
<td>29</td>
<td>100 (72)</td>
<td>98 (196)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5±1</td>
</tr>
<tr>
<td>30</td>
<td>95 (101)</td>
<td></td>
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</table>

The percentage of positive patients above the clinical cut-offs, percent of sample to reference ratio (SRR %) in bioassay and units of activity per liter (U/L) in TRAb immunoassays and the number of patients (n) are indicated for each referenced study. GD=Graves’ Disease, GO=Graves’ Orbitopathy and HT=Hashimoto’s Thyroiditis.

Figure 6. Meta-analysis of TSI and TRAb in correlation with disease activity of Graves’ Orbitopathy (GO). Forest plot of random effects from the correlations of the TSI or TRAb levels with GO clinical activity score (CAS) and NO SPECS classification. The literature reported coefficient of correlations Pearson (34,58) and Spearman rank (59-62). *p<0.0.5 and ** p<0.0.01 proptosis, soft tissue signs and symptoms, extraocular eye muscle involvement (OEM) and total eye score (TES). The size of square symbol is proportional to the GO patient sample size; N=55 chronic inactive (60), N=101 severe and active (62), N=155 active (34), and N=180 GO of which 30 had optic neuropathy (61). Total random effects of the GO population of the TRAb binding (vertical solid line) and TSI Bioassay (vertical dotted line). The calculated inconsistencies of the r values TSI Bioassay; f² 92% (95% CI 89-95), p<0.0.001 and TRAb binding f² 39% (95% CI 0-70%), p=0.0.8.

5. PERSPECTIVES

Highly efficient automated ECLIA are available for the diagnostics of GD with sensitivities and specificities >98% for untreated or newly cancer patients undergoing treatment with checkpoint inhibitors such as anti-CTLA and anti-PD1 (65-68). Thus, new applications of the TSH-R bioassays are likely in the future as demand increases to evaluate new groups of patients.
diagnosed GD. The TSH-R bioassays prevail over the TRAb binding for diagnostics of thyroid autoimmunity because they distinguish between stimulating and blocking anti-TSH-R autoantibodies. This feature allows the physician to properly assign the stimulating or blocking autoantibodies to clinical condition in GD/GO and HT patients undergoing therapy and moreover to make better decisions in neonatal hyperthyroidism in pregnancies. Any attempt to report functional anti-TSH-R autoantibodies using TSH-R immobilized on solid support i.e. any of the current TRAb immunoassays is incorrect, due to the fact that both stimulating and blocking immunoglobulins bind to the TSH-R ECD. Given the range of TSH-R autoantibody affinities in blood circulation and the mix of stimulator and blocker B cell clones which become activated in patients, the task of distinguishing a stimulator versus blocker autoantibody in the current static immobilized binding immunoassay format is untenable. Future nanotechnologies, micro-robotics and computer simulations of transmembrane helices and intracellular G-protein interactions need to emerge with sub-structures that accurately mimic the forces of ECD binding and transmit conformational changes leading to either activation or inhibition of GPCR cAMP signaling. Until this technology becomes available, the bioassay utilizing the TSH-R in live cells is expected to remain as the practical gold standard of functional TSH-R autoantibodies and continue as an integral part of the diagnostics and clinical practice of thyroid autoimmunity.

6. ACKNOWLEDGMENTS

We greatly appreciate the cooperation of Dr. Michaela Adam and Ms Ela Cernisova at Limbach Labor München Germany with measurements of M22 TRAb on Cobas e6000 and Mr. Benjamin Ferracin at the Laboratory of Prof Dr. Damien Gruson Brussels, Belgium with measurements of TRAb on Immulite XPi 2000.

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Abbreviations: TSH-R, thyroid stimulating hormone receptor; ECD, extracellular domain; LRD, leucine rich repeat domain; TRAb, autoantibodies against TSH-R detected in binding immunoassays; TSAb, functional stimulating autoantibodies against the TSH-R detected in cAMP bioassay; TBAb, functional blocking autoantibodies detected by the inhibition of the TSH stimulation in cAMP bioassay; GD, Graves’ disease; GO, Graves orbitopathy; MAb, monoclonal antibodies.

Key Words: Thyroid Stimulating Hormone Receptor, Autoantibodies, Bioassay, Immunoassay, Graves disease, Graves orbitopathy, Meta-analysis, Review

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