

The increasing clinical relevance of thyroid-stimulating hormone receptor autoantibodies and the concurrent evolution of assay methods in autoimmune hyperthyroidism

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Abstract: The thyrotropin receptor is a central stage for the thyroid function and growth and activates different signaling pathways for hormones synthesis and release by the thyrocyte. Stimulating, blocking and apoptotic autoantibodies directed against the extracellular domain of thyrotropin receptor (A subunit) are pathogenic for autoimmune hyperthyroidism or Graves' disease (GD). As outlined in the 2016 American Thyroid Association (ATA) guidelines, the measurement of thyrotropin receptor antibodies is now considered the first test for the management of hyperthyroidism. In the last 50 years different assay methods have been used to detect and measure these autoantibodies [bioassays and immunoassays (IMA)]. In this article a diagnostic model is proposed, taking account of the most recent refinements of laboratory assay methods: the IMA is considered the best solution to diagnose and monitor the overt cases of GD, while the bioassays are reserved for fine and complex diagnoses, in the cases of switch between stimulating and blocking antibodies in the same patients.

Keywords: Thyroid-stimulating hormone receptor (TSHR); TSH receptor autoantibodies (TRAbs); Graves' disease (GD); hyperthyroidism

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Introduction

For over half a century (1), the thyrotropin (thyroid-stimulating hormone, TSH) receptor (TSHR) appeared as a central stage of the thyroid function and the main, if not the only, example of autoantigen involved in autoimmune thyroid diseases (AITDs) and recognized by pathogenic autoantibodies (TSHR antibodies, TRAbs). TRAbs were able of inducing modifications of thyroid function with a similar but prolonged mechanism (long-acting thyroid stimulator, LATS) compared to that of the natural ligand (2). These modifications are the main cause of the clinical manifestations of autoimmune hyperthyroidism or Graves' disease (GD). In the last 60 years the detection/measurement of TRAbs was performed with biological and immunological laboratory methods, based on different assay principles.

When we consider the important role of laboratory tests, which are the cornerstone in the diagnostic pathway of any endocrine disease in the era of precision medicine, it is undeniable that until now the requests of TRAbs measurement in patients with hyperthyroidism was largely inappropriate by underutilization, due to controversies that surrounded the utility of these autoantibodies since its first reported use in clinical diagnosis (3). Recent advances and refinements of immunoassay (IMA) and bioassay methods have improved its specificity, reliability, and usability and now a wider use of the TRAb tests in clinical practice is a shared opinion.

The purpose of this review is to describe the ever-increasing diagnostic role of TRAbs in GD and other AITDs, to analyze the different methods for measuring these autoantibodies and to propose a rational approach for their use in clinical and laboratory practice.

Table 1 Proportion of clinician requesting TRAbs in Graves' disease in the last 25 years, in different surveys

Survey	Proportion (%)			Ref.
	Europe	North America	Asia	
Glinoe, 1987	38.0			(21)
Nagayama, 1989			28	(24)
Solomon, 1990		9.1		(23)
Burch, 2012	77.5	54.3	65.3	(22)
Bartalena, 2016	85.6			(20)

The TSHR and the economy of thyroid gland

TSHR, member of the class A G-protein-coupled receptors with the close relatives follitropin and lutropin/choriogonadotropin receptors (LH/CGR), is essential for the function and growth of the thyroid gland and activates different signaling pathways, required for thyroid hormones synthesis and release. The TSHR structure is constituted by interplaying domains located in different sites of the thyrotroph [the extracellular leucine-rich repeat domain (LRRD) and the hinge region, the intramembrane serpentine domain (SD) and the cytoplasmic tail] (4,5). After expression on the thickness of the plasma membrane, the TSHR undergoes cleavage within the hinge region. The loss of a C-peptide leads to an extracellular A subunit (comprising the LRRD and a part of the hinge region), and a B subunit (comprising the remainder of the hinge region, the SD and the cytoplasmic tail): the shed A subunit is the autoantigen initiating and driving the autoimmune response in GD. TSHR presents intermolecular interaction partners (e.g., TSH, small molecules, G-proteins, arrestin, or autoantibodies), the last of which is of fundamental importance for the pathogenic mechanisms underlying the major TSHR-associated human pathology, the autoimmune hyperthyroidism (6-8). Autoantibodies directed against the TSHR are pathogenic for GD, other AITDs (the atrophic silent form of Hashimoto's thyroiditis) and probably for GD-associated diseases (orbitopathy, dermopathy). Three types of TRAbs have been demonstrated, stimulating (S-TRAb or TSA), blocking (B-TRAb or TBA), and apoptotic antibodies (A-TRAb) (7-9) and their relative concentrations define the natural history and the clinical picture of GD (10).

GD is the main cause (60–80%) of hyperthyroidism, with a prevalence of 1.0–2.5% in the general population,

involving particularly women of reproductive age and in pregnancy (10,11).

Starting from the seminal experiments of Adams and Purves (2,12) (during development of a TSH assay at Otago University Medical School in Dunedin, New Zealand) 60 years ago, firstly demonstrating the presence of LATs in some thyrotoxic patients (12), the TSH receptor antibodies paved the way for the saga of the 'receptor autoimmunity' and for the laboratory methods for detecting and measuring autoantibodies (13).

The expanding clinical role of TRAbs in the management of hyperthyroidism

The increasing clinical relevance of TRAbs is defined by recent guidelines and surveys. Starting from the 2011 ATA guidelines (14), the clinical utility of TRAbs measurement was confined at a role of alternative way to diagnose GD.... *"when a thyroid scan and uptake are unavailable or contraindicated (e.g., during pregnancy and nursing)...., due to the statement:... 'most TRAb assays are specific for GD, but thyroid stimulating immunoglobulins and first-generation thyrotropin binding inhibitor immunoglobulin assays are less sensitive....'"* than thyroid scan or RAIU. This restriction is not shared by other non-American expert endocrinologists (15,16), creating a temporarily unresolved dualism between USA and Europe.

On the basis of the continuous improvement of assay methods for TRAbs measurement, some authors underlined the high accuracy of the IMA and bioassay methods (17,18): in reference of these data, Barbesino *et al.* underscored the clinical utility of TRAbs measurements and a wider utilization in the management of Graves' patients (19). Consequently, the percentage of clinicians requesting TRAb in the management of GD markedly increased in recent years worldwide: as demonstrated in a recent survey (20), with the use of a simple questionnaire, the proportion of European endocrinologists requesting TRAb measurement increased in 25 years from 38% to 85.6% (delta%: 47.6), compared to the previous survey (21); on the other hand in North America TRAbs are requested by the 54.3% of the US clinicians (22), compared with 9.1% of the previous survey (delta%: 45.2) (23) and in Asia the trend was similar (24), from 28% to 65% (delta%: 37.0) (Table 1).

As final point of this process, the recent ATA guidelines propose the use of TRAb testing as a first-line method for the determination of etiology of hyperthyroidism, "...

Table 2 Bioassay methods for detection/measurement of TRAbs

TSHR source	Read-out	Analyte	Assay time	Years
1st generation (animal models)				
Guinea pig	¹³¹ I T3, T4	LATS	Weeks	1956
Mouse	¹³¹ I T3, T4	LATS	Days	1958
2nd generation (TSHR primary cells)				
Mouse thyroid	¹³¹ I T3, T4 (chromatography)	LATS IgG	Weeks	1967
Porcine thyroid	cAMP, T3 uptake (RIA)	LATS IgG	Days	1973–1975
Human thyroid	cAMP (RIA)	TSAb	4 days	1973–1988
FTRL-5 rat thyrocytes	cAMP, T3 uptake (RIA)	TSAb, TSBAb	3 days	1983–1994
CHO wild type (JP09, JP14, JP26, JP28, JP209)	cAMP (RIA)	TSAb	2–4 days	1990–1998
CHO wild type (K1)	cAMP (RIA)	TSAb, TSBAb	20 hours	1997
3rd generation (TSHR luciferase reporter)				
CHO wild type (C6–13)	cAMP (luminescence)	TSAb	24 hours	1998
CHO wild type (JP09, JP26)	cAMP (luminescence)	TSAb, TSBAb	26 hours	1999
CHO wild type (K1)	cAMP (luminescence)	TSAb, TBAAb	24 hours	2001–2006
CHO chimera (K1)	cAMP (luminescence)	TSAb	20 hours	2010

LATS, long-acting thyroid stimulator; TSAb, thyroid stimulating antibody; TSBAb, thyroid stimulation blocking antibodies; cAMP, cyclic AMP; FTRL-5, Fisher rat thyroid cell line 5; CHO, Chinese hamster ovary.

if the diagnosis is not apparent based on the clinical presentation and initial biochemical evaluation...". Other alternative methods are the radioactive iodine uptake (RAIU) and ultrasonography (US): the choice depends on available expertise and resources (25). It's now possible to stay that the long run for the recognition of the TRAbs measurement as the main test for the management of hyperthyroidism has been completed.

But what are the reasons for this opinion that finally brings together endocrinologists all over the world? The first important reason is the use of TRAbs measurement not only for diagnosing GD, but also for monitoring disease activity, influencing treatment choices and improving the assessment of risk of relapse (25,26). Other reasons are surely cost, certainly lower than RAIU and US (27), the growing availability and local expertise, but in particular the improvement of the methods of detection/measurement.

The evolution of diagnostic technologies for the detection and measurement of TRAbs

In the last 60 years, a variety of laboratory methods have been proposed and employed to detect and measure TRAbs,

based on two different principles: bioassays and IMAs. The first measure functional activity of TRAbs, stimulating or blocking (28) (Table 2), while IMAs measure the binding to the receptor (total TRAbs, T-TRAbs), irrespective of functional discrimination (29) (Table 3).

Bioassays

Until 1958, the available methods for detection of TRAbs were the bioassays, based on the original principles of Adams and McKenzie (30–32). The bioassays, at first troublesome, poorly standardized and relatively insensitive for routine diagnostic use in GD, have been later developed through three generations, based on progressive technological improvement (33). The major innovations in the bioassay detection of TRAbs were the transfection of Chinese hamster ovary (CHO) cells with luciferase reporter gene, the availability of the TSHR-LH/CGR chimeric receptor, based on the substitution of aminoacid sequence of the wild-type TSHR with a similar sequence of the rat LH/CGR (34–36), on the use of CHO cells transfected with the recombinant human TSHR, and on the cyclic nucleotide-gated calcium channel and aequorin (37). TRAb

Table 3 Immunoassay methods for detection/measurement of TRAbs

TSHR source	Tracer	Assay time	Years	Procedure
1st generation (liquid phase)				
Solubilized porcine	¹²⁵ I bovine TSH	Days	1982–1995	Manual
Solubilized human	Enzyme-coated bovine TSH	Days	1990–1999	Manual
2nd generation (solid phase)				
Solubilized porcine	Peroxidase-avidin biotinylated bovine TSH	Hours	1999–2018	Manual
Immobilized recombinant human	¹²⁵ I-TSH, Acridinium-TSH	Hours	1999–2018	Manual
Immobilized recombinant human	Europium cryptate-TSH	Minutes	2010–2018	Full automated
3rd generation (solid phase)				
Solubilized porcine	Peroxidase-avidin biotinylated MoAb (M22)	Hours	2004–2018	Low automated
Solubilized porcine	Rutenium-avidin biotinylated MoAb (M22)	Minutes	2008–2018	Full automated
Immobilized chimera TSHR/LHCGR	Alkaline phosphatase-Dioxetan-phosphate-TSHR	Minutes	2016–2018	Full automated
Immobilized recombinant human	Galactosidase-4-methylumbelliferil-galactoside-MoAb (AB9)	Minutes	2017–2018	Full automated
Immobilized recombinant human	Aminobutyl-ethyl-isoluminol-TSHR	Minutes	2017–2018	Full automated

TSHR, thyrotropin receptor; LHCGR, lutropin-choriogonadotropin receptor; MoAb, monoclonal antibody.

Table 4 Different principles and related accuracy of immunoassay methods for measurement of TRAbs

Generation	Phase	IRP	Technology	Tracer	Clinical sensitivity, mean (range) (%)	Clinical specificity, mean (range) (%)	1st reference
1st	Liquid	MRC B65/122	RIA	Labeled TSH	79.8 (52.0–100.0)	99.2 (97.5–100.0)	Rees Smith, 1982
2nd	Solid	NIBSC 90/672	RIA, CLIA	Labeled TSH	96.4 (87.0–100)	98.1 (90.3–100.0)	Costagliola, 1999
3rd	Solid	NIBSC 90/672	CLIA, FIA	Labeled MoAb/ Labeled receptor	97.2 (95.0–100.0)	99.2 (97.3–100.0)	Hermesen, 2009

IMAs, immunoassays; IRP, international reference preparation; RIA, radioimmunoassay, CLIA, chemiluminescent immunoassay; FIA, fluoro-immunoassay; MRC, Medical Research Council; NIBSC, National Institute of Biological Standards and Controls.

levels, measured by such methods, are highly correlated with GD activity, but their use in clinical practice needs yet optimization and harmonization (36): this is the reason of the restriction of the use to a small number of specialized laboratories all over the world.

IMA

Following early experiments demonstrating that Graves' patient immunoglobulins inhibit the binding of radio-labeled TSH to human and guinea-pig thyroid membranes or solubilized receptors of human thyroid cells, Shewring *et al.* in the early 1980s firstly described a competitive radio-receptor IMA (38). The progressive improvement of the

analytical schemes (receptors of different species and tissues, preparation of antigenic source, types of tracers, etc.) has brought, through three generations of methods (*Table 3*), to the improvement of analytical (from 1.5 to 0.8 IU/L) and clinical (from 96.4% to 97.2%) sensitivity of IMAs (*Table 4*). Nevertheless, these assays cannot discriminate between the different types of TRAbs, present in patients with different AITDs (39–43). Also two recent IMA methods for the measurement of TRAbs, based on a distinctive technology and assay format, have been made available in automated commercial platforms/instruments (44–46), and show a high diagnostic accuracy. These methods shows similar results in discriminating GD patients from other hyperthyroid and non-hyperthyroid patients, but still need harmonization,

Table 5 Analytical findings of commercial automated IMAs methods for TRAb measurement

Product	Manufacturer	Method	Assay format	Solid phase	Tracer	Calibrator	Analyte
Kryptor TRAk human	ThermoFisher	FIA/TRACE	Competitive	rhTSHR-MoAb BA8	MoAb AB9-Cryptate	T-TRAb, 1st IRP NIBSC 90/672	T-TRAb
Elecsys-Cobas Anti-TSHR	Roche	ECLIA	Non-competitive (sequential)	rhTSHR-MoAb-M22	MoAb M22-Rutenium	T-TRAb, 1st IRP NIBSC 90/672	T-TRAb
Immulite TSI	Siemens	CLIA	Non-competitive (two sites)	Chimera TSHR/LHCGR-coated beads	TSHR/LHCGR-Alkaline phosphatase-Dioxetan phosphate	S-TRAb, 2nd IRP NIBSC 08/204	S-TRAb
EliA Anti-TSHR	Phadia	FIA	Competitive (sequential)	rhTSHR-coated wells	MoAb AB9- β -Galactosidase	S-TRAb, 2nd IRP NIBSC 08/204	T-TRAb
Maglumi TRAb	SNIBE	CLIA	Non-competitive (two sites)	rhTSHR-microbeads	Aminobutyl-ethyl-isoluminol-TSHR	S-TRAb, 2nd IRP NIBSC 08/204	T-TRAb

IMAs, immunoassays; T-TRAb, total TRAb, S-TRAb, stimulating TRAb; ECLIA, electrochemiluminescence immunoassay; FIA, fluoroimmunoassay; CLIA, chemiluminescence immunoassay; rhTSHR, recombinant human TSHR; MoAb, monoclonal antibody; IRP, international reference preparation; NIBSC, National Institute of Biological Standards and Controls.

particularly for the use of different reference preparations (Table 5).

In all 3rd generation IMA, it's now clear that animal (bovine, porcine) and human TSHR yielded similar functional and clinical sensitivities and specificities, as demonstrated in several experiences and studies in the last 15 years: such similarity is dependent to the identity of aminoacidic sequences in the animal and human TSHR regions for TRAb and TSH binding (47).

TRAb IMA automation passed through important breakthroughs represented by the use of new solid phases (microparticles), new tracers (fluorimetric or chemiluminescent), new assay schemes (non-competitive or two sites) and new reference preparations (NIBSC 08/204) (Table 5), that allow the reduction of assay time (minutes) and the improvement of analytical and clinical accuracy.

The role of bioassays and IMAs in the diagnostic/prognostic workup of ATDs

Defined the fundamental role of the measurement of TRAbs both in the diagnostic pathway and in the follow-up of the GD and other AITDs, the debate on which type of laboratory methods to use (IMA or bioassays) remains open and related to the different experiences of thyroidologists and researchers.

In this review a diagnostic model is proposed, which takes into account the current knowledge of commercial technologies for the measurement of TRAbs, in their

most recent refinements. It is clear that both technologies have significantly improved their analytical characteristics (29,36,44,46,48): the wide spread of the measurement of these autoantibodies within the thyroid test profile and their contained cost suggest the opportunity to use the IMA methods as the first choice in the current diagnostic approaches.

With the high automated IMA technologies (now sensitive, precise, and rapid), it is possible to complete in a single run the laboratory diagnostic procedure of the thyroid diseases, also in the presence of reflex and reflective thyroid tests. Notwithstanding the belief that, despite the latest technological innovations (44), IMAs mainly detect T-TRAbs and not only the S-TRAbs and consequently their measurement is able to meet the clinical needs in the overt case of hyperthyroid symptoms (given that in most cases autoantibodies exhibit characteristics of stimulating antibodies) not only for diagnostic purposes, but also during the follow up of patients (in predicting the outcome of GD after anti-thyroid drug treatment).

Bioassays should be reserved for fine and complex diagnoses, when clinical conditions suggest the identification of the functional activities of the TRAbs, for the possible switch between B-TRAb and S-TRAb and *vice versa* in the same patients (49-52):

- ❖ In predicting the type of relapse of GD;
- ❖ In predicting the likelihood of fetal/neonatal hyper- or hypothyroidism;
- ❖ In evaluating the concentration of B-TRAb and

S-TRAb in Hashimoto's thyroiditis and Graves' orbitopathy;

- ❖ In evaluating the risk of extrathyroidal manifestations of GD during pregnancy.

Conclusions

As a consequence of the recent quality improvement of laboratory methods, IMAs may be adopted in clinical practice for the initial diagnosis of hyperthyroidism and to follow disease activity and treatment, and bioassays may be used to assess particular type of patients with Graves' hyperthyroidism.

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Footnote

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