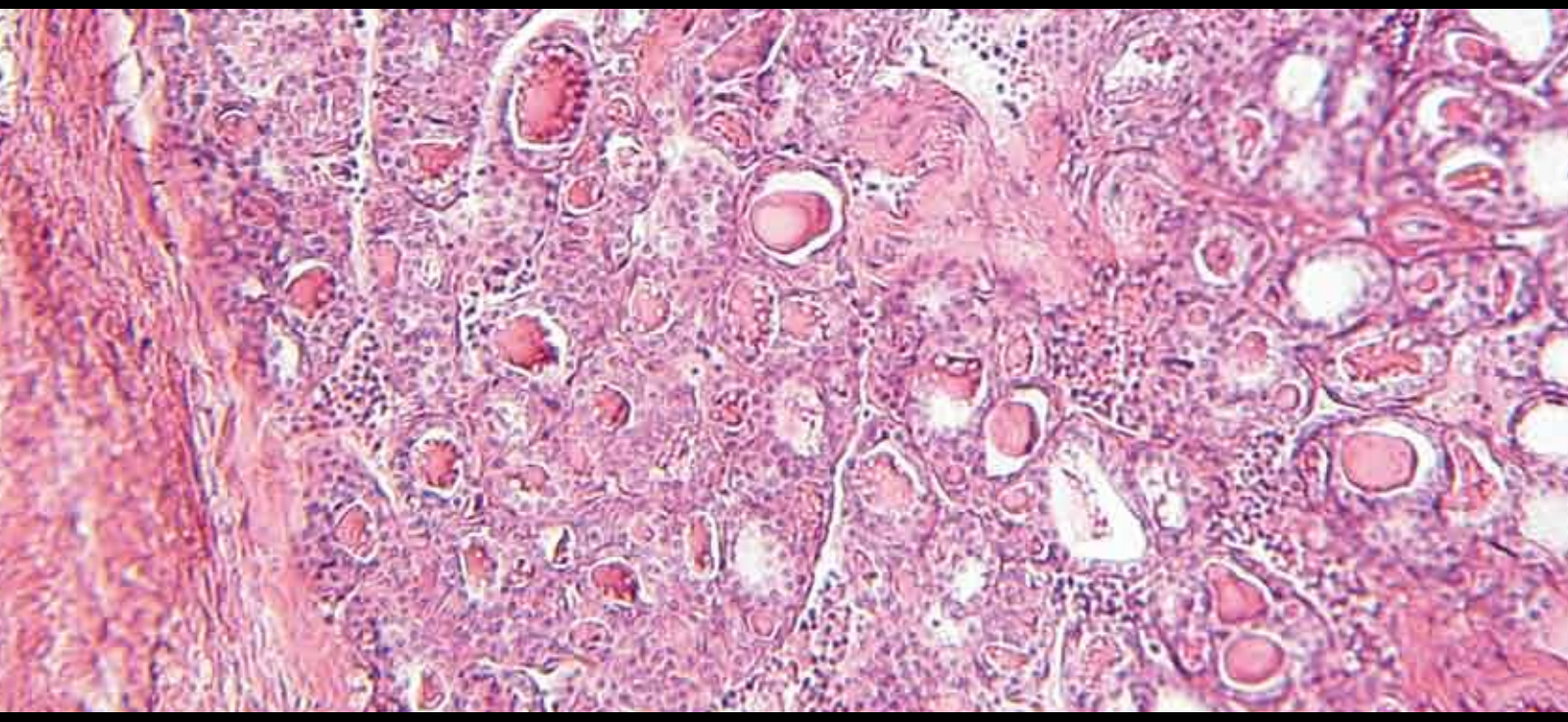
Journal of Thyroid Research

Advances in Graves' Disease

Guest Editors: Juan C. Galofré, Leonidas H. Duntas, L. D. Premawardhana, and Terry F. Davies



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Editorial Board

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Editorial
Advances in Graves' Disease

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"*From the very commencement the student should set out to witness the progress and effects of sickness and ought to persevere in the daily observation of disease during the whole period of his studies*."

It was Dr. Robert J. Graves who used to pronounce this statement at the inauguration of his yearly university lectures in Dublin. This was in the nineteen century and he had only just described Graves' disease, the most common hyperthyroid condition that is so widely recognized today. There could be at least two complementary ways of interpreting Dr. Graves' perennial advice. The first way is from a *practical* viewpoint. It emphasizes the importance of observation and monitoring clinical evolution. This is an important good-practice guide for doctors (and students) that are at a patient's bedside. This *practical* approach promotes a deep scrutiny of the disease, taking into consideration that it is not an abstract concept, but an ailment embodied in a given patient. The second interpretation of Graves' statement could be more *theoretical*. Thus the significance of the statement supports the concept of clinical and laboratory research. Physicians must participate in research at all levels: basic, translational, and clinical. Dr. Graves' counsel encourages efforts to achieve a deep knowledge of disease as individual entities. Therefore, both objectives, practical and theoretical, are closely entwined—laboratory advances connected to the bedside—what we now call translational medicine. Unfortunately, this link is often weak.

Since 1835, when Graves described his disease, dramatic progress has been made in our knowledge of the illness. During these nearly two centuries, we have come to understand a variety of molecular, genetic, and autoimmune mechanisms that give rise to and maintain the disease. However, it is also true that despite recent advances, the clinical management of Graves' disease has changed very little over the last few decades. Nevertheless, thanks to a group of outstanding physician-investigators able to integrate the laboratory with the bedside, we sense that exciting changes in the management of Graves' disease are at hand. Currently, for instance, there are several molecular target therapies under development that will significantly alter the clinical management of the disease within the next few years. This special issue is intended to highlight some of the most recent breakthroughs in this area. The issue includes a complete overview: from basic reviews to clinical papers through translational studies.

T. F. Davies et al. summarizes the new genetic insights into autoimmune thyroid diseases (AITDs), a complex topic that is actively being investigated. At present, more than twenty genes have been associated with AITD that can be categorized into two groups: immune regulatory genes (which are common to other autoimmune diseases) and thyroid-specific genes. Despite the described gene-AITD association, the individual gene contribution to AITD development is complex. Furthermore, no single polymorphism seems to contribute substantially to the development of the autoimmune reaction in thyroid diseases. The emerging evidence indicates that some environmental and/or epigenetic modifications over a predisposing genetic background could change individual gene expression, which subsequently elicits AITD manifestation. Although new genetic findings have emphasized the identification of the environmental components that interact with host genetic factors in other

autoimmune diseases, this approach has been elusive so far for AITD. Unfortunately for the clinician, the genetic profiling of AITD patients is unlikely to be productive in the near future, with the corresponding limitation in the development of new strategies in prevention and predictive treatment.

The role of microchimerism in Graves' disease is the subject of J. C. Galofré's review article. In this paper the author updates and reviews the main evidence that suggests a close relationship linking fetal microchimerism and the development of AITD. Certainly, the presence of intrathyroidal fetal cells within the maternal thyroid is an attractive candidate mechanism for the modulation of Graves' disease in pregnancy and the postpartum period. At present, however, microchimerism responsibility in the generation of AITD remains a hypothesis.

In their review articles, M. Žarković and L. H. Duntas address an important and emerging matter: the role of oxidative stress on the pathogenesis of Graves' disease and its specific treatment, respectively. M. Žarković describes how oxidative stress is indeed an environmental factor that induces and maintains the development of Graves' ophthalmopathy. Subsequently L. H. Duntas reviews the emerging role of selenium in the treatment of Graves' disease and ophthalmopathy. Both contributors tackle the question of the inflammatory process in AITD. The imbalance of the antioxidant-oxidant mechanism is described in detail. The authors illustrate how there is an increased production of radical oxygen species and cytokines, which sustain the autoimmune process and perpetuate the disease. It is stressed that selenium, a potent antioxidant, has been recently applied in patients with mild Graves' ophthalmopathy, slowing the progression of disease, decreasing the clinical activity score, and appreciably improving the quality of life. Questions remain open to further research such as whether enforced selenium nutritional supplementation has the same results on Graves' disease and whether prolonging selenium administration may have an impact on the prevention of disease.

S. El-Kaissi and J. R. Wall contribute with an original research article. The authors study the determinants of extraocular muscle volume (assessed by MRI) in 39 patients with Graves' disease. The study shows that patients with recently diagnosed Graves' disease and extraocular muscle volume enlargement have higher serum TSH and more severe hyperthyroidism at baseline than patients without extraocular muscle enlargement, with no difference in anti-TSH-R antibody positivity when comparing both groups.

C. Kamath et al. summarize the role of thyrotrophin receptor antibody (TR-Ab) assays in Graves' disease. TR-Ab assays commonly used and widely available to clinicians, measure thyroid-binding inhibiting immunoglobulins (TBII or receptor assays), and do not differentiate between stimulating (TRS-Ab), neutral, and blocking antibodies (TRB-Ab). This limitation can induce confusion in managing Graves' disease patients although the patient may be the best bioassay. The current 2nd-3rd generation receptor assays are highly sensitive and specific when used to differentiate between the functional types of TR-Ab. The authors also encourage measuring TR-Ab in pregnant women under appropriate circumstances. Unfortunately, current data are not conclusive about its use in predicting the outcome of Graves' disease after antithyroid drug therapy, as there is a significant variability in assay methodology, population characteristics (e.g., their iodine intake), and study design in published data.

An example of the inherent difficulties in interpretation of positive TR-Ab significance, as postulated in the C. Kamath et al. review article, is illustrated by N. Takasu and M. Matsushita original research article. The authors study the changes in serum TRB-Ab and TRS-Ab levels in 34 TRB-Ab-positive patients with hypothyroidism and in 98 TRS-Ab-positive Graves' patients with hyperthyroidism. The study covers a ten-year period. Serum TRB-Ab levels remained elevated during the entire study period in half of the patients with initial hypothyroidism. Interestingly, hypothyroid patients were divided according to the presence of atrophic or goitrous autoimmune thyroiditis. Despite the presence of positive TRB-Ab, all the patients with the goitrous form recovered from hypothyroidism whereas only 21% of the atrophic patients evolved to euthyroidism. Around 10% of the positive TRS-Ab patients remained with elevated circulating TRS-Ab levels at the end of the follow-up and these patients continued to have hyperthyroidism due to Graves' disease. On the other hand, remission of Graves' disease occurs in 82% of patients in whom TRS-Ab disappeared from the serum. The switch from TRB-Ab to TRS-Ab or vice versa took place in 5.8% and 2.0%, respectively, always inducing a change in the gland function. The authors' main conclusion is that positive TR-Ab may be associated with two manifestations: hyperthyroidism and hypothyroidism.

M. O. Hegazi and S. Ahmed review article focuses on atypical clinical manifestations of Graves' disease. Some of the atypical features are specifically related to Graves' disease (including anemia, vomiting, jaundice, and right heart failure), while others are also similarly found in patients with other forms of hyperthyroidism. Pulmonary hypertension is reported to be associated with Graves' disease and reportedly responds to its treatment. Such atypical signs and symptoms should be considered suspect and should not be allowed to delay diagnosis or unnecessary investigation.

We sincerely hope that the present volume will help clinicians who work in the stimulating field of thyroidology *to persevere in the daily observation of disease during the whole period of their studies* for the benefit of their patients.

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Clinical Study

Changes of TSH-Stimulation Blocking Antibody (TSBAb) and Thyroid Stimulating Antibody (TSAb) Over 10 Years in 34 TSBAb-Positive Patients with Hypothyroidism and in 98 TSAb-Positive Graves' Patients with Hyperthyroidism: Reevaluation of TSBAb and TSAb in TSH-Receptor-Antibody (TRAb)-Positive Patients

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Two TRAbs: TSBAb and TSAb. TSBAb causes hypothyroidism. TSAb causes Graves' hyperthyroidism. TSBAb and TSAb block TSH-binding to cells as TRAb, measured as TSH-binding inhibitory immunoglobulin (TBII). We reevaluate TSBAb and TSAb. We studied TSBAb, TSAb, and TBII over 10 years in 34 TSBAb-positives with hypothyroidism and in 98 TSAb-positives with hyperthyroidism. Half of the 34 TSBAb-positives with hypothyroidism continued to have persistently positive TSBAb, continued to have hypothyroidism, and did not recover from hypothyroidism. Ten of the 98 TSAb-positives with hyperthyroidism continued to have positive TSAb and continued to have hyperthyroidism. TSBAb had disappeared in 15 of the 34 TSBAb-positives with hypothyroidism. With the disappearance of TSBAb, recovery from hypothyroidism was noted in 13 (87%) of the 15 patients. TSAb had disappeared in 73 of the 98 TSAb-positives with hyperthyroidism. With the disappearance of TSAb, remissions of hyperthyroidism were noted in 60 (82%) of the 73. Two of the 34 TSBAb-positives with hypothyroidism developed TSAb-positive Graves' hyperthyroidism. Two of the 98 TSAb-positive Graves' patients with hyperthyroidism developed TSBAb-positive hypothyroidism. TSBAb and TSAb are TRAbs. TSBAb-hypothyroidism and TSAb-hyperthyroidism may be two aspects of one disease (TRAb disease). Two forms of autoimmune thyroiditis: atrophic and goitrous. We followed 34 TSBAb-positive patients with hypothyroidism (24 atrophic and 10 goitrous) over 10 years. All of the 10 TSBAb-positive goitrous patients recovered from hypothyroidism and 19 (79%) of the 24 TSBAb-positive atrophic patients continued to have hypothyroidism.

1. Introduction

There are two types of TSH receptor antibodies (TRAbs): thyroid stimulating antibody (TSAb) [1, 2] and TSH-stimulation blocking antibody (TSBAb) [3]. TSAb stimulates the thyroid and causes Graves' hyperthyroidism. TSBAb blocks TSH-stimulation of the thyroid and causes hypothyroidism. Both TSAb and TSBAb block TSH-binding to thyroid cells as TSH-receptor antibody (TRAb), which has been measured as TSH-binding inhibitory immunoglobulin (TBII) [1–3]. TBII indicates the inhibition of TSH-binding to TSH receptor but does not indicate the function of TRAb. TRAb can be stimulatory or inhibitory. To know whether TRAb is stimulatory or inhibitory, TSAb and TSBAb have been measured [1–3]. TRAb has been measured by different assay methods and given various names. Among them, TBII [1, 4, 5] and TSAb [1, 2, 6–9] have been measured as TRAb to diagnose Graves' disease and to follow the patients. TBII is measured as a receptor assay. TSAb is measured as a stimulator assay, using porcine thyroid cells. TSAb indicates the stimulation

activity of TRAb. TSBAb [3, 10–13] and TBII [3, 4, 10–13] have been measured as TRAb to diagnose TSBAb-positive hypothyroidism and to follow the patients. TSBAb has been measured as a TSH-stimulation blocking assay, using porcine thyroid cells [3, 10–13]. TSBAb indicates the inhibitory activity of TRAb. TSAb and TSBAb are TSH-receptor antibodies (TRAb). The former TRAb (TSAb) is a stimulating antibody [1, 2, 6–9], and the latter TRAb (TSBAb) is a blocking antibody [3, 10–13]. TSBAb blocks TSH-stimulation of the thyroid and causes hypothyroidism. TSBAb blocks TSH-binding to thyroid cells and is TRAb. TSBAb blocks TSH-stimulation of the thyroid and is measured as inhibition of TSH-stimulated cAMP synthesis of thyroid cells. TSBAb and TSAb are TRAb. TBII reflects TSBAb- and TSAb-activities.

TSAb stimulates the thyroid and causes Graves' hyperthyroidism. Treatment with antithyroid drugs (ATDs) decreases serum TSAb [14]. With the disappearance of TSAb, remissions of Graves' hyperthyroidism have been seen [14]. TSBAb blocks TSH-stimulation of the thyroid and causes hypothyroidism [3]. With the disappearance of TSBAb, recovery from hypothyroidism occurs [3].

It has been generally believed that Graves' patients have TSAb but do not have TSBAb, and that blocking antibody-(TSBAb-) positive patients with hypothyroidism have TSBAb but do not have TSAb. However, TSBAb-positive patients with hypothyroidism and TSAb-positive Graves' patients with hyperthyroidism could have both TSBAb and TSAb [13]. Some patients may have TSBAb and TSAb simultaneously or sequentially [13]. The balance of TSBAb and TSAb determines whether a patient has hypothyroidism or hyperthyroidism [13]. We have encountered TSBAb-positive patients with hypothyroidism, who developed TSAb-positive Graves' hyperthyroidism, and also TSAb-positive Graves' patients with hyperthyroidism, who developed TSBAb-positive hypothyroidism. Thyroid function can oscillate between hypothyroidism and hyperthyroidism as TSBAb or TSAb becomes dominant.

There are two forms of autoimmune thyroiditis: atrophic autoimmune thyroiditis and goitrous autoimmune thyroiditis [3]. It has become evident that hypothyroidism may occur as a result of the production of TSBAb. TSBAb has been said to cause hypothyroidism in the patients with atrophic autoimmune thyroiditis [3]. However, TSBAb has been found in patients with atrophic autoimmune thyroiditis, and also in patients with goitrous autoimmune thyroiditis [11]. TSBAb was detected in 25% of the patients with atrophic autoimmune thyroiditis and in 9% of those with goitrous autoimmune thyroiditis [3]. TSBAb causes hypothyroidism. With the disappearance of TSBAb, recovery from hypothyroidism has been reported [3]. Here, we followed 24 TSBAb-positive hypothyroid patients with atrophic autoimmune thyroiditis and 10 TSBAb-positive hypothyroid patients with goitrous autoimmune thyroiditis over 10 years. All of the 10 TSBAb-positive patients with goitrous autoimmune thyroiditis recovered from hypothyroidism and 19 (79%) of the 24 TSBAb-positive patients with atrophic autoimmune thyroiditis continued to have hypothyroidism.

We reevaluated TSBAb and TSAb in TRAb-positive patients. We studied serial changes of TSBAb and TSAb over 10 years in 34 TSBAb-positive patients with hypothyroidism and in 98 TSAb-positive Graves' patients with hyperthyroidism. With persistently positive TSBAb, recovery from hypothyroidism was not observed. With persistently positive TSAb, remissions of Graves' hyperthyroidism were not obtained. With the disappearance of TSBAb, recovery from hypothyroidism was seen. With the disappearance of TSAb, remissions of Graves' hyperthyroidism were also seen. Two of the 34 TSBAb-positive patients with hypothyroidism developed TSAb-positive Graves' hyperthyroidism. Two of the 98 TSAb-positive Graves' patients with hyperthyroidism developed TSBAb-positive hypothyroidism. TSBAb-positive hypothyroidism and TSAb-positive hyperthyroidism may be two aspects of one disease (TRAb disease).

2. Subjects and Method

2.1. Subjects. We studied 34 TSBAb-positive patients with hypothyroidism and 98 TSAb-positive Graves' patients with hyperthyroidism (Table 1). The 34 TSBAb-positive patients with hypothyroidism were treated with thyroxine (T4) and the 98 TSAb-positive Graves' patients with hyperthyroidism were treated with antithyroid drugs (ATDs). Serial changes of TSBAb and TSAb over 10 years were studied in 34 TSBAb-positive patients with hypothyroidism (I) and in 98 TSAb-positive Graves' patients with hyperthyroidism (II). TSBAb-positive patients with hypothyroidism were diagnosed on the basis of the history, signs of hypothyroidism, and the laboratory findings, including positive TSBAb (>+40%) and decreased serum-free thyroxine (fT4) and free triiodothyronine (fT3) with high TSH [3, 13]. The diagnosis of goitrous autoimmune thyroiditis was based on the finding of palpable goiter and that of atrophic autoimmune thyroiditis on the absence of goiter [3]. The 34 TSBAb-positive patients with hypothyroidism were treated with thyroxine (T4). Thyroxine was discontinued at 3 months after the disappearance of TSBAb. After the discontinuation of T4, the patients had been seen every 1–3 months. When the patients continued to be in euthyroid states and to have negative TSBAb and negative TBII for more than 1 year after the T4-discontinuation, they were considered to have recovery from hypothyroidism; otherwise, they had recurrence [3]. When serum TSH became higher than 10 mIU/L, T4-administration was restarted [3]. TSAb-positive Graves' patients with hyperthyroidism were diagnosed on the basis of the history, signs of hyperthyroidism with diffuse goiter, and the laboratory findings, including positive TRAb (TSAb and/or TBII) and elevated fT4 and fT3 with low TSH [1, 2]. The 98 Graves' patients were treated with antithyroid drugs (ATDs). They had been treated with ATD over several years. ATD was discontinued at 6 months after the TSAb-disappearance. After the discontinuation of ATD, the patients had been seen every 1–3 months. When the patients continued to be in euthyroid states and to have negative TSAb and negative TBII for more than 1 year after the ATD-discontinuation, they were considered to be in remission; otherwise, they had recurrence [14]. When they had recurrence, ATD-treatment was restarted. We had followed these 34 TSBAb-positive patients with hypothyroidism and

Table 1: Changes of TSBAb (TSH-stimulation blocking antibody) and TSAb (thyroid stimulating antibody) over 10 years in 34 TSBAb-positive patients with hypothyroidism and in 98 TSAb-positive Graves' patients with hyperthyroidism.

(I) 34 TSBAb-positive patients with hypothyroidism			34
Ia: Positive TSBAb persisted	Continued to have hypothyroidism	17	17
Ib: TSBAb disappeared	Ib1: Recovered from hypothyroidism	13	15
	Ib2: Continued to have hypothyroidism	2	
Ic: TSBAb → TSAb	TSBAb-positive hypo → Graves' hyper	2	2
(II) 98 TSAb-positive Graves' patients with hyperthyroidism			98
IIa: Positive TSAb persisted	Continued to have Graves' hyperthyroidism	10	10
IIb: Complex changes of TSAb	IIb1: Remission	1	13
	IIb2: Recurrence	12	
IIc: TSAb disappeared	IIc1: Remission	60	73
	IIc2: Recurrence	13	
IId: TSAb → TSBAb	Graves' hyper → TSBAb-positive hypo	2	2

Numbers of the patients are shown.
Serial changes of TSBAb and TSAb over 10 years were studied in 34 TSBAb-positive patients with hypothyroidism (I) and in 98 TSAb-positive Graves' patients with hyperthyroidism (II). The 34 TSBAb-positive patients with hypothyroidism were treated with thyroxine (T4) and the 98 TSAb-positive Graves' patients with hyperthyroidism were treated with antithyroid drugs (ATDs). Half (17) (Ia) of the 34 TSBAb-positive patients with hypothyroidism (I) continued to have positive TSBAb and continued to have hypothyroidism. Ten (IIa) of the 98 TSAb-positive Graves' patients with hyperthyroidism (II) continued to have positive TSAb and continued to have Graves' hyperthyroidism. With the disappearance of TSBAb, recovery from hypothyroidism was noted in 13 (Ib1) (87%) of the 15 patients, in whom TSBAb had disappeared (Ib). With the disappearance of TSAb, remissions of Graves' hyperthyroidism were noted in 60 (IIc1) (82%) of the 73, in whom TSAb had disappeared (IIc). Two of the 34 TSBAb-positive patients with hypothyroidism developed TSAb-positive Graves' hyperthyroidism (Ic), and two of the 98 TSAb-positive Graves' patients with hyperthyroidism developed TSBAb-positive hypothyroidism (IId).

98 TSAb-positive Graves' patients with hyperthyroidism over 10 years.

2.2. Porcine Thyroid Cell Cyclic AMP Production: TSBAb and TSAb. TSBAb and TSAb were measured as before [13, 14]. Cyclic AMP (cAMP) production was determined according to the instruction in commercial assay kit (Yamasa, Chosi, Chiba, Japan). Crude IgG, obtained as PEG (6000) 12.5% precipitated fraction- (final concentration) from 0.2 mL aliquot of test serum, was dissolved in modified Hanks' solution without NaCl. Porcine thyroid cells were incubated with test IgG in 0.25 mL Hanks' solution without NaCl, pH 7.5, containing 1.5% bovine serum albumin, 20 mM Hepes, and 0.5 mM 3-isobutyl-l-methylxanthine. Cyclic AMP production during 5 h incubation at 37°C was measured by radioimmunoassay (RIA), using a commercial kit (Yamasa). To measure TSBAb-activities, crude IgG was incubated with porcine thyroid cells in the presence of 25 μU bTSH (100 mU/L, final concentration), as before [3, 10–13, 15]. Cyclic AMP production during 5 h incubation was measured. TSBAb-activity was expressed as percentage inhibition of bTSH-stimulated cAMP production by test IgG. TSBAb-activity was calculated as follows:TSBAb (%) = $[1 - (c - b)/(a - b)] \times 100$ [3, 10–13, 15], where a: cAMP generated in the presence of normal IgG and bTSH, b: cAMP generated in the presence of normal IgG, and c: cAMP generated in the presence of test IgG and bTSH. Test IgG and normal IgG were the 12.5% PEG-precipitated fraction from test serum and normal human serum, respectively. TSBAb, described in this report, corresponds to TSBAb-A in the previous report [13]. TSBAb activities were studied in 95 normal subjects (normal values were less than +40%) [13]. TSBAb activities were more than +40% in all of the TSBAb-positive patients with hypothyroidism. TSAb activity was expressed as percentage cAMP production compared with the mean values for 125 normal subjects (normal values were less than 180%) [1, 2, 14]: TSAb (%) = $[d/b] \times 100$, where b: cAMP generated in the presence of normal IgG, and d: cAMP generated in the presence of test IgG.

2.3. TSH-Binding Inhibitory Immunoglobulin (TBII). TBII was measured by radioreceptor assay with a commercial kit (R. S. R. Limited, Cardiff, UK). Assay results were expressed as the percentage inhibition of I^{125}-TSH-binding to thyroid plasma membrane as before [1, 2, 5, 14]. Normal values were obtained from 128 normal control subjects and were less than l0% [1, 2, 14].

2.4. Statistical Analysis and Others. All samples were tested in duplicate or triplicate. Statistical analysis was performed using Student's t-test or χ^2-test. P values less than 0.05 were considered to be statistically significant. Serum-free T3, -free T4, and TSH were determined by electrochemiluminescence immunoassays (ECLIAs) (Roche Diagnostics, Tokyo, Japan). Normal reference ranges are as follows: fT3 3.5–6.6 nmol/L, fT4 11.6–21.9 pmol/L, and TSH 0.4–4.20 mIU/L. The study plan was reviewed and approved by our institutional review committee. Written informed consent was obtained from the patient prior to publication of this paper.

3. Resuls

Serial changes of TSBAb and TSAb over 10 years were studied in 34 TSBAb-positive patients with hypothyroidism and in 98 Graves' patients with hyperthyroidism (Table 1). The 34 TSBAb-positive patients with hypothyroidism (I) were treated with thyroxine (T4) and the 98 TSAb-positive

Table 2: Characteristics of the 34 TSBAb-positive patients with hypothyroidism and the 98 TSAb-positive Graves' patients with hyperthyroidism.

	Number of patients	Gender Men/Women	Age (years)	Before treatment TSBAb (%)	TSAb (%)	TBII (%)
(I) 34 TSBAb-positive patients with hypothyroidism						
Ia	17	5/12	42 ± 17	**94 ± 6**	146 ± 10	**95 ± 5**
Ib	15	4/11	45 ± 16	**90 ± 9**	136 ± 8	**92 ± 7**
Ic	2	1/1	38, 45	**98, 97**	100, 98	**96, 95**
Ia+Ib+Ic	34	10/24	43 ± 18	**92 ± 7**	140 ± 9	**94 ± 7**
(II) 98 TSAb-positive Graves' patients with hyperthyroidism						
IIa	10	3/7	40 ± 16	9 ± 8	**839 ± 421**	**76 ± 15**
IIb	13	3/10	42 ± 17	10 ± 11	**846 ± 195**	**68 ± 16**
IIc	73	18/55	44 ± 16	10 ± 10	**746 ± 390**	**56 ± 18**
IId	2	0/2	40, 48	2, 5	**1625, 852**	**76, 58**
IIa+IIb+IIc+IId	98	24/74	43 ± 17	10 ± 9	**775 ± 396**	**57 ± 17**

Values are means ± SD. I, Ia, Ib, Ic, II, IIa, IIb, IIc, and IId correspond to those in Table 1. No differences of gender and ages were noted among I, Ia, Ib, Ic, II, IIa, IIb, IIc, and IId. No differences of TSAb-, TSBAb-, and TBII-activities were noted among Ia, Ib, and Ic and among IIa, IIb, IIc, and IId.
All of the 34 TSBAb-positive patients with hypothyroidism had strongly positive TSBAb (85–103%, mean ± SD = 92 ± 7%) (Ia+Ib+Ic). Some of them had weakly positive TSAb. Their TSAb activity ranged from 92% to 240%. The TSAb activities were 180–240% in 7 (21%) of the 34 TSBAb-positive patients with hypothyroidism and were less than 180% in the other 27 patients (79%). Seven (21%) of the 34 TSBAb-positive patients with hypothyroidism had positive TSAb. TSBAb-positive patients with hypothyroidism had narrow distribution of TSBAb (82–104%, 92 ± 7%) and TSAb (92–240%, 140 ± 9%). All of the 98 Graves' patients with hyperthyroidism had positive TSAb (250–1795%, 775 ± 396%) (IIa+IIb+IIc+IId). Some of them had TSBAb. The TSBAb activities were +40–+52% in 11 (11%) and were less than +40% in the other 87 patients (89%). Graves' patients with hyperthyroidism had wide distributions of TSAb (250–1795%, 775 ± 396%) and TSBAb (−28–+52%, 10 ± 9%).

Graves' patients with hyperthyroidism (II) were treated with antithyroid drugs (ATDs). Among the 34 TSBAb-positive patients with hypothyroidism (I), 17 patients (Ia) continued to have persistently positive TSBAb and continued to have hypothyroidism. Half (17) (Ia) of the 34 TSBAb-positive patients continued to have persistently positive TSBAb, continued to have hypothyroidism, and did not recover from hypothyroidism. TSBAb disappeared in 15 (Ib) of the 34 TSBAb-positive patients with hypothyroidism. With the disappearance of TSBAb, recovery from hypothyroidism was seen in 13 (Ib1) (87%) of the 15 patients, in whom TSBAb had disappeared (Ib). Among the 98 TSAb-positive Graves' patients with hyperthyroidism (II), 10 patients (IIa) continued to have persistently positive TSAb and continued to have hyperthyroidism. Ten of the 98 TSAb-positive Graves' patients with hyperthyroidism continued to have persistently positive TSAb. They continued to have hyperthyroidism and did not get remissions of Graves' hyperthyroidism. They continued to take ATD. Complex changes of TSAb were noted in 13 TSAb-positive patients (IIb). One (IIb1) of the 13 patients with complex changes of TSAb got remissions, but the other 12 patients (IIb2) did not. TSAb disappeared in 73 (IIc) (74%) of the 98 TSAb-positive Graves' patients with hyperthyroidism. With the disappearance of TSAb, 60 (IIc1) (82%) of the 73 patients, in whom TSAb had disappeared (IIc), got remissions of Graves' hyperthyroidism. Two TSBAb-positive patients with hypothyroidism developed TSAb-positive Graves' hyperthyroidism (Ic). Two TSAb-positive Graves' patients with hyperthyroidism developed TSBAb-positive hypothyroidism (IId).

Table 2 shows characteristics of the 34 TSBAb-positive patients with hypothyroidism (I) and the 98 TSAb-positive Graves' patients with hyperthyroidism (II). I, Ia, Ib, Ic, II, IIa, IIb, IIc, and IId correspond to those in Table 1. No differences of gender and ages were noted among I, Ia, Ib, Ic, II, IIa, IIb, IIc, and IId. No differences of TSAb-, TSBAb-, and TBII-activities were noted among Ia, Ib, and Ic and among IIa, IIb, IIc, and IId. All of the 34 TSBAb-positive patients with hypothyroidism had strongly positive TSBAb (85–103%, mean ± SD = 92 ± 7%) (Table 2, Ia+Ib+Ic). Some of them had weakly positive TSAb. Their TSAb activity ranged from 92% to 240%. The TSAb activities were 180–240% in 7 (21%) of the 34 TSBAb-positive patients with hypothyroidism and were less than 180% in the other 27 patients (79%). Seven (21%) of the 34 TSBAb-positive patients with hypothyroidism had positive TSAb. TSBAb-positive patients with hypothyroidism had narrow distribution of TSBAb (82–104%, 92 ± 7%) and TSAb (92–240%, 140 ± 9%). All of the 98 Graves' patients with hyperthyroidism had positive TSAb (250–1795%, 775 ± 396%) (Table 2, IIa+IIb+IIc+IId). Some of them had TSBAb. The TSBAb activities were +40–+ 52% in 11 (11%) and were less than +40% in the other 87 patients (89%). Graves' patients with hyperthyroidism had wide distributions of TSAb (250–1795%, 775 ± 396%) and TSBAb (−28–+52%, 10 ± 9%).

3.1. 34 TSBAb-Positive Patients with Hypothyroidism (I) (Tables 1 and 2, I). All of the 34 TSBAb-positive patients with hypothyroidism had strongly positive TSBAb. Some of them had weakly positive TSAb. TSBAb-positive patients with hypothyroidism had narrow distributions of TSBAb (82–104%, 92 ± 7%) and TSAb (92–240%, 140 ± 9%) (Table 2, Ia+b+c). Figure 1 shows the changes of TSBAb

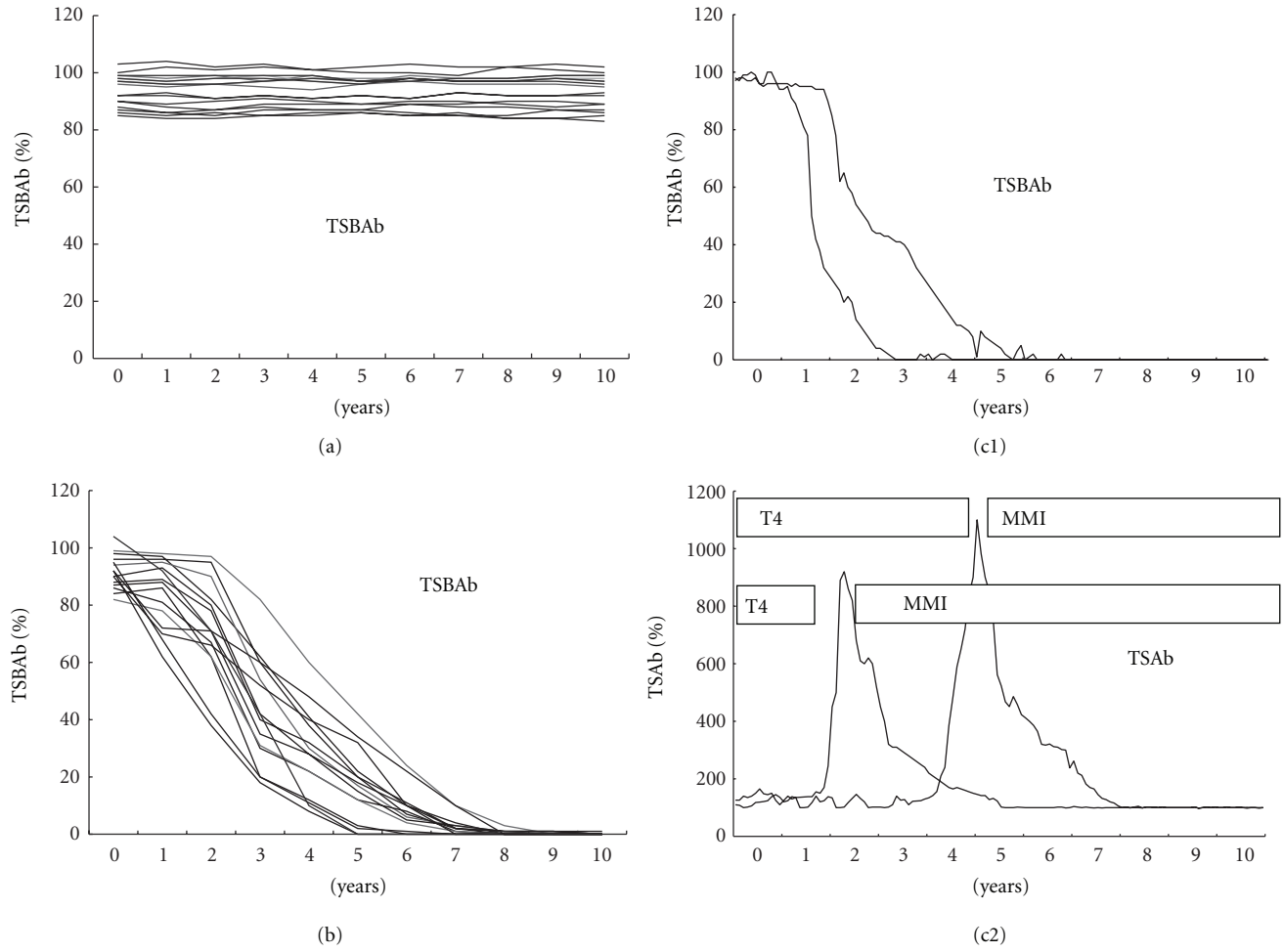

Figure 1: The changes of TSBAb in 34 TSBAb-positive patients with hypothyroidism (Table 1, I). Among the 34 TSBAb-positive patients with hypothyroidism, 17 patients continued to have persistently positive TSBAb and continued to have hypothyroidism (Table 1, Ia) (a). Half of the 34 TSBAb-positive patients continued to have persistently positive TSBAb, continued to have hypothyroidism, and did not recover from hypothyroidism. They continued to take thyroxine (T4). TSBAb disappeared in 15 of the 34 TSBAb-positive patients with hypothyroidism (Table 1, Ib) (b). Recovery from hypothyroidism was noted with the disappearance of TSBAb in 13 (87%) of the 15 patients, in whom TSBAb had disappeared. (c1, c2) show the changes of TSBAb and TSAb, respectively, in the 2 patients with TSBAb-positive hypothyroidism, who developed TSAb-positive Graves' hyperthyroidism (Table 1, Ic). In these 2 patients, TSBAb was dominant initially (c1), and then TSAb became dominant (c2); 2 patients with TSBAb-positive hypothyroidism developed TSAb-positive Graves' hyperthyroidism. Hypothyroidism was treated with thyroxine (T4). Graves' hyperthyroidism was treated with 1-methyl 2-mercapto imidazole (MMI). TSBAb: TSH-stimulation blocking antibody; TSAb: thyroid stimulating antibody.

in the 34 TSBAb-positive patients with hypothyroidism (Table 1, I). Among the 34 TSBAb-positive patients with hypothyroidism (I), 17 (Ia) (Table 1, Ia, Figure 1(a)) continued to have persistently positive TSBAb and continued to have hypothyroidism. Half (17) (Ia) of the 34 TSBAb-positive patients (I) continued to have persistently positive TSBAb, continued to have hypothyroidism, and did not recover from hypothyroidism. They continued to take T4. TSBAb disappeared in 15 (Ib) (Table 1, Ib, Figure 1(b)) of the 34 TSBAb-positive patients (I) with hypothyroidism. With the disappearance of TSBAb, recovery from hypothyroidism was noted in 13 (Ib1) (87%) of the 15 patients, in whom TSBAb had disappeared (Ib).

Figures 1(c1) and 1(c2) show the changes of TSBAb and TSAb, respectively, in the 2 patients with TSBAb-positive hypothyroidism, who developed TSAb-positive Graves' hyperthyroidism (Table 1, Ic). In these 2 patients, TSBAb was dominant initially (Figure 1(c1)), and then TSAb became dominant (Figure 1(c2)). These 2 TSBAb-positive patients had hypothyroidism and then developed TSAb-positive Graves' hyperthyroidism. They were treated with T4 and then treated with 1-methyl 2-mercapto imidazole (MMI). Figure 2 demonstrates the clinical course of one of these 2 patients with TSBAb-positive hypothyroidism, who developed TSAb-positive Graves' hyperthyroidism (Table 1, Ic). A 45-year-old woman with TSBAb-positive hypothyroidism developed TSAb-positive Graves' hyperthyroidism. TSBAb was dominant initially (Figure 2(a)), and then TSAb became dominant (Figure 2(b)). She had TSBAb-positive hypothyroidism with

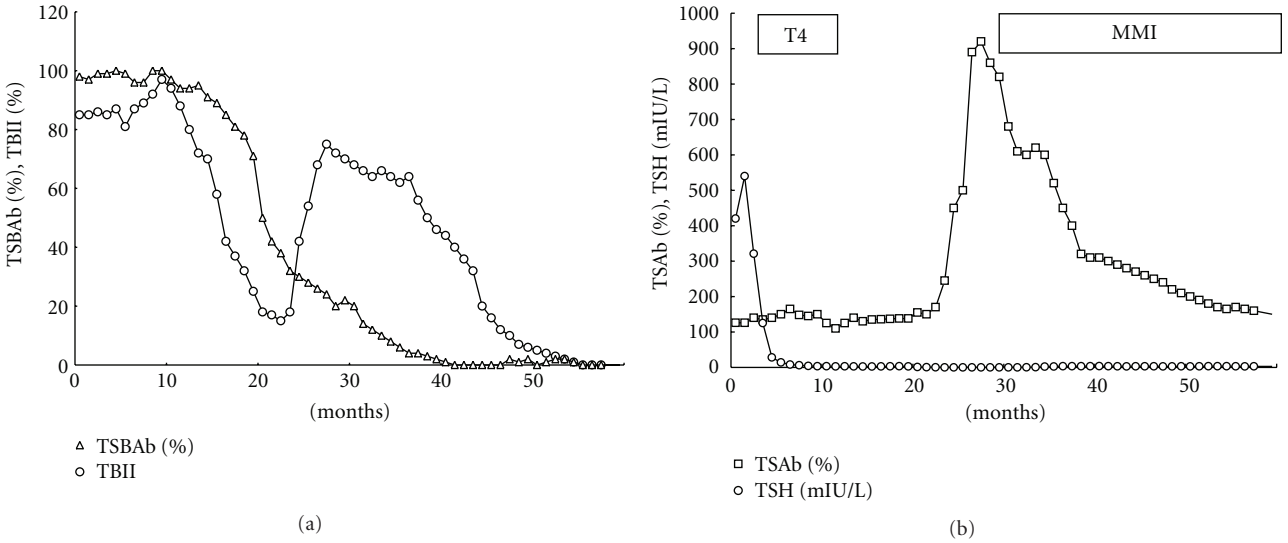

Figure 2: The clinical course of one of the 2 patients, who initially had TSBAb-positive hypothyroidism and then developed TSAb-positive Graves' hyperthyroidism (Table 1, Ic). A 45-year-old woman with TSBAb-positive hypothyroidism developed TSAb-positive Graves' hyperthyroidism. She had TSBAb-positive hypothyroidism ((a), △) with high serum TSH ((b), ∘) and then developed TSAb-positive Graves' hyperthyroidism ((b), □) with undetectable serum TSH ((b), ∘). TSBAb was dominant initially ((a), △), and then TSAb became dominant ((b), □). TBII (TSH-binding inhibitory immunoglobulin) ((a), ∘) reflects TSBAb- and TSAb-activity. A patient with TSBAb-positive hypothyroidism developed TSAb-positive Graves' hyperthyroidism. She was treated with T4 and then with MMI.

high serum TSH and then developed TSAb-positive Graves' hyperthyroidism with undetectable serum TSH. She was treated with T4 and then treated with MMI. She had a goiter initially and had goitrous autoimmune thyroiditis.

Among the 34 TSBAb-positive patients with hypothyroidism (Table 1, I), 24 had atrophic autoimmune thyroiditis and 10 had goitrous autoimmune thyroiditis (Table 3(a)). The 34 TSBAb-positive patients with hypothyroidism consisted of 17 patients (a: positive TSBAb persisited), 15 patients (b: TSBAb disappeared), and 2 patients (c: TSBAb → TSAb) (Table 3(a)). All of the 17 (a) patients continued to have positive TSBAb and continued to have hypothyroidism. All of the 17 (a) patients had atrophic autoimmune thyroiditis and none of them had goitrous autoimmune thyroiditis. TSBAb disappeared in the 15 (b) patients: 13 (b1) (87%) of the 15 (b) patients recovered from hypothyroidism and 2 (b2) (13%) of the 15 (b) patients continued to have hypothyroidism. Of the 13 (b1) patients, who recovered from hypothyroidism, 5 had atrophic autoimmune thyroiditis and 8 had goitrous autoimmune thyroiditis. The 2 (b2) patients, who continued to have hypothyroidism, had atrophic autoimmune thyroiditis. Of the 15 (b) patients, in whom TSBAb had disappeared, 7 [5 (b1) + 2 (b2)] had atrophic autoimmune thyroiditis and 8 [8 (b1)] had goitrous autoimmune thyroiditis. Two (c) patients of the 34 TSBAb-positive patients with hypothyroidism developed TSAb-positive Graves' hyperthyroidism had goitrous autoimmune thyroiditis.

Table 3(b) demonstrates recovery from hypothyroidism in the 34 TSBAb-positive patients with hypothyroidism (24 patients with atrophic autoimmune thyroiditis and 10 patients with goitrous autoimmune thyroiditis). Among the 34 TSBAb-positive patients with hypothyroidism, 19 [(17 (a) + 2 (b2)) in Table 3(a)] continued to have hypothyroidism over 10 years and 15 [13 (b1) + 2 (c)] recovered from hypothyroidism (13 (b1) recovered from hypothyroidism and had remissions and 2 (c) recovered from hypothyroidism and developed hyperthyroidism). All of the 19 TSBAb-positive patients with hypothyroidism, who continued to have hypothyroidism [17 (a) + 2 (b2)], had atrophic autoimmune thyroiditis, and none of them had goitrous autoimmune thyroiditis. Fifteen [13 (b1) + 2 (c)] of the 34 TSBAb-positive patients with hypothyroidism recovered from hypothyroidism. Five [5 (b1)] of the 15 patients, who recovered from hypothyroidism, had atrophic autoimmune thyroiditis and the other 10 [8 (b1) + 2 (c)] had goitrous autoimmune thyroiditis. Nineteen (79%) of the 24 TSBAb-positive hypothyroid patients with atrophic autoimmune thyroiditis continued to have hypothyroidism and the other 5 (21%) recovered from hypothyroidism. All (100%) of the 10 TSBAb-positive hypothyroid patients with goitrous autoimmune thyroiditis [8 (b1) + 2 (c)] recovered from hypothyroidism. Significant differences of recovery from hypothyroidism were noted between the patients with goitrous autoimmune thyroiditis and those with atrophic autoimmune thyroiditis ($\chi^2 = 17.9$, P value < 0.05). All of the 10 TSBAb-positive patients with goitrous autoimmune thyroiditis recovered from hypothyroidism and 19 (79%) of the 24 patients with atrophic autoimmune thyroiditis continued to have hypothyroidism.

3.2. 98 TSAb-Positive Graves' Patients with Hyperthyroidism (II) (Tables 1 and 2, II). All of the 98 Graves' patients

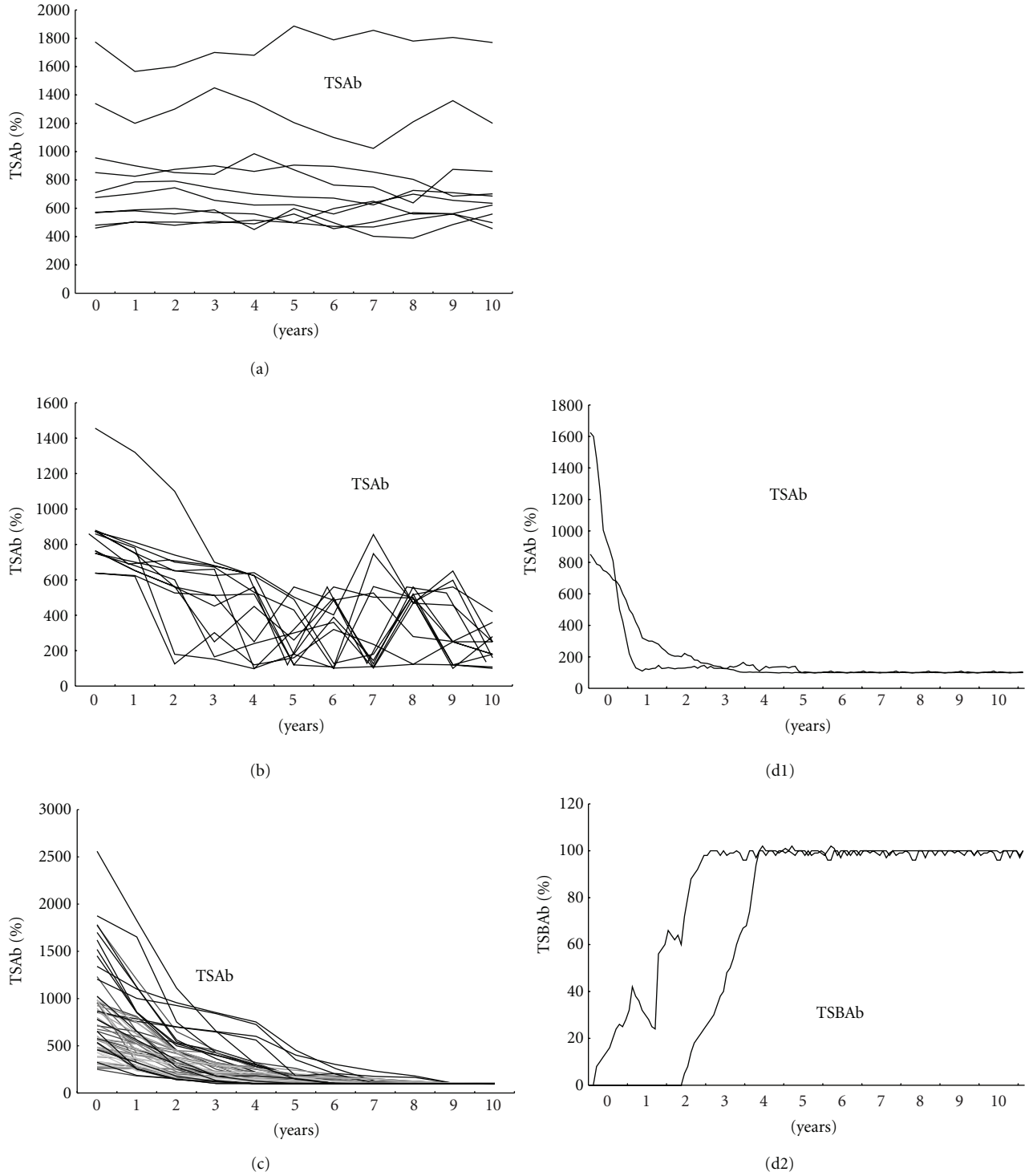

Figure 3: The changes of TSAb in 98 Graves' patients with hyperthyroidism (II) (Table 1, II). Among the 98 Graves' patients with hyperthyroidism, 10 patients continued to have persistently positive TSAb and continued to have hyperthyroidism (Table 1, IIa) (a). Ten of the 98 TSAb-positive Graves' patients with hyperthyroidism continued to have persistently positive TSAb. They continued to have hyperthyroidism and did not get remissions of Graves' hyperthyroidism. They continued to take MMI. Complex changes of TSAb were noted in 13 TSAb-positive patients (Table 1, IIb) (b). One of the 13 patients with complex changes of TSAb got remissions, but the other 12 patients did not get remissions. TSAb disappeared in 73 (74%) of the 98 TSAb-positive Graves' patients with hyperthyroidism (Table 1, IIc) (c). With the disappearance of TSAb, 60 (82%) of the 73 patients, in whom TSAb had disappeared, got remissions of Graves' hyperthyroidism. (d1, d2) show the changes of TSAb and TSBAb, respectively, in the 2 patients with TSAb-positive Graves' hyperthyroidism, who developed TSBAb-positive hypothyroidism (Table 1, IId). In these 2 patients, TSAb was dominant initially (d1), and then TSBAb became dominant (d2). Two patients with TSAb-positive Graves' hyperthyroidism developed TSBAb-positive hypothyroidism. Graves' hyperthyroidism was treated with MMI, and hypothyroidism was treated with T4.

Table 3: Atrophic autoimmune thyroiditis or goitrous autoimmune thyroiditis in the 34 TSBAb (TSH-stimulation-blocking-antibody)-positive patients with hypothyroidism (a) and recovery from hypothyroidism (b).

(a) Atrophic autoimmune thyroiditis (atrophic) or goitrous autoimmune thyroiditis (goitrous) in the 34 TSBAb-positive patients with hypothyroidism

34 TSBAb-positive patients with hypothyroidism (I in Table 1)†			34	
			Atrophic (24)	Goitrous (10)
a: Positive TSBAb persisted (Ia)		17	17	0
b: TSBAb disappeared (Ib) 15	b1: recovered (Ib1)	13	5	8
	b2: hypothyroid (Ib2)	2	2	0
c: TSBAb → TSAb (Ic)		2	0	2

Numbers of the patients are shown. (I in Table 1)† correspond to those in Table 1

Among the 34 TSBAb-positive patients with hypothyroidism (Table 1, I), 24 had atrophic autoimmune thyroiditis and 10 had goitrous autoimmune thyroiditis. The 34 TSBAb-positive patients with hypothyroidism consisted of 17 patients (a: positive TSBAb persisited), 15 patients (b: TSBAb disappeared), and 2 patients (c: TSBAb → TSAb). All of the 17 (a) patients continued to have positive TSBAb and continued to have hypothyroidism. All of the 17 (a) patients had atrophic autoimmune thyroiditis and none of them had goitrous autoimmune thyroiditis. TSBAb disappeared in the 15 (b) patients: 13 (b1) (87%) of the 15 (b) patients recovered from hypothyroidism and 2 (b2) (13%) of the 15 (b) patients continued to have hypothyroidism. Of the 13 (b1) patients, who recovered from hypothyroidism, 5 had atrophic autoimmune thyroiditis and 8 had goitrous autoimmune thyroiditis. The 2 (b2) patients, who continued to have hypothyroidism, had atrophic autoimmune thyroiditis. Of the 15 (b) patients, in whom TSBAb had disappeared, 7 [5 (b1) + 2 (b2)] had atrophic autoimmune thyroiditis and 8 [8 (b1)] had goitrous autoimmune thyroiditis. Two (c) patients of the 34 TSBAb-positive patients with hypothyroidism who developed TSAb-positive Graves' hyperthyroidism who had goitrous autoimmune thyroiditis.

(b) Recovery from hypothyroidism in the patients with atrophic autoimmune thyroiditis (atrophic) and in those with goitrous autoimmune thyroiditis (goitrous)

	Atrophic (24)	Goitrous (10)		
Continued to have hypothyroidism	19 (79%) [17 (a) + 2 (b2)]*	0 (0%)	19	$\chi^2 = 17.9$ P value < 0.05
Recovered from hypothyroidism	5 (21%) [5 (b1)]*	10 (100%) [8 (b1) + 2 (c)]*	15	
	24 (100%)	10 (100%)	34	

Numbers (%) of the patients are shown. [ ]* corresponds to Table 3(a).

Among the 34 TSBAb-positive patients with hypothyroidism (Table 1, I), 24 had atrophic autoimmune thyroiditis and 10 had goitrous autoimmune thyroiditis. Among the 34 TSBAb-positive patients with hypothyroidism, 19 [(17 (a) + 2 (b2)] (Table 3(a)) continued to have hypothyroidism over 10 years and 15 [13 (b1) + 2 (c)] recovered from hypothyroidism [13 (b1) recovered from hypothyroidism and had remissions and 2 (c) recovered from hypothyroidism and developed hyperthyroidism]. All of the 19 TSBAb-positive patients with hypothyroidism, who continued to have hypothyroidism [17 (a) + 2 (b2)], had atrophic autoimmune thyroiditis, and none of them had goitrous autoimmune thyroiditis. Fifteen [13 (b1) + 2 (c)] of the 34 TSBAb-positive patients with hypothyroidism recovered from hypothyroidism. Five [5 (b1)] of the 15 patients, who recovered from hypothyroidism, had atrophic autoimmune thyroiditis and the other 10 [8 (b1) + 2 (c)] had goitrous autoimmune thyroiditis. Nineteen (79%) of the 24 TSBAb-positive hypothyroid patients with atrophic autoimmune thyroiditis continued to have hypothyroidism and the other 5 (21%) of them recovered from hypothyroidism. All of the 10 TSBAb-positive hypothyroid patients with goitrous autoimmune thyroiditis [8 (b1) + 2 (c)] recovered from hypothyroidism. Significant differences of recovery from hypothyroidism were noted between the patients with goitrous autoimmune thyroiditis and those with atrophic autoimmune thyroiditis ($\chi^2 = 17.9$, P value < 0.05). All (100%) of the 10 TSBAb-positive patients with goitrous autoimmune thyroiditis recovered from hypothyroidism and 19 (79%) of the 24 patients with atrophic autoimmune thyroiditis continued to have hypothyroidism.

with hyperthyroidism had positive TSAb. Some of them had positive TSBAb. Graves' patients with hyperthyroidism had wide distributions of TSAb and TSBAb. Some of the Graves' patients had both positive TSAb and TSBAb. Figure 3 shows the changes of TSAb in 98 Graves' patients with hyperthyroidism (II) (Table 1, II). Among the 98 Graves' patients with hyperthyroidism, 10 patients continued to have persistently positive TSAb and continued to have hyperthyroidism (IIa) (Figure 3(a)). Ten of the 98 TSAb-positive Graves' patients with hyperthyroidism continued to have positive TSAb and continued to have Graves' hyperthyroidism. They did not get remissions of Graves' hyperthyroidism and continued to take ATD. Complex changes of TSAb were noted in 13 TSAb-positive patients (IIb) (Figure 3(b)). One (IIb1) of the 13 patients with complex changes of TSAb got remissions, but the other 12 patients (IIb2) did not get remissions. TSAb disappeared in 73 (IIc) (74%) of the 98 TSAb-positive Graves' patients with hyperthyroidism (IIc) (Figure 3(c)). With the disappearance of TSAb, 60 (IIc1) (82%) of the 73 patients, in whom TSAb had disappeared (IIc), got remissions of Graves' hyperthyroidism. Figures 3d1 and 3d2 show the changes of TSAb and TSBAb, respectively, in the 2 patients with TSAb-positive Graves' hyperthyroidism, who developed TSBAb-positive hypothyroidism (IId) (Table 1, IId). In these 2 patients, TSAb was dominant initially (Figure 3(d1)), and then TSBAb became dominant (Figure 3(d2)). The 2 patients had TSAb-positive Graves' hyperthyroidism and then developed TSBAb-positive hypothyroidism. They were treated with MMI, and then treated with T4. Figure 4 demonstrates the clinical course of one of these 2 patients with TSAb-positive Graves' hyperthyroidism, who developed TSBAb-positive hypothyroidism (Table 1, IId). A 40-year-old woman with TSAb-positive Graves' hyperthyroidism developed TSBAb-positive

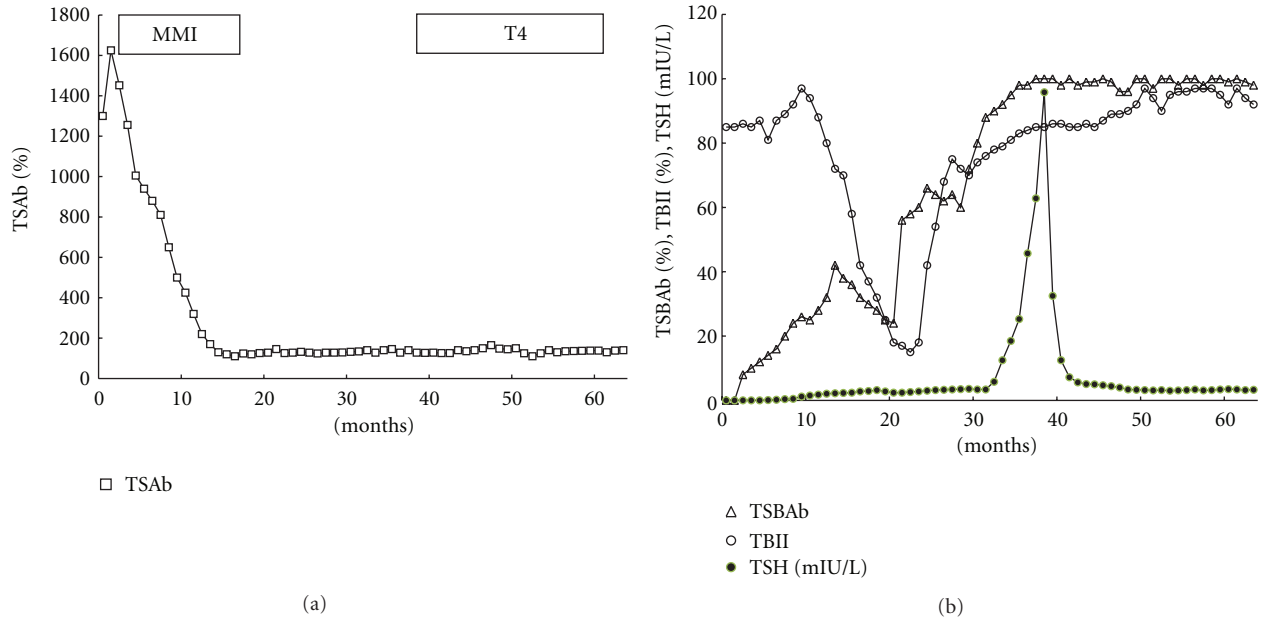

Figure 4: Clinical course of one of the 2 patients, who had TSAb-positive Graves' hyperthyroidism and then developed TSBAb-positive hypothyroidism (Table 1, IId). A 40-year-old woman with TSAb-positive Graves' hyperthyroidism developed TSBAb-positive hypothyroidism. She had TSAb-positive Graves' hyperthyroidism ((a), □) with undetectable serum TSH ((b), •) and then developed TSBAb-positive hypothyroidism ((b), △) with high serum TSH ((b), •). TSAb was dominant initially ((a), □), and then TSBAb became dominant ((b), △). TBII ((b), ◦) reflects TSBAb- and TSAb-activity. A patient with TSAb-positive Graves' hyperthyroidism developed TSBAb-positive hypothyroidism. She was treated with MMI and then with T4.

hypothyroidism. TSAb was dominant initially (Figure 4(a)), and then TSBAb became dominant (Figure 4(b)). She had TSAb-positive Graves' hyperthyroidism with undetectable serum TSH and then developed TSBAb-positive hypothyroidism with high TSH. She was treated with MMI and then treated with T4. She had a goiter over 10 years.

4. Discussion

We have reevaluated TSBAb and TSAb in 34 TSBAb-positive patients with hypothyroidism and in 98 TSAb-positive Graves' patients with hyperthyroidism. Half of the 34 TSBAb-positive patients continued to have persistently positive TSBAb, continued to have hypothyroidism and did not recover from hypothyroidism. Ten of the 98 Graves' patients continued to have positive TSAb. They continued to have hyperthyroidism, and did not get remissions of Graves' hyperthyroidism. TSBAb had disappeared in 15 of the 34 TSBAb-positive patients with hypothyroidism. With the disappearance of TSBAb, recovery from hypothyroidism was noted in 13 (87%) of the 15 TSBAb-positive patients. TSAb had disappeared in 73 of the 98 TSAb-positive Graves' patients with hyperthyroidism. With the disappearance of TSAb, 60 (82%) of the 73 TSAb-positive patients got remissions. Two of the 34 TSBAb-positive patients with hypothyroidism developed TSAb-positive Graves' hyperthyroidism. Two of the 98 TSAb-positive Graves' patients with hyperthyroidism developed TSBAb-positive hypothyroidism. TSBAb causes hypothyroidism. TSAb causes Graves' hyperthyroidism. TSBAb and TSAb are TRAb. TSBAb-positive hypothyroidism and TSAb-positive hyperthyroidism may be two aspects of one disease (TRAb disease).

TSBAb blocks TSH-stimulation of the thyroid and causes hypothyroidism. TSAb stimulates the thyroid and causes Graves' hyperthyroidism. Both TSBAb and TSAb block TSH-binding to thyroid cells as TSH receptor antibodies (TRAbs), which have been measured as TSH-binding inhibitory immunoglobulin (TBII) [1–3, 13]. TBII reflects TSBAb- and TSAb-activities. TBII measures the binding of antibody to TSH receptor by competition with radiolabeled TSH and does not distinguish between TSBAb and TSAb. TSBAb is measured as a TSH-stimulation blocking assay and TSAb as a stimulator assay. TSBAb is a blocking antibody [3, 13] and TSAb is a stimulating antibody [1, 2, 13].

TSBAb-activities were expressed as percentage inhibition of TSH-stimulated cAMP production by test IgG [3, 10–13, 15–20]. Two formulas (TSBAb-A and TSBAb-B) have been proposed to calculate TSBAb [3, 10–13]. TSBAb-A was used in the earlier reports [3, 10–13], and TSBAb-B in the later report [13]. TSBAb-A ignores TSAb activity in serum and might give low TSBAb activity. TSBAb-B considers TSAb activity in serum and might give high TSBAb activity. All of the TSBAb-positive patients with hypothyroidism had strongly positive TSBAb-A and TSBAb-B. Both TSBAb-A and TSBAb-B could be used to estimate TSBAb activities [13]. The details were discussed in the previous paper [13]. TSBAb, described in this paper, corresponds to TSBAb-A in the previous paper [13]. TSBAb-A [13] is used as TSBAb in this report.

All of the 34 TSBAb-positive patients with hypothyroidism and all of the 98 TSAb-positive Graves' patients had positive TBII (TRAb). TSBAb and TSAb are TSH-receptor antibodies (TRAbs), which have been measured as TBII. TBII does not distinguish between TSBAb and TSAb. TBII reflects TSBAb- and TSAb-activities [1–3, 13]. All of the 34 TSBAb-positive patients with hypothyroidism had strongly positive TSBAb. Some of them had positive TSAb [13]. All of the 98 Graves' patients had positive TSAb. Some of them had positive TSBAb [13]. TSBAb-positve patients with hypothyroidism had narrow distributions of TSBAb and TSAb, and Graves' patients with hyperthyroidism had wide distributions of TSBAb and TSAb [13]. TSBAb-positive patients with hypothyroidism have strongly positive TSBAb.

TBII reflects TSBAb- and TSAb-activities [1–3, 13]. Some of the TBII-positive patients have hypothyroidism, and the other TBII-positive patients have hyperthyroidism. The former TBII is TSBAb, and the latter TBII is TSAb. The numbers of the former TSBAb-positive patients with hypothyroidism are less than those of the latter TSAb-positive Graves' patients with hyperthyroidism. All of the TSBAb-positive patients with hypothyroidism have high titers of TBII, which is TSBAb [3]. Almost all of the untreated Graves' patients with hyperthyroidism have TBII, which is TSAb [1, 2]. TSBAb- (TRAb-) positive hypothyroidism and TSAb- (TRAb-) positive Graves' hyperthyroidism may be two aspects of one disease (TRAb disease).

Hypothyroidism may result from the production of TSBAb [3]. In 1992, we followed 21 TSBAb-positive patients with hypothyroidism over 10 years and found that with the disappearance of TSBAb, recovery from hypothyroidism was noted in 6 (40%) of the 15 TSBAb-positive patients [3]. Here, we followed 34 TSBAb-positive patients with hypothyroidism over 10 years and found that with the disappearance of TSBAb, recovery from hypothyroidism was noted in 13 (87%) of the 15 patients. The frequency of recovery from hypothyroidism with the disappearance of TSBAb in this paper is much higher than that in the previous one [3]. With the disappearance of TSBAb, recovery from hypothyroidism is observed. The production of TSBAb may subside, producing remissions of hypothyroidism.

It is important to know whether a patient with Graves' disease gets remission or not during ATD treatment. Disappearance of TSAb predicted the remissions of Graves' hyperthyroidism [14]. With the disappearance of TSAb, 36 (82%) of the 44 patients were reported to get remissions in the previous paper [14] and 60 (82%) of the 73 patients are reported to get remissions in this paper. Disappearance of TSAb predicts the remissions of Graves' hyperthyroidism.

Two of the 34 TSBAb-positive patients with hypothyroidism developed TSAb-positive Graves' hyperthyroidism (Ic). Two of the 98 TSAb-positive Graves' patients with hyperthyroidism developed TSBAb-positive hypothyroidism (IId). In the former, TSBAb was dominant initially and then TSAb became dominant. In the latter, TSAb was dominant initially and then TSBAb became dominant. Thyroid function can oscillate between hypothyroidism and hyperthyroidism as TSBAb or TSAb becomes dominant. TSAb and TSBAb can be used to document the functions of TRAb [13]. TBII-positive patients with strongly positive TSBAb have hypothyroidism. TBII-positive patients with positive TSAb have hyperthyroidism. TSBAb-positive patients with hypothyroidism and TSAb-positive Graves' patients with hyperthyroidism may have both TSBAb and TSAb [1, 2, 13, 21–26]. TSBAb-positive patients with hypothyroidism may develop TSAb-positive hyperthyroidism. TSAb-positive Graves' patients with hyperthyroidism may develop TSBAb-positive hypothyroidism. TSBAb and TSAb are TRAb. TSBAb- (TRAb-) positive hypothyroidism and TSAb- (TRAb-) positive hyperthyroidism may be two aspects of one disease (TRAb disease).

In Japan, TRAb has been measured as TBII and TSAb [14]. TSAb is a bioassay, using porcine thyroid cells. We usually measure TSAb, using a commercially available kit [14]. In Japan, TSAb-assay kit is available, but TSBAb-assay kit is not. When a patient has hypothyroidism with elevated TSH and positive TBII, this TBII is thought to be TSBAb. We usually do not measure TSBAb. Practically, when a patient with hypothyroidism has positive TBII, this TBII may be TSBAb. When a patient with hyperthyroidism has positive TBII, this TBII may be TSAb. TSAb and TSBAb can be used to document TRAb-function. TBII, measuring the antibody-binding to the receptor by competition with radiolabeled TSH, does not distinguish between TSAb and TSBAb. A positive TBII result in a patient with hypothyroidism is evidence for the presence of TSBAb. A positive TBII result in a patient with hyperthyroidism is evidence for the presence of TSAb. These bioassays (TSAb and TSBAb) are useful to detect transient neonatal hyperthyroidism and hypothyroidism [10] and are also important to confirm the causes of hyperthyroidism and hypothyroidism [13]. TBII-positive patients may have TSBAb or TSAb. Thyroid function can oscillate between hypothyroidism and hyperthyroidism as TSBAb or TSAb becomes dominant. TSAb and TSBAb can be used to document TRAb-function [13].

There are two forms of autoimmune thyroiditis: atrophic autoimmune thyroiditis and goitrous autoimmune thyroiditis [3]. We followed 34 TSBAb-positive patients with hypothyroidism (24 patients with atrophic autoimmune thyroiditis and 10 with goitrous autoimmune thyroiditis) over 10 years. TSBAb has been found in patients with atrophic autoimmune thyroiditis, and also in patients with goitrous autoimmune thyroiditis [11]. All of the 10 TSBAb-positive patients with goitrous autoimmune thyroiditis recovered from hypothyroidism and 19 (79%) of the 24 with atrophic autoimmune thyroiditis continued to have hypothyroidism. With the disappearance of TSBAb, recovery from hypothyroidism has been seen. TSBAb-positive hypothyroid patients with goitrous autoimmune thyroiditis may recover from hypothyroidism, and those with atrophic autoimmune thyroiditis may continue to have hypothyroidism.

Conflict of Interests

The authors have accepted no funding or support from an organization that may gain or lose financially from the results of their study. They have not been employed by any organization that may gain or lose financially from the result of their study.

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Review Article

The Role of Thyrotrophin Receptor Antibody Assays in Graves' Disease

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Thyrotrophin receptor antibodies (TRAb) exist as stimulating or blocking antibodies in the serum (neutral TRAb have been identified recently). The clinical features of GD occur when stimulating TRAb predominate. But the relationship of TRAb to clinical phenotype and outcome is not clear when current assay methods are used. Therefore no consensus exists about its utility in diagnosing and predicting outcome in GD. The most commonly used TRAb assays, measure thyroid binding inhibiting immunoglobulins (TBII or "receptor assays") and don't differentiate between stimulating and blocking antibodies. However, the more expensive, technically demanding and less freely available "biological assays" differentiate between them by their ability to stimulate cyclic AMP or failure to do so. Failure to differentiate between TRAb types and its heterogeneous molecular and functional properties has limited TBII use to GD diagnosis and differentiating from other forms of thyrotoxicosis. The current 2nd-3rd generation receptor assays are highly sensitive and specific when used for this purpose. TRAb assays should also be done in appropriate pregnant women. Current data do not support its use in outcome prediction as there is a significant variability of assay methodology, population characteristics and study design in published data, resulting in a lack of consensus.

1. Introduction

The immunopathogenesis of Graves' disease (GD) is a story that continues to evolve. GD is unique amongst autoimmune endocrine diseases as the underlying immune perturbation results in thyroid stimulation rather than its functional or structural inhibition. The contribution of genetic (MHC, CTLA-4, and PTPN22) and environmental influences (smoking, stress, drugs, micronutrients) to the aetiology of GD has been described extensively [1–6]. This complex genetic/environmental interaction results in the production of Thyrotrophin Receptor Antibodies (TRAb) which stimulate the TSH receptor (TSHR) and are the proximate cause of GD. Their precise role in the extrathyroidal manifestations of GD is currently being investigated [7].

The earliest description of a thyroid stimulator in GD was by Adams and Purves in 1956 [8]. The discovery of this "long-acting thyroid stimulator (LATS)" led to further attempts to characterize it [9]. The target antigen for LATS was the TSHR [10], and research showed these "thyroid stimulators" in GD were in fact autoantibodies to the TSHR; that is, TRAb. The complex nature of the interaction between TSHR and TRAb has been elegantly demonstrated using advanced techniques, and the molecular and crystalline structure of TRAb has been described in detail [11–14]. It would seem intuitive therefore that measurement of TRAb, the proximate cause of GD and so intimately involved in its pathogenesis, would assist in its diagnosis and management. However, neither contention is consistently borne out in clinical practice. The relationship between TRAb measured using currently available assays and GD is complex and needs to be understood by clinicians if they are to be correctly interpreted in clinical practice.

Current assays detect TRAb in 95-96% of subjects with GD although only some can demonstrate their functional characteristics [15]. However, there is no consensus about its role in diagnosing and managing GD, and its utility in predicting outcome. The inherent functional properties of TRAb, the variability in study design, and assay methodology have contributed to this uncertainty.

Table 1: A comparison of TBII and biological assays.

	TBII assays	Biological assays
Advantages	Freely available commercially	Differentiate between stimulating and blocking activities of TRAb
	Relatively cheap	
	Easy to perform	
	Sensitive 2nd-3rd generation assays available	
Disadvantages	Do not differentiate between stimulating and blocking activities of TRAb	Most are technically complex and time consuming
	Lack absolute correlation with clinical phenotype	Relatively expensive
	No correlation with severity of illness	
	Lack predictive value for GD outcome	

TBII are easy to perform, cheap and are highly sensitive. They remain the preferred assay method of choice in clinical practice. Bioassays have the ability to differentiate between stimulating and blocking TRAb, but the utility of this property in day-to-day clinical practice is unclear. Furthermore, they require greater technical expertise to perform and currently are more expensive.

2. The Structure of TRAb and Their Interaction with TSHR in GD

TRAb are heterogeneous in both molecular structure and biological activity with a propensity to change during the course of the disease. They may stimulate the TSHR (thyroid stimulating antibodies-TSAb) or block its activity (thyroid blocking antibodies-TBAb) [16]. The clinical phenotype is thus determined by the balance between their opposing actions-thyrotoxicosis when TSAb predominate, and hypothyroidism when TBAb predominate. Neutral TRAb have also been isolated recently and their role in GD is yet to be defined [17]. TSAbs probably undergo affinity maturation and bind TSHR with high affinity, although details are not accurately known [18, 19]. A new classification has been proposed for TRAb based on their ability to stimulate or block both classical cyclic AMP (cAMP) and nonclassical non-cAMP signalling pathways. This classification is functionally more accurate and intellectually more attractive [16].

The TSHR is a G protein-coupled receptor and has a molecular structure consistent with this. The extracellular component consists of a Leucine-rich repeat domain (LRD) and a hinge region (HR), which links to the 7 domain transmembrane and intracellular components. The increasingly important role and the structure and function of the HR are currently being defined [20, 21]. There have been major recent studies of the synthesis, post translational modification, shedding of the α-subunit and the effect of the unbound α-subunit on the TSHR [22–25]. The α-subunit appears to be the primary autoantigen for TRAb formation [23, 26].

TRAb, in common with TSH, bind to the concave surface of the LRD. Recent crystallization studies using the TSHR stimulating human monoclonal antibody M-22 have shown the importance of several residues on this concave surface to the binding process [27] which seemed to be specific to this antibody [13]. These residues may not be specific for native TSH signalling. After binding to the TSHR, TRAb stimulate cAMP-dependent signal transduction (and also non-cAMP-dependent signalling pathways) resulting ultimately in increased thyroid hormone secretion [28]. The clinical features of GD are thus produced when TSAb predominate. Predominant TBAb have the opposite effect.

3. Measuring TRAb

3.1. Assay Methodology and Sensitivity. There are two currently available methods for measuring TRAb [29].

(1) "Receptor assays" using I^{125} labelled TSH are freely available commercially for clinical use.

(2) "Bioassays" using cultured cells, which measure cAMP production as an indicator of TSHR stimulation or inhibition, are still most often used in a research setting (Table 1).

3.1.1. Receptor Assays. Receptor assays measure "thyroid-binding inhibiting immunoglobulins" (TBIIs); that is, antibodies that block binding of TSH to an in vitro TSHR preparation and do not therefore differentiate between TSAb and TBAb in serum samples. Some who do not advocate routine testing of TRAb in GD insist that this is of minor consequence as clinical and biochemical features will identify functional characteristics of the predominant TRAb in a patient with GD. The lack of correlation between TRAb in these assays and the clinical and biochemical severity of GD and its outcome may indeed be related to this inability to differentiate between the functional properties of TRAb. They therefore do not accurately predict GD phenotype in

every patient. These assays also have wide intermethod variability. It has been estimated that the interassay coefficient of variation between various commercially available assays is 15.2–21.6% [30]. They are commercially freely available and are easy to perform (Table 1).

While first-generation TBII assays using porcine cells and bovine labelled TSH had a sensitivity of only 50–80% [31], second-generation assays using recombinant human TSHR are said to be 90–99% sensitive and 95–100% specific [32–34]. Third-generation assays using human monoclonal TSHR stimulating antibodies are said to be even better [35] with improved sensitivities (97%) compared to second generation assays (94%) in one study [36].

There are still a minority of individuals who have GD who remain TRAb negative even when modern TBII assays are used. They usually have mild disease, smaller goitres, and minimal RAI uptake on scintigraphy [37]. In a recent study only 1.4% of an untreated group of thyrotoxic patients were in this group when a third-generation assay was used [38]. It is speculated that they have intrathyroidal TRAb production which does not spill over to the circulation, or that even third-generation TBII assays are too insensitive. Fully automated TBII assays are now available and should improve their use [39].

3.1.2. Biological Assays. Biological assays in contrast measure the ability of TRAb to stimulate or inhibit TSHR activity. They measure the production of cAMP when sera containing TRAb are exposed to TSHR on cell preparations such as FRTL-5 or CHO [40, 41]. Therefore, they are able to differentiate between TSAb and TBAb. However, their sensitivity at predicting GD recurrence is still surprisingly poor as some studies indicate [42]. This may relate to inherent properties of TRAb (e.g., antibodies with both blocking and stimulating activities, very similar receptor-binding characteristics and affinity for the TSHR) or to antibodies that interfere with these assays that make results difficult to interpret. More recent bioassays using a luciferase reporter gene on cell lines expressing the TSHR are technically less demanding and more rapidly done [43, 44].

Assays utilising modified TSHR, substituting some amino acid residues from the luteinizing hormone receptor (LHR), have produced encouraging results. These chimaeric TSHR-containing assay systems, for instance using the Mc4 TSHR where amino acid residues 262–368 of the human wild type receptor have been replaced by residues 262–334 of the rat LHR, seem to perform well under experimental conditions [45, 46].

Biological assays are currently limited to research in many centres. Although they provide information about the functional status of TRAb, their use has been restricted because of expense, and technical expertise and time required to perform them. Furthermore, the current utility of TBII assays in association with clinical and biochemical features to predict the functional status of TRAb in GD confers on them an advantage over biological assays. However, with advancing technology some of the above disadvantages should be overcome [26].

3.2. TRAb Assays and Specificity. Current TRAb assays lack specificity and may be positive in other thyroid disease. Recent studies have shown that a significant minority with painless thyroiditis (9.2%) and subacute thyroiditis (6.7%), hypothyroidism (9%) and multinodular goitre (17.2%) is TRAb positive using receptor assays [36, 52]. The inability of current assays to functionally define TRAb may account for this lack of specificity.

4. TRAb in the Diagnosis of Thyrotoxic States

4.1. Establishing a Diagnosis of GD and Differentiating from Other Causes of Thyrotoxicosis. Some argue that TRAb assays are not necessary to diagnose GD and for its differential diagnosis from other causes of thyrotoxicosis. If clinical symptoms and signs are nonspecific, they advocate the use of radioiodine (RAI) scintigraphy to differentiate GD from other thyrotoxic states [53]. In some centres about 20% remained of "indeterminate origin" even after RAI scintigraphy [54, 55], despite a retrospective cost effectiveness analysis comparing ultrasound to radioiodine scintigraphy in GD, which found a high sensitivity (97.4%) and specificity (98.8%) for RAI with equally good positive and negative predictive values [56]. Some argue that assays for other antibodies such as thyroid peroxidase antibodies (TPOAbs), present only in about 80% of GD but which are easier to perform and freely and more cheaply available, could be used instead of TRAb. TPOAb has a low sensitivity and specificity in this context and therefore is not very helpful in our opinion. Thus RAI uptake scans and TPOAb assays are inadequate for routine clinical use for the differential diagnosis of thyrotoxic states.

GD is difficult to diagnose in the minority of patients where goitre, overt clinical features, and GO are absent. The proponents of TRAb agree that the availability of sensitive and easy to perform, comparatively cheap assays should make TRAb an essential tool in the diagnostic work-up. Its high sensitivity ensures that virtually all subjects with GD are picked up. This is important from a practical point of view in centres where first line therapy for GD and other thyrotoxic states differs. Most clinicians treat GD initially with thionamides, before giving RAI therapy for a recurrence [57]. They also treat toxic nodular disease (almost all TRAb negative) with RAI as first line therapy (usually after making them euthyroid with thionamides) [58]. The use of TRAb would therefore help this decision-making process at an early stage. There is also an economic argument for using relatively cheap TRAb assays without using more expensive and cumbersome thyroid scintigraphy. In centres where TRAb assays have been established as routine and are cheaper to do, this differential in expense is even greater. The current use of TRAb in diagnosing GD seems to be governed by tradition, expense, and the availability of suitable assays.

5. Special Situations

5.1. Pregnancy. GD is responsible for nearly 85% of the 0.1–0.4% of pregnancies that are complicated by hyperthyroidism [59, 60]. Transplacental passage of TRAb causes

foetal or neonatal thyrotoxicosis in 1–5% of pregnancies in women with current or past GD [61]. In the majority of pregnant women, TRAb levels begin to decline at around 20 weeks of gestation because of gestational immune modulation; the immune milieu is consistent with the Th2 paradigm during pregnancy and the important roles of hormones and regulatory T cells in this process are not within the scope of this paper [62]. The persistence of high levels of TRAb in the third trimester (measured between 22–26 weeks) increases risk to the foetus and indicates the need for close monitoring in association with obstetricians and neonatal specialists. Some would limit third trimester TRAb testing only to those mothers who had high titres in the first trimester [63]. Although investigators have attempted to correlate TRAb activity in the mother and neonate with foetal and neonatal GD, there has been no consensus. Some investigators found maternal TRAb of >40 U/L (using human recombinant receptor assays) predicted neonatal GD [64]. Japanese investigators also found that in mothers who had RAI for GD, TRAb levels at delivery were significantly higher in those who delivered infants with neonatal hyperthyroidism compared to those who did not [65].

The current indications for TRAb testing in pregnancy are as follows [66].

(a) Current GD that is, those on thionamide therapy.

(b) Previous radioiodine treatment or surgery for GD even if euthyroid—2–10% risk of foetal and neonatal hyperthyroidism.

(c) Previous history of delivering an infant with neonatal hyperthyroidism.

Subjects who have had previous GD who are in remission (i.e., on no drug therapy), do not need TRAb testing as their euthyroid state implies the absence of significant levels of TRAb and therefore no risk to the foetus.

5.2. Immune Reconstitution Syndromes. Modern lymphocyte depleting agents such as Alemtuzumab (CAMPATH), an anti-CD52 monoclonal antibody, cause thyroid dysfunction in a significant minority of patients, as many as 30% when used to treat multiple sclerosis. This immune reconstitution syndrome may also occur in highly active antiretroviral therapy (HAART) for HIV infection, and bone marrow transplantation from a GD patient [67]. These subjects develop GD and have detectable TRAb. The mechanisms in Alemtuzumab and HAART induced GD seem to be naive CD4 T-cell expansion, while a graft versus host disease may account for it in bone marrow recipients [67].

5.3. Orbitopathy. A significant proportion of subjects with GD have clinically evident Graves' orbitopathy (GO), estimated to be between 30–50% in various studies. Sight threatening disease occurs in about 5% [68]. The coexistence of symptoms and signs of GD in the majority of them helps establish an accurate diagnosis.

However, TRAb assays are mandatory in two circumstances: (a) to diagnose the minority where GO occurs as an isolated disorder without symptoms or signs of GD and (b) rarely when GO occurs in a hypothyroid patient.

6. What Happens to TRAb When GD Is Treated?

Both thionamide therapy and thyroid surgery reduce TRAb in GD. Thionamides, reduce TRAb primarily by their immunomodulatory effects [69, 70]. Surgery does so by removing the antigen, TSHR [71], and possibly by T and B lymphocytes apoptosis following high level antigen release during surgery [25]. The effects of RAI therapy on TRAb are different. An initial rise in TRAb after RAI is followed by a gradual fall [72]. This initial rise is probably a result of the release of TSHR antigen following tissue destruction by RAI. RAI-induced inhibition of T regulatory cells (TReg) may also contribute [73]. The modulation of TRAb levels after the three modalities of treatment described above would suggest that the persistence of TRAb at significant levels would predict further recurrences. However, the story is far from clear.

7. TRAb in Predicting Recurrences of GD

The inability of currently available TRAb assays to predict remission and recurrences of GD remains a great shortcoming in this area. A prediction tool such as TRAb could spare patients from long and sometimes complicated drug regimes with potentially serious side effects. The ability to predict the course of GD would also facilitate early definitive therapy with RAI or surgery. Clinical utility at predicting recurrences was inadequate when using clinical data (goitre volume, family history of GD, age, gender, smoking, etc.) and biochemical/immunological data (thyroid hormone levels, TRAb levels, rate of TRAb decline during treatment, etc.) either singly or in combination.

Early attempts at using TRAb to predict remission of GD followed a meta-analysis which suggested that the absence of TRAb after antithyroid drug therapy predicted remission [47]. But the practical value of this analysis was questionable and limited as nearly 25% of subjects were misclassified [29]. Although large scale, well-powered prospective studies addressing this question are lacking, a brief examination of the data from the last decade for the use of TRAb as a predictor of GD outcome is warranted (Table 2).

The predictive value for TRAb at the assay diagnostic cutoff value of 1.5 U/L, was low and not of high clinical utility in an early study published in 2002 [74]. Subsequent studies attempted to use TRAb thresholds that were higher, and measured at various points during the course of the disease to improve its predictive value. A cutoff above 10 IU/L at 6 months increased the positive predictive value (PPV) to 97% in one study. But its negative predictive value (NPV) was too low for clinical utility [48]. In a subsequent multicentre prospective study it was found that within 2 years of stopping antithyroid drugs (ATD) 49% of 96 patients relapsed. In this study TRAb at a level of 10 U/L measured at 4 weeks after stopping ATD had a PPV 0f 83% and NPV of 62% (specificity 92%). But TSH also measured at 4 weeks after stopping ATD

Table 2: Recent clinical studies examining the utility of TRAb assays in predicting GD outcome.

Author (year, (ref))	Assay (n)	Study design	TRAb cutoff value	% Relapse	PPV %
Zimmermann-Belsing et al. (2002, [47])	TBII (129)	TRAb assays at diagnosis (122) and at withdrawal of drugs (129): median followup 18 months	1.5 U/L	45	49
Quadbeck et al. (2005, [48])	TBII (96)	TRAb assays done 4 weeks after withdrawal of drugs: followup for 2 years	1.5 U/L	49	49
			10 U/L		83
Quadbeck et al. (2005, [48])	Bioassay (96)	As above	1.5 U/L		49
					TSAb-51
Schott et al. (2007, [49])	TBII (131)	TRAb and TPOAb assays done 4.3 months (mean) after GD diagnosis	>2 and <6 U/L	71.8	66.7–90
			>6 + >5000		100
			>6 + >500		93.7–96
Cappelli et al. [2007, [50]]	TBII (216)	TRAb assays done at diagnosis and 6 monthly for 120 months	>46.5 U/L at diagnosis or	67.1	52%
			>30.7 U/L at 6 months		53.2
Massart et al. (2009, [51])	TBII (128)	TRAb assays compared after 18 months of treatment: 3-year followup	0.94–3.2 IU/L	48	53–66%

Most recent studies are small and retrospective. They were variable in their study design (e.g., timing of TRAb measurement), assay methodology and TRAb cutoff values used for analysis, and population characteristics (i.e., geographically disparate). Although there was a high relapse rate (45–71.8%), TRAb assay by itself had a poor PPV and was a poor predictor of relapse even when different cutoff values were used.

had a PPV of 70% and a negative predictive (NPV) value of 62% for a relapse [49]. Another study made use of the fact that thyroid peroxidase antibodies (TPOAb) which are detectable in GD may be used to advantage in combination with TRAb to increase the predictive value of a relapse. 71.8% of 131 patients with GD relapsed during followup for between 10–77 months [49]. The PPV for relapse was 100% when a cutoff of >5000 U/mL was used for TPOAb and of >6 U/L for TRAb (Table 2). Cappelli and his colleagues studied 216 patients with GD prospectively for 120 months. They measured TRAb at diagnosis and every 6 months thereafter for the duration of the study. TRAb at >46.5 U/L at diagnosis had a PPV of 52% and NPV 0f 77% and at >30.7 U/L at 6 months had a PPV of 53.2% and NPV of 79% for a relapse [50]. A study comparing human monoclonal antibody M22-based TRAb assays and second generation TRAb assays by Massart and colleagues was not conclusive either [51]. They measured TRAb after 18 months of antithyroid drug treatment and found that the newer M22-based assays did not improve the predictive value of relapse. They also commented on high intermethod variability.

Defining a consensus is therefore difficult and relates to several pertinent issues. The above studies were variable both in relation to TRAb assay methodology and study design. Some studies were retrospective (with all their associated problems) and some prospective. In the retrospective studies attempts were made to find the most sensitive and specific cutoff values for TRAb and in one its use in combination with TPOAb was examined. They were also variable in the timing of TRAb assay, being measured at diagnosis or at different points in the course of their disease. Furthermore, population genetics and iodine status may also have influenced these studies as they were done in geographically disparate areas. Therefore, it is our view that till further good quality evidence is forthcoming, TRAb assays seem a rather blunt tool to predict remission or relapse of GD using current methodology.

8. Conclusions and Indications for TRAb Testing

The clinical utility of TRAb as an important tool in the differential diagnosis of thyrotoxic states is established in our opinion. Although some experts doubt its value in subjects with typical features of GD, we believe that TRAb assays should be done in all patients to positively establish a diagnosis and to help in differentiating between the various causes of thyrotoxicosis. Most such experts base their argument for selective TRAb testing, on the basis of cost, availability of assays, and traditional practice. However, TRAb measurements using modern 2nd-3rd generation receptor assays are increasingly more freely available, quickly done and cheap (certainly in high volume laboratories). They offer a greater advantage over TPOAb and thyroid scintigraphy, in terms of higher sensitivity and specificity, logistical considerations and cost savings. Furthermore, newer automated 3rd-generation assays provide excellent sensitivity and specificity with high PPV and NPV in subjects with biochemical hyperthyroidism [75]. Table 3 illustrates the current indications for performing TRAb tests.

Table 3: Current indications for TRAb testing.

Indications for TRAb testing
Establishing diagnosis of GD and differentiating from other thyrotoxic states
Thyrotoxicosis complicating the Immune reconstitution syndrome (CAMPATH and HAART)
Euthyroid or unilateral orbitopathy
Orbitopathy with hypothyroidism
Pregnancy in women: (a) currently on ATD therapy (b) who have had previous ablative therapy (RAI or surgery) (c) with previous children who had neonatal thyrotoxicosis
In the first trimester and at 22–26 weeks gestation

The current indications for TRAb testing are detailed above. Its use is limited to diagnostic indications. There is no clinical utility of TRAb in predicting outcome at present.

However, its utility in predicting GD remission/relapse is still unproven. An ideal prediction tool would be easy and cheap to measure, sensitive with high PPV and NPV, when measured early in the course of the disease. The lack of large, reproducible, well-designed, prospective studies is a shortcoming in this area of thyroidology. Furthermore, the variability of study design, TRAb assay methodology, and target study populations in currently published studies, added to the variability of intrinsic molecular and functional characteristics of the TRAb molecule, make this aspect of GD management frustrating and lacking in consensus.

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Review Article

Microchimerism in Graves' Disease

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Microchimerism is the presence of cells from one individual in another genetically distinct individual. Pregnancy is the main cause of natural microchimerism through transplacental bidirectional cell trafficking between mother and fetus. The consequences of pregnancy-related microchimerism are under active investigation. However, many authors have suggested a close relationship linking fetal microchimerism and the development of autoimmune diseases. It has been more than ten years now since the demonstration of the presence of a significant high number of fetal microchimeric cells residing in thyroid glands from operated patients with Graves' disease. This intrathyroidal fetal microchimerism is an attractive candidate mechanism for the modulation of Graves' disease in pregnancy and the postpartum period.

1. Introduction

Microchimerism is defined by the presence of alien cells within an individual tissue with genetically different background [1]. Microchimeric cells have two possible origins: natural and artificial. Examples of the former are pregnancy, miscarriage, and twinning or sexual intercourse, whereas the most common cases for the later are tissue transplant or blood transfusion. Pregnancy is the major source of natural microchimerism.

Contrary to previous expectations, the placental trophoblastic physical barrier effect is not a perfect cutoff system. A certain cell leakage is present between mother and fetus during gestation, and this transplacental cell trafficking is a two-way process. Fetal cells movement into maternal circulation starts very early during pregnancy. Circulating fetal cells have been found in maternal blood as soon as the fourth week of gestation [2], whereas maternal microchimerism has been detected in a newborn thyroid autopsied at day 2, but so far has not been reported in thyroid diseases [3]. Earlier evidences concluded that fetal cells transfer into maternal circulation was more intense than maternal cells into fetal blood [4]. However, subsequent investigations estimated that fetus-to-maternal transfer should be as frequent as maternal-to-fetus trafficking because maternal DNA has been detected in 40–100% of cord blood samples when polymerase chain reaction (PCR) techniques were used [5, 6]. The extent of this phenomenon is universal since fetal cells can be found in the peripheral blood of almost 100% of women during pregnancy [7]. Although the level of circulating cells has been reported to be very low (1 : 500,000 fetal : maternal cells) [8], such fetal cells can remain after delivery for more than 38 years postpartum [9]. The most plausible explanation for this long cell persistence is that fetal microchimeric cells can engraft into maternal bone marrow and provide a renewing source of fetal cells in maternal blood for decades after delivery [10].

So far, only the human leukocyte antigen (HLA) compatibility between mother and fetus has been identified as a factor influencing the persistence of microchimeric cells. Microchimerism and chronic graft versus host disease resemble each other. Both diseases share many clinical and pathological features with autoimmune diseases. Since autoimmune disease are more frequent in childbearing age females, it has been hypothesized that fetal microchimerism maybe involved in their etiology. For a successful pregnancy, the maternal immune system must not overreact to the fetus. Dramatic changes throughout gestation make possible maternal tolerance of the fetus and permit fetal cells to move into maternal circulation and settle in maternal tissues. As a

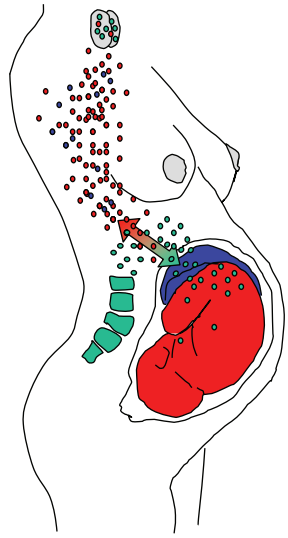

Figure 1: Pregnancy is the major source of natural microchimerism. Pregnancy related microchimerism results from deficiencies in the natural placental physical barrier tissue that divide the maternal circulation from the fetal circulation. As a consequence, there is mutual and bidirectional maternal and fetal cell traffic during pregnancy. Microchimeric cells enter the circulation and persist for many years in the host tissues. These cells are tolerated while acquire specific and diverse biological actions. (Copy of Figure 5 in [1]).

result, maternal tolerance allows the persistence of fetal microchimerism (Figure 1).

Intrathyroidal fetal microchimerism has been reported using different techniques. PCR-based analysis identifies Y-chromosomal and merely demonstrates the presence of male cells in maternal tissues [11, 12]. Other methodologies, such as immunohistochemistry, fluorescence in situ hybridization (FISH) and HLA typing identify location, cellular progeny, and immunogenic properties of microchimerism in different tissues [13, 14].

2. Effects of Microchimerism

According to cellular characterization, fetal microchimeric cells could cover a wide spectrum of action. Experimental data support a variety of important hypotheses concerning their biological implications. The types of cells crossing the placenta into the mother include both immune cells and cytokeratin-positive epithelial cells. Such cells have been identified as hematopoietic progenitor cells, nucleated erythrocytes, trophoblast cells, and leukocytes [15]. Therefore, microchimeric cells could potentially operate as effectors' cells or as targets of an immune response. Other possibilities include reactivity of microchimeric T-cell clones to the nonshared maternal human leukocyte antigen (HLA) antigens and presentation of microchimeric peptides by one host cell to another host cell [16].

We have previously proposed a three-role division for fetal microchimerism, which covers *pathogenic*, *beneficial,* and *neutral* microchimerism [1]. The concept of *pathogenic microchimerism* initially suggested by Nelson [17], hypothesizes that fetal cells following gestation may lead to a graft versus host-like reaction in women. Accordingly, maternal immune response to these foreign cells may support an autoimmune reaction. It is also plausible the existence of a *beneficial microchimerism*, where persistent fetal cells may have a beneficial effect as a new source of progenitor cells potentially capable to contribute to maternal tissue repair processes. The third possibility could be *neutral microchimerism*, where fetal cells may act as innocent bystanders playing no role in biology at all.

In line with our proposed microchimeric fetal cells varied effects, Fugazzola et al. [10] have recently speculated about three new possible roles of these foreign cells in relation with cancer, which are cell cancer destruction, tissue repair, and promotion of cell cancer progression. Apparently, a given fetal cell could act differently according to the particular tissue environment or depending on the type of malignancy.

3. Immune Changes during Pregnancy

For a successful pregnancy, the maternal immune system must not overreact to the fetus. The mechanisms through which the immune systems between mother and fetus interact to induce and maintain this immune tolerance are not fully understood, but several changes have been described. Trophoblast cells serve both as physical barrier as well as immune modulator by expressing several molecules and secreting specific cytokines. This involves the expression of Fas-ligand, cytokines, indoleamine 2,3-dioxygenase, immune-modulating sex steroids, and HLA-G. Fas-ligand is involved in the removal of maternal T-cell clones that react to fetal antigens. In animal models, indoleamine 2,3-dioxygenase catabolizes tryptophan from maternal immune cells in the placental area, which is essential for a successful pregnancy in murine models, whereas HLA-G, exclusively expressed on trophoblast cells, inhibits natural killer cell-mediated cellular immunity [11].

These changes also include a reduction of T-regulatory cells activity and affect maternal T-helper (Th) cell differentiation. Placental immune modulation promotes suppression of Th1 (cellular), whereas relative enhancement of Th2 (humoral) immunity occur. Thus placental immune suppression helps establish fetal microchimerism. Immune tolerance to fetal implant allows pregnant woman to accept fetal circulating cells [14]. Consequently, once fetal cell migrate and take up residence in maternal tissues, they may survive. This immune suppression may remain some months after delivery [18], allowing fetal cells to establish themselves and to survive the postpartum period [19]. Such dramatic changes throughout gestation make possible maternal tolerance of the fetus and permit fetal cells to move into maternal circulation and settle in maternal tissues. As a result, maternal tolerance allows the persistence of fetal microchimerism.

This complex modulation of the maternal immune system by pregnancy is also reflected by the variation of circulating levels of autoantibodies. Furthermore, there is compelling evidences that circulating thyroid autoantibodies are predictors for the increased risk of pregnancy complications like miscarriage, breech presentation, or prematurity [20]. Probably thyroid autoimmunity represents a more profound abnormal immune state, which induces an unstable implant [21]. For instance, a recent Italian study showed that 37.2% of thyroid peroxidase (TPO) antibody-positive women had postpartum thyroiditis, versus 1.7% of the TPO antibody-negative women. Furthermore, 20% of the TPO antibody-positive women remained hypothyroid at the end of the first postpartum year versus 1% of TPO antibody-negative women [22]. Another classic study described that up to 60% of reproductive Graves' disease women reported the onset of the disease within one year after delivery [23].

At the same time, it has been hypothesized that pregnancy-related immune changes have important positive effects for the fetal immune system. There is evidence suggesting that maternal immune cells instruct fetal cells how to balance the requirement for self-defense on one hand and the need for immunologic tolerance on the other. There is still much to understand about tolerogenic versus immunogenic forms of microchimerism, both of which have been reported [24].

Taken together, these different observations a question may be raised whether the sequence of events in autoimmunity is at least partly due to alloimmunity rather than autoimmunity.

4. Pregnancy and Autoimmune Diseases

It is well recognized the highest prevalence of autoimmune diseases in childbearing-age women than in men. It is also well known that autoimmune diseases have a profound influence on pregnancy outcome [19, 25–27]. Hormonal and genetic factors are probably involved, but a clear explanation for this type of preponderance is currently lacking. However, the role of fetal microchimerism as a contributing factor for the starting or maintenance of the autoimmune reaction in women is an attractive hypothesis [1].

Fetal cells have been found not just in peripheral blood but also within a variety of damaged tissues, where the autoimmune reaction is taking place [11, 28–31]. The presence of activated immune fetal cells within the maternal tissues may trigger susceptible women to develop autoimmune disorders. As the placental-induced immune suppression is vanished, the fetal immune cells may indeed become activated and initiate the autoimmune reaction [19]. The cause of this relationship has been based on the degree of HLA discrepancy between host and alien cells which may determine the status of any potential graft versus host reaction.

On the other hand, it should be emphasized that a positive effect of pregnancy on autoimmune disease is generally observed. As previously mentioned, the placenta induces immune suppression and so lessens autoimmune activity. Actually, the amelioration of autoimmune clinical manifestations along gestation is a usual clinical observation. Experimental data suggest that, despite the relative enhancement of Th2 reaction, both arms of the immune response (Th1 and Th2) are globally reduced during pregnancy. The observation is supported by the increase in T-regulatory cells observed in pregnancy and because autoantibodies greatly decrease during pregnancy [32].

Assuming that part of microchimeric fetal cells could be progenitor cells of the fetal immune system, the pathogenic effect of fetal microchimeric cells in autoimmune diseases can be perpetuated. These cells could survive in bone marrow or move to maternal different organs, where they could proliferate, differentiate, and activate. The activation of fetal immature T cells, monocytes, macrophages, and natural killer cells and the production of inflammatory cytokines and chemokines are believed to initiate then autoimmune diseases [33, 34]. Alternatively, these cells could be recognized as partially alloimmune and in consequence give rise to the autoimmune reaction [10].

Finally, this potential association is also supported by the observation that, albeit with few exceptions, the resetting to normal immune status in the postpartum period is usually coincidental with a clinical exacerbation of many autoimmune diseases.

5. Microchimerism and Autoimmune Thyroid Disease

Several evidences have shown that female subjects with autoimmune thyroid diseases frequently have microchimeric fetal cells residing within their thyroid glands [3, 11, 12]. This has been described both in animals [27, 35] and in humans [11, 12, 14, 36, 37]. Whether the presence of fetal cells increases maternal thyroiditis or a previous episode of thyroiditis increases the recruitment of these fetal cells remains unknown. A number of studies revealed this association demonstrating that the prevalence of male cells is higher in women with autoimmune thyroid diseases who previously had given birth to a son than in women without autoimmune thyroid diseases who previously had given birth to a son [10, 14]. These results generate the attractive hypothesis of a causal relationship between microchimerism and autoimmune thyroid diseases.

Davies' laboratory has extensively investigated the influence of pregnancy in autoimmune thyroid diseases, including the relationship between fetal microchimerism and autoimmune thyroid diseases [27, 35]. The group initially found that experimental autoimmune thyroiditis in mice enhanced the accumulation of intrathyroidal fetal cells during pregnancy [35]. The murine model of experimental autoimmune thyroiditis was established in female mice using murine thyroglobulin (Tg) as antigen. Tg-treated mice developed a florid lymphocytic infiltration by 4–6 weeks after immunization [27]. In addition, the results showed that there were no significant differences in thyroid function between nonimmunized and Tg-immunized pregnant mice.

Davies' group, going forward in the study of fetal microchimerism as a cause involved in the development

of autoimmune thyroid disease, used their experimental autoimmune thyroiditis model using sex-determining region Y (SRY) gene as the marker of presence of male microchimeric cells within maternal thyroids. The results demonstrated the presence of fetal cells in 46% of Tg-immunized pregnant mice, whereas few male fetal cells were detected in only 20% of controls or nonimmunized pregnant mice. Subsequent studies of cell characterization revealed the immune origin of the cells that accumulate within the thyroid of mice with experimental autoimmune thyroiditis during pregnancy and early postpartum [35]. These fetal cells were identified as regulatory and cytotoxic CD4+ (in a significant proportion), CD11c+, and weakly CD8+, but not from B220/CD45R+, CD11b+, or Sca-1+, which indicates that intra-thyroidal fetal cells included T-cell and dendritic cell lineage. This was accompanied by high titers of antibodies to Tg. Furthermore, mice postpartum followup revealed that intra-thyroidal fetal cells were most easily seen in experimental autoimmune thyroiditis animals during pregnancy, whereas the presence of these cells decreased in the postpartum period. So, a fetal cell-induced modulation during pregnancy and postpartum was highly feasible.

As aforementioned, several clinical studies have found male cells in thyroid samples of women previously diagnosed of Hashimoto's thyroiditis [12–14, 37]. The presence of alien cells in autoimmune involved thyroids ranged between 38 and 83%. This wide spectrum of percentages probably reflects the discrepancies in study design. Interestingly, the percentage differences between the Hashimoto's thyroiditis and control groups in individual studies are constant. Although the presence of fetal microchimerism was not identified in normal thyroids or patients with nodular goiters by all authors [12, 13], nowadays it is considered that microchimeric cells are present in normal glands and around 20% of follicular adenomas [10]. A recent Italian report has revealed fetal microchimeric cells in normal maternal thyroid tissue [38]. The authors explain the discrepancy due to the different origin of normal samples, from the normal tissue contralateral, to a neoplastic lesion. This finding further supports the idea that, in the presence of a neoplastic process, microchimeric cells could migrate to the thyroid and participate in the repair process [38]. All in all, these results indicate a higher number of fetal microchimeric cells in autoimmune thyroid diseases than in normal thyroids or benign proliferative disorders [11, 14, 19, 37]. Therefore taken together, these findings strongly support a possible pathogenic role for fetal microchimeric cells in the development autoimmune thyroid disease. However, there is still an important missing link that is the presence of maternal microchimerism in male patients with Graves' disease, which has been poorly studied.

6. Fetal Microchimerism and Graves' Disease

6.1. Graves' Disease in Pregnancy. Pregnancy-related factors have a strong influence in Graves' disease [25]. Normally, the clinical course of Graves' disease improves as pregnancy progresses, paralleling the reduction in serum TSH receptor (TSH-R) autoantibodies level. The reflection of this clinical improvement could be not only the quantity of serum anti-body concentration but also the quality (or the biological action) of these antibodies. Some authors have hypothesized that the equilibrium between TSH-R antibodies blocking and stimulating activities may shift in favor of blocking antibodies [39], though not all the experts share this opinion [40]. In any event, a fluctuant Graves' disease clinical evolution is a common finding during pregnancy with exacerbation during approximately the first three months and improvement in the last trimester [41].

In the postpartum period as the pregnancy-associated immune-privileged state disappears, a relapse, exacerbation, or new onset of Graves' disease may occur. This outbreak normally happens between 4–12 months after delivery [42]. In fact, epidemiological studies show that around 60% of childbearing age women develop Graves' disease during the first year after delivery [29, 43], whereas the frequency of relapses varies from 30% to 70% of cases [44]. Likewise, an increased risk of developing Graves' disease after pregnancy may be greater in older patients (>35 years), and this risk lasts for several years after delivery [21, 45].

6.2. Experimental Evidence. It was also Davies' group who in 2002 first demonstrated that intrathyroidal fetal microchimerism was common and profound in female patients with Graves' disease [11]. Renné et al. confirmed these findings two years later [14]. Since then, more information has arisen in the scientific arena [10].

Davies' group research was conducted in a sample of 27 thyroid glands from patients with a past medical history of Graves' disease [11]. The investigators analyze the presence of male-specific SRY gene in maternal thyroid glands by ELISA-PCR technique for the detection of DNA. This was a two-step designed study. Initially, male cell assay was applied to screen for circulating peripheral blood fetal micro-chimerism in 20 females and lastly in stored thyroid tissue. The preliminary results showed that none of 16 never-pregnant females had male cells detected. However, in previous pregnant women, male cells were easily detected in 28.6% of blood samples. Furthermore, 47% of female Graves' blood specimens contained significant male cells. These results were similar to prior reports indicating that peripheral blood fetal microchimerism is a common finding in childbearing-age women.

A subsequent analysis was focused on thyroid samples from females previously diagnosed of Graves' and in a group of thyroid adenomas as control. The storage of the thyroid samples had been diversed. Twenty glands had been prepared in paraffin blocks, whereas the remaining seven were frozen. Interestingly, SRY gene was found in only 20% of paraffin embedded tissues, while 86% of the frozen samples were positive for the Y chromosome gene. In the former group, the ratio of male to female cells ranges from 14 to 295 by 10^5, with a median of $37/10^5$ male/female cells. SRY gene was searched in 10 thyroid adenoma specimens (6 in paraffin and 4 frozen). None of the 6 paraffin-embedded thyroid adenoma was positive for the SRY gene analysis, whereas 1 out

Table 1: Summary of the main experimental and clinical findings of Graves' disease and microchimerism.

Thyroid tissue origin	Storage	*N*	Gene and technique	Presence of microchimeric cells (%)	Reference
Graves' disease	Paraffin-embedded	20	SRY gene by ELISA-PCR	20%	Ando et al. [11]
	Frozen	7	SRY gene by ELISA-PCR	86%	Ando et al. [11]
	Paraffin-embedded	15	X and Y chromosomes by FISH	40%	Renné et al. [14]
Thyroid adenoma	Paraffin-embedded	6	SRY gene by ELISA-PCR	0%	Ando et al. [11]
	Frozen	4	SRY gene by ELISA-PCR	25%	Ando et al. [11]
	Paraffin-embedded	9	X and Y chromosomes by FISH	22%	Renné et al. [14]
Hashimoto's thyroiditis	Paraffin-embedded	25	X and Y chromosomes by FISH	60%	Renné et al. [14]

SRY: sex-determining region Y; PCR: polymerase chain reaction; FISH: fluorescence in situ hybridization.

of 4 frozen samples with thyroid nodules showed male cells. Authors speculate that the greater detection of the SRY gene in frozen female thyroid tissues was probably due to DNA fragmentation in the paraffin-derived samples. Unexpectedly, many of the patients with male cell-positive thyroids had no history of earlier male pregnancies at the time of surgery. However, as the authors stated in the discussion, this did not necessarily exclude the possibility of undetected first trimester pregnancies because it has been demonstrated that fetal microchimerism can be established in the first month of pregnancy [11, 46].

Renné et al. study compared three entities: Graves' disease, Hashimoto's thyroiditis, and nodular or diffuse follicular adenomas from women whose childbirth history was positive for sons [14]. These investigators screened by fluorescence *in situ* hybridization for X chromosome and Y chromosome specific staining from paraffin-embedded thyroid specimen taken at surgery. The results showed that 23 out of 49 thyroids (47%) were positive for Y-chromosome-specific staining. These authors found no Y-chromosomes-positive thyrocytes. The proportion of Y-chromosome-positive thyroid section was highest in Hashimoto's thyroiditis (15/25; 60%), lower in Graves' disease (6/15; 40%), and infrequent in follicular adenomas (2/9; 22%) [14]. The results indicate a higher degree of microchimerism in autoimmune thyroid disease than in benign proliferative disorder. The results were consistent with previous reports also showing that microchimeric cells are found in more subjects with autoimmune thyroid diseases than in any other thyroid diseases [11, 13].

A summary of these findings is presented in Table 1.

7. Controversial Studies about the Relationship between Microchimerism and AITD

Those dissenting from the appraisal of the relation between autoimmune thyroid disease and microchimerism mainly base on data from epidemiological studies. An Australian study over a large community-based female population (1,045 participants) showed no association between parity and presence of thyroid autoantibodies or thyroid dysfunction. Hence, the authors suggested a lesser role of fetal microchimerism in autoimmune thyroid diseases [47]. A year later, a Danish group also investigated the association between the presence of circulating thyroid autoantibodies and previous pregnancy, parity, and the use of estrogens in an even larger population cohort of 3,712 women. These authors reinforce the Australian conclusion, as they also did not find any association between thyroid antibodies and pregnancy. No association was observed between hormonal replacement therapy and serum TPO antibodies levels either. In line with the Australian authors, the Danish investigators concluded that there was no association between previous pregnancy and serum thyroid Abs level, which argues, in their opinion, against the role of microchimerism as a trigger factor of thyroid autoimmunity [48]. These two studies seem to indicate that the risk of having TPO antibodies or Tg antibodies was similar in nulliparous women compared with women with one or more previous pregnancies. However, recently in 2011, an American study was published with a confronting conclusion [49]. The authors analyze the relationship between TPO antibodies and increasing parity in a population of 17,298 women, larger than the two previous samples. The authors analyze the relationship of serum TPO antibody levels and increasing parity. Despite that the incidence of abnormally elevated TPO antibody levels increased with advancing parity, this trend was not significant after adjustment for maternal characteristics. However at higher TPO antibody levels, a significant relationship with advancing parity persisted after adjustments. Therefore, it was concluded that advancing parity is associated with an increased risk for high serum concentrations of TPO antibodies, suggesting that fetal microchimerism may play a role in development of autoimmune thyroid disorders [49]. The two main results from this large American study could help to explain why Australian and Danish authors could not find any relationship between parity and presence of TPO antibody levels. Nevertheless, it remains to be demonstrated that the establishment of microchimeric cells openly influences the natural history of autoimmune thyroid diseases.

8. Conclusions

Currently, there is a large body of evidence showing that maternal thyroid gland is a significant organ where a variety of fetal cells settle and persist for decades. These foreign

immune cells may be activated after delivery once placental immune suppression is over. This remains an attractive hypothesis for the postpartum increase of prevalence of Graves' disease.

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Review Article

New Genetic Insights from Autoimmune Thyroid Disease

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The autoimmune thyroid diseases (AITDs) (Graves' disease and Hashimoto's thyroiditis) are complex genetic diseases which most likely have more than 20 genes contributing to the clinical phenotypes. To date, the genes known to be contributing fall into two categories: immune regulatory genes (including *HLA, CTLA4, PTPN22, CD40, CD25, and FCRL3*) and thyroid-specific genes (*TG and TSHR*). However, none of these genes contribute more than a 4-fold increase in risk of developing one of these diseases, and none of the polymorphisms discovered is essential for disease development. Hence, it appears that a variety of different gene interactions can combine to cause the same clinical disease pattern, but the contributing genes may differ from patient to patient and from population to population. Furthermore, this possible mechanism leaves open the powerful influence of the environment and epigenetic modifications of gene expression. For the clinician, this means that genetic profiling of such patients is unlikely to be fruitful in the near future.

1. Introduction

Many diseases have a tendency to run in families, and we know that this may be due to either environmental influences, or family genetics, or both. The autoimmune thyroid diseases (AITDs), Graves' disease and Hashimoto's thyroiditis, are typical examples of such complex diseases and have been recognized for many years as having an important genetic component. In the last 10 years we have learned many new insights into the way genetic influences can enhance thyroid autoimmunity, but there remain large gaps in our knowledge which are unlikely to be filled without major theoretical and technical advances. This brief review examines the current state of knowledge and what new insights we have gained from exploring the genetics of the AITDs, and in particular Graves' disease.

2. Thyroid Autoantibodies

Autoantibodies to thyroid peroxidase (TPO) and thyroglobulin (Tg) are reflections of thyroid disease rather than causative agents [1]. Hence, such thyroid autoantibodies may develop before the onset of clinical AITD and have been long known to increase the risk of developing clinical AITD [2]. The recognition of a familial association for the production of thyroid antibodies [3] led to studies of first-degree relatives of probands with AITD and indicated a dominant pattern of inheritance. Indeed, up to 50% of the siblings of patients with AITD are thyroid antibody positive [4, 5] in contrast to ~15% in the general population [6]. Several segregation analyses have also shown a Mendelian dominant pattern of inheritance for the expression of thyroid autoantibodies [7, 8], and genetic transmission of TPO antibody subclass "fingerprints" has suggested that the pattern of autoantibody recognition of the TPO antigen was also genetically transmitted [9].

3. Genetic Susceptibility to AITD

The recognition of an association between AITD and certain human leukocyte antigens (HLA) first provided a mechanism for the genetic contribution to Graves' disease and Hashimoto's thyroiditis [10]. This association has been especially well seen in identical twins [11]. The HLA antigens provide a means for the immune system to recognize thyroid antigenic peptides, and recent data have demonstrated this enhanced association as secondary to the presence of particular residues in the HLA class II binding pocket such as Arg 74 [12]. In addition, as the pathological and molecular

Table 1: Methods of genetic analysis.

(A) *Linkage analysis*
This is based on the principle that the chance for a recombination event between 2 loci (i.e., a marker, such as the candidate gene, and the true disease gene) is proportional to the chromosomal distance between them. Therefore, if a marker is close to a disease susceptibility gene, this marker will cosegregate with the disease in families.
The logarithm of odds (LOD) score is a measure of the evidence for or against linkage between a marker and a trait or disease [13]. LOD score analysis has had important advantages for the study of AITD because it has allowed a way to test for the presence of heterogeneity within the data set and allowed deduction of the mode of inheritance and the degree of penetrance from the linkage data.
Linkage studies are highly specific but have been clearly shown not to be highly sensitive.
(B) *Association studies*
These studies simply compare the presence of a disease marker (such as the candidate gene) in the disease population with the presence of the marker in a control population without the disease.
Here, the difficulty may lie in the appropriate control population, which needs to be comparable and large.
If this difficulty is overcome, association studies can reveal a genetic influence, and with large patient groups, this type of study can be highly sensitive.

Table 2: Methods for whole-genome screening.

(A) *Microsatellites*
These are regions in the genome that are composed of repetitive sequences. The most common microsatellites are the CA (dC-dA)n repeats. Microsatellite loci are highly polymorphic because of variation in the number of repeats (usually there are 5 to 15 alleles per locus), and they are uniformly distributed throughout the genome at distances of fewer than 1 million base pairs [15]. Therefore, microsatellites served as useful markers in linkage studies designed to search for unknown disease susceptibility genes. Investigators then further narrowed the suspected gene region with more dense markers, and the gene could be identified.
(B) *Single-nucleotide polymorphisms* (SNPs)
Without having to enlist families, it is now possible to use genome-wide association studies involving up to 10^6 SNPs (on a microchip), each of which is in linkage disequilibrium with large segments of the genome, and then analyze their association with any disease.

mechanisms involved in AITD became known, many of which were not only common to all autoimmune diseases but also highly variable between individuals; this allowed the recognition of candidate genes responsible for disease susceptibility. Such genes could then be assessed by either linkage analysis or association studies (see Table 1).

4. Detecting Susceptibility Genes in AITD

The candidate HLA gene complex was first associated with AITD in association studies but then failed to show linkage with AITD [14]. This showed that the genetic contribution of HLA to AITD was not strong enough to be seen in linkage analyses [13]. This indicated that association studies were more likely to detect genes contributing small effects on disease susceptibility. As a consequence of the Human Genome Project, it became possible to identify genes for diseases that had a complex genetic basis without resorting to the candidate gene approach. This was achieved by "typing" individuals using a genome screen of genetic markers, at first with microsatellites (1 microsatellite per 10 cM DNA) and later single-nucleotide polymorphisms (SNPs) (~1 SNP per < 1 cM DNA), which covered the entire genome (Table 2) [15]. Then investigators observed which markers segregated with the disease. However, the reduced sensitivity of linkage analyses, compared to association studies, made it more difficult to perform these analyses for the complex traits characteristic of a non-Mendelian pattern of inheritance and with variable clinical phenotypes. However, using large numbers of SNPs, developed as a result of the HapMap project [16, 17], and which had a much greater degree of coverage of the whole genome, it was easier to decipher which markers segregated with the disease using association analyses. These SNP markers occur more frequently than microsatellite markers and are easy to detect, allowing for greater genetic sensitivity. The suspected gene region can then be further narrowed with more dense SNPs and the gene can be identified. Results are now available for a variety of autoimmune diseases including rheumatoid arthritis and type 1 diabetes mellitus [18] and most recently for AITD [19].

It is obviously essential that whole-genome association study results must be reliably and repeatedly reproduced, but the complexity of this type of analysis and the high cost have raised problems [20, 21]. If common diseases are associated with common risks, then replication across populations can be expected. But common diseases may be related to population-specific risks, and, therefore, such data can only be reproduced in the same population as that which was studied in the original report. Reproducibility had been a problem for studies that used microsatellite screening, including the studies in patients with AITD, and this problem has persisted in the much larger studies using whole-genome association studies such as in those analyzing Parkinson's disease and also obesity. Hence, all reports of genetic linkage and association require confirmation by independent studies before they can be accepted.

5. Genes for AITD

The *HLA* and *CTLA4* genes were the first genes identified by the candidate approach [22, 23] (Table 3).

As discussed earlier, the *HLA* genes make up the major histocompatibility complex (MHC) which contains many genes related to immune system function in humans. These include HLA class I (A, B, and C), HLA class II (DP, DM, DOA, DOB, DQ, and DR), and HLA class III (coding for

Table 3: Genes linked and/or associated with autoimmune thyroid disease.

Gene symbol	Gene name	Chromosome location	Odds ratio
HLA	Major histocompatibility complex	6p21	2.0–4.0
CTLA4	Cytotoxic T-lymphocyte-associated protein 4	2q33	1.5–2.2
PTPN22	Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	1p13	1.4–1.9
CD40	CD40 molecule, TNF receptor superfamily member 5	20q11	1.3–1.8
IL2RA (*CD25*)	Interleukin 2 receptor, alpha	10p15	1.1–1.4
FCRL3	Fc receptor-like 3	1q23	1.1–1.3
TG	Thyroglobulin	8q24	1.3–1.6
TSHR	Thyroid-stimulating hormone receptor	14q31	1.4–2.6

other immune proteins). The major GD-associated *HLA*, *HLA-DR3*, locates at the *HLA DR* locus and plays a key role in the normal immune response by binding peptide antigens and presenting them to T-cell receptors.

The cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*) gene is an immune regulatory molecule, which is expressed on the surface of Helper T cells and transmits an inhibitory signal to T cells. In addition to the *HLA* and *CTLA4* gene loci, there are confirmed associations (2 or more reports) for a number of genes also common to many autoimmune diseases: *PTPN22*, *CD40*, *IL2RA* (*CD25*), and *FCRL3* (Table 3).

The gene for protein tyrosine phosphatase, non-receptor type 22 (lymphoid), also known as just *PTPN22*, encodes a protein tyrosine phosphatase expressed primarily in lymphoid tissues. This enzyme associates with the molecular adapter protein CBL and may be involved in regulating CBL function in the T-cell receptor signaling pathway. A variant of the *PTPN22* encodes Lyp phosphatase (Lyp620W) and confers risk for multiple autoimmune diseases. Most recently, Zhang et al. [24] reported that levels of the Lyp620W variant were decreased in human T and B cells, and its calpain binding and cleavage were increased relative to wild-type Lyp620R. Therefore, calpain-mediated degradation with consequently reduced Lyp expression and lymphocyte and dendritic cell hyperresponsiveness represents a potential mechanism for unregulated autoimmunity. The LypR620W variant, with an arginine to tryptophan substitution, loses its function and influence on immune responses, which increases the risk for autoimmune disease.

The *CD40* molecule, or TNF receptor superfamily member 5 gene, encodes a costimulatory receptor which is essential in mediating a broad variety of immune and inflammatory responses including T-cell-dependent immunoglobulin class switching, memory B-cell development, and germinal center formation [25]. The interleukin 2 (IL2) receptor alpha gene (*IL2RA* or *CD25*) encodes one of the subunits of the IL-2 receptor that binds IL-2 and is vital in the regulation of T-cell function. The Fc receptor-like protein 3 (*FCRL3*) gene encodes a protein containing an immunoreceptor-tyrosine activation motif and immunoreceptor-tyrosine inhibitory motif in its cytoplasmic domain and may play a role in immune regulation.

To date, the only thyroid-related genes associated with AITD are *TG* (the gene encoding thyroglobulin) [26], in both Graves' disease and Hashimoto's thyroiditis, and *TSHR* (the gene encoding the thyrotropin receptor) restricted to Graves' disease [27, 28] (Table 3).

The thyroglobulin (*TG*) gene encodes a large glycoprotein homodimer produced exclusively by the thyroid gland. It acts as a substrate for the synthesis of thyroid hormones thyroxine (T4) and triiodothyronine (T3) as well as the storage of the inactive forms of thyroid hormone and iodine. How this gene influences susceptibility is unclear but Stefan et al. [29] have recently described a genetic/epigenetic mechanism by which a newly identified *TG* promoter SNP variant predisposes to AITD. Sequencing analyses followed by case control and family-based association studies identified a SNP ($-1623\text{A} \rightarrow \text{G}$) that was associated with AITD in the Caucasian population, and the associated nucleotide substitution SNP (-1623A/G) modified a binding site for interferon regulatory factor-1 (IRF-1), a major interferon-induced transcription factor, indicating enhanced sensitivity to this inflammatory cytokine [29].

The thyroid stimulating hormone receptor (*TSHR*) gene encodes a membrane protein that signals through binding TSH ligand and is a major controller of thyroid cell growth and metabolism. SNPs in intron 1 (in Caucasians) and intron 7 (in Japanese) have been associated with Graves' disease in a number of studies [27, 28, 30]. Recent data suggest that TSHR-associated SNPs are related to defective thymic tolerance for the TSHR as shown by reduced expression within the thymus gland where it is needed to delete TSHR autoreactive T cells [31].

Because all the identified susceptibility genes found to date appear to have a low level of contribution to genetic susceptibility, a number of whole-genome screening studies have also been attempted in AITD to find more important genes [32–36]. One whole-genome association study using only 10^4 nonsynonymous SNPs (those involving parts of a gene likely to affect the product character) showed a number of the previously recognized genes, as well as locating some new sites, but the new sites could not subsequently be confirmed [37, 38]. Most recently, the first full genome-wide study of Graves' disease with 660 K SNPs has now been reported from China [19]. This study again identified many of the known genes for AITD, but also described two new sites on chromosomes 6q and 4p. These await further confirmation. Again, however, no very highly associated new genes have emerged.

6. The Degree of Enhanced Susceptibility Remains Low

All the genes associated with AITD are individually able to confer only modest degrees of disease susceptibility (expressed as odds ratios, see Table 3). Hence, these data only allow us to conclude that the AITDs, both Graves' disease (including Graves' ophthalmopathy) and Hashimoto's thyroiditis, are complex genetic disorders involving multiple genes that may interact to provide a susceptible background for disease development. Furthermore, there appear to be disease-specific genes, such as the gene encoding the TSHR in Graves' disease and a larger group of susceptibility genes, such as *CTLA4*, which are common to many autoimmune diseases. This combination of gene polymorphisms likely allows epigenetic phenomena, subsequent to a variety of influences such as infection and the environment, to initiate disease.

7. The Controversy over Major Genes in AITD

After the clarification that multiple genes are at work in AITD, it is likely that more than 20 potential genes contribute to the AITD phenotypes. But major genes, those essential to disease development, have not been found [39]. A major gene should be involved in the majority of patients with the disease, and the risk ratios, even for HLA, do not reveal such a gene (Table 3). This most likely means that different combinations of genes may produce similar clinical phenotypes or that epigenetic phenomena are dominant. So far, in the whole-genome screening of families, siblings, and populations with AITD, a number of sites have been established for Graves' disease and Hashimoto's thyroiditis susceptibility, but none of them have had very high statistical values (LOD scores) [32, 33, 35]. This finding has been true not just for AITD, but also for other autoimmune diseases including type 1 diabetes mellitus. This is best understood by thinking of HLA once again. Not every patient with Graves' disease has the associated HLA-DR3 subtype and not even the associated Arg74 in its binding pocket, irrespective of the HLA-DR subtype [12]. Hence, the disease can occur in the absence of the expected HLA association.

8. A Note on Epigenetics

One mechanism by which environmental factors may combine with genetic risk to promote AITD is by altering the epigenetic control of gene expression as seen, for example, in the pancreas [40] and as shown for a virus interacting with a susceptibility gene in Crohn's disease [41]. While little is known about such interactions with AITD, there has been wide confirmation of a role for X chromosome inactivation (XCI) [42, 43]. Patients with AITD more often than expected showed a biased expression of a maternal or paternal X chromosome leading to the hypothesis that the poorly expressed chromosome could become active in certain tissues such as the thyroid and express new antigenic sequences not previously recognized by the immune system. These potential mechanisms for enhanced susceptibility to AITD require further exploration.

9. Conclusions

How environmental factors combine with genetic risk at the molecular level to promote complex genetic diseases such as AITD is largely unknown. The genes that are linked to and/or associated with AITD are each small contributors to genetic risk. Multiple-gene polymorphisms (combinations of haplotypes) appear to be needed to develop AITD and may differ between geographic populations secondary to epigenetic influences. Much remains to be learned.

Abbreviations

AITD: Autoimmune thyroid disease
LOD: Logarithm of odds
SNP: Single-nucleotide polymorphism.

Disclosure

TFD is a Board Member of Kronus Inc., Star, Idaho (a distributor of thyroid antibody test kits). The other authors have no conflict of interests to disclose.

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Research Article

Determinants of Extraocular Muscle Volume in Patients with Graves' Disease

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Background. To examine factors contributing to extraocular muscle (EOM) volume enlargement in patients with Graves' hyperthyroidism. *Methods*. EOM volumes were measured with orbital magnetic resonance imaging (MRI) in 39 patients with recently diagnosed Graves' disease, and compared to EOM volumes of 13 normal volunteers. Thyroid function tests, uptake on thyroid scintigraphy, anti-TSH-receptor antibody positivity and other parameters were then evaluated in patients with EOM enlargement. *Results*. 31/39 patients had one or more enlarged EOM, of whom only 2 patients had clinical EOM dysfunction. Compared to Graves' disease patients with normal EOM volumes, those with EOM enlargement had significantly higher mean serum TSH (0.020 ± 0.005 versus 0.007 ± 0.002 mIU/L; *P* value 0.012), free-T4 (52.9 ± 3.3 versus 41.2 ± 1.7 pmol/L; *P* value 0.003) and technetium uptake on thyroid scintigraphy (13.51 ± 1.7% versus 8.55 ± 1.6%; *P* value 0.045). There were no differences between the 2 groups in anti-TSH-receptor antibody positivity, the proportion of males, tobacco smokers, or those with active ophthalmopathy. *Conclusions*. Patients with recently diagnosed Graves' disease and EOM volume enlargement have higher serum TSH and more severe hyperthyroidism than patients with normal EOM volumes, with no difference in anti-TSH-receptor antibody positivity between the two groups.

1. Introduction

Thyroid-associated ophthalmopathy (TAO) is an autoimmune disorder of uncertain aetiology. While the involvement of extraocular muscles (EOMs) in patients with Graves' disease may seem infrequent on clinical examination, orbital magnetic resonance imaging (MRI) studies suggest that the majority of such patients have EOM enlargement [1].

Along with the orbital fibroblast, the EOM is likely to be a primary target in TAO. This is supported by evidence of T-cell reactivity against both orbital fibroblast and EOM cells *in vitro* [2] and muscle fibre damage in electron microscopic studies of EOM from patients with recent onset TAO [3, 4]. In addition, expression of the thyrotropin-receptor (TSH-R) in EOM [5, 6], as opposed to the widespread distribution of TSH-R in adipose tissues throughout the body [7], may indicate that EOMs have a more direct and specific role in TAO than previously thought [8].

In this study, we investigated potential factors affecting EOM volume enlargement as measured by orbital MRI in patients with recently diagnosed Graves' disease.

2. Materials and Methods

A total of 39 patients diagnosed with Graves' hyperthyroidism within the preceding 3 months were selected for this study. The patients were involved in a larger study looking at potential risk factors for TAO [9].

The diagnosis of Graves' disease was based on the presence of biochemical hyperthyroidism, a symmetrical goitre and positive thyroid autoantibodies, and/or diffuse uptake on ^{99m}Technetium thyroid nuclear scan. The abbreviated clinical activity score (CAS) model was employed for the diagnosis of active ophthalmopathy. This model assigns one point for each of the following: spontaneous retrobulbar

pain, pain on eye movement, eyelid erythema, eyelid oedema, chemosis, conjunctival injection, and swelling of the caruncle [10]. A total score ≥4 out of 7 was defined as active ophthalmopathy [11]. EOM function was evaluated by asking the patient to move their eyes in an H-shaped pattern, and proptosis was assessed using a Hertel exophthalmometer. The ophthalmic examination was performed by a trained clinical nurse.

Patients who were pregnant, less than 18 years of age, and those with a history of radioactive iodine therapy, orbital surgery, orbital irradiation, or significant loss of vision were excluded. The study was conducted at an outpatient endocrine practice in Victoria, Australia. Written, informed consent was obtained, and the study was approved by the Barwon Health Research and Ethics Advisory Committee.

EOM volumes were measured by a single investigator (SEK) from T_1-weighted, 2 mm slice orbital MRI scans using the digital software MRIcro (Version 1.38 Beta; Chris Rorden) as previously described [9]. Briefly, the volumes of the medial, inferior, and lateral recti were measured manually by circling the muscle perimeter on each slice. The superior rectus muscle, the superior ophthalmic vein, and the levator palpebrae superioris were measured together as the superior muscle group (SMG) because of difficulties in delineating these structures from each other. Orbital measurements were expressed as a percentage of the mean globe volume for each patient in order to adjust for interindividual variation in EOM volumes.

3. Statistical Analysis

Statistical analysis was performed with the software programs Minitab 14.12 and SPSS 13.0. Proportions were compared with Fisher's exact test while the sample means were evaluated with the 2-sample t-test. The effect of TSH-R antibody positivity on EOM volume was examined with binary logistic regression. Significance was set at P value less than 0.05.

The cutoff values of MRI-measured EOM volumes were determined with receiver-operating-characteristic analysis by comparing patient EOM volumes to those of 13 normal volunteers with no history of thyroid or eye disease. The cutoff values, their sensitivities and specificities, and the coefficients of variation of each measurement were detailed in an earlier publication [9].

4. Results

Based on the EOM volume cutoff value, only 8 patients had normal volumes in all 4 EOM groups. Of the 31/39 patients with at least one enlarged EOM volume, 3 patients had one enlarged EOM, 3 patients had 2 enlarged EOM, 7 patients had 3 enlarged EOM, and all 4 muscles were enlarged in 18 patients. The most frequently affected EOMs were the medial and lateral ($n = 27$ each) followed by the inferior recti and SMG ($n = 24$ each).

Assessment of baseline characteristics in patients with and without EOM volume enlargement on MRI showed no significant differences in the proportion of males, tobacco smokers, those with active ophthalmopathy (CAS ≥ 4), or elevations in anti-TSH-R, antithyroid peroxidase (TPO), or antithyroglobulin autoantibodies (Table 1). Only two patients, both with EOM volume enlargement, had clinically evident EOM dysfunction.

However, patients with enlarged EOM volumes had significantly higher ^{99m}technetium uptake on thyroid scintigraphy and greater serum free-T4 and thyrotropin (TSH) levels (Table 2). Importantly, there were no significant differences between the two groups in the proportion of patients who received anti-thyroid medications prior to recruitment into the study. Overall, 8/31 patients with enlarged and 2/8 patients with normal EOM volumes received anti-thyroid medications for a mean 1.06 ± 0.45 weeks and 1.13 ± 0.79 weeks, respectively. Exclusion of those patients from the analysis resulted in persistent elevations of free-T4 and TSH levels in patients with EOM enlargement, although the differences in mean TSH became of borderline significance (mean free-T4 42.4 ±1.9 versus 52.3±3.9 pmol/L (P value 0.034); mean TSH 0.021 ± 0.006 versus 0.008 ± 0.003 mIU/L (P value 0.054)).

In further analysis using binary logistic regression, EOM volumes were not associated with elevated anti-TSH-R, anti-TPO, or antithyroglobulin autoantibodies, smoking, or the presence of active ophthalmopathy.

5. Discussion

This study shows that patients with newly diagnosed Graves' disease and EOM enlargement have higher serum TSH and more severe hyperthyroidism, as suggested by the higher serum free-T4 and greater uptake on thyroid scintigraphy, than patients without EOM enlargement.

While more severe hyperthyroidism has not been identified as an independent risk factor for TAO [12], a greater serum free-T3 at baseline is associated with an increased risk of TAO after radioiodine therapy for Graves' hyperthyroidism [13]. In this study, EOM volume enlargement was associated with higher free-T4 levels (P value 0.003; Table 2) and greater uptake on thyroid scintigraphy (P value 0.045; Table 2). The serum free-T3 level was greater in patients with EOM volume enlargement without reaching statistical significance (P value 0.062; Table 2), perhaps due to the small sample size. The mechanism whereby more severe hyperthyroidism leads to greater EOM volumes is uncertain, but we speculate that it may be related to higher levels of the shared thyroid-orbital antigen(s).

In this study, the mean serum TSH was significantly higher in patients with EOM volume enlargement. The role of serum TSH in the initiation and propagation of TAO is well documented after RAI therapy [13–16], and empirical thyroid hormone replacement after RAI ablation, but before the onset of biochemical hypothyroidism, has been shown to reduce the incidence of TAO after RAI [17]. It is possible that EOMs, which express TSH-R [5, 6], are sensitive to seemingly minor elevations in serum TSH in patients with Graves' hyperthyroidism, leading to greater EOM volumes. The higher TSH levels in patients with enlarged EOM volumes occurred despite higher free-T4 and free-T3 levels in this group. While the serum TSH usually changes in a reciprocal fashion to

Table 1: Comparison between patients with and without EOM volume enlargement. The number of patients is shown, and the proportions *P* value was calculated using Fisher's exact test.

	Enlarged EOM volume ($n = 31$)	Normal EOM volume ($n = 8$)	*P* value
Males	3	1	1.0
Smokers	9	3	0.7
Active ophthalmopathy	15	3	0.7
Clinical EOM dysfunction	2	0	1.0
Elevated TSH-R antibodies	23	7	0.6
Elevated thyroglobulin antibodies	14	5	0.4
Elevated TPO antibodies	19	7	0.2

Table 2: Means ± SEM of measurements for patients with and without EOM volume enlargement. Where the measurement was not performed on all patients, the number of patients is shown in square brackets. *P* values calculated using 2-sample *t*-test.

	Enlarged EOM volume ($n = 31$)	Normal EOM volume ($n = 8$)	*P* value
Age (yrs)	44 ± 2.1	40.5 ± 4.1	0.5
^{99m}Technetium uptake (%)	13.51 ± 1.7, ($n = 28$)	8.55 ± 1.6, ($n = 6$)	0.045
TSH (mIU/L)	0.020 ± 0.005	0.007 ± 0.002	0.012
Free-T4 (pmol/L)	52.9 ± 3.3	41.2 ± 1.7	0.003
Free-T3 (pmol/L)	22.8 ± 1.9, ($n = 29$)	18.3 ± 1.3	0.062
CAS	2.97 ± 0.53	3.25 ± 1.2	0.8
Proptosis (mm)	17.46 ± 0.46	16.00 ± 0.71	0.097

the serum free-T4 and free-T3 levels, it is worth noting that this relationship is attenuated or "flattened" in hyperthyroid patients with a suppressed serum TSH below 0.01 mIU/L [18]. Upon starting treatment with anti-thyroid medications, the serum free-T4 and free-T3 fall rapidly whereas the serum TSH typically "lags" behind and remains undetectable for up to 3 months [18]. It is therefore unlikely that the greater serum TSH in patients with EOM volume enlargement was related to treatment with anti-thyroid medications prior to recruitment into the study, especially because the mean duration of treatment with anti-thyroid medications was 1.08 ± 0.39 weeks, and did not exceed 3 months in any patient. In addition, exclusion of patients who received anti-thyroid medications from the analysis did not abolish the differences in serum TSH between the two groups, although the differences became of borderline significance (*P* value 0.054), possibly due to the smaller sample size.

While the majority of patients in this study had elevated anti-TSH-R antibody levels, there were no significant differences in the prevalence of anti-TSH-R antibody positivity between patients with and without EOM volume enlargement, and in binary logistic regression analysis there was no association between antibody positivity and EOM volumes. TAO is thought to occur following sensitization of T-lymphocytes to a common thyroid and orbital antigen. The identity and location of this antigen remains unknown, but the TSH-R is the most likely candidate [7, 19]. Autoimmunity against other antigens particularly the skeletal muscle protein calsequestrin [20] is of potential importance, but is not well understood. The role of TSH-R in the initiation and propagation of TAO is supported by the close temporal relationship between the onset of ophthalmopathy and Graves' disease which is caused by stimulating anti-TSH-R antibodies [12], and the positive correlation between these antibodies and the prevalence of TAO in untreated Graves' disease [21]. In addition, TSH-R antibody levels are closely associated with CAS readings, the severity of the eye disease [22], and to a lesser extent with proptosis [23]. Therefore, the lack of an association between TSH-R antibody positivity and EOM volume enlargement in this study should be interpreted with caution, especially because of the small sample size and the increased risk of a type 2 error.

Similarly, the small sample size may account for nonsignificant differences in measures of proptosis, which was greater in patients with enlarged EOM volumes without reaching statistical significance (*P* value 0.097; Table 2). In contrast, the lack of an association between active ophthalmopathy and EOM enlargement may be related to the use of the CAS model which measures soft tissue and periorbital inflammation rather than EOM involvement [10, 24].

6. Conclusions

In patients with newly diagnosed Graves' disease, EOM volume enlargement is associated with greater serum TSH levels and more severe hyperthyroidism, as suggested by greater serum free-T4 levels and more avid uptake on thyroid scintigraphy. There was no association between EOM volumes and anti-TSH-R antibody positivity, although the small sample size may have contributed to this negative finding. Larger studies are needed to examine the relationship between serum TSH, anti-TSH-R antibodies, and EOM enlargement.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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Review Article

The Evolving Role of Selenium in the Treatment of Graves' Disease and Ophthalmopathy

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Graves' disease (GD) and ophthalmopathy (GO) are organ-specific autoimmune-inflammatory disorders characterized by a complex pathogenesis. The inflammatory process is dominated by an imbalance of the antioxidant-oxidant mechanism, increased production of radical oxygen species (ROS), and cytokines which sustain the autoimmune process and perpetuate the disease. Recently, selenium, which is a powerful antioxidant, has been successfully applied in patients with mild GO, slowing the progression of disease, decreasing the clinical activity score, and appreciably improving the quality of life. The mechanisms of selenium action are variable. The aim of this review is to summarize the actions of selenium in GD and GO. Selenium as selenocysteine is incorporated in selenoproteins, such as glutathione peroxidase which catalyzes the degradation of hydrogen peroxide and lipid hydroperoxide that are increasingly produced in hyperthyroidism. Moreover, selenium decreases the formation of proinflammatory cytokines, while it contributes, in synergy with antithyroid drugs, to stabilization of the autoimmune process in GD and alleviation of GO. It is now to be clarified whether enforced nutritional supplementation has the same results and whether prolonging selenium administration may have an impact on the prevention of disease.

1. Introduction

Observed and briefly described, though not published, by Parry in the late 1700s, Graves' disease (GD) was definitively identified and documented by Robert Graves in 1835 and classically described by von Basedow in 1840 [1–3]. GD is an autoimmune disease characterized by the activation of autoantibodies against the TSH receptor (TRAB), leading to excessive thyroid hormone production [4]. GD manifests, interalia, via thyrotoxicosis and extrathyroid involvement often entailing orbitopathy (GO) and, rarely, dermopathy (pretibial myxedema) and acropathy. Moreover, the TRAB, by stimulating cyclic adenosine monophosphate (AMP), cause proliferation and hyperplasia of the thyroid follicular cells resulting in enlargement of the gland, frequently the first sign of the disease, the swelling ranging from slight to marked [5]. Clinically, the thyroid is firm in consistency and tender in patients with a greatly enlarged goiter, while palpation lobulations are also commonly detected which can be mistaken for nodules.

No single gene has been pinpointed as causing GD, a disease which is most prevalent in women between the ages of 20 and 50 years. However, it has been associated with certain MHC Class II HLA alleles depending on the racial group, for example, HLA-DR3 in whites [4]. An association of GD with polymorphisms of the cytotoxic T-lymphocyte antigen 4 (CTLA-4) gene has also been established, suggesting a functional role of CTLA-4 in autoreactive T cells [4, 5].

A combination of genetic and environmental factors is responsible for the initiation of autoimmunity. Interactions between genetic and environmental factors are underscored by the existing associations linking age at diagnosis, goiter, disease severity, smoking, and family history [6]. In addition, iodine repletion in iodine-deficient areas is usually accompanied by an increased incidence of GD due to the Jod-Basedow phenomenon. Stress is also thought to be a significant factor precipitating GD in susceptible individuals [7], while smoking is well established as being linked to GO but not to GD [8].

Treatment modalities of GD consist of administration of antithyroid drugs, radioiodine therapy, or surgery. Radioiodine therapy, is favored only in USA, whereas antithyroid drugs, including methimazole, carbimazole, and propylthiouracil, comprise first choice treatment in the rest of the

world. Nevertheless, according to a recent study examining the frequency of antithyroid drug prescription in USA, methimazole (MMI) has lately become the most frequently prescribed antithyroid drug, indicating a clear shift towards pharmacological treatment as the primary treatment option in GD [9]. Treatment should be planned for a period of at least 12 months, and patients are usually becoming euthyroid within this timeframe; nevertheless, the duration of the remission period is unpredictable, since the disease is marked by cycles of remission and relapse of variable duration [4].

Recently, evidence has emerged indicating that selenium administration could be effective and safe in patients with GD and with mild forms of GO [10].

The aim of this paper is to briefly evaluate the current knowledge concerning the pathogenesis of GD and GO and discuss the evolving role of selenium within the context of its potential as a therapeutic means of intervention in these disorders.

2. Pathogenesis of GD and GO

Hyperthyroidism is caused by the binding of TSH-stimulating antibodies to the TSH receptor, a G-protein-coupled receptor. However, the first step in this process is considered to be precipitation by environmental factors of an HLA-related organ-specific defect in suppressor T-lymphocyte function [5]. This leads to decreased suppression of thyroid-directed helper T-lymphocytes which, in the presence of dendritic cells and macrophages, produce the cytokines γ-interferon (IFNγ) and interleukin-1 (IL-1), subsequently differentiating B cells to plasma cells and generating TRAB. Concomitantly, IFNγ enhances the expression of HLA-DR antigens on the surface of thyroid cells (Figure 1). Thus, IFNγ modulates the autoimmune process and, by stimulating chemokine production by thyroid follicular cells, contributes to the maintenance of the autoimmune process [10]. The contribution of dendritic cells and B cells is apparently crucial for the initiation of disease since they express the costimulatory molecules, CD80 and CD86, that are key triggers for the reaction of T lymphocytes to thyroid cell presenting antigens [4]. TRAB stimulate the TSHR on the thyroid follicular cells, resulting in increased thyroid hormone production, which may further reduce the number and function of suppressor T lymphocytes and stimulate helper T lymphocyte, thus, perpetuating the cyclicity of disease [4, 5].

GO is a complex autoimmune disease. Whereas the cycle of GD consists of two components, immunological and hormonal, that perpetuate the process, the progression of GD to GO, and rarely to dermopathy, is likely to be a positive feedback cycle composed of three interrelated components: mechanical, immunological, and cellular [5]. Comprehensive reviews on the pathophysiology of GO have recently been published [11–14]. Briefly, the loss of tolerance of T cells to the TSHR, via as yet unknown mechanisms, ignites the autoimmune process. The TSHR is internalized and presented by antigen-presenting cells to helper T cells. Subsequently, the TRAB, which are secreted by activated B cells, recognize the TSHR on the fibroblasts of the orbita, where they initiate the ocular changes [12, 13]. The fibroblasts have been recognized as target cells in GO. Orbital fibroblasts stimulated by IFNγ, tumor necrosis factor-α (TNF-α), growth factors and oxygen reactive species (ROS), secrete hyaluronic acid, and prostaglandin E_2, known mediators of inflammation, while a subgroup may differentiate into mature adipocytes presenting TSHR [13, 14]. The subsequent proliferation of adipocytes and fibroblasts results in increased synthesis of glycosaminoglycans (GAG), which causes edema of orbital structures, extraocular muscle enlargement, and adipose tissue expansion; these events are constituting the signs of disease [15].

Concerning the recent enquiry as to whether autoimmunity against IGF-1R is primarily involved in the pathogenesis of GO, it is likely that it is not specific but instead constitutes a secondary reaction of the autoimmune process [16]

The mechanisms promoting oxidative stress have also been implicated in the pathogenesis of GO. Hyperthyroidism increases oxidants and decreases antioxidants leading to oxidative stress, this process is dominated by the production of ROS which have long been recognized as intermediates of various essential biological redox reactions [17, 18]. The adverse effects induced by ROS have been suggested as being partly responsible for the tissue injury. Mitochondria are a major source of superoxide anion (O_2^-) and hydrogen peroxides (H_2O_2), while a number of intracellular enzymes, xanthine oxidase being the best known, are involved in oxidation reactions in which molecular oxygen (O_2) is reduced to O_2^- [19].

Ongoing autoimmunity may contribute to increased oxidative stress even in euthyroid GD patients, while patients who have relapsed present increased markers of oxidative stress [20]. Moreover, the content of 8-hydroxy 2′-deoxyguanosine (8-OHdG), an important biomarker of oxidative DNA damage, was found significantly higher in orbital fibroblasts together with O_2^- and H_2O_2, underscoring the major role that ROS play in the pathogenesis of GO [21].

Recently, increased 11β-hydroxysteroid dehydrogenase (11β-HSD1) expression, induced by cytokines, was described in orbital adipose cells, a condition leading to elevated local generation of cortisol by 11β-HSD1, which may suppress cytokine synthesis and resolve the inflammation [22]. 11β-HSD1 activates cortisone to cortisol in peripheral and visceral adipose tissues. According to the authors, since failure to produce adequate levels of local glucocorticoids in the orbita may signify persistence of the disease, 11β-HSD1 could provide a new therapeutic target of disease [22].

3. Presentation and Treatment Novelties of GD and GO

TRAB levels in serum are pathognomonic for GD, predicting the course of disease and response to antithyroid treatment; they do not, on the other hand, predict the development of GO [23]. In conjunction with the high levels of TRAB, the risk of relapse is related to young age, male gender, and large goiter [24]. Tobacco smoking has been consistently linked to development or deterioration of GO [8, 25]. Since RAI

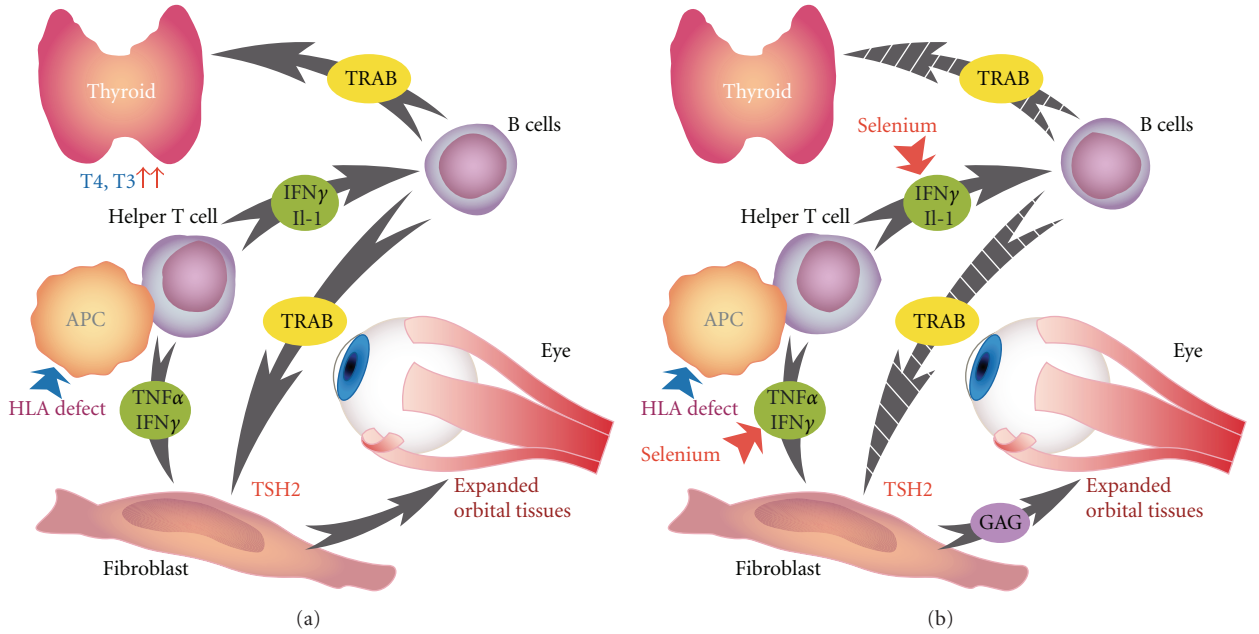

Figure 1: (a) Schematic presentation of the cascade of events in the pathogenesis of Graves' ophthalmopathy. Secretion of cytokines, such as IFN-γ and IL-2, by activated helper cells result in activation of B cells and secretion of TSH receptor antibodies. These bind to the TSH receptor in the orbital fibroblast and on the thyroid follicular cells, thereby, extending muscle enlargement resulting in oedema. (b) Selenium by suppressing cytokines production considerably attenuates the inflammation leading to alleviation of symptoms and signs. Abbreviations: HLA: human leukocyte antigen; APC: antigen presenting cell; IFN-γ: interferon-γ; IL-1: interleukin-1; TRAB: TSH-receptor antibodies; GAG: glycosaminoglycans.

treatment for GD is associated with a worsening of GO, patients, and particularly those who are smokers, should be administered oral steroids [26]. Interestingly, a recent study from Varese has suggested that steroid prophylaxis can be achieved by applying lower prednisone doses, that is, 0.2 mg/kg BW, than had previously been reported [27]. Moreover, RAI when applied for treatment for GD results more frequently in aggravation or appearance of GO than after antithyroid treatment [28]. Nevertheless, choice of the best treatment for hyperthyroidism in patients with active GO remains a dilemma [29]. In a recent prospective analysis of the data of 108 patients with Graves' hyperthyroidism and severe orbitopathy, it was reported that prolonged treatment applying partial block therapy with low-dose thionamides plus LT4, over a median duration of 80 months, led to euthyroidism and stabilized the orbitopathy [30]. Within this context, a retrospective study proposes block-replacement treatment of GD patients with GO as a feasible treatment option until the orbitopathy becomes inactive, and no further treatment is required [31].

Neither antithyroid drug treatment nor thyroidectomy has any impact on the course of GO, and treatment in the active phase is based on the clinical activity score (CAS) [32, 33]; introduced by Mourits et al. in 1989, the CAS remains a reliable and easily applied scoring system enabling the classification of patients into those with active or inactive disease [33].

Recently, rituximab, a CD-20 antibody which blocks the differentiation of B cells and potentially inhibits B-cells-mediated immunity, was applied with encouraging results in patients with GO [34]. The compound was shown to improve GO without, however, affecting the TRAB levels [35]. Serum cytokine IL-6 levels did not change, while chemokine ligand 10 (CXCL10) increased at B-cell depletion.

Based on the knowledge of the crucial role of the oxidants in the pathogenesis of GD as well as in the development of GO, several studies have been conducted administrating antioxidants as the treatment modality in patients with GD and GO.

In a nonrandomized study, 82% of the 11 patients with active GO responded to antioxidant treatment with nicotinamide and allopurinol as compared to only 27% of the control group. Soft tissue inflammation parameters responded better than any other component of disease [36].

Supplementation with a mixture of antioxidants, including selenium, beta-carotene, and vitamins C and E, in addition to methimazole, in 29 patients with GD led to euthyroidism faster than in 28 patients taking only methimazole and who served as the control group [37]. Serum selenium levels as well as glutathione peroxidase activity were statistically significantly elevated in the supplemented patients, validating treatment with antioxidants, especially when this incorporated selenium.

In a more recent, randomized, double-blind, and placebo-controlled study recruiting 159 patients with mild GO, the effects of selenium administration for 6 months in the form of selenite were assessed versus an anti-inflammatory agent [10]. Selenium improved quality of life and significantly slowed the progression of GO, while it greatly decreased the CAS when compared with the pentoxifylline or

placebo group. A 6-month followup confirmed the results of the 6-month treatment. The authors hypothesized a reversal of the disturbed antioxidant-oxidant balance in GD and GO although the exact mechanisms of selenium action are not elucidated.

In another study assessing the selenium levels in patients going into remission ($n = 24$) and relapses ($n = 59$), no statistically significant differences were detected between the two groups. However, patients in remission of GD had the highest (>120 μg/L) serum selenium levels, while it is of interest that TRAB levels and selenium were negatively correlated [38].

4. Mechanisms of Selenium Action in GD and GO

Selenium is vital for a wide range of biological processes; hence, the state of "selenostasis" is essential for wellbeing and human health [39]. The many biological and clinical benefits conferred by selenium are achieved by virtue of its remarkable antioxidative effects mediated mainly by the selenoproteins GPx and TRx reductase. TRx is a stress- and iodine-induced protein, possessing strong redox activities, and it has been postulated that it may be implicated in the regulation of T3 production in GD. It has been reported highly produced in GD and expressed in the thyroid follicular cells. Nevertheless, its precise role, though of considerable interest due to its characteristics, remains as yet unraveled [40].

The hypermetabolic state in acute GD, the intracellular ATP, and increased oxygen consumption lead to mitochondria dysfunction, which generates ROS and disrupts the oxidant and antioxidant balance, thereby, causing oxidative stress and tissue injury [41]. By activating GPxs, selenium ignites the "second line" of antioxidant defense, behind the enzymatic "first line" defense system composed of the superoxide dismutase (SOD) and catalase (CAT) [42]. Thus, SOD and CAT synthesize an efficient antioxidative mechanism capable of neutralizing the biologic effects of free radicals; when this mechanism is saturated, the "second line," regulated by selenium availability, is activated. Experimental studies in hyperthyroidism have documented an enhanced activity of the TRx and GPx systems, stimulated by the calcium phosphatidylinositol cascade which is usually activated in hyperthyroidism, as well as increased levels of SOD and of glutathione in erythrocytes [43, 44]. These findings provide evidence of an upregulation of the antioxidative and protective systems in acute GD, depending, however, on the duration and severity of the disease; these system(s) might become saturated, following which supplementation or nutritional intervention is required.

The induced oxidative stress enflames lipid peroxidation and activates various inflammatory pathways. ROS may stimulate the NF-κB pathway, a cornerstone of immune and inflammatory response, which has been associated with increased production of TNF-α and IL-6 cytokines [45]. Selenium inhibits NF-κB from binding to its gene promoters and consequently diminishes cytokine production and attenuates the inflammation; by contrast, selenium is likely not to interfere with the translocation of NF-κB and its subunits to the nucleus [46]. This could be one of the most important anti-inflammatory effects of selenium supplementation and thus be of potential benefit for patients suffering from GD and, especially, GO.

In GO, the balance of T helper (Th) 1/Th2 lymphocytes shifts to a prevalence of Th1 type CD4+, which plays a pivotal role in the development of disease [47]. Consequently, the ratio Th1/Th2 has been proposed as a biomarker of disease activity and as a target for specific immune therapy of GO. The subsequent overproduction of cytokines, such as TNF-α and IFNγ, sustains the inflammatory process. It is of interest that treatment with a mixture containing selenium-suppressed Th1 while upregulating Th2 [48]. Th1 predominate in eye muscles (EM) and IFN-γ, TNF-α, IL-1β, and IL-6 mRNA have been abundantly detected in EM in contrast to orbit fat where IL-4 and IL-10 mRNA, with significant variations within patients, were more frequently detected [49]. Thus, mediated by the suppression of Th1-like cytokines, selenium alleviates the soft tissue inflammation and improves eye motility.

ROS, such as H_2O_2, may also activate p38 mitogen-activated protein kinase (p38MAPK) and induce expression of high levels of cyclooxygenase (COX)-2; this reaction is depending on the severity of GO, in orbital fibroadipose tissues [50]. Recently, it has been shown that selenium was able to reduce H_2O_2-mediated expression of COX-2 in vascular endothelial cells by inhibiting the p38 MAPK pathway [51].

In summary, selenium influences the inflammatory process in GD and GO by inhibiting various pathways though its mechanism of action is not completely clarified. It is nonetheless possible that, in synergy with antithyroid drugs or immune modulators, selenium might offer an alternative therapeutic approach in patients with severe disease. It also remains to be established whether enforced nutritional supplementation has the same effects and whether long-term selenium administration in the form of selenomethionine or as nutritional intervention may have an impact on the incidence of relapse of GD and GO.

Conflict of Interests

The author declares that there is no conflict of interests.

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Review Article

The Role of Oxidative Stress on the Pathogenesis of Graves' Disease

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Graves' disease is a most common cause of hyperthyroidism. It is an autoimmune disease, and autoimmune process induces an inflammatory reaction, and reactive oxygen species (ROSs) are among its products. When balance between oxidants and antioxidants is disturbed, in favour of the oxidants it is termed "oxidative stress" (OS). Increased OS characterizes Graves' disease. It seems that the level of OS is increased in subjects with Graves' ophthalmopathy compared to the other subjects with Graves' disease. Among the other factors, OS is involved in proliferation of orbital fibroblasts. Polymorphism of the 8-oxoG DNA N-glycosylase 1 (hOGG1) involved in repair of the oxidative damaged DNA increases in the risk for developing Grave's disease. Treatment with glucocorticoids reduces levels of OS markers. A recent large clinical trial evaluated effect of selenium on mild Graves' ophthalmopathy. Selenium treatment was associated with an improved quality of life and less eye involvement and slowed the progression of Graves' orbitopathy, compared to placebo.

1. Introduction

Graves' disease is a most common cause of hyperthyroidism in iodine sufficient areas [1]. It is characterized by diffuse goitre and hyperthyroidism. Graves' orbitopathy represents orbit involvement and is clinically relevant in about half of the patients with the Graves' disease. In 3 to 5% of the patients, orbitopathy is severe [2]. Graves' disease is an autoimmune disease characterized by the presence of the serum autoantibodies. TSH receptor antibody represents the major autoantibody in Graves' disease [3].

Autoimmune process induces an inflammatory reaction and reactive oxygen species (ROSs) are among its products. ROSs are formed as normal metabolic products and are important in normal cellular functioning, but their production can be increased under pathological conditions and cause damage [4, 5]. Therefore, a large number of antioxidant systems act as protective mechanism. Among them are superoxide dismutase which catalyses dismutation of superoxide to peroxide, catalase which catalyses the decomposition of hydrogen peroxide to water and oxygen, while glutathione peroxidise which reduces lipid hidroperoxides while simultaneously oxidizing glutathione [6]. Situation in which balance between oxidants and antioxidants is disturbed in favour of the oxidants is termed "oxidative stress" (OS) [4].

2. Oxidative Stress and the Thyroid Gland

Synthesis of thyroid hormones requires formation of the hydrogen peroxide, a highly reactive oxidant. Hydrogen peroxide and oxidized iodine are immediately used in peroxidation reaction that is catalysed by thyroid peroxidase [7]. To protect thyroid cells from reactive oxygen species (ROSs) a potent antioxidant system exists in thyroid. Peroxiredoxin, glutathione peroxidase, thioredoxin, and catalase are involved in this antioxidant system [8]. Peroxiredoxins belong to a family of antioxidant proteins that are well conserved during evolution. Peroxiredoxin 5 (PRDX5) is expressed in the thyroid, mostly in the cytoplasm. The level of expression is correlated with the functional status of thyroid cells, being higher in multinodular goitres, and even higher in hyperthyroid tissues [9]. Catalase and glutathione peroxidases are also increased in hyperthyroid tissues [10].

Some level of oxidative load is necessary for thyroid function and proliferation. In a healthy thyroid, ROSs are

produced in an area that is located at the apical pole of the cell in microvilli, where H_2O_2 is consumed either during the hormone synthesis or by antioxidant systems. However, Th1-induced ROS production causes ROS accumulation both in the cytoplasm and in nuclei, where it can become toxic. Interestingly, *in vivo*, both the antioxidant N-acetylcysteine (NAC) and the anti-inflammatory prostaglandin 15deoxy-12,14-prostaglandin J2 (15dPGJ2) protect the thyroid against toxic effects of the OS. It seems that NAC and 15dPGJ2 mainly act on infiltrating inflammatory cells, reducing the extrafollicular ROS load [11]. As hydrogen peroxide and iodine are cosubstrates in thyroid hormone production, iodine inhibits hydrogen peroxide production [12]. Tobacco smoke contains thyocyanate that blocks iodine transport into thyrocite. This could increase H_2O_2 production and oxidative load, especially when associated with other environmental factors [13, 14].

Poncin and coworkers suggested that thyroid interstitial inflammation depends on the balance of the OS and the antioxidative defences (AODs). In basal, healthy conditions, both OS and AOD are low, and there is no inflammation. Increase in OS balanced by the increase in AOD would lead to minimal inflammation, but unopposed increase in OS would lead to strong inflammation and cell necrosis. Reducing OS would lead to inflammation reduction and vice versa [11, 15].

3. Oxidative Stress in Graves' Disease and Peripheral Tissues

Graves' disease is characterized by increased oxidative stress. Abalovich at all found increased markers of OS and decrease in markers of AOD in erythrocytes of patients with Graves' disease. All analysed markers normalized when euthyroidism was achieved after treatment with methimazole. However, after treatment with radioactive iodine, levels of tert-butyl hydroperoxide initiated chemiluminiscence and superoxide dismutase levels did not normalize [16]. Increased markers of OS were found in plasma of Graves' disease patients, even when they are rendered euthyroid. Levels of OS and AOD markers were higher, both in plasma and in thyroid tissue in patients whose treatment was shorter than 6 months [17]. However, thyroid hormones, per se, induce OS, which is tissue and species specific [18]. Even in subclinical hyperthyroidism, oxidative stress and antioxidative response seem to be increased [19]. It seems that the oxidative stress-induced activation of the NF-kappaB pathway might play a role in the autoimmune response in hyperthyroidism [20, 21]. Therefore, when antioxidant supplementation is added to methimazole, euthyroidism is more rapidly achieved [22]. However, it seems that the level of OS is increased in subjects with Graves' ophthalmopathy compared to the other subjects with the Graves' disease. Methimazole treatment normalizes markers of oxidative stress in plasma in subject with Graves' disease, but not in subjects with Graves' ophthalmopathy [23].

Hyperthyroidism is associated with increased lipid peroxidation products in rat liver and with increased activities of glutathione peroxidase, superoxide dismutase, and catalase in the liver [24]. Liver oxidative stress increases quickly after increase of thyroid hormones [25]. In rat kidney and testis, hyperthyroidism is associated with increased oxidative stress and lipid peroxidation [26–28].

Hyperthyroidism is also associated with increased oxidative stress and oxidative damage to lipids and genomic DNA in the aortic wall [29]. During hyperthyroidism, there is an increase in myocardial oxidative stress that is associated with lipid peroxidation and protein oxidation. Myocardial antioxidant enzyme activities elevation accompanied by protein expression induction occurs after four weeks of hyperthyroidism [30]. It seems that oxidative stress plays an important role in cardiac hypertrophy, by the redox activation of AKT1 and JUN/FOS signaling pathways [31]. Redox imbalance due to hyperthyroidism induces adaptation of antioxidant systems, also inducing ERK1/2 activation and leading to development of cardiac hypertrophy [32]. It is interesting to note that although long-term thyroxin administration causes cardiac hypertrophy, it is also associated with enhanced tolerance of the myocardium to ischemia and reperfusion. This response may involve the thyroid hormone-induced upregulation of HSP70 [33]. In skeletal muscle, hyperthyroidism causes increased oxidative stress associated with oxidative modification in myosin heavy chain causing the decrease in force production [34].

4. Oxidative Stress in Graves' Disease and Retroorbital Tissues

Graves' orbitopathy is caused by inflammation in the orbital connective tissue. Enhanced adipogenesis and overproduction of glycosaminoglycans causes an increase in orbital volume and fibrosis of the extraocular muscles [35]. Among the other factors, OS is involved in proliferation of orbital fibroblasts. In orbital fibroblasts, obtained from subjects with severe grave orbitopathy, superoxide radicals induce a dose-dependent cellular proliferation. This effect is not observed in fibroblast cultures obtained from control subjects [36]. However, superoxide-induced fibroblast proliferation could be prevented by methimazole, the xanthine oxidase inhibitor allopurinol, and nicotinamide [36, 37]. In orbital tissue samples, there is increased level of lipid hydroxyperoxide, superoxide dismutase, glutathione peroxidise, and glutathione reductase in Graves' orbitopathy patients, compared to controls. Furthermore, there is strong negative correlation between the ophthalmopathy index and glutathione level [38].

IL-1β is produced by activated macrophages and is an important mediator of the inflammatory response. Adding IL-1β to cultures of retroorbital fibroblasts causes an increased oxygen-free radical production in a dose-dependent manner. This is observed both in Graves' and in control cultures. Total intracellular superoxide dismutase (SOD) activity was stimulated by IL-1β, both in control and in Graves' cultures. However, in Graves' cultures SOD activity was increased at rest and less responsive to IL-1β stimulation. IL-1β was a potent stimulator of glycosaminoglycan

(GAG) accumulation in both normal and GO retroocular fibroblasts. IL-1β significantly stimulated the GAG synthesis in both normal and Graves' fibroblasts cells in a dose-dependent manner. Adding SOD and catalase partially blocked accumulation of the GAG induced by IL-1β [39].

HSP72 is a stress inducible form of cytosolic HSP70. Its expression is induced by the environmental stress, such as heat shock, anoxia, and ischemia. HSP72 has cytoprotective effects and functions as a molecular chaperone in protein folding, transport, and degradation. Moreover, HSP72 can inhibit apoptosis by several different mechanisms. In addition, HSPs are potent activators of the innate immune system and they stimulate the production of proinflammatory cytokines. In retroorbital fibroblasts obtained from GO patients, both H_2O_2 and heat stress significantly increased HSP72 expression. Antioxidants, methimazole, and PTU reduced H_2O_2-induced HSP72 expression, and to a lesser degree heat-induced HSP72 expression [40–42].

Oxidative DNA damage was found to be significantly elevated in cultured orbital fibroblasts, but only slightly increased in fibroadipose tissues of patients with Graves' orbitopathy. In patients with Graves' orbitopathy, there was significant correlation between TSH receptor antibody levels and 8-hydroxy-2′-deoxyguanosine (a biomarker of DNA damage) content [43]. The presence of oxidative stress parameters in cultured orbital fibroblasts and its correlation with TSH receptor antibody levels represents a good indication that oxidative stress exerts action in GO.

Urinary 8-hydroxy-2′-deoxyguanosine (8-OhdG) is also a marker of oxidative DNA damage. The study by Tsai et al. found that the urinary level of 8-OHdG was significantly increased in GO patients (1.9-fold compared with normal subjects). This increase was pronounced in patients with active GO (2.4-fold compared with normal subjects). Moreover, urinary 8-OhdG level significantly correlated with both clinical activity score and ophthalmopathy index. However, this association becomes nonsignificant after adjustment for other parameters, particularly the smoking status. It should be noted that smoker had higher urinary 8-OhdG level than never-smokers, and that smoking was significant factor in multivariate analysis [44]. It is well known, from epidemiological studies, that strong evidence for a causal association between smoking and development of Graves' orbitopathy exists [45]. Study by Tsai et al. implies that smoking-induced oxidative stress contributes to the pathogenesis of Graves' orbitopathy [44].

One of the major forms of DNA damage induced by OS is 7, 8-dihydro-8-oxoguanine, referred in an abbreviated way as 8-oxoguanine (8-oxoG). This type of DNA damage is repaired by the base excision repair pathway. This pathway is initiated by the recognition and excision of the oxidized guanine by a DNA glycosylase. In humans, the major glycosylase is 8-oxoG DNA N-glycosylase 1 (hOGG1). The hOGG1 is located on chromosome 3p25/26 and is highly polymorphic. The C to G substitution at position 1245 in exon 7 results in substitution of serine with cysteine in codon 326 has been associated with a reduced capacity to repair oxidative DNA damage. Tanrikulu et al. assessed hOGG1 Ser326Cys polymorphism (rs1052133) as a candidate risk factor for GD. They found that Cys/Cys genotype had a 3.5-fold (95% CI: 2.10–6.01, $P < 0.001$) and the Cys allele had 1.83-fold (95% CI: 1.43–2.34, $P < 0.001$) increase in the risk for developing Grave's disease in their population [46]. The Ser326Cys polymorphism in hOGG1 gene was shown to reduce the hOGG1 activity in both *in vitro* and *in vivo* studies [46]. As the production of 8-oxoG is increased both in retroorbital fibroblasts and in urine of patients with GD and correlates with the disease activity, it could be argued that reduced hOGG1 activity causes increased DNA damage and increased OS making subject more susceptible to development of Graves' orbitopathy [43, 44].

5. Antioxidants as Treatment for Graves' Disease

Treatment of the Graves' disease reduces OS both by rendering patients euthyroid and by the direct effect of antithyroid drugs, particularly methimazole, on OS. Methimazole completely normalized parameters of OS in peripheral erythrocytes, while radioactive iodine did not [16]. In cultured fibroblasts methimazole prevented superoxide-induced fibroblast proliferation, while propylthiouracil had little effect [36]. Other forms of treatment for Graves' disease also influence parameters of OS. In euthyroid patients treatment of Graves' ophthalmopathy with oral glucocorticoids significantly reduced urinary level of 8-OhdG (a marker of oxidative DNA damage). It was noted that in patients who had recurrence of GO urinary level of 8-OhdG was high [47]. In the study by Akarsu et al. serum levels of serum level malondialdehyde (MDA, a product of ROS degradation of degrade polyunsaturated lipids) were higher in patients with GO, compared to controls and Graves' disease patients without GO. On the other hand, level of glutathione (GSH, a nonenzymatic antioxidant) was decreased in GO patients. Treatment with intravenous or oral methylprednisolone reduced MDA level. However, intravenous methylprednisolone induced more rapid therapeutic response and more rapid reduction in MDA level (in 4 weeks). Twelve weeks after the end of the treatment, clinical activity score and serum level of MDA were the same in both methylprednisolone-treated groups [48].

Treatment of Graves' disease with antioxidants is based on a premise of role of the OS in its' pathogenesis. A small trial using allopurinol and nicotinamide showed effectiveness of antioxidant treatment of mild and moderately severe Graves' ophthalmopathy [49].

Selenium is a trace element and is essential for selenoproteins synthesis where selenium functions as a redox centre. Some of selenoproteins like thioredoxin reductase and glutathione peroxidases play the key role in antioxidative defences. Most of the European countries are selenium deficient [50]. Previous clinical trials showed some effect of selenium on thyroid autoimmunity [51]. A recent large clinical trial evaluated effect of selenium on mild Graves' ophthalmopathy. Patients from several European countries were treated with sodium selenite in a dose of 100 μg twice daily. Selenium treatment was associated with an improved quality

of life and less eye involvement and slowed the progression of Graves' orbitopathy, compared to placebo [52].

6. Concluding Remarks

Evidence from previous studies suggests that oxidative stress plays a role in the pathogenesis of Graves' disease. *In vitro* and *in vivo* studies showed that antithyroid drugs and antioxidants influence parameters of oxidative stress both in retroorbital tissue and in the whole organism. However, until recently, all studies were small, nonrandomized, or uncontrolled, and large, controlled study was asked for [53]. Now, a large, randomized, controlled study proved that selenium supplementation significantly improves quality of life and reduces ocular involvement in patients with mild Graves' orbitopathy. Although it seems that antioxidative therapy will not play a major role in the treatment of Graves' disease, further trials are necessary to define its place as adjunctive therapy, or as the therapy for mild and moderate Graves' ophthalmopathy.

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Review Article

Atypical Clinical Manifestations of Graves' Disease: An Analysis in Depth

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Over the past few decades, there has been an increase in the number of reports about newly recognized (atypical or unusual) manifestations of Graves' disease (GD), that are related to various body systems. One of these manifestations is sometimes the main presenting feature of GD. Some of the atypical manifestations are specifically related to GD, while others are also similarly seen in patients with other forms of hyperthyroidism. Lack of knowledge of the association between these findings and GD may lead to delay in diagnosis, misdiagnosis, or unnecessary investigations. The atypical clinical presentations of GD include anemia, vomiting, jaundice, and right heart failure. There is one type of anemia that is not explained by any of the known etiological factors and responds well to hyperthyroidism treatment. This type of anemia resembles anemia of chronic disease and may be termed GD anemia. Other forms of anemia that are associated with GD include pernicious anemia, iron deficiency anemia of celiac disease, and autoimmune hemolytic anemia. Vomiting has been reported as a presenting feature of Graves' disease. Some cases had the typical findings of hyperthyroidism initially masked, and the vomiting did not improve until hyperthyroidism has been detected and treated. Hyperthyroidism may present with jaundice, and on the other hand, deep jaundice may develop with the onset of overt hyperthyroidism in previously compensated chronic liver disease patients. Pulmonary hypertension is reported to be associated with GD and to respond to its treatment. GD-related pulmonary hypertension may be so severe to produce isolated right-sided heart failure that is occasionally found as the presenting manifestation of GD.

1. Introduction

Graves' disease (GD) accounts for up to 80% of hyperthyroidism cases and is estimated to affect 0.5% of the population [1]. It usually presents with the common well- known symptoms and signs (goiter, ophthalmopathy, weight loss, nervousness, tremors, palpitations, sweating, etc.) which are the distinctive features of the disease (Table 1). We can observe another group of manifestations, such as periodic paralysis, apathy, or psychosis, which are less common and less distinctive despite being well documented in relation to GD (Table 1). Over the past few decades, there has been an increase in the number of reports about newly recognized (atypical or unusual) manifestations of hyperthyroidism that are related to various body systems and may create a wide range of differential diagnosis [2, 3]. Most of these atypical manifestations are mainly reported in patients with GD (Table 1), either due to a specific relation to the autoimmune thyroid disorder, or because GD accounts for the majority of hyperthyroidism cases. Occasionally, one of the atypical manifestations is the main presenting feature of GD [2]. Lack of knowledge of the association between these findings and GD may lead to delay in diagnosis, misdiagnosis, or unnecessary investigations.

The atypical manifestations of GD represent a wide spectrum of clinical and laboratory findings, and in this review we will focus on the clinical part of that spectrum. For example, while hematological manifestations of GD include thrombocytopenia, leucopenia, anemia, and pancytopenia; we will discuss anemia as the clinical presenting feature. Other atypical clinical presentations of GD that will be discussed here are vomiting, jaundice, and right heart failure. These manifestations can be attributed to a wide variety of hematological, gastrointestinal, and cardiopulmonary

Table 1: Manifestations of Graves' disease (GD).

Well recognized/common	Recognized/Less common	Unusual/atypical (estimated prevalence in GD patients)
Weight loss	Agitation/psychosis	Jaundice (mild hyperbilirubinemia in up to 30%)
Anxiety/nervousness	Apathy/depression	Vomiting (up to 44%)
Tremors	Confusion/delirium	Anemia (up to 33%)
Goiter	Myopathy	Pancytopenia
Tachyarrhythmia	Paraparesis or quadriparesis	Leukopenia/thrombocytopenia
Breathlessness	Abnormal liver function tests	Heart block
Left ventricular failure		Myocardial infarction
Increased bowel movements		Pulmonary hypertension (up to 43%)
Sweating		Right heart failure
Heat intolerance		Angioedema
Staring gaze/exophthalmos		Erythema annulare centrifugum

causes, and each of them represents a very common clinical condition.

2. Anemia

Anemia is not uncommonly found in association with GD. It has been found in 33% of GD patients [4], and was a presenting manifestation in up to 34% of cases with hyperthyroidism [5]. It is somewhat challenging to face anemia as the presenting manifestation of GD, especially when the typical clinical features of hyperthyroidism are subtle or overlooked. Regardless of the incidental association of GD with other forms of anemia (e.g., iron deficiency anemia, thalassemia, etc.), there are specific types of anemia that are directly or indirectly related to GD (Table 2). As an autoimmune disease, GD was found to be associated with other autoimmune diseases that include pernicious anemia, celiac disease, and autoimmune hemolytic anemia [6, 7]. Moreover, there is a certain type of anemia that occurs with Graves' disease and remains unexplained after excluding all other possible causes [4, 8]. Because of its clear relation to GD, and its cure following hyperthyroidism treatment, this type of anemia may be termed GD anemia [4].

2.1. Graves' Disease Anemia. In the study by Gianoukakis et al., GD anemia was found in 22% of GD patients [4]. In GD anemia the mean corpuscular volume (MCV) could be normal [8] or, probably more commonly, low [4, 9]. Generally the anemia that coexists with GD is observed to be mild and is commoner with severe disease [5] When GD anemia is microcytic, iron indices are normal and hereditary hemoglobinopathies are readily excluded [10]. Anemia may be the sole haematological abnormality, or it may be combined with thrombocytopenia, or leucopenia; and occasionally it may be present as a part of a GD-associated pancytopenia [9, 11, 12]. Erythropoietin levels are within normal reference ranges [4] and bone marrow, if examined, is hypercellular or, less commonly, normocellular; with normal iron stores [9, 13]. The exact pathogenesis of GD anemia remains unclear [8]; however an effect of the excess thyroid hormones has been postulated [10]. The hypercellular marrow may indicate that erythropoiesis is enhanced due to hyperthyroidism, but in the same time it is ineffective, hence the finding of anemia with low MCV [10]. Hematologically, anemia in the presence of hypercellular marrow could be related to either organ sequestration such as observed in hypersplenism, an enhanced removal of circulating red blood cells by an immune or toxic mechanism, or a hemopoietic stem cell dysfunction such as myelodysplasia [9]. One or both of the latter 2 mechanisms could be responsible for the GD anemia, with myelodysplasia being the most widely accepted explanation [9, 10, 13]. The finding that thyroid-stimulating hormone (TSH) receptor antibodies nonspecifically attach to the surface of the red blood cells, may suggest an autoimmune basis for GD anemia [14]. However, the rare occurrence of GD anemia with hyperthyroid nodular goiter (toxic multinodular goiter and toxic adenoma) makes the effect of thyroid hormones on hemopoiesis a more likely explanation than the autoimmune mechanism [12, 13]. Generally, GD anemia resembles anemia of chronic disease in many aspects including red cell morphology, iron status, erythropoietin levels, and association with markers of inflammation [4]. GD anemia was observed to correct promptly with return to the euthyroid state following hyperthyroidism treatment [4, 9, 10, 12, 13]. Correction included normalisation of the haemoglobin concentration and also of the MCV [4, 9, 10]. This improvement was observed regardless of the mode of therapy of hyperthyroidism, with antithyroid drugs being the more commonly used agents in this regard [4, 9, 10, 13].

2.2. Pernicious Anemia. Pernicious anemia is a well-known form of the autoimmune diseases that may occur in association with GD [6, 7, 15]. In the study by Boelaert et al., the prevalence of pernicious anemia among patients with GD was 1.4% compared to 0.13% in the UK general population [7]. The finding of megaloblastic anemia (marked macrocytosis with hypersegmanted polymorphonuclear leukocytes) in the peripheral blood film of a GD patient should raise the suspicion of this association. Anemia may be associated with

Table 2: Types of Anemia Associated with Graves' disease (GD).

	MCV[¥]	Iron status[#]	Prevalence in GD patients	Response to GD treatment
GD Anemia	Low or normal	Normal or high	22%	Y
Pernicious Anemia	High	Normal	1.4%	N
Iron deficiency Anemia of Celiac Disease	Low	Low	0.9%	N
Autoimmune Hemolytic Anemia	Normal or high	Normal	Only single case reports	Y*

Y: Yes; N: No, [¥] mean corpuscular volume, [#] serum iron, serum ferritin, ± bone marrow iron stores, * may respond to thionamide drug therapy alone.

leukopenia or thrombocytopenia; or it could form a part of the pancytopenia of pernicious anemia [16]. The diagnostic workup is a straight forward one and includes checking serum vitamin B12 concentration, red cell or serum folate concentration (to rule out folate deficiency), anti-intrinsic factor antibody gastric parietal cell antibody and the Schilling test.

2.3. Iron Deficiency Anemia due to Celiac Disease. In general, the major cause of iron deficiency anemia (microcytic anemia with a low iron status) is blood loss, either overt or occult [17]. Lack of evidence of blood loss, or the refractoriness to treatment with oral iron may lead to the suspicion of celiac disease. In GD patients, the presence of an iron deficiency anemia may indicate an associated celiac disease, but of course it does not mean omitting blood loss as a common possible cause. In the study by Boelaert et al., the prevalence of celiac disease was 0.9% in GD patients compared to 0.047% in the general UK population [7]. Review of the literature also showed that asymptomatic cases of celiac disease were detected when patients with autoimmune thyroid disease (including GD) were screened by autoantibody testing and duodenal biopsy [18]. However, Sattar et al. stated that screening for celiac disease in patients with autoimmune thyroid disease may not be justified without comorbidities or symptoms [19]. When GD and celiac disease co-exist, it is not clear whether the treatment of one of them affects the course of the other, but it is interesting to mention that treatment with a gluten-free diet has been associated with improvement in the coexistent Hashimoto's hypothyroidism, with reduction of the required thyroxine doses an effect probably related to enhanced drug absorption [18].

2.4. Autoimmune Haemolytic Anemia. The association of GD with autoimmune haemolytic anemia has been described in single case reports in the English and non-English literatures [20–23]. It appears that autoimmune haemolytic anemia is much less commonly found in association with GD when compared with immune thrombocytopenia and pernicious anemia [24]. In some of the case reports, autoimmune haemolytic anemia was present as a part of Evans' syndrome (autoimmune haemolytic anemia and idiopathic thrombocytopenic purpura) in association with GD [25, 26]. In the study by Rajic et al., on 362 subjects with autoimmune haematological disorders, there was no evidence of simultaneous autoimmune thyroid disease in the subgroup of patients with autoimmune haemolytic anemia [24]. Ikeda et al. reported a case of Evans' syndrome in a patient with GD that was not hyperthyroid after treatment with radioiodine, and suggested that an underlying immunological mechanism could be responsible for the association [25]. In this regard it was very interesting to get an effective control of hemolysis with the use of an antithyroid drug alone (namely, propylthiouracil) that was observed in a case of autoimmune haemolytic anemia [20], and in another one with Evan's syndrome [26]. This finding might be related to the earlier observation that microsomal antibodies and TSH receptor antibodies decreased in parallel, while patients with GD were taking carbimazole, whereas no significant changes were observed during treatment with placebo or propranolol [27]. The changes in autoantibody levels during carbimazole treatment were independent of changes in serum thyroxine and could have been due to a direct effect of the drug on autoantibody synthesis [27].

3. Vomiting

Vomiting is one of the most common symptoms of gastrointestinal disease. Patients with GD may present mainly with gastrointestinal symptoms that include diarrhea, frequent defecation, dyspepsia, nausea, vomiting, and abdominal pain [28]. A special clinical situation arises when a thyrotoxic patient, who lacks the typical unique features of hyperthyroidism, presents with severe and persistent vomiting. In one of the earliest reports, Rosenthal et al. described 7 patients with thyrotoxic vomiting with a delay in the detection of hyperthyroidism of 8 & 17 months in two of the cases [29]. Lack of awareness about the association between vomiting and hyperthyroidism may lead to a more marked delay in the diagnosis; that was 7 years in one case report [30]. In a review of 25 newly diagnosed thyrotoxicosis cases 44% of subjects were complaining of vomiting [31]. The mechanism by which vomiting develops in hyperthyroid patients remains uncertain [32]. Researchers have documented increased levels of estrogens in patients of both sexes with thyrotoxicosis [32]. Estrogens may act as an emetic agent with individual variation in susceptibility between patients [32]. Another postulated mechanism is through an increase in beta adrenergic activity due to an increased number of beta adrenergic receptors in hyperthyroid patients [32]. This mechanism has been concluded from the finding of increased adrenergic activity in hyperthyroidism [33], and from the observation that starting treatment with

beta blockers ameliorates the vomiting in some cases [32]. However, such an explanation may be debated, as vomiting is more likely to be linked to hypo-, rather than hyper-adrenalism. In addition, the beneficial effect of beta blockers could be due to the reduced thyroid hormone activity (reduced T3 concentration) and not due to a decrease in beta adrenergic activity. Another possible mechanism is through the effect of excess thyroid hormones on gastric motility. Thyroid hormones are thought to decrease gastric emptying secondary to a malfunction of the pyloric sphincter [32]. In a study on 23 patients with hyperthyroidism, 50% had delayed gastric emptying [34]. In another study, a slight but a statistically significant increase in the rate of gastric emptying occurred in patients after restoration of euthyroidism as compared with healthy control subjects [35]. In almost all reports, thyrotoxic vomiting showed an excellent improvement either within several days after the initiation of antithyroid treatment, or in temporal relation with the return to the euthyroid state [29, 30, 32].

3.1. Hyperthyroidism with Vomiting in Pregnancy. Vomiting is common in pregnancy and pregnant women are frequently checked for thyroid disorders [36, 37]. Hyperemesis gravidarum (HG) is known to be associated with mild transient hyperthyroidism probably due to the thyroid stimulating effect of human chorionic gondotropin [36–39]. On the other hand, frank hyperthyroidism is not infrequently discovered for the first time during pregnancy with GD being the most common cause [36, 40, 41]. Moreover, hyperthyroidism occurs in pregnancy with clinical presentation similar to HG and pregnancy itself [36, 41].

A common, challenging scenario develops when a pregnant lady gets severe vomiting together with a biochemical evidence of hyperthyroidism. Here she could be having either transient hyperthyroidism that is associated with HG, or overt hyperthyroidism that manifests with vomiting. It is important to differentiate between the two conditions (Table 3) because transient hyperthyroidism with HG is usually mild, self-limited, and requires no treatment [36, 37]; while frank hyperthyroidism (due to GD in 90% of cases) confers high maternal and fetal morbidity and mortality, and needs to be early detected and treated [36, 40, 41]. The presence of marked tachycardia, tremors, muscle weakness, and ophthalmopathy make the diagnosis of frank hyperthyroidism more likely (Table 3). Goiter especially if associated with a thyroid bruit may point to GD, but one should bear in mind that the thyroid gland may physiologically enlarge during normal pregnancy [41]. The presence of severe vomiting makes HG the likely diagnosis only with the exception of the unusual situation when vomiting is the main presenting symptom of thyrotoxicosis. Biochemically, transient hyperthyroidism of HG usually shows a picture of subclinical hyperthyroidism (Low TSH and normal free T4). The diagnosis of overt hyperthyroidism in pregnant women should be based primarily on a serum TSH value <0.01 mU/L and also a high serum-free T4 value [42]. Free T3 measurements may be useful in women with significantly suppressed serum TSH concentrations and normal or minimally elevated free T4 values [42]. Thyroid peroxidase antibodies are markers of autoimmune thyroid disease in general and will not differentiate as they are found in a considerable percentage of pregnant women. TSH receptor antibodies may help to indicate that GD is the cause of the overt hyperthyroidism. Finally, if the clinical and/or the biochemical hyperthyroidism persist beyond the first trimester, causes of hyperthyroidism other than HG should be actively sought, putting in mind that some 10% of women with HG may continue to have symptoms throughout pregnancy [40].

Table 3: Comparison between Graves' disease hyperthyroidism (GD) and Transient hyperthyroidism of hyperemesis Gravidarum (THHG).

	GD	THHG
Hyperthyroidism symptoms[1]	Y	N
Ophthalmopathy	Y	N
Goiter	Y[2]	N[3]
Significant weight loss	Y	N[4]
Severe vomiting	N[5]	Y
TSH	Low (usually <0.01 mU/L)	Low (usually not <0.01 mU/L)
free T4	High (significant rise)	Normal (or mild rise)
Free T3	High	Normal
Persistence >1st trimester	Y	N
Treatment required	Y	N

Y: Yes; N: No, [1]tremors, marked tachycardia, muscle weakness. [2]especially with a bruit. [3]Thyroid gland may enlarge during normal pregnancy. [4]may be 5% or more in severe cases of HG. [5]Rarely severe vomiting is a hyperthyroidism feature.

4. Jaundice

The spectrum of liver affection in GD extends from asymptomatic biochemical abnormality to frank hepatitis [3, 43]. In the vast majority of cases it is only the biochemical abnormality that attracts the physician rather than the clinically obvious liver disease [3, 43, 44]. Liver function derangement in hyperthyroid patients can be mainly subdivided into either transaminases elevations (hepatocellular pattern), or intrahepatic cholestasis [3, 43, 45]. In a study by Gürlek et al., at least one liver function test abnormality was found in 60.5% of hyperthyroid patients [44]. Elevations of alkaline phosphatase, alanine aminotransferase, and gamma-glutamyl transpeptidase levels were observed in 44%, 23%, and 14% of the patients, respectively [44]. The mechanism of hepatic injury appears to be relative hypoxia in the perivenular regions, due to an increase in hepatic oxygen demand without an appropriate increase in hepatic blood flow [46]. One theory suggests that the liver is damaged by the systemic effects of excess thyroid hormones [47]. The hypermetabolic state makes the liver more susceptible to injury, and, in addition, thyroid hormones might also have a direct toxic effect on hepatic tissue [47]. In almost

all the reported cases, the relation of the intrahepatic cholestasis to hyperthyroidism was documented when the jaundice has resolved with hyperthyroidism treatment, and after excluding all other possible causes of cholestasis [45–47]. Histologically, there are mild lobular inflammatory cellular infiltrates in addition to centrilobular intrahepatic cholestasis [46]. In a case series analysis by Fong et al. the liver histology changes due to hyperthyroidism were not characteristic and nonspecific [48].

Jaundice due to intrahepatic cholestasis may be a prominent symptom in GD patients, and very occasionally it is the presenting manifestation of thyrotoxicosis [44, 48]. Very high-serum bilirubin levels (up to 581 μmol/L) were occasionally noted in patients with hyperthyroidism [45, 47, 48].

The relation of jaundice to GD (or hyperthyroidism in general) can be presented in three clinical scenarios. GD may be the underlying cause of jaundice that develops in a previously healthy subject [47, 49]. The presentation of GD for the first time with jaundice may lead to unnecessary investigations and a delay in management [47]. It is prudent to look carefully for clinical stigmata of thyroid dysfunction, and to consider checking thyroid hormone levels while investigating patients with jaundice of unknown cause. The second clinical scenario develops when a patient with a preexisting chronic liver disease gets deterioration of his liver function tests with deep jaundice. Numerous possibilities are usually considered in this situation including a complicating hepatocellular carcinoma, viral reactivation or superinfection, sepsis, and drug side effects. In this setting, hyperthyroidism should not be omitted as a possible cause. Hegazi et al. reported a case of deep jaundice caused by hyperthyroidism due to a toxic adenoma in a patient with hepatitis B cirrhosis, with return of serum bilirubin to baseline level after treatment with radio-iodine [45]. Thompson et al. reported a patient with primary biliary cirrhosis who had dramatic deterioration of liver functions with jaundice due to the development of GD [50]. The patient's jaundice entirely reversed with treatment of the hyperthyroidism [50]. Thirdly, when a GD patient develops jaundice, a list of possible causes should be considered. These include, an unrelated biliary or hepatic disease [48, 51], an autoimmune liver disease that is known to be associated with GD [46], hepatic congestion due to concomitant congestive cardiac failure [48], hepatic manifestations of hyperthyroidism [47, 49], and hepatotoxic side effects of antithyroid drugs [52]. In the analysis made by Fong et al., severe liver test abnormalities, including deep jaundice occurred in patients with hyperthyroidism alone and with hyperthyroidism with congestive cardiac failure [48]. Drug-induced hepatotoxicity should be considered in those who present with hepatic dysfunction after initiation of thionamide therapy [46, 53].

Treatment of a hyperthyroid patient with jaundice needs to be considered and therefore, it will be discussed here. Review of the literature showed that treatment options other than thionamide drugs might have been preferably used in cases of jaundice and hyperthyroidism. In many of the cases the mode of antithyroid therapy was radio-iodine [45, 54], or thyroidectomy [51, 55]. Antithyroid drugs have hepatotoxic side effects in 0.5% of cases with methimazole and carbimazole mainly producing cholestasis, and propylthiouracil mainly causing hepatocellular damage [52]. These side effects are idiosyncratic rather than dose related [46]. Methimazole therapy may deteriorate a GD-related cholestatic jaundice [53]. However, it has been reported that carbimazole and methimazole were successfully used in restoring euthyroidism as well as ameliorating the hyperthyroidism-related jaundice [47, 56].

In the absence of another evidence of liver disease, and when jaundice is purely due to the hyperthyroidism, thionamide drugs may be used with monitoring of serum bilirubin and liver function tests. In patients with acute or chronic liver disease who develop GD that aggravates their jaundice, the small probability of hepatotoxic side effects of thionamide drugs may carry the risk of inducing fulminating hepatic failure [51], so that alternative GD treatment options are preferred.

5. Right Heart Failure

Thyroid hormone effects on the cardiovascular system include increased resting heart rate, left ventricular contractility, blood volume, and decreased systemic vascular resistance [57, 58]. Cardiac contractility is enhanced and cardiac output may be increased by 50% to-300% over that of normal subjects [57, 58]. The well-recognized cardiovascular manifestations of hyperthyroidism include palpitations, tachycardia, exercise intolerance, dyspnea on exertion, widened pulse pressure, and atrial fibrillation [57, 58]. In spite of the increased cardiac output and contractility, the left ventricular failure that may occur in severe and chronic cases of hyperthyroidism could be explained by a tachycardia-related left ventricular dysfunction, and/or a thyrotoxic cardiomyopathy [57, 58]. The higher prevalence of hyperthyroid heart failure in older age groups signifies the contribution of other cardiovascular comorbidities that include hypertension and coronary artery disease [57].

In addition to the well-known presentations, a variety of unusual cardiovascular manifestations are increasingly being reported in association with hyperthyroidism. These include pulmonary arterial hypertension (PH) [59, 60], right heart failure [61, 62], myocardial infarction [63], and heart block [64]. Clinically, isolated right-sided heart failure may be the presenting feature of GD.

In an echocardiographic study by Marvisi et al., mild PH was found in 43% of the 114 hyperthyroid patients and in none of the healthy control group [59]. In another study by Mercé et al., there was a high prevalence of PH in hyperthyroid patients [60]. Additional studies [65], case series [66], and case reports [61] have shown similar findings. The pathophysiologic link between thyroid disease and PH remains unclear [67]. Possible explanations include immune-mediated endothelial damage or dysfunction, increased cardiac output resulting in endothelial injury, and increased metabolism of intrinsic pulmonary vasodilator substances [60]. Review of the literature reveals some support for the immune-mediated mechanism [68, 69]. In a review by Biondi and kahaly, PH was more linked to GD than to other causes of hyperthyroidism [68]; and in a study

by Chu et al., there was a high prevalence of autoimmune thyroid disease in patients with PH [69]. However, in a study by Armigliato et al., the immune mechanism has been questioned because 52% of hyperthyroid subjects with PH did not have evidence of autoimmune thyroid disease [70]. Also in the study by Mercé et al., pulmonary hypertension did not correlate with the cause of hyperthyroidism [60]. Furthermore, Marvisi et al. found no statistical difference in thyroid antibody levels between the hyperthyroid study group and the euthyroid control group and stated that PH could be due to a direct influence of thyroid hormones on pulmonary vasculature [59]. We tend to believe that an effect of excess thyroid hormones may be responsible for the development of PH, especially with the finding of PH also in patients with hyperthyroid nodular goiter.

In spite of the observation that PH was mild in most of the studied hyperthyroid patients [58], cases of severe PH leading to right-sided heart failure are increasingly being recognized [71]. GD occasionally presents with frank isolated right heart failure due to the severe PH [61, 72, 73]. All other possible causes of right ventricular failure including left-sided systolic and/or diastolic dysfunctions have been excluded in reported cases [61]. PH as well as right heart failure showed improvement after the treatment of the concomitant hyperthyroidism [58, 61, 71, 73]. It may take several months for the pulmonary artery pressure to normalize following the initiation of antithyroid treatment [61, 66]. In one case report, the severe pulmonary hypertension has dropped to a near-normal value, only after 14 months from initiation of carbimazole therapy, in spite of a long period of clinical and biochemical euthyroidism [61].

6. Conclusions and Recommendations

The unusual manifestations of GD are diverse and affect various body systems. They include hematological, cardiovascular, gastrointestinal, hepatic, and dermatological manifestations (Table 1). Reports of other less frequent or rare presentations like venous thromboembolism [74] and cerebral vasculitis [75] may need further support and documentation. One or more of the unusual manifestations may be the main presenting feature of GD. Awareness about the relation of these presentations to GD or hyperthyroidism is essential to avoid wrong diagnosis and unnecessary investigations.

The mechanism remains uncertain in the majority of the atypical manifestations. However, a good response to hyperthyroidism treatment is almost guaranteed. The response to hyperthyroidism treatment is either rapid or quite delayed. In the case of vomiting the response occurs within days, however, in the case of right heart failure the improvement occurs within several months from starting the treatment. The excellent recovery that occurs in response to the restoration of euthyroidism makes the effect of excess thyroid hormones the likely underlying mechanism in most of the cases. With the exception of the autoimmune conditions that are associated with GD, the occurrence of the atypical manifestations also in patients with hyperthyroid nodular goiter stands against an autoimmune basis of pathogenesis.

Such atypical presentations appear to affect significant percentages of GD patients; however, most of the studies conducted in this respect were small. For instance, vomiting was a symptom in 44% of 25 thyrotoxic patients [31], and alkaline phosphatase was raised in 44% of 43 hyperthyroid patients [44]. Larger studies to further evaluate the prevalence of each of the atypical features in GD patients are needed to confirm that some of these findings are not unusual, but are rather under-recognized. The widespread hyperthyroidism manifestations that influence all body systems make us believe that the thyroid hormone effects on various body tissues are not yet fully unveiled.

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