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TSH-receptor autoantibodies in patients with chronic thyroiditis and hypothyroidism

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Abstract

Objectives: The reported prevalence of TSH-receptor (TSHR) autoantibodies (TRAb) in patients with chronic thyroiditis (CT) range from 0 to 48%. The objective was to study the prevalence of TRAb in patients with CT and hypothyroidism and to correlate it with gender, age, thyroid dimensions, TSH levels, and autoimmune diseases.

Methods: The study comprised 245 patients with CT and hypothyroidism (median age 42 years, 193 females, 52 males) and 123 Italian healthy subjects matched for sex and age as controls. TRAb were tested with ELISA using a >2.5 IU/L cut off for positivity. TSHR blocking (TBAb) and TSHR stimulating autoantibodies (TSAb) were measured in 12 TRAb-positive patients using bioassays with Chinese hamster ovary (CHO) cells expressing wild-type or R255D-mutated TSHR.

Results: TRAb positivity was found in 32/245 (13.1%) patients and significantly correlated ($p < 0.05$) with TSH levels. TRAb positivity was significantly higher in males vs. females ($p = 0.034$), in females 16–45 years of age vs. >45 years of age ($p < 0.05$) and in patients with reduced vs. normal/increased thyroid dimensions ($p < 0.05$). Linear

regression analysis showed a correlation between TRAb concentrations with age ($p < 0.05$) and TRAb concentrations with TSH ($p < 0.01$). In bioassay with TSHR-R255D all 12 patients tested were TBAb-positive while 33% were also TSAb-positive suggesting the presence of a mixture of TRAbs with different biological activities in some patients.

Conclusions: TRAb have been found in patients with CT and hypothyroidism. A mixture of TBAb and TSAb was found in some patients and this may contribute to the pathogenesis of thyroid dysfunction during the course of the disease.

Keywords: autoimmune thyroid diseases; chronic thyroiditis; hypothyroidism; TSH-receptor autoantibodies; TSH-receptor blocking antibodies; TSH-receptor stimulating antibodies.

Introduction

Chronic thyroiditis (CT) is one of the most prevalent autoimmune diseases [1–3] and is associated with autoantibodies to thyroid peroxidase (TPOAb) and thyroglobulin (TgAb) [4–6]. TSH-receptor (TSH-R) autoantibodies (TRAb) with thyroid stimulating (TSAb) or blocking activity (TBAb) were also reported in some patients with CT [4, 5, 7–10]. The prevalence of TRAb reported between 1978 and 2021 in a total of 2,308 patients with CT varied between 0 and 48% with a similar prevalence for TBAb [11–34] (Supplemental Table 1). On Italian population two studies were performed and in the first were evaluated 38 patients with CT and hypothyroidism and TRAb were found in 15% [19], in the second none of the 83 patients with CT was found TRAb-positive (32). In previous studies TRAb were found in CT patients with atrophic/non-goitrous primary myxoedema or goitrous CT and were more often associated with CT without goitre [12, 15, 20, 22–24]. Patients' TSH levels and treatment were not always stated and differences in patient sample size (often small groups), age, gender, and geographical location could account for the differences in the reported prevalence of TRAb in CT patients. Furthermore, the majority of the studies reported the prevalence of

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TRAb measured using antibody binding assays rather than the bioassays designed to detect TRAb functional activities.

The early studies were carried out using the radioimmunoassay (RIA) of Shewring and Rees Smith [35] with many variations in preparations of solubilised or membranous thyroid tissues and porcine, bovine or human 125I-TSH. The measurements carried out using the early TRAb RIAs have since been surpassed in sensitivity, specificity and precision by more modern second generation TRAb assays using immobilised TSH-R coated onto either tubes or enzyme-linked immunosorbent assay (ELISA) plate wells [25, 36]. More recently the use of a TSH-R stimulating human monoclonal autoantibody M22 in a third generation TRAb assay has brought about further improvements in the sensitivity and the specificity of TRAb measurements [36]. For example, the second generation DYNOTestTRAK human (Thermo Scientific) and third generation Version 3TRAb ELISA (RSR Ltd, Cardiff) assays were recently reported to have 95 and 94.1% assay sensitivity with 99 and 99% specificity, respectively [37]. This is similar to a previously reported sensitivity and specificity for the third generation V3 TRAb ELISA (RSR Ltd., Cardiff) and second generation V2 TRAb ELISA (RSR Ltd. Cardiff) of 95 and 89% sensitivity at 100% specificity, respectively [37].

Similarly, bioassays for the detection of TSAb and TBAb activity have improved since the early reports and have been replaced with assays using cultured cells stably expressing the TSH-R [38, 39]. Recently it has been shown that a TSH-R mutation from arginine to aspartic acid (R255D) at the amino acid 255 reduces the stimulating activity of TRAb [40]. Consequently, bioassays with TSH-R R255D allow determination of TBAb in samples containing a mixture of both blocking and stimulating TRAb [40–42].

This study aimed to evaluate the prevalence of TRAb in a cohort of 245 Italian patients with CT with various grades of hypothyroidism and to correlate the presence and levels of TRAb with gender, age, thyroid dimensions, TSH levels, TPOAb, TgAb and concurrent presence of other autoimmune diseases. TSAb and TBAb were measured in bioassays with wild type or R255D mutated human TSH-R in selected 12 TRAb positive patients with CT and different grades of hypothyroidism.

Materials and methods

Patients

A total of 245 patients were recruited from the Endocrine Unit at the Azienda Ospedaliera-Universitaria in Padova between January 2013 and December 2015 with informed consent and according to the

ethical principles of the Declaration of Helsinki. Patients were diagnosed with CT associated with hypothyroidism based on serum TSH levels >7 mIU/L and the presence of TPOAb and/or TgAb and/or a typical hypoechoic pattern on thyroid ultrasound scans [43]. None of the patients had Graves' disease. Patients were also assessed for other clinical, subclinical or potential autoimmune diseases based on clinical presentation and appropriate autoantibodies and functional tests. The patients' median age was 42 years (range 12–89 years). There were 193 females (median age 40 years; range 12–89 years) and 52 males (median age 44.5 years; range 16–85 years) with a female/male ratio 3.7/1. The group included 10 patients below the age of 16 years, 219 patients between 16 and 69 years and 16 patients >69 years of age. Eleven females (median age 30 years; range 22–39 years) presented with post-partum thyroiditis. Five patients had ophthalmopathy (two males and three females). Sex and age matched 123 healthy Italian subjects without personal or family history of CT, negative for TPO and/or TgAbs and with normal TSH were used as controls.

Methods

TSH was measured in all patients with ECLIA (Elecsys 2010 Cobas[®], Roche Diagnostic, Mannheim, Germany; normal range 0.27–4.2 mIU/L).

TRAb were measured using the third generation TRAb-Test ELISA Rapid IgG (Euroimmun, Medizinische Labordiagnostika AG, Lubeck, Germany) with calibrators between 1 and 40 IU/L (National Institute for Biological Standards and Control; NIBSC code 90/672). In the assay binding of serum TRAb to TSHR-coated ELISA plate wells is inhibited by a TSHR human monoclonal autoantibody M22 labelled with peroxidase and a signal is then developed by addition of a colour-generating substrate. The concentration of TRAb in test samples is inversely proportional to the signal in the assay. All 245 patients were tested for TRAb at the time of diagnosis and in the majority of patients before the start of thyroxine replacement. TRAb concentrations in samples above 40 IU/L were determined by sample dilution as appropriate. The cut-off for positivity of ≥ 2.5 IU/L specific for our study population was selected using 123 control samples as recommended by kit the manufacturers. TPOAb and TgAb were measured using a chemiluminescence test (LIASION[®] Anti-TPO and Anti-TG, Dia Sorin, Saluggia [VC], Italia) and values above 16 IU/L and 100 IU/L, respectively were considered positive. The dimensions of thyroid were measured and rated as reduced, normal, or increased by measuring antero-posterior diameter during ultrasound examination.

TBAb and TSAb bioassays

Twelve selected CT patients with a broad range of TSH levels mIU/L or (7.8–278 mIU/L) and different levels of TRAb (3.3–231 IU/L) gave their consent to measure TSAb and TBAb in bioassays using CHO cells expressing the wild type and R255D mutated human TSH-R [38–42]. The intracellular cyclic AMP concentration in the cell lysates was determined using the Direct Cyclic AMP Correlate-EIA Kit (Enzo Life Sciences, Farmingdale, New York, USA) according to manufacturer's instructions. Samples that produced an increase of cyclic AMP concentrations $\geq 150\%$ relative to the healthy blood donor pool were considered positive for TSAb activity. Samples that showed $\geq 30\%$ inhibition of stimulating activity of TSH were considered positive for TBAb.

Statistical analysis

All statistical analyses were performed using MedCalc software (version 19.5.3). Distribution of data was assessed by D'Agostino-Pearson test for normal distribution. The Mann-Whitney and the Kruskal-Wallis tests were used to analyse differences between not normally distributed variables. Chi-squared or Fisher's exact tests were used to assess possible associations between qualitative variables. Linear regression analysis was used to assess the relationship between two variables considered. The multivariate analysis, analysis of covariance (ANCOVA) was used to analyse the relationship between TRAb levels as dependent parameter and patient characteristics as independent variables. Meta-analysis of proportions was used to calculate an overall proportion from a set of proportions. For all tests, a $p < 0.05$ was considered statistically significant.

Results

TRAb were detected in 32/245 (13.1%) patients with CT and hypothyroidism and ranged from 2.5 IU/L to 231 IU/L; 14/245 (5.7%) had TRAb between >2.5 and 7.5 IU/L, 18/245 (7.3%) had TRAb >7.5 IU/L while 213/245 (86.9%) had TRAb levels <2.5 IU/L. The median levels of TSH in TRAb-positive and negative patients are summarized in Table 1. TSH levels were significantly higher in TRAb-positive (median 51.3 mIU/L; range 7.0–300 mIU/L) compared to TRAb-negative patients (median 17.1 mIU/L, range 7.0–434 mIU/L); ($p < 0.01$; Mann-Whitney test). TSH levels (median 86.8 mIU/ml) in 18 patients with TRAb >7.5 IU/L were significantly higher ($p = 0.018$; Mann-Whitney) compared to 14 patients with TRAb levels >2.5–7.5 IU/L (median TSH 26.8 mIU/L) (Table 1).

Patients were analysed based on TSH levels in a subgroup A including 124 patients with TSH levels 7–19 mIU/L and a subgroup B including 121 patients with TSH levels >19 mIU/L. In group A, 8/124 (7.3%) patients were TRAb positive while 24/121 (19.8%) were positive in group B. TRAb-positivity was significantly higher ($p = 0.0022$

Table 1: TRAb concentrations in 245 patients with chronic thyroiditis and hypothyroidism.

| TRAb levels, IU/L | no. of patients (%) | TSH, mIU/L, median (range) |
|-------------------|---------------------|-----------------------------|
| <2.5 | 213/245 (87) | 17.1 (7.0–434) ^a |
| ≥2.5 | 32/245 (13.1) | 51.3 (7.0–300) |
| ≥2.5–7.5 | 14/245 (5.71) | 26.8 (7.0–128) |
| >7.5 | 18/245 (7.36) | 86.8 (7.8–300) ^b |

^aTSH levels were significantly increased in the patients with TRAb ≥2.5 IU/L compared to patients with TRAb <2.5 IU/L ($p < 0.01$; Mann-Whitney test). ^bTSH levels were significantly increased in the patients with TRAb >7.5 IU/L compared to patients with TRAb ≥2.5–7.5 IU/L ($p = 0.025$; Mann-Whitney test).

Table 2: Prevalence and concentration of TRAb in patients with different serum TSH levels.

| Group | TSH, mIU/L | Patients with TRAb ≥2.5 IU/L | TRAb levels, IU/L, median (range) | Median age, years, median (range) |
|-------|------------|------------------------------|-----------------------------------|-----------------------------------|
| A | 7–19 | 8/124 (7.3%) | 7.0 (2.5–40 IU/L) | 39 (12–81) |
| B | >19 | 24/121 (19.8%) ^a | 9.0 (2.8–231 IU/L) | 44 (15–89) |

^aThe prevalence of TRAb was significantly higher ($p = 0.0022$; Fischer's exact test) in the group B compared to group A.

Fischer's exact test) in patients with TSH >19 mIU/L compared to patients with TSH levels 7–19 mIU/L (Table 2).

TRAb were found in 22/193 (11.4%) females (mean 8 IU/L ± 2.9–23 IU/L) and in 10/52 (19%) males (mean 9 IU/L ± 2.5–88 IU/L). The prevalence of TRAb was significantly higher in males ($p = 0.0339$; Chi-squared test) than in females (Table 3).

Two of 11 (18.2%) females with post-partum thyroiditis were TRAb positive (3.9 IU/L and 231 IU/L, respectively).

None of the 10 patients below 16 years of age (six females and four males; median TSH 11.5 mIU/L) had TRAb ≥2.5 IU/L. In older age groups, 29/219 (13.2%) of 16–69 years old patients (median TSH 19 mIU/L) had detectable TRAb levels (median 8 IU/L) and 3/16 (19%) of patients >69 years of age (median TSH 34.73 mIU/L) were TRAb positive (median 26.6 IU/L). One of five patients with CT and ophthalmopathy had high TSH (100 mIU/L) and was TRAb positive (17.8 IU/L). After excluding the patients with ophthalmopathy 31/240 (12.9%) of CT patients had TRAb >2.5 IU/L.

The prevalence and concentrations of TRAb in CT patients was further analysed in females and in males aged 16–45 years and >45 years. In 16–45 year old females TRAb were detected in 16/106 (15%) of patients (Table 4); 9/106 had TRAb levels ≥2.5–7.5 IU/L and 7/106 had TRAb levels >7.5 IU/L. In females >45 years of age, 6/77 (7.8%) patients were TRAb positive (Table 4); one had TRAb levels ≥2.5–7.5 IU/L and five had TRAb levels >7.5 IU/L. TRAb prevalence was significantly different between females <45 years and >45 years of age ($p < 0.05$; Chi-squared test). There were no significant differences in TSH concentrations between females <45 years and >45 years of age ($p > 0.05$; Mann-Whitney test) (Table 4).

TRAb >2.5 IU/L were found in 6/27 (22.2%) of 16–45 years old males (Table 4); 3/27 had TRAb levels ≥2.5–7.5 IU/L and 3/27 had TRAb levels >7.5 IU/L. In males >45 years of age, 4/25 (16%) patients had TRAb >2.5 IU/L (Table 4); one had TRAb levels ≥2.5–7.5 IU/L and three had TRAb levels >7.5 IU/L. There were no significant differences between the prevalence of TRAb or TSH concentrations between

Table 3: Prevalence and concentration of TRAb in females and males with chronic thyroiditis and hypothyroidism.

| Gender | Patients with TRAb ≥ 2.5 IU/L | TRAb levels, IU/L, median (range) | Median age, years, median (range) | TSH, mIU/L, median (range) |
|---------|------------------------------------|-----------------------------------|-----------------------------------|----------------------------|
| Females | 22/193 (11.4%) | 8.0 (2.9–231 IU/L) | 40 (12–89) | 18.0 (7.0–434) |
| Males | 10/52 (19.2%) ^a | 9.0 (2.5–88 IU/L) | 44 (12–85) | 23.6 (7.0–300) |

^aThe prevalence of TRAb was significantly higher in males ($p=0.034$; Chi-squared test) than in females.

Table 4: Prevalence of TRAb in females and males in different age groups.

| Gender | Age, years | Patients with TRAb ≥ 2.5 IU/L | Median age, years, median (range) | TSH, mIU/L, median (range) |
|---------|------------|------------------------------------|-----------------------------------|----------------------------|
| Females | 16–45 | 16/106 (15%) ^a | 32 (16–45) | 17 (7–434) ^b |
| | >45 | 6/77 (7.8%) | 56 (46–89) | 20 (7–316) |
| Males | 16–45 | 6/27 (22.2%) ^c | 35 (16–45) | 18 (7–300) ^c |
| | >45 | 4/25 (16%) | 60 (47–85) | 52 (7–185) |

^aThe prevalence of TRAb in females 16–45 years old was significantly higher than in the females >45 years old ($p<0.05$, Chi-squared test).

^bThere was no significant differences in the concentration of TSH between age groups in females ($p>0.05$ Mann Whitney test). ^cNo significant differences were found between the prevalence or concentration of TRAb ($p>0.05$, Chi-squared test) or of TSH between the different age groups in males ($p>0.05$, Mann Whitney test).

males <45 years and > 45 years of age ($p>0.05$; Chi-square test and Mann Whitney test, respectively) (Table 4).

Ultrasound evaluation carried out in 45 patients showed reduced thyroid dimensions in 15 and normal or increased dimensions in 30 subjects. TRAb >2.5 IU/L were detected in 10/15 (66.7%) of patients with reduced thyroid dimensions and in 9/30 (30%) of patients with normal/increased thyroid dimensions (Table 5). The prevalence of TRAb in patients with reduced thyroid dimensions was significantly higher ($p<0.05$; Kruskal-Wallis test) compared to patients with normal/increased thyroid dimensions (Table 5).

TPOAb and TgAb were measured in 210 patients and 190 (90.5%) were found positive of whom 26 (13.7%) had TRAb >2.5 IU/L (median 9.25 IU/L). Furthermore, 2/20

Table 5: TRAb in 45 patients who had thyroid assessed by antero-posterior diameter in ultrasound examination.

| Thyroid dimensions | Patients with TRAb ≥ 2.5 IU/L, n (%) | TRAb levels, IU/L, median (range) |
|--------------------|---|-----------------------------------|
| Reduced | 10/15 (67) ^a | 3.30 (1.0–231) |
| Normal/Increased | 9/30 (30) | 1.25 (1.17–2.05) |

^aThe prevalence of TRAb was significantly higher ($p<0.05$; Kruskal-Wallis test) in patients with reduced compared to normal/increased thyroid dimensions.

TPOAb and/or TgAb-negative patients were TRAb positive (2.9 and 6.0 IU/L, respectively). Consequently, at least one type of thyroid autoantibody (TPOAb and/or TgAb and/or TRAb) was found in 192/210 (91.4%) of patients with CT and hypothyroidism.

Linear regression analysis showed a correlation between age and TRAb concentrations ($p<0.05$) with an F-ratio 4.026 and an R^2 value 0.05 (Figure 1A), a correlation between TSH levels and TRAb levels ($p<0.01$; F-ratio 21.087, R^2 0.476) (Figure 1B) and a correlation between TSH levels and age in males ($p=0.0196$; F-ratio 5.81, R^2 0.10), but not in females ($p>0.05$; F-ratio 0.15, R^2 0.001) (Figure 1C).

In the multivariate analysis (ANCOVA) using TRAb levels as dependent parameter and patient characteristics as independent variables only TSH and age showed significant correlation ($p<0.0001$; F-ratio 9.461, R^2 0.288) consistent with the linear regression analysis.

In this study 38/245 (18.4%) patients with CT and hypothyroidism also presented with one or more autoimmune diseases including autoimmune gastritis, type 1 diabetes mellitus, rheumatoid arthritis, premature ovarian failure, Addison's disease, undifferentiated connective tissue disease, celiac disease, alopecia areata, myasthenia gravis, Sjogren's disease, scleroderma and multiple sclerosis. TRAb >2.5 IU/L (median 8.3 IU/L) were found in 6/38 (15.8%) patients with other associated autoimmune diseases and in 26/207 (12.6%) without associated diseases (median 8.25 IU/L). These differences were not statistically significant.

TSAb and TBAb bioactivity

Results of TSAb and TBAb measurements in 12 selected patients aged 27–85 years (seven females and five males) are shown in Table 6. One patient (Table 6, number 9) had CT, hypothyroidism and ophthalmopathy.

In the bioassay using the mutated TSHR-R255D, 12/12 (100%) patients were positive for TBAb and 4/12 (33.3%) also showed TSAb activity (Table 6). In the bioassays with wild type TSHR, 9/12 (75%) were positive for TBAb while 10/12 (83.3%) were positive for TSAb and overall 7/12

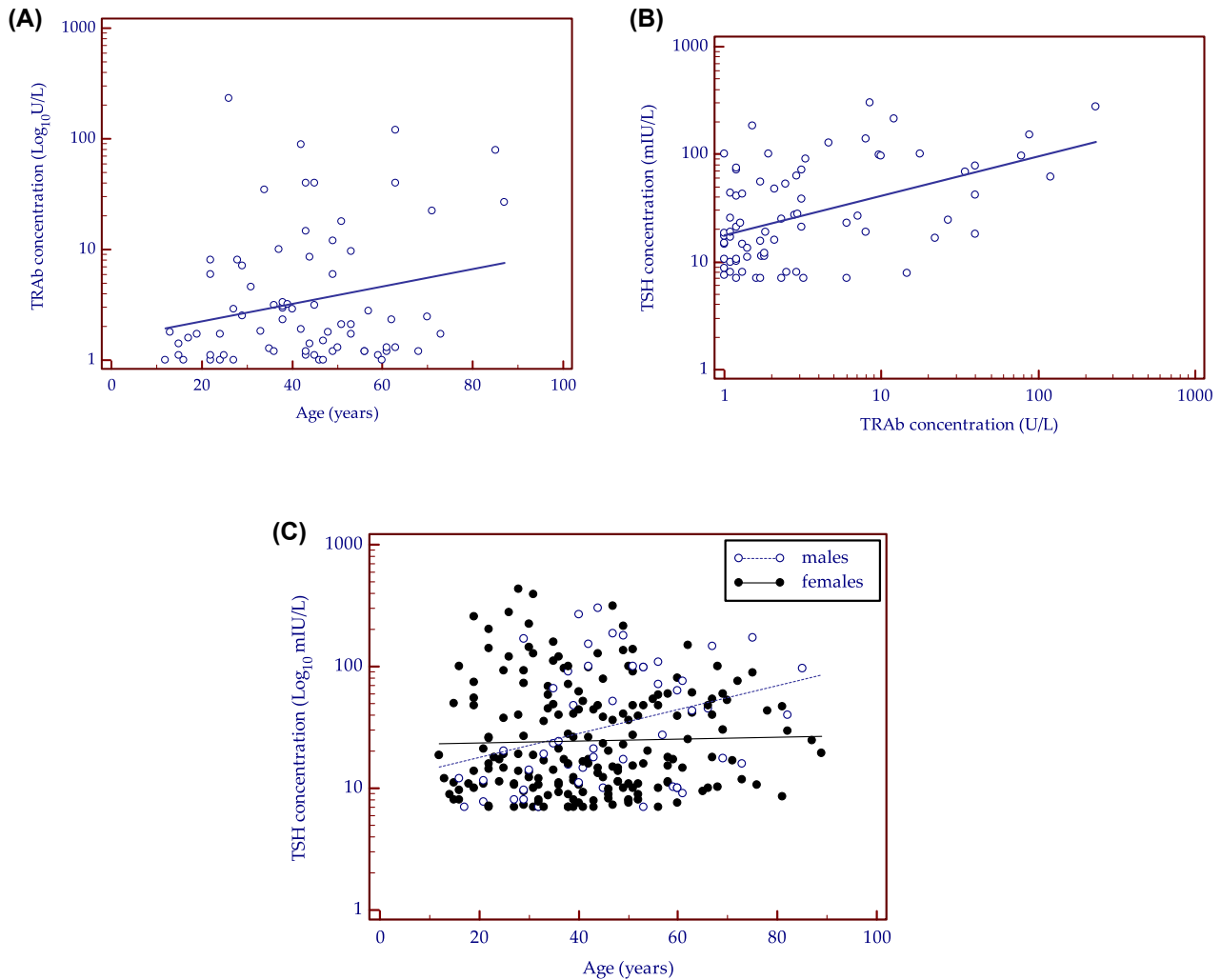


Figure 1: Linear regression analysis.

(A) Between age and TRAb levels ($p < 0.05$, F-ratio=4.026, $R^2=0.05$). (B) Between TSH and TRAb concentration ($p < 0.01$, F-ratio=21.087, $R^2=0.476$). (C) Between age and TSH concentration (in males $p < 0.05$, F-ratio=5.81, $R^2=0.10$; in females $p > 0.05$, F-ratio=0.15, $R^2=0.001$).

(58.3%) patients were positive for both TSAb and TBAb, 3/12 (25%) were positive for TSAb only and 2/12 (16.7%) were positive for TBAb only (Table 6).

Patients with CT and hypothyroidism tested for TSAb and TBAb

Six patients (numbers 2, 3, 4, 6, 7, and 10; Table 6) remained on L-thyroxine replacement with TSH within the normal range during the follow up. Two patients (numbers 5 and 12; Table 6) had post-partum thyroiditis. One patient (number 9; Table 6) had CT, hypothyroidism and ophthalmopathy. Three patients (numbers 1, 8, and 11; Table 6) treated with L-thyroxine experienced fluctuating thyroid function. Six patients are described in more detail below.

Patient 5: a 38 year old female delivered a baby with elevated TSH (67.9 mIU/L) detected on neonatal screening. The baby tested positive for TRAb (3.9 IU/L), positive for TBAb (100% inhibition) and negative for TSAb and was started on L-thyroxine. The mother was diagnosed with CT with hypothyroidism at one month post-partum with TSH 71.4 mIU/L, positive TPOAb (400 IU/L), negative TgAb, positive TRAb (3.7 IU/L), positive TBAb in both bioassays and negative TSAb in both bioassays (Table 6) and was started on L-thyroxine. After 6-months the baby became negative for TRAb and TBAb, L-thyroxine was discontinued and TSH remained in the normal range. However, the baby presented permanent neurological defects attributed to foetal hypothyroidism. The mother remained persistently positive for TRAb and TBAb and continued on L-thyroxine.

Patient 12: a 27 year old female delivered a healthy baby with normal TSH on neonatal screening. At 5 months

Table 6: Analysis of TBAb and TSAb in 12 TRAb-positive patients using CHO transfected with wild TSH-R and mutated TSH-R255D.

| Patient no. | Age (sex) | TSH, mIU/L | TRAb levels ≥ 2.5 IU/L | Wild type TSH-R | | Mutated TSH-R-R255D | |
|--------------------------|-----------|------------|-----------------------------|---------------------|---------------------|---------------------|---------------------|
| | | | | TBAb % ^a | TSAb % ^b | TBAb % ^a | TSAb % ^b |
| 1 | 43 (F) | 7.8 | 14.5 | 31 | 1,128 | 36 | 128 |
| 2 | 71 (F) | 16.8 | 22.1 | 32 | 1,867 | 39 | 276 |
| 3 | 63 (F) | 61.0 | 120.0 | 59 | 435 | 94 | 124 |
| 4 | 34 (F) | 68.1 | 34.5 | 14 | 200 | 77 | 139 |
| 5 | 38 (F) | 71.4 | 3.7 | 81 | 81 | 99 | 76 |
| 6 | 38 (M) | 89.7 | 3.3 | 18 | 152 | 34 | 107 |
| 7 | 85 (M) | 95.6 | 78.0 | 40 | 605 | 92 | 117 |
| 8 | 53 (M) | 98.0 | 9.6 | 32 | 1,576 | 62 | 406 |
| 9 ^c | 51 (M) | 100.0 | 17.8 | 0 | 3,230 | 46 | 1,401 |
| 10 | 31 (F) | 128.0 | 4.6 | 39 | 107 | 54 | 97 |
| 11 | 42 (M) | 152.0 | 88.0 | 79 | 235 | 95 | 171 |
| 12 | 27 (F) | 278.0 | 231.0 | 44 | 1,342 | 97 | 130 |
| Positive patients | | | 12/12 (100%) | 9/12 (75%) | 10/12 (83%) | 12/12 (100%) | 4/12 (33%) |

^aIn the blocking assay, samples that showed $\geq 30\%$ inhibition of stimulating activity of TSH were considered positive for TBAb (shown in bold).

^bSamples that caused increase of cyclic AMP concentrations $\geq 150\%$ relative to the healthy blood donor pool were considered positive for TSAb activity (shown in bold). ^cPatient with ophthalmopathy.

post-partum she developed severe hypothyroidism (TSH 278 mIU/L) and was started on L-thyroxine. At diagnosis she had high TRAb (231 IU/L) with positive TBAb activity in both bioassays and positive TSAb activity in the bioassay with wild type TSH-R (Table 6). She remained persistently positive for TRAb and continued on L-thyroxine.

Patient 9: a 51 year old man presented with CT, hypothyroidism (TSH 100 mIU/L) and ophthalmopathy with positive TRAb (17.8 U/L). Moderate TBAb activity (46%)

was detected in the bioassay with TSH-R R255D and highly positive TSAb activity (1401% and 3230%) in both bioassays (Table 6).

Patient 1: a 43 year old female with hypothyroidism (TSH 7.8 mIU/L) was on L-thyroxine for 2 years. Thereafter she stopped L-thyroxine and remained euthyroid for the following 2 years. TRAb levels, TSAb and TBAb activities at the time when she was in a hypothyroid and euthyroid state are summarized in Table 7.

Table 7: Thyroid parameters in the three patients with CT and fluctuating thyroid function. The Patient number refers to those in Table 6 (bold for positive values of TRAb, TBAb and TSAb).

| Patient (gender, age) | Disease phases and duration | Therapy | TSH (mIU/L) | TRAb levels (IU/L) ^a | Wild type TSHR | | R255D TSHR | |
|-----------------------|-----------------------------|---|-------------|---------------------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| | | | | | TBAb (%) ^b | TSAb (%) ^c | TBAb (%) ^b | TSAb (%) ^c |
| 1 (F, 43) | Hypothyroidism | Started L-thyroxine and continued for 2 years. | 7.8 | 14.5 | 31 | 1,128 | 36 | 128 |
| | Euthyroidism | Stopped L-thyroxine for 2 years | 2.15 | 11.6 | 34 | 731 | 34 | 137 |
| 8 (M, 53) | Hypothyroidism | Started L-thyroxine and continued for 1 year. | 98 | 9.6 | 32 | 1,576 | 62 | 406 |
| | Subclinical hyperthyroidism | Stopped L-thyroxine | 0.01 | 29.4 | 0 | 2,492 | 74 | 524 |
| | Euthyroidism | No treatment for the subsequent 2 years | 3.8 | 9.8 | 0 | 1,957 | 49 | 469 |
| 11 (M, 42) | Hypothyroidism | Started L-thyroxine and continued for 2 years | 152 | 88 | 79 | 235 | 95 | 171 |
| | Euthyroidism | Stopped L-thyroxine for 1 year | 0.99 | 183 | 55 | 627 | 89 | 327 |
| | Hyperthyroidism | Started methimazole | 0.01 | 30 | 12 | 1,188 | 78 | 546 |
| | Hyperthyroidism | Continued with methimazole for the subsequent 5 years | 1–3 | 28.6 | 36 | 629 | 81 | 195 |

^aTRAb concentration positive ≥ 2.5 IU/L (shown in bold). ^bIn the blocking assay, samples that showed $\geq 30\%$ inhibition of stimulating activity of TSH were considered positive for TBAb (shown in bold). ^cSamples that caused increase of cyclic AMP concentrations $\geq 150\%$ relative to the healthy blood donor pool were considered positive for TSAb activity (shown in bold).

Patient 8: a 53 year old male with hypothyroidism (TSH 98 mIU/L) was on L-thyroxine for one year. He then progressed to subclinical hyperthyroidism, stopped L-thyroxine, became euthyroid and remained euthyroid for the following 2 years. Results of TRAb, TSAb and TBAb measurements in different phases of his thyroid function are summarized in Table 7.

Patient 11: a 42 year old male with hypothyroidism (TSH 152 mIU/L) was on L-thyroxine for 2 years. After 2 years he stopped L-thyroxine and remained euthyroid for the following year. Thereafter he developed hyperthyroidism and was treated with methimazole for the following 5 years. The measurements of TRAb, TBAb, and TSAb at different phases of his thyroid function are summarized in Table 7.

Discussion

In this study 13.1% of patients with CT and hypothyroidism had detectable TRAb. The meta-analysis of the results reported in 24 previous studies including this report revealed that there was a significant heterogeneity in TRAb prevalence (Test for heterogeneity: $Q\ 135.4$, $p < 0.0001$, $I^2 84.5\%$) (Supplementary Table 2).

The present study included the largest cohort of Italian patients with CT and hypothyroidism described to date and the largest cohort of patients with CT and hypothyroidism from one nation tested for TRAb.

In a recent study 35/656 (5%) of patients with CT without thyroid associated ophthalmopathy had detectable TRAb [30]. In contrast, 22/44 (50%) of patients with CT and ophthalmopathy were TRAb positive and 30/44 (68.2%) were TSAb positive [30]. In our study 1/5 (20%) patient presenting with CT, hypothyroidism and ophthalmopathy was TRAb-positive with both TBAb and TSAb activities in the bioassays (patient 9; Table 6). Overall, 31/240 (12.9%) of patients with CT and hypothyroidism without ophthalmopathy in our study were TRAb positive and this prevalence is higher than reported by Kahaly et al. [30]. However, our study population included all CT patients with associated hypothyroidism while the majority of CT patients in the other study had normal thyroid function [30].

The levels of TRAb in patients in our study varied and approximately half had TRAb >7.5 IU/L. TRAb positivity was significantly higher in patients with high TSH levels (>19 mIU/L) than in those with moderate TSH levels. Furthermore, TRAb positivity was significantly higher in males than in females. TSH levels were significantly higher in TRAb-positive than in TRAb-negative patients and TSH levels were significantly higher in the patients with high

levels of TRAb (>7.5 IU/L) compared to patients with lower levels of TRAb (<7.5 IU/L).

The linear regression analyses showed a correlation between age and TRAb concentrations ($p < 0.05$), between TSH levels and TRAb levels ($p < 0.01$) and between TSH levels and age in males ($p = 0.0196$) but not in females (Figure 1). Furthermore, the prevalence of TRAb in patients with reduced thyroid dimensions was significantly higher than in patients with normal/increased thyroid dimensions (Table 5) and this is in agreement with a previous study [44].

In this study, 15% of females of child bearing age (16–45 years old) were TRAb positive and a large proportion had high TRAb concentrations (>7.5 IU/L). Maternal TRAb may be responsible for foetal and neonatal transient thyroid dysfunction particularly if present at high concentrations [45–55]. Foetal and neonatal thyroid dysfunctions (both hypothyroidism and hyperthyroidism) are often associated with serious clinical symptoms and negative long-term developmental outcomes [44, 48, 51, 53–59]. In view of a potential harm to the developing foetus caused by maternal TRAb we suggest that TRAb should be tested in all females of child-bearing age at the time of first diagnosis of CT and hypothyroidism. TRAb testing should also be offered to women of child bearing potential with a CT and hypothyroidism diagnosis who are on L-thyroxine and may be in a euthyroid state at the time of planning a pregnancy or in the early stages of pregnancy. Further to the guidelines on testing for thyroid autoimmune disease in pregnancy [60] TRAb test would be a helpful addition to TPOAb/TgAb in females with confirmed CT and hypothyroidism. In our study 11 women developed CT and hypothyroidism post-partum and 2/11 (18.2%) had high TRAb levels. This was most likely related to the rebound immune response to the TSH-R post-partum.

At least one type of thyroid autoantibody (TPOAb and/or TgAb and/or TRAb) was found in 192/210 (91.4%) of patients with CT and hypothyroidism and TRAb positivity correlated with TPOAb/TgAb positivity. However, 10% of patients negative for TPOAb/TgAb were positive for TRAb only. Consequently, TRAb testing may be a useful diagnostic tool in TPOAb/TgAb negative patients with hypothyroidism and normal echogenic pattern to exclude rare causes of hypothyroidism such as TSH-R gene mutations [61].

A previous study reported that the presence of TRAb was correlated to both thyroid function and to reduced thyroid volume in euthyroid or hypothyroid patients with CT [62]. Our study confirmed a significant correlation between reduced thyroid dimensions and the presence of TRAb. In our study population a proportion of patients also had coexisting clinical or subclinical autoimmune diseases

as described previously [63, 64]. However, the prevalence of TRAb was not significantly different in patients with CT with or without other autoimmune disorders.

TRAb functional activity was tested in bioassays with the wild type and mutated TSH-R. The bioassay with mutated TSHR R255D allows determination of TBAb with reduced interference of stimulating TRAb [40–42]. The majority of patients studied had a mixture of TRAb with both activities, some had TBAb only and some had TSAb only (Table 6). In patients with a mixture of TSAb and TBAb the prevailing activity and/or concentration of TBAb may result in clinical hypothyroidism while prevailing activity of TSAb may result in hyperthyroidism or the net effect may be clinical euthyroidism [10]. Patient 11 (Table 7) after 2 years of hypothyroidism became spontaneously euthyroid for one year and then developed persistent clinical hyperthyroidism. This patient's TBAb activity remained unchanged throughout the various phases of the disease while TSAb activity varied with the highest activity coinciding with the presentation of hyperthyroidism. Similar mechanisms may be responsible for changes in thyroid function in patients one and eight (Table 7). The transition of thyroid function from hypothyroidism to hyperthyroidism/euthyroidism, as seen in our patients, has been reported previously [24, 41, 65–69]. However, the activities of TRAb are unlikely to be solely responsible for thyroid function in autoimmune thyroid disease. The effects of T-lymphocyte infiltration and autoimmune inflammation/destruction on the ability of the follicular cells to respond to TSH, TSAb, and TBAb also play an important role in the pathogenesis of thyroid dysfunction [7–10, 65].

The study limitation is that this is not a prospective study. Furthermore, only a limited number of patients had the TRAb functional activity assessed in the bioassays.

Two recent reviews have discussed about the use of TRAb in patients with CT. The first have suggested that testing for TRAb including their bioactivity is currently recommended for the diagnosis and management of pregnant patients and patients with thyroid eye disease [70]. The second have suggested that the evaluation of TRAb is recommended in case of patients with thyroid associated orbitopathy not associated with hyperthyroidism. At present, however, the most relevant recommendation for the use of TRAb assay is in patients with CT secondary to a known agent; in particular, after treatment with alemtuzumab for multiple sclerosis. However, the routine use of TRAb or TSAb/TBAb assay

cannot be suggested at the present for diagnosis/follow up of patients affected by CT; there are, however, several conditions where their detection can be clinically relevant [71].

Our study, in addition the above recommendations, suggests that TRAb should be extended to women of child bearing potential with a CT and hypothyroidism diagnosis at the time of planning a pregnancy or in the early stages of pregnancy also if they are in euthyroidism under replacement therapy, in the phases of thyroid dysfunction in the post-partum thyroiditis. The measurement of TRAb should also be helpful for differential diagnosis in TPOAb and/or TgAb negative patients with hypothyroidism. Furthermore, TRAb testing and the assessment of TSAb and TBAb should also be helpful in patients with fluctuating thyroid function.

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