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The association of TSH-receptor antibody with the clinical and laboratory parameters in patients with newly diagnosed Graves' hyperthyroidism: experience from a tertiary referral center including a large number of patients with TSH-receptor antibody-negative patients with Graves' hyperthyroidism

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Abstract

Introduction: Although the TSH-receptor antibody (TRAb) plays a central role in the pathogenesis of Graves' disease (GD), the association between TRAb at first diagnosis and clinical and laboratory parameters is not well known. On the other hand, a minority of patients with GD may be TRAb negative, and there is a lack of adequate evidence to demonstrate the clinical and laboratory characteristics of these patients. Therefore, we aimed to investigate the association of TRAb at the initial diagnosis of GD with the clinical and laboratory parameters in a large number of patients with GD and to compare the clinical and laboratory parameters between patients with high TRAb levels and TRAb-negative patients.

Material and methods: This study included 440 patients [326 (74%) female, 114 (26%) male]. All patients were classified according to gender, age, smoking habit, and TRAb levels.

Results: TRAb levels were significantly higher in male compared to female patients and in smokers compared to non-smokers. Smoking male patients had the highest TRAb levels. In regression analysis, goiter size, male gender, cigarette smoking, Graves' orbitopathy, fT3, and anti-TPO antibody levels were independently associated with high TRAb levels, while age at diagnosis and fT4 levels were not independently associated with high TRAb levels. TRAb-negative GD was diagnosed in 80 (18%) patients. TRA-negative patients had markedly less severe clinical and laboratory hyperthyroidism compared to patients with high TRAb levels. Moreover, the smoking habit was significantly lower in patients with TRAb-negative GD.

Conclusions: According to our study results, TRAb levels at the initial diagnosis of GD are differently associated with clinical and laboratory parameters. Male patients and smoking patients with GD tended to have markedly higher TRAb levels and more severe clinical hyperthyroidism. Therefore, besides other contributing factors, male gender and smoking may affect TRAb levels and consequently the severity of hyperthyroidism in patients with GD. Furthermore, male gender and smoking may have a synergistic effect on TRAb levels and consequently on the severity of hyperthyroidism in patients with GD. **(Endokrynol Pol 2021; 72 (1): 14–21)**

Key words: *Graves' disease; hyperthyroidism; TRAb; TRAb-negative; smoking*

Introduction

Graves' disease (GD) is a chronic autoimmune disease of the thyroid gland with an incidence of 21 cases per 100,000 per year and is characterized by diffuse goiter, hyperthyroidism, Graves' orbitopathy (GO), and rarely by localized dermopathy and thyroid acropachy [1, 2]. Although the pathogenesis of GD is not fully understood, the hyperthyroidism and goiter in GD are related to the TSH-receptor antibody (TRAb), which binds to and activates the TSH receptors (TSHR) on the surface of the follicular thyroid cells [3].

Although the measurement of TRAb is the easiest way to diagnose GD accurately, in recent American Thyroid Association (ATA) and European Thyroid Association (ETA) Guidelines for the management of hyperthyroidism, measurement of TRAb levels at first admission is not routinely suggested in all patients with GD and is only suggested in patients with thyrotoxicosis in whom a differential diagnosis could not be accurately

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made based on the clinical and laboratory results [4, 5]. Therefore, the number of studies evaluating the relationship between TRAb and clinical and laboratory parameters during the initial diagnosis of GD is relatively low. Most of the existing studies focus only on the relationship between TRAb and GO.

One of the important clinical factors associated with the development of GD, the occurrence of relapse after ATD treatment, and the development and severity of GO is cigarette smoking [4, 6–8]. Evidence suggests that, after total thyroidectomy, TRAb persists for a longer time in the sera of smoking patients with GD compared to non-smoking patients [9]. Therefore, smoking may prolong the half-life of TRAb. However, the answer to the question *Does smoking have any association with TRAb levels at the initial diagnosis of GD?* is not clear yet.

Autoimmune diseases are more frequent among females. However, some autoimmune diseases such as autoimmune hepatitis and systemic lupus erythematosus (SLE) are associated with worse outcomes in male patients [10]. Evidence suggests that compared to female patients, male patients with GD have more severe disease, a higher frequency of GO, and a higher frequency of relapse after ATD discontinuation [11]. The age of the patient during the first diagnosis is another factor associated with the outcome of GD. Patients < 40 years old at diagnosis have also a higher risk of relapse after treatment with ATDs [12, 13]. However, the association between gender as well as age with TRAb at the initial diagnosis of GD is not well known.

The sensitivity and specificity of TRAb for diagnosis of GD differ according to the TRAb detection method. The sensitivity and specificity of TRAb measured by a third-generation electrochemiluminescence immunoassay method for diagnosis of GD have been reported to be 96.2% and 95.2%, respectively [14]. However, even assessed by a third-generation method, TRAb could be lower than cut-off values in 5–10% of the patients with GD [15], a condition known as TRAb-negative GD. Although TRAb-negative GD is a rare condition, the assessment of the TRAb by older laboratory methods has generally been associated with negative results [16]. On the other hand, TRAb-negative GD patients have been demonstrated to have markedly different histopathological features compared to patients with high TRAb levels [17]. Therefore, TRAb-negative GD has been suggested to be a subtype of GD, but due to the rarity of the condition, most studies have been conducted in a small number of patients, and therefore there is a lack of evidence to demonstrate the clinical and laboratory characteristics of the patients with TRAb-negative GD, and to compare the clinical and laboratory parameters between TRAb-negative patients with GD and those with high TRAb levels. Therefore, in the present study, we aimed to investigate the association of TRAb at the initial diagnosis of GD with clinical and laboratory parameters in a large number of patients with GD and to compare the clinical and laboratory parameters between patients with high TRAb levels and TRAb-negative GD as well.

Material and methods

Patients

This retrospective study included 440 patients [326 (74%) female, 114 (26%) male] who were newly diagnosed with GD from January 2015 through January 2020 in a tertiary endocrinology referral center. Patients under treatment with antithyroid drugs, patients with a previous history of GD, patients < 17 years old, patients with a prior history of radioiodine ablation therapy or thyroidectomy, pregnant patients with hyperthyroidism of any kind, patients with amiodarone-induced hyperthyroidism, patients with toxic multinodular goiter and toxic adenoma, patients with any kind of thyroiditis, and patients in whom the exact cause of hyperthyroidism could not be determined were not included in the study. Graves' disease with high TRAb levels was defined as previously reported [11]. In brief, GD with high TRAb levels was diagnosed according to clinical hyperthyroidism with or without extrathyroidal manifestations associated with a suppressed serum levels of thyroid-stimulating hormone (TSH), elevated serum of free thyroxine (fT4), and/or free tri-iodothyronine (fT3) levels, a diffuse hypoechoic ultrasonographic appearance of the thyroid gland, and high TRAb titers. Nevertheless, TRAb-negative GD was diagnosed according to the clinical and biochemical findings of hyperthyroidism associated with a high uptake of ^{99m}Tc pertechnetate, in which a diffuse thyroid overactivity with a homogeneous distribution of the radiotracer and a decreased uptake in salivary glands were accepted as diagnostic for GD, elevated thyroid gland vascularity on color-Doppler ultrasonography and/or assessment of the inferior thyroid artery peak-systolic velocity by color-flow Doppler ultrasonography [15,18], the presence of anti-thyroid peroxidase (anti-TPO) and/or anti-thyroglobulin (anti-TG) antibodies, history of other autoimmune diseases, family history of autoimmune thyroid disease, follow-up data (relapse after antithyroid drug treatment, and TRAb levels after relapse), and exclusion of the other causes of hyperthyroidism stated above. The study protocol was approved by the Local Ethics Committee.

Clinical and laboratory data

All clinical and laboratory data of the patients were obtained through the computer records of our center. The following data of the patients at first admission were recorded: gender, age, serum TRAb, TSH, fT3, fT4, anti-TPO, and anti-thyroglobulin (anti-TG) antibody levels, goiter size by palpation according to the WHO classification (0–III), smoking status (yes and no) and GO. In the case of missing data, we complemented missing data by phone calls to patients. GO in this study was classified according to Werner's criteria as absent (class 0–1) or present (class 2–6: the presence of inflammatory signs, proptosis, extraocular muscle, and corneal involvement) [19]. To assess the TRAb levels according to age, the age of the patients was divided as < 40 and ≥ 40 years old, due to a higher frequency of relapse observed in patients < 40 years old. However, to assess also the possible effect of menopause on TRAb levels, the age of the female patients was also divided as < 50 and ≥ 50 years old.

After blood withdrawal, samples for the TSH, fT3, ft4, anti-TPO, and anti-TG were centrifuged and analyzed while serum samples for the measurement of TRAb were stored at –20°C and analyzed weekly. Serum TRAb levels were detected using an electrochemiluminescence immunoassay method (Cobas e 801 analyzers, Roche

Diagnostics, Mannheim, Germany). This method uses a biotinylated mouse monoclonal antibody that binds to the C-terminal moiety of the TSH receptor to immobilize solubilized porcine TSH receptor using magnetic microparticles. This capture antibody does not interfere with the binding of another monoclonal antibody (M22) to the TSHR [14]. TRAb is detected by its ability to inhibit the binding of ruthenium-labeled M22 to the TSH receptor. In this study, the cut-off value of TRAb for untreated GD was accepted as 1.75 IU/L, as recommended by the manufacturer, with a maximum detection capacity of 40 IU/mL (inter-and intra-assay coefficient of variation < 17.3% and < 10.3%). Serum TSH, fT3, fT4, anti-TPO, and anti-TG antibody levels were also detected using a chemiluminescence immunoassay method (Cobas e 801 analyzers, Roche Diagnostics, Mannheim, Germany). The normal reference values and inter- and intra-assay coefficient of variations for TSH, fT3, fT4, anti-TPO, and anti-TG antibody levels were as follows: 0.27–4.2 μIU/mL (< 7.2% and < 3%), 2.0–4.4 pg/mL (< 7.2% and < 6.5%), 0.93–1.7 ng/dL (< 78.4 and < 4.3%), 5–600 IU/mL (< 9.5% and < 6.3%), and 10–4000 IU/mL (< 6.3% and 4.9%), respectively.

Statistical analysis

PASW Statistics 18 for Windows was used for data input and statistical analysis. Mean ± standard deviation and frequencies were used to state results. The Independent Sample T-test was used for the comparison of normally distributed data, and the Mann-Whitney U test was used for the comparison of data that did not follow a normal distribution. Analysis of variance (ANOVA) followed by post-hoc analysis was used for multiple comparisons. χ^2 analysis was used for categorical data comparison. Pearson correlation analysis was used to determine the correlation between variables. A multiple regression analysis was used to assess the association between each clinical and laboratory variable with TRAb. All tests were two-tailed and a p-value < 0.05 was considered statistically significant.

Results

A total of 440 patients (326 [74%] female, 114 [26%] male; mean age 41.84 ± 13.26 years) were included in this study. The clinical and biochemical characteristics of the patients as a whole group and according to gender are shown in Table 1.

In the present study, when all patients were analyzed as a single group, TRAb levels at the initial diagnosis were significantly higher in male compared to female patients (13.83 ± 12.17 *vs*. 9.56 ± 10.31 IU/L, p < 0.001). As demonstrated in Table 1, compared to female patients, male patients at the initial diagnosis more commonly had GO, were more often smokers, and tended to have larger goiter sizes. Although a mild inverse correlation was found between TRAb levels and age (r = 0.10, p = 0.029) when the age of the patients was divided as < 40 and ≥ 40 years old, TRAb levels were significantly higher in patients < 40 compared to the patients ≥ 40 years old (12.07 ± 11.43 *vs*. 9.45 ± 10.31 IU/L, p = 0.012). Nevertheless, when TRAb levels were separately analyzed according to age and gender, TRAb levels were only significantly

Table 1. *Clinical and laboratory characteristics of the study participants as a single group and according to gender*

Variables	All patients 440 (100%)	Female 326 (74%)	Male 114 (26%)	p-value*
Age	41.8 ± 13.26	40.8 ± 12.9	44.6 ± 13.9	0.01
TRAb [IU/L]	10.66 ± 10.97	9.56 ± 10.31	13.83 ± 12.17	< 0.001
TSH [IU/mL]	0.009 ± 0.047	0.011 ± 0.055	0.006 ± 0.004	0.32
fT3 [pg/mL]	10.95 ± 6.52	10.65 ± 6.17	11.82 ± 7.41	0.09
fT4 [ng/dL]	3.45 ± 1.73	3.36 ± 1.70	3.70 ± 1.79	0.07
Anti-TPO [IU/mL]	210.1 ± 208.1	197.5 ± 204.7	246.4 ± 214.6	0.03
Anti-TG [IU/mL]	339.5 ± 710.5	338.4 ± 698.1	342.4 ± 747.8	0.96
Smoking status (Yes/No)	179/261 (41/59)	112/214 (34/66)	67/47 (59/41)	< 0.001
Goiter size				
0	39 (9)	29 (9)	10 (9)	0.05
1	198 (45)	158 (49)	41 (36)	
2	147 (33)	102 (31)	45 (39)	
3	55 (13)	37 (11)	18 (16)	
Orbitopathy (Yes/No)	118/322 (27/73)	76/250 (23/77)	42/72 (37/63)	0.004

TRAb — TSH-receptor antibody; TSH — thyroid-stimulating hormone; fT3 — free tri-iodothyronine; fT4 — free thyroxine; anti-TPO — anti-thyroid peroxidase; anti-TG — anti-thyroglobulin

Mean ± standard deviation; *the difference between male and female patients.

The laboratory results for anti-TPO and anti-TG were missed in 5 and 11 patients, respectively

Normal reference values: TRAb < 1.75 IU/L, TSH 0.27–4.2 μIU/mL, fT3 2.0–4.4 pg/mL, fT4 0.93–1.7 ng/dL, anti-TPO 0–34 IU/mL, and anti-TG 0–115 IU/mL.

higher in male patients < 40 compared to the male patients > 40 years old (18.48 ± 12.24 *vs*. 10.44 ± 11.02 IU/L, p <0.001), while TRAb levels were not significantly different between females < 40 and ≥ 40 as well as < 50 and ≥ 50 years old (10.09 ± 10.4 *vs*. 9.07 ± 10.2 IU/L, p = 0.37 and 9.59 ± 10.27 *vs*. 9.47 ± 10.46 IU/L, p = 0.92, respectively).

Taking into consideration the smoking habits of the patients, TRAb levels at the initial diagnosis were significantly higher in smoking patients compared to non-smoking patients (12.05 ± 11.61 *vs*. 9.71 ± 10.42 IU/L, p = 0.028). However, when TRAb levels were analyzed according to the smoking habits in male and female patients separately, smoking male patients had the highest TRAb levels compared to the non-smoking male, and smoking and non-smoking females (14.96 ± 10.9, 12.2 ± 11.9, 10.3 ± 10.8, and 9.1 ± 10 IU/L; p = 0.049, p = 0.029, and p = 0.001, respectively). Furthermore, although statistically it did not reach significance, in post-hoc analysis, smoking male patients < 40 years old had the highest TRAb levels compared to smoking males ≥ 40 years old and smoking female patients < 40 and ≥ 40 years old (17.2 ± 12.3, 12.4 ± 11.9, 12 ± 11.7, and 8.5 ± 9.7 IU/L; p = 0.064, p = 0.056, and p = 0.003, respectively).

In this study, although a strong linear correlation was found between TRAb levels at diagnosis and goiter size (r = 0.66, p < 0.001), in post-hoc analysis, TRAb levels were significantly higher in patients with grade 3 compared to the patients with grade 0, 1, and 2 goiters, (21.8 ± 12.03, 1.87 ± 1.92, 5.5 ± 5.83, 15.82 ± 11.43 IU/L, respectively; p < 0.001 for all comparisons). Weak linear correlations were also found between TRAb and fT3, fT4, and anti-TPO antibody levels (r = 0.33, p < 0.001, r = 0.23, p = 0.01, and r = 0.29, p < 0.001, respectively). In regression analysis, goiter size, male gender, GO, cigarette smoking, fT3, and anti-TPO antibody levels were independently associated with high TRAb levels (OR = 6.42, 95% CI: 5.33–7.83, p < 0.001; OR = 2.59, 95% CI: 1.53–4.52, p = 0.009; OR = 2.91, 95% CI: 1.01–4.82, p = 0.003, OR = 1.27, 95% CI: 1.03–1.56, p = 0.046; OR = 3.5, 95% CI: 2.08–10.98, p = 0.004; OR = 11.11, 95% CI: 7.6–20.12, p < 0.001, respectively), while age at diagnosis as well as fT4 levels were not independently associated with high TRAb levels (OR = 1.11, 95% CI: 0.69–1.64, p = 0.29 and OR = 1.13, 95% CI: 1.03–2.53, p = 0.37).

Among the participants, 80 (18%) patients were found to have TRAb-negative GD (< 1.75 IU/L). Due to the higher frequency of TRAb-negative GD among the patients included in our study, all of the patients diagnosed as TRAb-negative GD were carefully assessed by clinical evaluation, and laboratory and imaging studies to avoid a false diagnosis of GD. ^{99m}Tc pertechnetate uptake was performed in all of these patients, and 76 patients (95%) had high uptake values, while the remaining four patients (5%) had typical color Doppler ultrasonography appearances of thyroid inferno (markedly increased vascularity with homogeneous distribution within the thyroid parenchyma) and an increased (> 30 cm/s) inferior thyroid artery peak-systolic velocity on color-flow Doppler ultrasonography. Moreover, 39 (49%) and 29 (37%) of the patients with TRAb-negative GD had increased anti-TPO and anti-TG antibody levels, respectively, and 10 (12.5%) patients had a mild GO that improved spontaneously. Among the patients with TRAb-negative GD, 16 (20%) experienced a relapse during the follow-up. TRAb levels became positive (> 1.75 IU/L) in two patients after relapse, while they remained negative (< 1.75 IU/L) in the remaining patients. On the other hand, 14% of the TRAb-negative patients had a history of another autoimmune disease, including rheumatoid arthritis, type-1 diabetes, psoriatic arthritis, primary biliary cirrhosis, and vitiligo, and 33% of them had a family history of autoimmune thyroid disease. As in patients with high TRAb levels, TRAb-negative GD was also more common among females compared to male patients, while age at the initial diagnosis was not different between patients with high TRAb levels and TRAb-negative GD (Tab. 2). However, as demonstrated in Tab. 2, fT3, fT4, anti-TPO, and goiter sizes of the patients were significantly different between TRAb-negative patients and patients with high TRAb levels (p < 0.001, p = 0.001, p < 0.001, and p < 0.001, respectively). As shown in Table 2, the smoking habit and GO were also more frequent among GD patients with high TRAb levels as compared to TRAb-negative patients. The difference between clinical and laboratory parameters of the GD patients with TRAb-negative disease and high TRAb levels are shown in Table 2. Conversely, 18 (4%) patients [8 (7%) male and 10 (3%) female] had TRAb levels > 40 IU/L. However, although the frequency of male patients with TRAb levels > 40 IU/L was higher than female patients, the number of patients with TRAb levels > 40 IU/L was not adequate for comparison of clinical and laboratory parameters.

Discussion

In this study, TRAb levels at the initial diagnosis of GD were differently associated with clinical and laboratory parameters. TSH-receptor antibody levels were significantly higher in male patients, and male patients tended to have markedly larger goiter sizes at diagnosis as well as more common GO, as compared to female patients.

Autoimmune diseases are generally more prevalent in females than males; however, the severity of each autoimmune disease could be different between gen-

Table 2. *The difference between clinical and laboratory characteristics of patients with high TRAb levels and patients with TRAb-negative Graves' disease (GD)*

Variables	Patients with high TRAb* 360 (82%)	TRAb-negative patients† 80 (18%)	p-value*
Age	41.4 ± 13.03	43.8 ± 14.13	0.14
Gender (female/male)	263/97 (73/27)	63/17 (79/21)	0.18
TRAb [IU/L]	12.81 ± 11.03	0.99 ± 0.43	< 0.001
fT3 [pg/mL]	11.55 ± 6.75	8.25 ± 4.55	< 0.001
fT4 [ng/dL]	3.57 ± 1.78	2.88 ± 1.35	0.001
Anti-TPO [IU/mL]	231.5 ± 213.6	114.4 ± 148.34	< 0.001
Anti-TG [IU/mL]	370.4 ± 748.9	203.7 ± 489.2	0.06
Smoking status (Yes/No)	155/205 (43/57)	24/56 (30/70)	0.02
Goiter size			
0	16 (4.4)	23 (28.7)	< 0.001
1	147 (40.8)	51 (63.7)	
2	142 (39.4)	5 (6.3)	
3	54 (15)	1 (1.3)	
Orbitopathy (Yes/No)	108/252	(30/70) 10/70	(12.5/87.5) 0.001

TRAb — TSH-receptor antibody; fT4 — free thyroxine; fT3 — free tri-iodothyronine; anti-TPO — anti-thyroid peroxidase; anti-TG — anti-thyroglobulin

*Patients with TRAb level of > 1.75 IU/L, †patients with TRAb levels < 1.75 IU/L. The laboratory results for anti-TPO and anti-TG were missed in 5 and 11 patients, respectively. Normal reference values: TRAb < 1.75 IU/L, TSH 0.27–4.2 μIU/mL, fT3 2.0–4.4 pg/mL, fT4 0.93–1.7 ng/dL, anti-TPO 0–34 IU/mL, and anti-TG 0–115 IU/mL

ders [10]. For instance, studies suggest that course of some autoimmune diseases such as psoriasis, autoimmune hepatitis, SLE, and multiple sclerosis are more severe in males compared to females. Although the reason is not exactly clear yet, some genetic, environmental, and hormonal causes have been suggested as the reasons for the different gender prevalence, and a different course of autoimmune disease between genders [10]. Although the cause is not clear yet, evidence suggests that compared to female patients, male patients with GD have a significantly higher frequency of relapse after antithyroid drug discontinuation, as well as a more severe disease course [11, 20]. In the present study, the male gender was found to be a significant independent contributor to TRAb levels in patients with GD. However, although TRAb levels were significantly higher in younger male patients, in regression analysis, age at diagnosis was not found to be an independent contributor to TRAb levels. We believe that higher TRAb levels observed in male patients at the initial diagnosis of GD in our study could support the results of prior studies reporting a higher frequency of relapse after discontinuation of the antithyroid drugs as well as a more severe disease course in male patients with GD [11, 12, 20].

The risk of developing GD is higher among smokers. Cigarette smoking affects innate and adaptive immune systems with both pro-inflammatory and immunosuppressive effects, but it is still unknown how smoking might enhance immune pathways like T helper-2 responses, leading to GD [6]. In a meta-analysis, the odds ratio (OR) of developing GD was 3.3 (95% CI: 2.09–5.22) in current smokers compared with 1.41 (95% CI: 0.77–2.58) in never smokers [8]. Smoking has been suggested to have a modifying immunological consequence and an adverse impact on the course of GD after the withdrawal of antithyroid drug treatment [21]. In a study by Yoshioka et al., smoking was associated with a prolonged half-live of the serum TRAb levels after total thyroidectomy in patients with GD [9]. Although the underlying mechanism by which smoking contributes to the high serum level of TRAb is not clear, smoking might influence cytokine secretion and consequently increase TRAb levels [22, 23]. However, there is a lack of evidence in terms of the association between TRAb levels at the initial diagnosis of GD and smoking. In our study, TRAb levels at the initial diagnosis were significantly higher in smokers compared to non-smokers (12.05 ± 11.61 *vs.* 9.71 ± 10.42 IU/L, p = 0.028), and the highest TRAb level was observed in smoking male patients. Moreover, in regression analysis, smoking was also found to be an independent contributor to TRAb levels. As demonstrated in Table 1, 59% of the male and 34% of the female patients

were current smokers. Furthermore, 73% of the male patients < 40 years old, the group with the highest TRAb levels were current smokers compared to 37% of the female patients of the same age. Therefore, the higher TRAb levels found in younger male patients may be related to the higher frequency of smoking among these patients. In contrast, some studies could not find a relationship between smoking and TRAb. For instance, in a retrospective study, Nyirenda et al. could not find a difference in TRAb levels between smoking and non-smoking patients with GD [24]. Nevertheless, it should be emphasized that only 24 patients in their study were current smokers. According to our study results, cigarette smoking can affect TRAb levels and consequently the severity of hyperthyroidism in patients with GD. Moreover, smoking and male gender may have a synergistic effect on TRAb levels as well as on the severity of hyperthyroidism in patients with GD.

Most of the patients with GD have a total T3/T4 ratio of > 20 [4, 25]. The higher T3 levels in patients with GD are thought to be related to activation of type-II deiodinase by TRAb, an enzyme that is abundantly expressed in human thyroid tissues [26]. Our study results are in line with previous study results. Although a weak linear correlation was found between TRAb and fT3 levels, the correlation between TRAb and fT4 was even weaker. However, in regression analysis, only fT3 levels were independently associated with high TRAb levels, and therefore this result of our study may explain, in part, the higher fT3/fT4 ratio observed in the majority of the patients with GD [4, 25].

TRAb assays methods have improved over the years. The first-generation TRAb assay detects TRAb levels in a competition assay in which the patient's sera is mixed with radiolabeled TSH and TRAb competes for binding to the TSH receptors in the liquid phase. The second-generation TRAb assay improved the sensitivity of the assay by fixing the TSH receptors to the bottom of the wells (solid phase), which reduces the interference with the labeled TSH. Nowadays, TRAb is generally measured by third-generation assays, in which TRAb competes with anti-TSH receptor monoclonal antibody (M-22) rather than TSH [14]. However, as stated previously, even assessed by a third-generation method, TRAb could be lower than the predetermined cut-off values in 5–10% of patients with GD, a condition known as TRAb-negative GD [15], and the clinical and laboratory characteristics of these patients are not fully understood. In a study conducted by Kawai et al., TRAb-negative GD patients in whom TRAb remained negative throughout treatment with anti-thyroid drugs had a markedly lower risk of relapse than patients with high TRAb levels at diagnosis and those who become TRAb positive during treatment [27]. In another study by Kawai et al., TRAb-negative GD patients had markedly different histopathological features as compared to GD patients with high TRAb levels [17]. Papillate hyperplastic epithelia were significantly less severe, and enlarged colloid was less common in patients with TRAb-negative GD compared to patients with high TRAb levels. Likewise, moderate or marked lymphocytic infiltrations were also observed in all patients with TRAb-negative GD but were virtually absent in patients with high TRAb levels [17]. In a study by Mukuta et al., untreated TRAb-negative patients with GD had a mild elevation of thyroid hormones, mildly elevated ^{123}I uptake, and small goiters as compared to patients with high TRAb levels [28]. However, in their study, the patients with high TRAb levels used for the comparison were randomly selected among a huge number of patients with GD [28]. Although in our study almost all patients who were TRAb-negative and those with high TRAb levels were included, the results are in line with those of the study by Mukuta et al. and suggest markedly lower thyroid hormones, anti-TPO, and anti-TG antibody levels in patients with TRAb-negative GD as compared to GD patients with high TRAb levels (Tab. 2). As demonstrated in Tab. 2, our study results also suggest that TRAb-negative GD may be a less severe subtype of GD with mild to moderate hyperthyroidism, very mild GO in a minority of the patients, and smaller goiter sizes, as compared to GD patients with high TRAb levels. Although no difference in terms of age and gender was found between TRAb-negative patients and patients with high TRAb levels, the smoking habit was more common among patients with high TRAb levels (Tab. 2). This outcome of our study demonstrates again that cigarette smoking could affect TRAb levels in patients with GD. The high number of TRAb-negative patients observed in our study may be due to different factors, including the health care system in our country in which all patients can directly apply to tertiary referral centers without being treated in primary care health centers. This factor may decrease the number of patients with mild disease, particularly TRAb-negative cases in referral centers in some parts of the world. Another important factor that could be related to a higher frequency of TRAb-negative GD in our study is the cut-off value of TRAb used in our institution. In the present study, the cut-off value of TRAb for untreated GD was accepted as 1.75 IU/L, as recommended by the manufacturer. In a recent study conducted by Scappaticcio et al., the optimal cut-off value for TRAb assessed by a second-generation method (Thermo Scientific BRAHMS Kryptor) was suggested as 0.7 IU/L (sensitivity and specificity 93% and 88.8%, respectively), despite the manufacturer recommended cut-off value of 1.5 IU/L [29]. In that study six (7%) out of the 86 patients

were reported to have TRAb-negative GD, despite using a cut-off value of 0.7 IU/L. Although in the recent ETA guidelines for the management of Graves' hyperthyroidism the sensitivity and specificity of TRAb assessed either by a second- or a third-generation assay method were indicated as 97 and 98%, respectively, they do not suggest any specific cut-off value for TRAb assessed either by a second- or a third-generation method [5]. In the present study, if a cut-off value of 0.7 IU/L instead of the manufacturer recommended cut-off value of 1.75 IU/L for TRAb had been used, only 23 patients (5%) would have been diagnosed as TRAb-negative GD. Therefore, the manufacturer-recommended cut-off value for TRAb may not be appropriate for all populations. On the other hand, the different genetic backgrounds of our patients may also have contributed to the higher frequency of TRAb-negative patients. For instance, in a study by Vos et al., CTLA4–49 polymorphism was not associated with relapse of GD in Dutch patients [13], while it was suggested as a risk factor for relapse in Turkish patients with GD [30]. However, the exact causes of the higher number of TRAb-negative GD in our patients have to be investigated further.

Our study has several limitations. The main limitation of our study is the retrospective design of the study that may have caused a selection bias. Therefore, the inclusion of some patients with GD may have been missed, and the number of TRAb-negative patients may not reflect the true frequency of TRAb-negative patients among the patients with GD. Secondly, because our study was retrospective, we could not assess thyroid-stimulating immunoglobulin levels in TRAb-negative patients with GD. Thirdly, the GO was not assessed according to the current guidelines, and we could not determine the association between the severity scores of the GO and TRAb levels. And finally, the goiter sizes of the participants were assessed by manual palpation rather than the assessment of the thyroid volume by ultrasonography. Although all patients admitted for hyperthyroidism in our center undergo thyroid ultrasonography and color-Doppler ultrasonography, the thyroid volume is not measured routinely in all patients. Therefore, we could not assess the relationship between TRAb and thyroid volume. However, the strength of this study derives from the inclusion of a large number of patients, particularly a large number of TRAb-negative GD patients with appropriate clinical and laboratory data. Therefore, we believe that our study is a clear example of daily practice.

Conclusions

Results from this study suggest TRAb levels at the initial diagnosis of GD are differently associated with clinical and laboratory parameters. Male patients and smoking patients with GD tended to have significantly higher TRAb levels at the initial diagnosis and more severe clinical hyperthyroidism. Furthermore, smoking male patients tended to have markedly higher TRAb levels compared to all other patients. TRAb-negative GD patients tended to have significantly less severe clinical and biochemical hyperthyroidism compared to patients with high TRAb levels. However, the smoking habit was also significantly lower among TRAb-negative GD patients. Therefore, our study results suggest that besides the other factors, male gender and smoking habit may affect TRAb levels and consequently the severity of hyperthyroidism in patients with GD. Furthermore, male gender and smoking may have a synergistic effect on TRAb levels and consequently on the severity of hyperthyroidism in patients with GD. Further studies are required to confirm our results.

Declaration of interest

No potential conflict of interest was reported by the authors.

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