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patients with Graves' disease

## Age may influence the impact of TRAbs on thyroid function and relapse-risk in patients with Graves' disease

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Age, thyroid hormones and relapse in Graves' disease

**Context:** Thyrotropin receptor antibodies (TRAbs) play a crucial role in the pathogenesis of Graves' disease (GD). However, factors that influence the association of TRAbs with thyroid hormones and relapse risk in GD remain unclear.

**Objective:** We investigated: (i) the associations of TRAbs at diagnosis with thyroid hormones and relapse risk; (ii) potential factors that can influence these associations in GD.

**Design and Setting:** A prospective study in GD patients from a single endocrine centre in the north-east of England, seen between January 2008 and March 2018.

**Patients and Main outcome measures:** Consecutive GD patients (n=384) who had measurements of TRAbs, free thyroxine and free triiodothyronine at diagnosis. The association of TRAbs with thyroid hormones and relapse risk was assessed through linear regression and Cox proportional hazard models, adjusted for potential confounders.

**Results:** TRAbs were non-linearly associated with thyroid hormones, following a curve with an initial positive slope and a subsequent flattening (p-values <0.0001). Higher TRAbs were also associated with greater relapse risk (Hazard ratio [HR], 1.05; 95% confidence interval [95% CI], 1.02-1.08, per 1 U/L increase in TRAb; p-value 0.001). These associations were modified by age, but not by sex, race, smoking or thyroid peroxidase antibody levels. In younger participants, increasing TRAbs were associated with higher thyroid hormones, and greater relapse risk (HR, 1.13; 95% CI, 1.04-1.23, per 1 U/L increase in TRAb; p-value 0.005). In older participants, increasing TRAbs were not associated with meaningful increases in thyroid hormones or relapse risk (HR, 0.99; 95% CI, 0.93-1.05, per 1 U/L increase in TRAb, p-value 0.7).

**Conclusions:** In GD, age can influence the impact of TRAbs on thyroid function and relapse risk. TRAbs at diagnosis have better predictive value in younger patients with GD.

In a prospective analysis of 384 patients with Graves' disease, we found that age influences the association between TRAb levels and thyroid hormone levels as well as relapse risk.

## Introduction

Graves' disease (GD) is a common autoimmune disorder characterised by thyrotoxicosis, goitre, and in some patients, ophthalmopathy. In iodine sufficient areas, it accounts for 70-80% of all cases of thyrotoxicosis.(1) As in most autoimmune diseases, GD is more frequent in women and can be observed at any age although its incidence peaks between the fifth and sixth decades.(2) No consistent differences by ethnicity in the incidence of GD have been observed, but a higher prevalence of the disease in Caucasians and Asians than in Africans has been noted.(3)

GD is caused by circulating antibodies that bind to and stimulate the thyroid stimulating hormone receptor (TSHR), resulting in increased synthesis and release of thyroid hormones and hypertrophy of thyroid follicular cells. Antibodies against the TSHR (TRAbs) are pathognomonic for GD. They are detectable in the serum of about 98% of untreated GD patients using a second-generation assay,(4) and in an even higher proportion of patients using a third-generation assay.(5) TRAb measurement is useful to differentiate between GD and thyrotoxicosis due to other causes.(6) Furthermore, circulating TRAb levels correlate with the clinical course and severity of GD and are useful predictors of relapse risk.(7) Although the exact aetiology of GD remains largely unclear, it is thought that a complex interaction between genetic and environmental factors in susceptible individuals leads to the breakdown of immune tolerance to thyroid antigens, and to the initiation of an immune reaction against the TSHR.(8)

Antithyroid drugs (ATDs) are widely used to manage Graves' thyrotoxicosis and are safe and usually effective, but recurrence is common after their withdrawal with recurrence rates of 40–50% in Europe and 70–80% in the United States.(9) A number of risk factors for recurrence of GD after ATD cessation have been identified including younger age,(10) male sex,(10) large goitre size,(11) biochemical severity of thyrotoxicosis at diagnosis,(12) cigarette smoking,(6, 10-12) and high TRAb levels, both at diagnosis(13) and at cessation of therapy.(7)

Although the central role of TRAbs in the pathogenesis of GD has been known for a number of years, it still remains unclear which socio-demographic, environmental or immunological factors influence their relationship with thyroid function and also affect the risk of relapse. We, therefore, studied patients with GD with the aim: (i) to investigate the association of TRAb at diagnosis with thyroid hormones and risk of relapse, and (ii) to explore whether age, sex, race, smoking, or thyroid peroxidase antibody (TPOAb) influence the association of TRAb with thyroid hormones and risk of relapse.

## Material and methods

### Patients:

Consecutive patients with hyperthyroidism referred to an outpatient endocrine clinic in Gateshead, England were included prospectively. All patients provided informed consent to participate in the study. The diagnosis of GD was confirmed after a clinical examination was performed and the typical biochemical picture of low serum thyroid-stimulating hormone (TSH) and high thyroid hormone levels in the presence of elevated TRAb levels or uniform uptake on Tc99m scans was noted. All patients had TRAb levels measured at diagnosis but Tc99m scans were only performed if the TRAb levels were negative or borderline (< 1.0 or between 1.0 – 2.0 U/L, respectively). Clinical and biochemical information was collected at diagnosis and prior to commencement of ATD. Graves' orbitopathy (GO) was noted as either present or absent based on clinical guidelines.(14) None of the patients included in this analysis were taking any medications that could affect thyroid function. Patients with GD who were pregnant, either at diagnosis, during ATD treatment or during follow-up after ATD

cessation, were not included in this analysis. The median (interquartile range) duration of treatment with ATD was 12 (11 – 14) months. The majority of patients were treated with carbimazole (90.0%). After the baseline visit, a number of patients were not included in the follow-up analysis: lost to follow-up or moved to a different area (n=13), opted for definitive treatment with either surgery or radioactive iodine (n=8), did not require ATD treatment due to presentation with subclinical hyperthyroidism (n=34) and still continuing treatment with ATD therapy (n=98). Overall, the baseline characteristics of participants with data available on relapse were similar to those without follow-up data available (Table 1). Relapse was defined as recurrent hyperthyroidism after ATD cessation. The start date of follow-up was considered the date of ATD cessation. The end-date of follow-up was considered the date of relapse, the date of death, or the date of 12 months after ATD cessation, whichever came first.

### Biochemical analyses

Thyroid function tests, TRAb, and TPOAb were analyzed using the Roche Elecsys electrochemiluminescence immunoassay on the Cobas e602 analytical platform. The reference ranges were as follows: TSH (0.4–4.0 mIU/L), free thyroxine (FT4) (10–25.0 pmol/L), free triiodothyronine (FT3) (3.0–6.8 pmol/L), TPOAb (<35 IU/mL), and TRAb (<1.0 IU/L). In our laboratory, the coefficient of variation for all analyses were <5% except TPOAb where it was <10%.

### Technetium uptake scan

Anterior views of the thyroid were obtained using a gamma camera 20 minutes following injection of 100 MBq 99mTc pertechnetate.

### Statistical analyses

Linear regression and Cox proportional hazard models were utilised to investigate the association of TRAb at diagnosis with thyroid hormones and the risk of relapse. We first investigated the cross-sectional association of TRAb at diagnosis with FT4 and FT3 levels, by performing ordinary least-squares linear regression. Restricted cubic splines with three knots were used to allow for potential nonlinearity. Moreover, we investigated the prospective association of TRAb at diagnosis with the risk of relapse by using Cox proportional hazard models. All analyses were tested for potential effect modification by several factors. We separately added product interaction terms of TRAb with age, sex, race, smoking status, and TPOAb levels. Furthermore, we stratified the analyses by age, sex, race, smoking status, and TPOAb levels.

Potential confounders were selected based on biological plausibility and previous literature. All analyses were adjusted for age, sex, race, smoking status, and TPOAb levels. Several sensitivity analyses were performed: (A) To account for a potential influence of GO in our results, we additionally adjusted our analyses for the presence of GO. (B) We restricted the cross-sectional analysis to participants with data available on relapse. (C) Longitudinal analyses were additionally adjusted for FT4 levels at diagnosis, average daily dose of ATD treatment, and duration of ATD treatment in months. (D) We extended the follow-up time to 24 months in cases with available data on relapse after 12 months of follow-up. (E) To evaluate the role of age on the bioactivity of TRAb, we performed a *post-hoc* analysis investigating the association of age with FT4/TRAb ratio as a marker of TRAb potency, adjusting for sex, race, smoking status and TPOAb levels. In addition, we investigated the associations of age with TRAb and FT4 levels, respectively, adjusting for sex, race, smoking status and TPOAb levels.

The assumption of normally distributed residuals was checked and met. The proportional hazards assumption was assessed by Schoenfeld test and plots. No violation of the proportional hazards assumption was observed. A p value of <0.05 was deemed to indicate

statistical significance. Statistical analyses were conducted using R statistical software (rms package, R project, Institute for Statistics and Mathematics, R Core Team, version 3.2.2) and IBM SPSS version 21 (IBM Corp, Chicago, Ill).

## Results

Baseline characteristics of 384 eligible participants are presented in Table 1. The median age was 48.0 (interquartile range, 35.0-58.0) years, 85.2% were women, 27.1% were current cigarette smokers, 93.5% were of Caucasian origin and 19.3% had active GO. Of these participants, 231 had data available on relapse. Over a median follow-up time of 12 months (range 3-12) after ATD cessation, a total of 45 participants (19.5%) relapsed to overt hyperthyroidism.

### Cross-sectional association of TRAb at diagnosis with thyroid hormones

TRAbs at diagnosis were non-linearly associated with FT4 (p-value <0.0001, Figure 1a) and FT3 levels (p-value <0.0001; Figure 2a), following a curve with an initial positive slope and a subsequent flattening (p for nonlinearity, 0.0003 and 0.007, respectively). The association was independent of age, sex, race, smoking status, and TPOAb levels. Additional adjustments for the presence of GO provided consistent results. Also, results remained similar after restricting the analysis to participants with data available on relapse. In the cross-sectional analyses of TRAb at diagnosis with FT4 and FT3, we found differences by age (p for interaction, 0.0008 and 0.0006, respectively), but no differences by sex, race, smoking status, and TPOAb levels. Further stratification by age tertiles showed differences among categories (Figure 1b, Figure 2b). In the youngest participants (i.e, 17 to 39 years old, first tertile of age) and middle-aged group (i.e, 40 to 54 years old, second tertile of age), TRAb levels at diagnosis were positively associated with higher FT4 and FT3 concentrations in a linear manner (p for non-linearity  $\geq 0.05$ ) (Figure 1b, Figure 2b). In the oldest participants (i.e, 55 to 92 years old, third tertile of age), TRAb at diagnosis lower than 10 U/L were positively associated with FT4 and FT3 levels in a linear manner, but there were no significant changes in FT4 or FT3 levels with TRAb at diagnosis higher than 10 U/L (Figure 1b, Figure 2b). Interaction terms of TRAb with sex, race, smoking status, and TPOAb levels were not statistically significant in relation to FT4 (p for interactions: 0.5, 0.9, 0.6, and 0.3, respectively) and FT3 (p for interactions: 0.1, 0.7, 0.7, and 0.1, respectively). After further stratifying by sex, race, smoking status, and TPOAb levels, we did not observe meaningful differences in the direction or magnitude of the associations across categories. In our *post-hoc* analyses, age was not associated with TRAb levels (p value 0.2) or FT4/TRAb ratio as a marker of TRAb potency (p value 0.8). Increasing age was associated with lower FT4 levels at diagnosis ( $\beta$ , -0.29; 95% confidence interval [95%CI], -0.43 to -0.16; per 1 year increase in age, p value < 0.0001).

### Prospective association of TRAb at diagnosis with the risk of relapse

Higher TRAb levels at diagnosis were associated with a higher risk of relapse, independent of age, sex, race, smoking status, and TPOAb levels (HR, 1.05; 95%CI, 1.02 to 1.08, per 1 U/L increase in TRAb, p-value 0.001; Figure 3a, Table 2). No evidence of non-linearity was observed. Results did not change after additional adjustments for the presence of GO (HR, 1.05; 95%CI, 1.02 to 1.08, per 1 U/L increase in TRAb). Results remained similar after additionally adjusting for FT4 levels at diagnosis, average daily dose of ATD treatment, and duration of ATD treatment (HR, 1.06; 95%CI, 1.02 to 1.09, per 1 U/L increase in TRAb), or after extending the follow-up time to 24 months (HR, 1.04; 95%CI, 1.01 to 1.08, per 1 U/L increase in TRAb). Out of 231 GD patients, we observed 66 relapse (28.6%) cases after extending the follow-up time to 24 months (interquartile range 12-24).

In the prospective analyses of TRAb at diagnosis with the risk of relapse, we found significant differences by age ( $p$  for interaction, 0.01), but no differences by sex, race, smoking status, and TPOAb levels. Further stratification by age tertiles showed differences among categories (Figure 3b). In the youngest participants (i.e., 18 to 41 years old, first tertile of age) and middle-aged group (i.e., 42 to 56 years old, second tertile of age), higher TRAb at diagnosis were associated with a higher risk of relapse (HR, 1.13; 95%CI, 1.04 to 1.23, per 1 U/L increase in TRAb,  $p$ -value 0.005, and HR, 1.05; 95%CI, 1.01 to 1.09, per 1 U/L increase in TRAb;  $p$ -value 0.01, respectively; Table 2). In the oldest participants (i.e., 57 to 90 years old, third tertile of age), TRAb at diagnosis were not associated with the risk of relapse (HR, 0.99; 95%CI, 0.93 to 1.05, per 1 U/L increase in TRAb,  $p$ -value 0.7; Table 2). Interaction terms of the TRAb with sex, race, smoking status, and TPOAb levels were not statistically significant ( $p$  for interactions: 0.3, 0.9, 0.9, and 0.8, respectively). After further stratifying by sex, race, smoking status, and TPOAb levels, we did not observe meaningful differences in the direction or magnitude of the associations across categories.

## Discussion

In a prospective cohort study of GD patients, we found that TRAb levels at diagnosis were positively associated with circulating thyroid hormones and relapse risk. These associations were modified by age, but not by sex, race, smoking or TPOAb. Importantly, increasing TRAb levels were associated with higher concentrations of thyroid hormones and increased relapse risk in the younger patients (aged <55 years old, first and second tertile of age), but not in the older patients (aged  $\geq$ 55 years old, third tertile of age).

Previous studies in GD patients have also reported that increasing TRAb levels at diagnosis are associated with higher circulating thyroid hormones(15) and increased relapse risk.(7, 13, 16) These results are not surprising, given that TRAbs can directly stimulate thyroid epithelial cells and lead to various degrees of Graves' thyrotoxicosis. Our study extends these previous findings by showing that the impact of TRAbs on thyroid function and risk of relapse can essentially depend on age. Indeed, we observed a gradual change in the pattern of the associations throughout ageing. This suggests that there is no specific inflection point for age, but the effect of TRAb levels on relapse risk changes gradually with increasing age. In addition, our data exhibits good concordance between FT4 and FT3 with regards to their association with TRAbs and the modifying effect of age. The ageing process has a complex role on the pathophysiology of GD. The onset of GD at younger ages has been so far linked with an increased degree of thyrotoxicosis(17, 18) and greater risk of relapse.(10, 19) In our study, older patients with TRAb levels greater than 10 U/L resulted in a milder degree of biochemical thyrotoxicosis compared with younger patients. For example, TRAb levels of 40 U/L in a younger patient were associated with a FT4 of approximately 80 pmol/L whereas the same TRAb levels resulted in a FT4 of around 50 pmol/L in an older person with GD. Furthermore, our data suggests that circulating TRAb levels may not be a good predictor of relapse risk in the older GD patients. Therefore, we recommend a more cautious interpretation of thyroid parameters in the older GD patients. The choice of treatment and the course of follow-up may also be influenced by these findings.

TRAbs are auto-antibodies that bind to the TSHR with either a stimulating, blocking or neutral effect. Over the last few decades TRAb assays have evolved substantially and undergone several improvements.(6) Current TRAb assays in use are either competition-based or functional assays. The competition-based assays detect TRAb in serum by competing for binding of the TSH receptor with a known ligand, whereas the functional assays detect cAMP production in cells incubated with the patient's sera. The third generation of TRAb competition-based assay – that was utilised in our study – has high sensitivity and specificity in diagnosing GD, but does not differentiate the stimulating from

the blocking or neutral variety. It is possible that older age could lead to altered TRAb bioactivity and/or increase the non-stimulatory to stimulating autoantibody ratio. However, our *post-hoc* analyses did not show a link between age and TRAb levels or FT4/TRAb ratio, suggesting that the ageing process is unlikely to have affected TRAb bioactivity. On the other hand, our *post-hoc* analyses revealed a negative association of age with FT4 levels independent of TRAb concentrations, indicating a milder degree of biochemical thyrotoxicosis in older people with GD. It is therefore likely that ageing may modify the response of the thyroid gland to stimulation. Indeed, ageing is known to alter the set point of the hypothalamus-pituitary-thyroid axis.(20) Even in euthyroid subjects, older age has been related to increasing circulating TSH without substantial changes in FT4 concentrations.(21) This suggests that in older people, the thyroid gland may become less responsive to stimulation by TSH.(22) Similar to TSH, TRAbs also target the TSHR in the thyroid gland. Thus, it can be assumed that in the older GD patients, the TSHR and the thyroid gland may become less responsive to TRAbs stimulation. Interestingly, our data suggests that the association of TRAbs with FT4 and FT3 levels across the oldest age tertile consistently diverges at a TRAb level of approximately 10 U/L. One explanation may be that the thyroid gland of older individuals becomes less responsive to stimulation only at relatively high TRAb levels. However, additional studies are warranted to clarify the exact mechanisms through which ageing influences the action of TRAbs on the thyroid gland.

To the best of our knowledge, this is the first study reporting an effect modification by age on the associations of TRAb at diagnosis with thyroid hormones and relapse risk in GD. Our well-characterised study sample was adequately powered to inform the predictive value of TRAbs in Graves' relapse. Another strength is the prospective study design. Baseline measurements of thyroid function and TRAb levels were collated prior to the evaluation of relapse risk. Moreover, we had extensive and detailed information on covariates, including exposures, outcomes, and potential confounders.

Several limitations should also be considered. The follow-up time of our study was restricted to a maximum of 12 months. However, our results remained consistent after we extended the follow-up time to 24 months in cases where data was available. Another limitation is that we had no information on whether TRAbs were stimulatory, inhibitory or neutral in character. Nevertheless, the lack of an association between age and FT4/TRAb ratio suggests that age does not influence TRAb potency. Lastly, given the observational character of our study, we cannot rule out the possibility of residual confounding.

In summary, our study provides novel insights into the influence of age on the predictive value of TRAbs in GD. TRAbs at diagnosis may have a better predictive value in younger patients than in the older patients with GD, in terms of biochemical parameters and relapse risk. This conclusion can help inform clinical decision in GD. Future studies need to confirm our results in other populations of GD patients. Also, the exact mechanisms that underlie our findings need to be further investigated.

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Disclosure summary:

The authors have nothing to disclose.

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Figure 1. Cross-sectional association of TRAb at diagnosis with FT4 levels. a. Association of TRAb with FT4. We used linear regression models with restricted cubic splines. Predicted means of FT4 (black lines) with 95% confidence intervals (gray areas) were plotted against TRAb, adjusting for age, sex, race, smoking status, and TPOAb. The *P* for interactions of TRAb with age, sex, race, smoking status, and TPOAb levels were 0.0008, 0.5, 0.9, 0.6, and 0.3, respectively. b. Association of TRAb with FT4 among age tertiles. Abbreviations: TRAb, thyrotropin receptor antibody levels; FT4, free thyroxine; TPOAb, thyroid peroxidase antibody levels; y, years.

Figure 2. Cross-sectional association of TRAb at diagnosis with FT3 levels. a. Association of TRAb with FT3. We used linear regression models with restricted cubic splines. Predicted means of FT3 (black lines) with 95% confidence intervals (gray areas) were plotted against TRAb, adjusting for age, sex, race, smoking status, and TPOAb. The *P* for interactions of TRAb with age, sex, race, smoking status, and TPOAb levels were 0.0006, 0.1, 0.7, 0.7, and 0.1, respectively. b. Association of TRAb with FT3 among age tertiles. Abbreviations: TRAb, thyrotropin receptor antibody levels; FT3, free triiodothyronine; TPOAb, thyroid peroxidase antibody levels; y, years.

Figure 3. Prospective association of TRAb at diagnosis with the risk of relapse. a. Association of TRAb with the risk of relapse. Log relative hazards of relapse risk (black lines) with 95% confidence intervals (gray areas) were plotted against TRAb, adjusting for age, sex, race, smoking status, and TPOAb. The *P* for interactions of TRAb with age, sex, race, smoking status, and TPOAb levels were 0.01, 0.3, 0.9, 0.9, and 0.8, respectively. b. Association of TRAb with the risk of relapse among age tertiles. Abbreviations: TRAb, thyrotropin receptor antibody levels; HR, hazard ratio; TPOAb, thyroid peroxidase antibody levels; y, years.

Table 1. Baseline characteristics

	Study participants (n=384)	Data available on relapse risk (n=231)	Data not available on relapse risk (n=153)
Age, years	48.0 (35.0-58.0)	50.0 (36.5-60.0)	45 (33-54)
Female	327 (85.2%)	195 (84.4%)	132 (86%)
Smoking			
<i>current</i>	104 (27.1%)	64 (27.7%)	40 (26.1%)
<i>former</i>	90 (23.4%)	60 (26.0%)	30 (19.6)
<i>never</i>	190 (49.5%)	107 (46.3%)	83 (54.2%)
Race			
<i>Caucasian</i>	359 (93.5%)	217 (93.9%)	142 (92.8%)
<i>Black</i>	3 (0.8%)	1 (0.4%)	2 (1.3%)
<i>Asian</i>	22 (5.7%)	13 (5.6%)	9 (5.9%)
Active Graves orbitopathy	74 (19.3%)	43 (18.6%)	31 (20.3%)
TPOAb, IU/ml	128.0 (22.9-365.2)	129.2 (26.6-379.9)	124 (10.5-130.2)
TRAb, U/L	7.0 (3.6-14.3)	7.0 (3.7-13.6)	7.1 (3.2-16.0)
FT4, pmol/L	39.8 (28.3-58.8)	39.3 (29.1-58.4)	40.0 (23.5-59.4)
FT3, pmol/L	15.2 (9.7-26.0)	15.1 (10.2-25.4)	16.1 (8.0-27.7)

Data are presented as numbers (%) or median (interquartile range).

Abbreviations: TPOAb, thyroid peroxidase antibody levels; TRAb, thyrotropin receptor antibody levels; FT4, free thyroxine; FT3, free triiodothyronine.

Table 2. Association of TRAb at diagnosis with the risk of relapse

	HR (95%CI)	p-value
All participants	<b>1.05 (1.02; 1.08)</b>	0.001
<i>P</i> for interaction with age		0.01
First tertile of age	<b>1.13 (1.04; 1.23)</b>	0.005
Second tertile of age	<b>1.05 (1.01; 1.09)</b>	0.01
Third tertile of age	0.99 (0.93; 1.05)	0.7

Adjusted for age, sex, race, smoking status, and TPOAb levels at diagnosis. HRs are denoted per one unit (U/L) increase in TRAb.

Abbreviations: HR, hazard ratio; CI, confidence interval; TRAb, thyrotropin receptor antibody levels; TPOAb, thyroid peroxidase antibody levels.





