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EDITED BY

Bernadette Biondi,
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REVIEWED BY

Ricardo Pujol Borrell,
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Jose Luis Fachi,
Washington University in St. Louis,
United States
Viktor Kravchenko,
National Academy of Sciences of Ukraine,
Ukraine

*CORRESPONDENCE

Willem A. Dik

✉ w.dik@erasmusmc.nl

Gijsbert J. Hötte

✉ g.hotte@oogziekenhuis.nl

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Thyroid stimulating immunoglobulin concentration is associated with disease activity and predicts response to treatment with intravenous methylprednisolone in patients with Graves' orbitopathy

Gijsbert J. Hötte^{1*}, P. Martijn Kolijn², Maaïke de Bie^{1,2}, Ronald O. B. de Keizer¹, Marco Medici³, Kim van der Weerd⁴, P. Martin van Hagen^{2,5}, Dion Paridaens^{1,6} and Willem A. Dik^{2*}

¹Department of Oculoplastic, Lacrimal & Orbital Surgery, Rotterdam Eye Hospital, Rotterdam, Netherlands, ²Laboratory Medical Immunology, Department of Immunology, Erasmus MC University Medical Center Rotterdam, Rotterdam, Netherlands, ³Academic Center for Thyroid Diseases, Department of Internal Medicine, Erasmus MC University Medical Center Rotterdam, Rotterdam, Netherlands, ⁴Department of Internal Medicine, Franciscus Gasthuis & Vlietland, Rotterdam, Netherlands, ⁵Department of Internal Medicine, Section Clinical Immunology, Erasmus MC University Medical Center Rotterdam, Rotterdam, Netherlands, ⁶Department of Ophthalmology, Erasmus MC University Medical Center Rotterdam, Rotterdam, Netherlands

Background: Thyroid stimulating immunoglobulins (TSI) play a central role in the pathogenesis of Graves' orbitopathy (GO), while soluble interleukin-2 receptor (sIL-2R) is a marker for T-cell activity. We investigated TSI and sIL-2R levels in relation to thyroid function, disease activity and severity and response to treatment with intravenous methylprednisolone (IVMP) in patients with GO.

Methods: TSI (bridge-based TSI binding assay), sIL-2R, TSH and fT4 levels were measured in biobank serum samples from 111 GO patients (37 male, 74 female; mean age 49.2 years old) and 25 healthy controls (5 male, 20 female; mean age 39.8 years old). Clinical characteristics and response to treatment were retrospectively retrieved from patient files.

Results: Higher sIL-2R levels were observed in GO patients compared to controls ($p < 0.001$). sIL-2R correlated with fT4 ($r = 0.26$), TSH ($r = -0.40$) and TSI ($r = 0.21$). TSI and sIL-2R concentrations were higher in patients with active compared to inactive GO ($p < 0.001$ and $p < 0.05$, respectively). Both TSI and sIL-2R correlated with total clinical activity score (CAS; $r = 0.33$ and $r = 0.28$, respectively) and with several individual CAS items. Cut-off levels for predicting active GO were 2.62 IU/L for TSI (AUC = 0.71, sensitivity 69%, specificity 69%) and 428 IU/mL for sIL-2R (AUC = 0.64, sensitivity 62%, specificity 62%). In multivariate testing higher TSI ($p < 0.01$), higher age ($p < 0.001$) and longer disease duration ($p < 0.01$) were associated with disease activity. TSI levels were higher in patients with a poor IVMP response ($p = 0.048$), while sIL-2R levels did not differ between responders

and non-responders. TSI cut-off for predicting IVMP response was 19.4 IU/L (AUC = 0.69, sensitivity 50%, specificity 91%). In multivariate analysis TSI was the only independent predictor of response to IVMP ($p < 0.05$).

Conclusions: High TSI levels are associated with active disease (cut-off 2.62 IU/L) and predict poor response to IVMP treatment (cut-off 19.4 IU/L) in GO. While sIL-2R correlates with disease activity, it is also related to thyroid function, making it less useful as an additional biomarker in GO.

KEYWORDS

graves' orbitopathy, disease activity, treatment response, methylprednisolone, TSI, sIL-2R

Introduction

Graves' orbitopathy (GO) is an autoimmune condition characterized by inflammation and volume expansion of the soft tissues that surround the eye, resulting in proptosis, eyelid retraction, edema, restricted ocular motility and diplopia (1). It arises from a complex cellular interplay involving T-cells, B-cells, mast cells and orbital fibroblasts (OF) (2, 3). Orbital fibroblasts express the thyroid-stimulating hormone (TSH) receptor (TSH-R) and activation of the OF is, among other stimuli, triggered by binding of TSH-R autoantibodies (TSH-R-Ab or TRAb) (4, 5).

TSH-R-Ab can be further divided into stimulating autoantibodies (TSAb, also referred to as TSH-R stimulating immunoglobulins [TSI]), blocking autoantibodies (TBAb, also known as TSH-R blocking immunoglobulins [TBI]), or neutral autoantibodies that do not interfere with TSH binding to the receptor (5, 6). Competitive-binding immunoassays that detect the total of stimulatory and blocking TSH-R-Ab, also referred to as TSH-R-binding inhibitory immunoglobulins (TBII), are commonly used in clinical practice (5). Although these TBII assays display good sensitivity and specificity, they do not provide information on the actual functionality of the antibodies (i.e. stimulating or blocking) (5). Selective detection of autoantibodies with either TSH-R stimulating or blocking properties is possible with technically more challenging functional cell-based bioassays (5–7). More recently, an automated bridge-based binding assay has become commercially available (Immulite[®] 2000 TSI immunoassay) (8, 9). This assay uses a TSH-R chimera and has been designed to detect TSI more specifically, although not exclusively since a number of studies show that certain TBI are also detected (10–12).

Because of its central role in the pathogenesis, the use of TSH-R-Ab as a biomarker for GO has been investigated. Research herein has primarily focused on the use of different assays as a biomarker for disease activity and severity, showing that TSI bioassays, outperform TBII assays (4, 5, 7, 13–25). So far, only a limited number of studies investigated the applicability of the bridge-based TSI binding assay as a biomarker for disease activity and severity in

GO, with conflicting results (26–30). Although identifying patients with active disease is important, it may be even more relevant to identify patients who will not respond to intravenous methylprednisolone (IVMP). This treatment is still considered the first-line treatment during the phase of active inflammation, but a large portion of patients respond insufficiently (31, 32). Unfortunately, reliable biomarkers to identify these non-responders before IVMP initiation are lacking and so far studies that explored the relation between TSH-R-Ab assays and IVMP response are scarce (26, 33–35).

While research strongly focused on the use of TSH-R-Ab as biomarker for GO, soluble interleukin-2 receptor (sIL-2R) is a biomarker for T-cell activity that is used to evaluate disease activity and treatment response in a variety of immune-mediated diseases, including autoimmune diseases (36, 37). Higher levels of sIL-2R have also been described in patients with Graves' disease (GD), which correlated with thyroid function (38–43). Interestingly, sIL-2R levels were found to be higher in GD patients with orbitopathy as compared to GD patients without orbitopathy, which may reflect more profound activation of the immune system in the first group (44, 45). However, only few studies have explored the clinical relevance of sIL-2R as a biomarker for GO activity and severity (42, 43, 46, 47) and none have tested the relation between sIL-2R levels and response to IVMP.

The goal of the present study was to investigate serum TSI levels measured with the bridge-based TSI binding assay (Immulite[®] 2000 TSI immunoassay) and serum sIL-2R levels in relation to thyroid function, disease activity, disease severity, and IVMP treatment response in patients with GO.

Materials and methods

Patients and controls

For this study, serum samples that were stored at -80°C in the Combined Ophthalmic Research Rotterdam Biobank (CORRBI)

were used. Ethical approval for CORRBI in general was granted by the local medical ethical committee (MEC-2012-031). Informed consent was obtained for all CORRBI participants after being informed on the ethical issues regarding storage and use of samples (48). The use of samples for our study was approved by the biobank committee. All files from patients whose samples were stored under the (tentative) diagnosis of GO were selected for further review. Clinical characteristics, laboratory tests and orbital imaging were evaluated to confirm diagnosis of GO, ultimately resulting in 111 patients that were included. In addition, serum samples from a cohort of 25 healthy individuals were obtained as a control group, as approved by the local medical ethical committee (MEC-2021-0251).

Clinical evaluation

Medical history and demographic features were recorded for all patients and healthy controls. For GO patients, results from ophthalmological and orbital examination were retrospectively obtained from the patient files. Severity of the condition was determined using the EUGOGO classification (mild, moderate-to-severe, and sight-threatening GO) (31). Disease activity was assessed using the clinical activity score (CAS) of 7 items: spontaneous retrobulbar pain, gaze evoked pain, eyelid erythema, conjunctival hyperemia, eyelid swelling, chemosis and inflammation of the caruncle/plica (49). Active disease was defined as a total CAS of ≥ 3 points in one or both eyes. Patients who were treated with IVMP after the biobank sample was obtained, were evaluated for treatment response. IVMP dosing schemes were based on EUGOGO guidelines and tailored in selected cases depending on comorbidity and side effects (31). For severe disease, the standard scheme included 1000mg of IVMP for three consecutive days, which was repeated if indicated. For moderate-to-severe disease, the standard dosing regimen consisted of a cumulative dose of 4500mg of IVMP in 12 weekly infusions. As part of a recent study by our group, a small subset of patients with moderate-to-severe disease was treated with a regimen of prednisolone-encapsulated liposomes (two times 150mg intravenously with a 2-week interval), that is potentially associated with a more specific targeted delivery of the drug at the inflamed areas and requires fewer hospital visits while typical steroid-related adverse events are reduced (50). A beneficial response to IVMP treatment was defined as (1) achievement of a total CAS < 3 in both eyes, or (2) an improvement of ≥ 2 points in one eye without concomitant deterioration in the fellow eye.

Serologic evaluation

Serum samples were defrosted and analyzed for free thyroxine (fT4), TSH, TSI and sIL-2R. The analyses were performed under strict quality rules (ISO15189) by the clinical chemistry facility and medical immunology laboratory at Erasmus MC. Reference range was 14 - 29 pmol/L for fT4 and 0.56 - 4.27 mIU/L for TSH. Based on fT4 and TSH results, thyroid function was further classified as hyperthyroid, subclinical hyperthyroid, hypothyroid, subclinical

hypothyroid and euthyroid. TSI was measured with Immulite[®] 2000 TSI immunoassay (Siemens Healthineers AG, Erlangen, Germany) and sIL-2R was measured with Immulite[®] 2000 IL2R immunoassay. For TSI a cut-off value for positivity of ≥ 0.55 IU/L, as defined by the manufacturer, was used. Our institution's current cut-off value for elevated sIL-2R is 555 IU/ml.

Statistical analyses

Castor EDC was used as a clinical data management system (51). Data were subsequently exported to SPSS v.28 (IBM corp., Armonk, New York, USA) and R Statistical Software (v4.2.2, R Core Team 2021) (52) for statistical analysis. Differences in continuous variables between groups were evaluated using an independent sample t-test or Mann-Whitney U test. For categorical variables Fisher's exact test was used. Spearman rank correlation coefficient was used for correlation analyses. Receiver operator curve (ROC) analysis was performed using the pROC R package (v1.18.0) (53). Cut-off values based on Youden's indices were used for dichotomous distribution and subsequently applied to a multivariate logistic regression model.

Results

Patient characteristics

Table 1 summarizes the demographic and clinical data of GO patients (n = 111) and healthy controls (n = 25). The clinical data of the GO patients correspond to the visit at which the biobank sample was obtained. In total, 39 patients were treated with IVMP. Of these, 11 patients were treated for severe disease with daily high doses of IVMP (median cumulative dose of 3000mg; IQR = 1000). Furthermore, 24 patients with moderate-to-severe disease were treated with weekly infusions of IVMP, with a median cumulative dose of 4500mg (IQR = 0). Another four patients with moderate-to-severe disease from this cohort received treatment with prednisolone-encapsulated liposomes, with a cumulative dose of 300mg (50). Median time between obtaining the serum sample and the start of IVMP treatment was 11 days (IQR = 39.50). The median duration between completion of IVMP treatment and subsequent clinical evaluation was 16.5 days (IQR 38.50).

Serologic results

Serologic results from patients and controls are shown in Table 2. As expected, median TSI was significantly higher in patients compared to controls ($p < 0.001$) and TSI levels above the reference range were found in 97 GO patients (87.4%) but in none of the healthy controls. Interestingly, GO patients also showed significantly higher median sIL-2R concentrations than the control group ($p < 0.001$; Table 2; Supplementary Figure 1). sIL-2R levels above the reference range were observed in 26 patients (23.4%) but not in any of the controls.

TABLE 1 Demographic and clinical data of GO patients and healthy controls.

		Graves' orbitopathy	Healthy Controls	p-value
		(n = 111)	(n = 25)	
Sex	Male	37 (33.3%)	5 (20%)	0.236 ^a
	Female	74 (66.7%)	20 (80%)	
Age (years)	Mean	49.2 (± 15.7)	39.8 (± 11.5)	< 0.01 ^b
Smoking status	Smoker	34 (30.6%)	0 (0%)	< 0.001 ^a
	Non-smoker	57 (51.4%)	25 (100%)	
	Unknown	20 (18.0%)	0 (0%)	
Thyroid disease history	Hyperthyroidism	103 (92.8%)	N/A	N/A
	Hypothyroidism	8 (7.2%)		
Previous thyroid treatment	Complete or partial thyroidectomy	11 (9.9%)	N/A	N/A
	Radioactive iodine	25 (22.5%)		
Current thyroid medication	Block & replace	35 (31.5%)	N/A	N/A
	Titration	16 (14.4%)		
	Thyroid hormone	37 (33.3%)		
	None	23 (20.7%)		
Duration of symptoms (months)	Median	10 (IQR 22)	N/A	N/A
CAS	Median	2.0 (IQR 3)	N/A	N/A
	Active (CAS ≥ 3)	45 (40.5%)		
	Inactive (CAS < 3)	65 (58.6%)		
	Unknown	1 (0.9%)		
Severity	Mild	14 (12.6%)	N/A	N/A
	Moderate-to-severe	87 (78.4%)		
	Severe	10 (9.0%)		
Response to IVMP	Responder	22 (56.4%)	N/A	N/A
	Non-responder	16 (41.0%)		
	Unknown	1 (2.6%)		

CAS (Clinical Activity Score); IVMP (intravenous methylprednisolone); IQR (interquartile range); N/A (not applicable).

a: Fisher's exact test, Freeman-Halton extension when appropriate.

b: Independent sample t-test.

Relation between sIL-2R levels and TSI and thyroid function

In patients with GO, we observed a positive correlation between sIL-2R levels and fT4 ($r = 0.26$) and TSI levels ($r = 0.21$). A negative correlation was found between sIL-2R and TSH ($r = -0.40$) and duration of symptoms ($r = -0.19$) (Figure 1).

Relation between TSI and sIL-2R levels and disease severity

TSI and sIL-2R concentrations did not differ between severity groups (mild, moderate-to-severe and severe), although a trend

towards lower sIL-2R levels was observed in moderate-to-severe compared to severe GO (Supplementary Figure 2).

Relation between TSI and sIL-2R levels and disease activity

As for disease activity, we observed increased TSI concentrations for a subset of patients with active disease compared to those with quiescent disease (Figure 2A). Similar results were found for sIL-2R, showing higher levels in active GO (Figure 2B).

Moreover, a weak correlation was observed for TSI levels with total CAS ($r = 0.33$) and with the individual items of gaze evoked

TABLE 2 Serologic results in GO patients and healthy controls.

		Graves' orbitopathy	Healthy controls	p-value
Current thyroid status	Euthyroidism	44 (39.6%)	20 (80%)	< 0.001 ^a
	Subclinical hypothyroidism	13 (11.7%)	4 (16%)	
	Overt hypothyroidism	11 (9.9%)	1 (4%)	
	Subclinical hyperthyroidism	28 (25.2%)	0 (0%)	
	Overt hyperthyroidism	15 (13.5%)	0 (0%)	
fT4	Median	20.7 (IQR 8.5)	18.4 (IQR 3.82)	< 0.05 ^b
(normal range 14 - 29 pmol/L)	Below normal limits	11 (9.9%)	1 (4%)	0.064 ^a
	Within normal limits	85 (76.6%)	24 (96%)	
	Above normal limits	15 (13.5%)	0 (0%)	
TSH	Median	1.12 (IQR 3.0)	1.84 (IQR 1.16)	< 0.05 ^b
(normal range 0.56 - 4.27 mIU/L)	Below normal limits	44 (39.6%)	0 (0%)	<0.001 ^a
	Within normal limits	46 (41.4%)	21 (84%)	
	Above normal limits	21 (18.9%)	4 (16%)	
TSI	Median	3.36 (IQR 8.29)	<0.10 (IQR 0.0)	< 0.001 ^b
(normal range < 0.55 IU/L)	Within normal limits	14 (12.6%)	25 (100%)	< 0.001 ^a
	Above normal limits	97 (87.4%)	0 (0%)	
sIL-2R^c	Median	418 (IQR 225)	286 (IQR 149)	< 0.001 ^b
(normal range < 555 IU/mL)	Within normal limits	85 (76.6%)	25 (100%)	< 0.01 ^a
	Above normal limits	26 (23.4%)	0 (0%)	

fT4 (free T4, thyroxine); sIL-2R (soluble interleukin-2 receptor); TSH (thyroid-stimulating hormone); TSI (thyroid-stimulating immunoglobulins).

a: Fisher's exact test, Freeman-Halton extension when appropriate.

b: Mann-Whitney U test.

c: see also [Supplementary Figure 3](#).

pain ($r = 0.20$), conjunctival hyperemia ($r = 0.28$), eyelid swelling ($r = 0.25$) and chemosis ($r = 0.23$) ([Figure 3](#)). sIL-2R also displayed a weak correlation with the total CAS ($r = 0.28$) and with the individual items of gaze evoked pain ($r = 0.25$) and eyelid erythema ($r = 0.29$) ([Figure 3](#)).

For TSI, ROC analysis showed an area under the curve (AUC) of 0.71 for identifying active disease. With Youden's index, a TSI cut-off value of 2.62 IU/L was associated with a sensitivity of 69% and specificity of 69% ([Figure 4A](#)). For sIL-2R, the AUC for identifying active disease was 0.64 and a cut-off value of 428 IU/mL displayed a 62% sensitivity and 62% specificity ([Figure 4B](#)). The cut-off values for TSI and sIL-2R were used for dichotomous distribution (i.e. low or high levels). With these cut-off values, multivariate logistic regression analysis showed that higher TSI, older age and longer disease duration were independently associated with active disease, while sIL-2R was not ([Table 3](#)).

Relation between TSI and sIL-2R levels and response to treatment with intravenous methylprednisolone

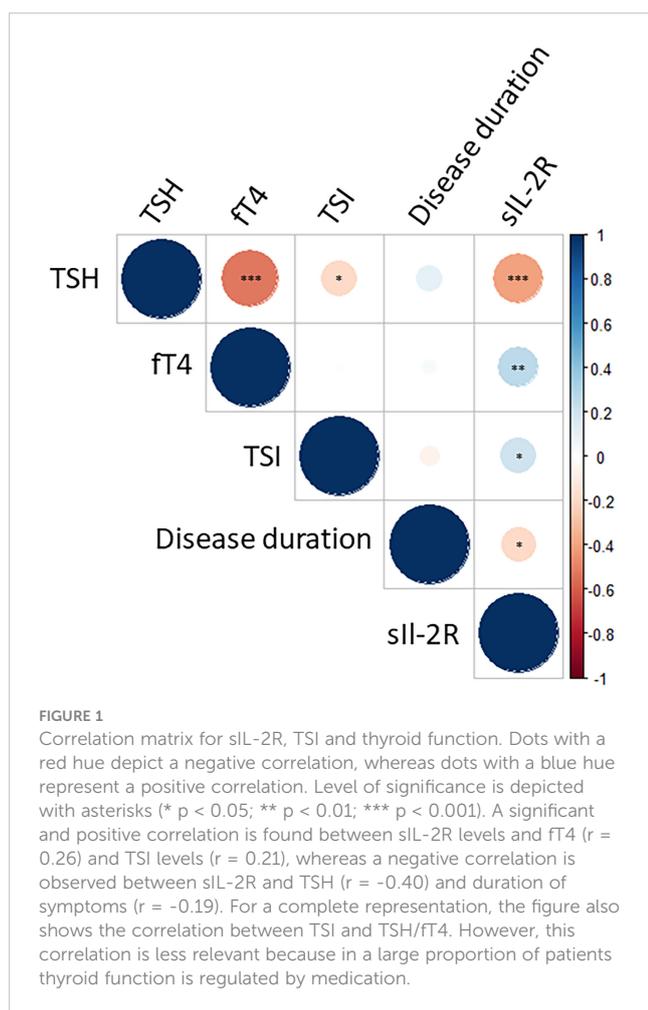
Median TSI levels at baseline were significantly lower in patients that responded to IVMP treatment (3.36 IU/L; IQR = 7.78)

compared to non-responders (14.2 IU/L; IQR = 30.46; $p = 0.049$; [Supplementary Figure 3A](#)). The difference between these groups remained statistically significant when the four patients treated with prednisolone-encapsulated liposomes were omitted from the analysis ($p = 0.046$). When evaluating only patients with moderate-to-severe disease, the analyses lost statistical significance. No difference was observed in sIL-2R levels between IVMP responders and non-responders ([Supplementary Figure 3B](#)). No difference in baseline CAS was found between responders (median 4; IQR 2) and non-responders (median 5; IQR 2; $p = 0.43$).

ROC analysis for TSI as a marker to identify patients responding to IVMP showed an AUC of 0.69. A cut-off value of 19.4 IU/L was associated with a sensitivity of 50% and a specificity of 91% ([Figure 5](#)). Based on this cut-off, TSI was the only independent predictor of response to IVMP treatment in multivariate logistic regression ([Table 4](#)).

Discussion

This is the first study to demonstrate that high TSI levels (measured with the bridge-based TSI binding assay: Immulite[®] 2000 TSI immunoassay) are associated with poor response to IVMP treatment in patients with GO, as observed in both



univariate and multivariate analyses. The calculated TSI cut-off value of 19.4 IU/L for predicting a favorable treatment response showed low sensitivity (50%) but high specificity (91%), indicating half of responders could be accurately identified, with limited false positives among non-responders. These findings suggest that switching to alternative immunosuppressive treatment at an earlier stage, or starting additional immunosuppression simultaneously with IVMP, may be beneficial in patients with high TSI levels. Also, while antithyroid drug treatment and thyroidectomy are generally associated with a reduction of TSH-R-Ab levels, radioactive iodine (RAI) may lead to an increase (54). Therefore, our study provides additional support for the consensus that RAI should be avoided in patients with active orbitopathy, especially in those requiring IVMP treatment. Interestingly, the only other study that investigated the relation between the bridge-based TSI binding assay and IVMP response could not identify such a difference (26). However, in their study patients who experienced an improvement in CAS of ≥ 1 were considered responders, which may explain the discrepancies in outcome. As for other types of antibody assays, a limited number of studies have explored the potential of TSH-R-Ab to predict IVMP response. Studies by Leo et al. and De Bellis et al. did not identify TBII as an independent predictor for treatment response (33, 34). On the other hand, Park et al. demonstrated that higher TSI bioassay levels were in fact

linked to a poor response to IVMP, although with low magnitude (OR 1.005; $p = 0.038$) (35).

The mechanisms through which high TSI levels result in poor response to IVMP are currently unclear. Presumably, high TSI levels are associated with a more profound disease process and overactive inflammatory state. In this biological context, the suppressive signal exerted by IVMP may potentially be less effective. This assumption is strengthened by our observation that high TSI levels are also associated with disease activity in both univariate and multivariate analysis. However, it appears to be in contrast to a study by Mourits et al. in which an association between high pre-treatment CAS and a favorable response to oral prednisolone, was observed (55). This association could not be confirmed in our study and this discrepancy may be explained by the fact that they also included patients with pre-treatment CAS < 3 . Although they used the NOSPECS score to define treatment response, it has many similarities with the CAS in terms of soft tissue signs. With both scoring systems it is perhaps more likely that an improvement is obtained when, for example, the initial CAS is 7. After all, there will be 7 items available for improvement. This is in contrast to when, for example, the CAS is 2, which puts only 2 items available for improvement, while 5 others are eligible to deterioration.

The degree of correlation that we observed between TSI and total CAS was weak and the cut-off value of 2.62 IU/L for active disease showed limited discriminative value. These results are inferior to those from studies that determined true functional biological activity of TSH-R-Ab with specific bioassays (5, 18), possibly because the bridge-based binding assay used in our study still detects certain TBAb/TBI (10–12). Interestingly, although the bridge-based assay presumably detects TSI more specifically than TBII assays, studies found that TBII assays correlate equally or even stronger with disease activity than the bridge-based assay (26, 30). Furthermore, our study did not find any correlation between bridge-based TSI levels and the severity of the disease, which is consistent with some previous reports (26, 28), but differing from another (27).

Although the AUC that we found for TSI in predicting activity and IVMP response is regarded to be sufficient (56), it is desirable to identify additional biomarkers associated with disease activity and IVMP response. Because T-cells contribute to GO pathogenesis, we hypothesized that sIL-2R, which is shed by activated T-cells, could serve as another biomarker in GO (36, 37). Consistent with other studies, we demonstrated that sIL-2R is elevated in GO patients compared to healthy controls and that serum levels correlate with thyroid function (38–43). Furthermore, we observed higher sIL-2R concentrations in patients with active GO compared to inactive disease, which is in line with results from Mariotti et al., but in contrast to a study by Wakelkamp et al. (42, 43) Comparable to TSI, serum sIL-2R also showed a weak correlation with total CAS. However, after correcting for thyroid function, sIL-2R was not independently associated with disease activity. These results support previous reports indicating that elevated sIL-2R levels in GD are related to thyroid function per se, rather than the underlying autoimmune process (38, 41). High levels of sIL-2R in the context of hyperthyroidism can be explained by the interplay

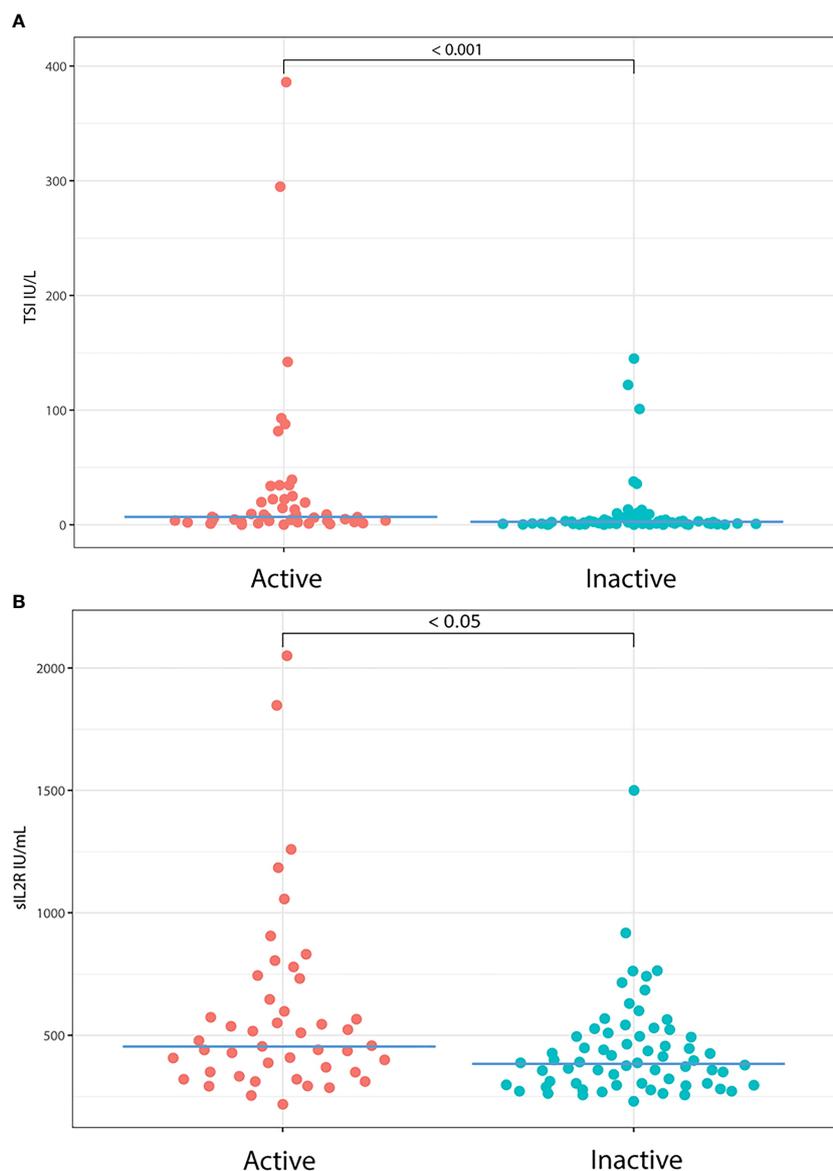


FIGURE 2

TSI and sIL-2R levels in patients with active and inactive disease. We observed increased TSI and sIL-2R values for a subset of patients with active disease compared to inactive disease. **(A)** Median TSI was 6.80 IU/L (IQR = 21.14) in active GO and 2.53 IU/L (IQR = 4.53) in inactive GO ($p < 0.001$). **(B)** Median sIL-2R was 457 IU/mL (IQR = 340) in patients with active orbitopathy compared to 387 IU/mL (IQR = 202) in patients with quiescent disease ($p < 0.05$).

between thyroid hormones and T-cells. Thyroid hormones are known to cause increased numbers of activated T-cells in GD patients, which normalize after restoration of thyroid function (41, 57–60). Because of the relation with thyroid function, sIL-2R is not a valuable biomarker for immune activity in GO. Additionally, we found no statistically significant association between sIL-2R and disease severity, which is consistent with other studies, although a trend towards lower sIL-2R levels was observed in moderate-to-severe compared to severe GO ($p = 0.059$) (43, 46).

Furthermore, we could not identify a relation between sIL-2R serum levels and response to IVMP. While our study is the first to investigate such an association, a prior study by Prummel et al.

found that GO patients with elevated sIL-2R tended to have greater probability to respond to oral prednisone, although this did not reach statistical significance ($p = 0.081$) (46).

Interestingly, although both serum TSI and sIL-2R showed a weak correlation with total CAS, the levels did not correlate separately with all individual items of the CAS, which is consistent with the findings of a recently published study that was conducted at the same time as ours (61). However, as it is unlikely that a single biomarker would be able to capture all facets of the disease process, the lack of correlation between some of the CAS items and TSI or sIL-2R does not necessarily imply that these items have no clinical value. As such, our data do not suffice to present a proposal regarding a modified CAS. Nonetheless, these results,

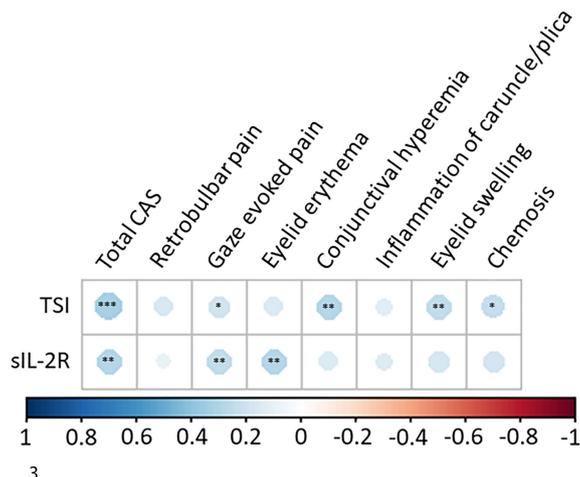


FIGURE 3 Correlation matrix for sIL-2R and TSI levels and clinical activity score (CAS). Dots with a blue hue depict a positive correlation. Level of significance is depicted with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). TSI levels correlated with total CAS ($r = 0.33$) and with the individual items of gaze evoked pain ($r = 0.20$), conjunctival hyperemia ($r = 0.28$), eyelid swelling ($r = 0.25$) and chemosis ($r = 0.23$). sIL-2R levels correlated significantly with the total CAS ($r = 0.28$) and with the individual items of gaze evoked pain ($r = 0.25$) and eyelid erythema ($r = 0.29$).

together with known limitations of the current CAS, do suggest that it is valuable to further investigate whether all items of the CAS have equal clinical and biological relevance, and whether some elements could be replaced or supplemented with biomarkers (31, 49, 62).

Our study has several limitations. sIL-2R concentrations increase with age and in our study GO patients were significantly older than the healthy controls (49.2 vs. 39.8 years old, respectively) (63). However, because the age effect is seen particularly after 65 years, we consider it unlikely that this has had a major impact on our observations (63). Studies have also shown that sIL-2R is higher in smokers and this may also have affected the comparison between GO patients and healthy (non-smoking) controls (63). Nonetheless,

we did not find a difference in sIL-2R levels when we compared smokers and non-smokers within our GO group (443.5 IU/mL, IQR = 200 vs. 428 IU/mL, IQR = 247; $p = 0.68$).

A further consideration is the heterogeneity of our study population. The dosing scheme of IVMP varied depending on the specific indication (severe or moderate-to-severe) and our cohort also included four patients who, as part of a trial, were treated with prednisolone-encapsulated liposomes. The differences in TSI levels between responders and non-responders remained statistically significant when these four patients were removed from the analysis. However, when evaluating patients with moderate-to-severe disease only, significance was lost, which may be due to the low number of patients.

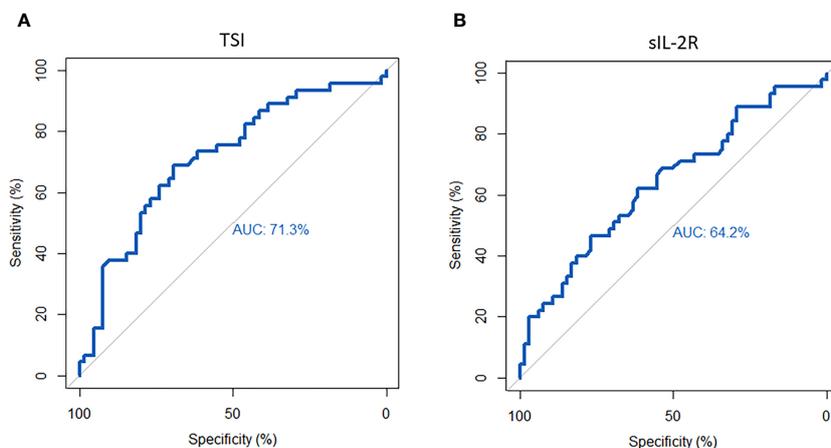


FIGURE 4 Receiver operator curve (ROC) for sIL-2R and TSI in identifying active disease. **(A)** For TSI, ROC analysis showed an area under the curve (AUC) of 0.71. The cut-off value of 2.62 IU/L represented a 69% sensitivity and 69% specificity. **(B)** For sIL-2R, the AUC was 0.64 and a cut-off value of 428 IU/mL showed a sensitivity of 62% and a specificity of 62%.

TABLE 3 Multivariate logistic regression analysis for disease activity.

	OR	CI (95%)	p-value
(Intercept)	0.0062	0.00024 - 0.094	< 0.001
sIL-2R elevated ^a	2.38	0.68 - 8.82	0.18
TSI elevated ^b	9.28	2.20 - 52.7	< 0.01
Age	1.10	1.05 - 1.16	< 0.001
Sex (male)	0.68	0.14 - 3.07	0.61
smoking (unknown)	0.67	0.099 - 3.93	0.66
Smoking (yes)	1.14	0.26 - 5.14	0.86
Hyperthyroidism	1.05	0.12 - 9.95	0.96
Hypothyroidism	2.46	0.27 - 22.6	0.42
Subclinical hyperthyroidism	1.35	0.29 - 6.50	0.70
Subclinical hypothyroidism	3.96	0.42 - 37.9	0.22
Disease duration	0.996	0.993 - 0.999	< 0.01

a: Elevated sIL-2R is based on Youden's index (> 428 IU/mL).

b: Elevated TSI is based on Youden's index (> 2.62 IU/L).

Bold refers to statistically significant results.

TABLE 4 Multivariate logistic regression analysis for response to treatment with methylprednisolone.

	OR	CI (95%)	p-value
(Intercept)	6.31	0.16 - 442.7	0.34
TSI (low) ^a	13.8	1.97 - 171.2	< 0.05
Age	0.95	0.89 - 1.01	0.099
Sex (male)	0.68	0.098 - 4.33	0.68
Smoking (unknown)	3.33	0.18 - 136.8	0.45
Smoking (yes)	0.23	0.031 - 1.35	0.12

a: low TSI is based on Youden's index (< 19.4 IU/L).

Bold refers to statistically significant results.

Moreover, in this retrospective study TSI and sIL-2R levels were only measured at a single point during the disease course. Although disease duration was added in the multivariate model, it would be ideal to include longitudinal serologic data. By doing so, serum levels can be correlated to disease activity along the course of Rundle's curve more precisely.

A final point of attention is that a relation between serum levels and disease severity in our study may have been missed because of the small numbers of patients with mild or severe disease. While severe disease is rather rare in general, the number of mild cases in our cohort is limited because the study reflects a tertiary referral center.

In summary, high TSI levels measured with the bridge-based binding assay are independently associated with active disease (cut-off 2.62 IU/L) and predict poor response to treatment with IVMP (cut-off 19.4 IU/L) in GO patients. However, because of the limited AUC for TSI in predicting activity and IVMP response, it is desirable to identify additional biomarkers for this clinically important application. While sIL-2R correlates with disease activity in univariate analysis, it is also related to thyroid function, making it less useful as an additional biomarker in GO. A prospective study is currently underway validating the results of our current study and exploring the applicability of other biomarkers.

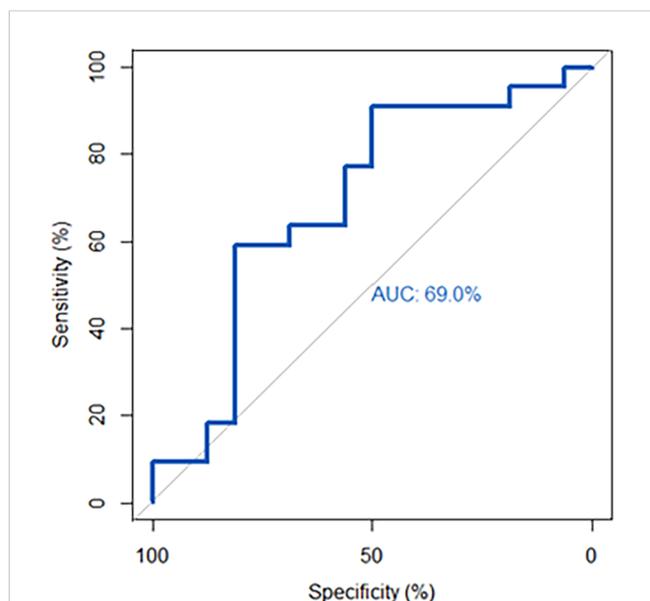


FIGURE 5

Receiver operator curve (ROC) for TSI in identifying responders to IVMP. ROC-analysis showed an area under the curve (AUC) of 0.69. The cut-off of 19.4 IU/L was associated with a sensitivity of 50% and a specificity of 91%.

While the CAS is the most commonly used and best validated scoring system for disease activity and response to treatment, it has several limitations (31). Although we were unable to do so in our current retrospective study, we plan to validate our findings in future studies using other modalities to grade disease activity, such as magnetic resonance imaging (MRI).

Data availability statement

The datasets presented in this article are not readily available because na. Requests to access the datasets should be directed to g.hotte@oogziekenhuis.nl.

Ethics statement

The studies involving humans (biobank and healthy controls) were approved by the local medical ethical committee of the Erasmus

MC (MEC-2012-031 and MEC-2021-0251). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

GH: Writing – original draft, Formal analysis, Data curation. PK: Writing – review & editing, Visualization, Formal analysis. MB: Writing – review & editing, Investigation. RD: Writing – review & editing, Funding acquisition. MM: Writing – review & editing. KV: Writing – review & editing. PV: Writing – review & editing, Supervision. DP: Writing – review & editing, Supervision, Conceptualization. WD: Writing – review & editing, Supervision, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fendo.2024.1340415/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

sIL-2R levels in GO patients and healthy controls. sIL-2R levels are significantly higher in GO patients (418 IU/mL, IQR = 225) compared to healthy controls (286 IU/mL, IQR = 149; $p < 0.001$).

SUPPLEMENTARY FIGURE 2

TSI and sIL-2R levels among severity groups. (A) Median TSI levels were 1.92 IU/L (IQR = 13.21) in mild disease, 3.36 IU/L (IQR = 8.27) in moderate-to-severe and 4.27 IU/L (IQR = 13.6) in severe GO. The differences were not statistically significant. (B) Median sIL-2R concentrations were 392 IU/mL (IQR = 245) in mild GO, 409 IU/mL (IQR = 219) in moderate-to-severe and 543.5 IU/mL (IQR = 236) in severe disease. The trend towards lower sIL-2R levels in moderate-to-severe compared to severe GO did not reach statistical significance ($p = 0.059$).

SUPPLEMENTARY FIGURE 3

TSI and sIL-2R levels in patients responders and non-responders to IVM. (A) Higher TSI levels were observed in non-responders (14.2 IU/L; IQR = 30.46) compared to responders (3.36 IU/L; IQR = 7.78 $p = 0.049$). (B) No difference in sIL-2R levels was found between responders (459.5 IU/mL; IQR = 261) and non-responders (474.5 IU/mL, IQR = 394; $p = 0.35$).

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