Prevalence of Thyroid Peroxidase and Thyroglobulin Autoantibodies in the Swedish Population

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Key words
Antibodies , Autoimmunity, Thyroid Autoimmunity

received 04.12.2022
revised 21.02.2023
accepted 27.03.2023
published online 28.06.2023

Bibliography
Exp Clin Endocrinol Diabetes 2023; 131: 456–462
DOI 10.1055/a-2096-9641
ISSN 0947-7349

Georg Thieme Verlag, Rüdigerstraße 14,
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ABSTRACT

Autoimmune thyroid disease (AITD) may be detected prior to clinical symptoms through the presence of autoantibodies against thyroid peroxidase (TPOab), thyroglobulin (TGab), or both.

The present study aimed to develop a novel radiobinding assay (RBA) for TPOab and to determine the prevalence of TPOab and TGab in the Swedish population.

Patient samples from 27 newly diagnosed Graves' disease patients in longitudinal follow-up and 124 AITD autoantibody-positive children in prospective follow-up for increased risk of type 1 diabetes were included to validate the novel RBA for TPO. The results of RBA were compared with those obtained by commercial radioimmunoassay (RIA) and electrochemiluminescence (ECL). Furthermore, 476 serum samples from adult blood donors and 297 from 13-year-old school children were analyzed for the presence of TPOab and TGab.

Receiver operating characteristics analysis for the novel TPOab resulted in an area under curve (AUC) value of 0.82 ($p < 0.0001$), a sensitivity of 77.8 %, and a specificity of 91.9 % in adult blood donors, and an AUC value of 0.70 ($p < 0.0001$), a sensitivity of 53.2 % and a specificity of 95.3 % in the 13-year-old school children, respectively. TPOab levels in RBA correlated with both ECL ($r = 0.8950$, $p < 0.0001$) and RIA ($r = 0.9295$, $p < 0.0001$). The prevalence of TPOab and TGab was 6.3 % and 7.6 % in adult blood donors and 2.9 and 3.7 % in 13-year-old school children.

In conclusion, a novel RBA for the determination of TPOab was developed and validated with current methodologies. This study also reports an increasing prevalence of thyroid autoantibodies from adolescence to adulthood.

Introduction

Autoimmune thyroid disease (AITD) is an organ-specific disease and the most common human autoimmune endocrine disorder. AITD may present with hyperthyroidism, as in Graves' disease, or as hypothyroidism in Hashimoto's thyroiditis, the latter being much more common [1, 2].

AITD is characterized by the presence of autoantibodies towards thyroid peroxidase (TPOab) and thyroglobulin (TGab). Thyroid peroxidase is a membrane-anchored enzyme in the thyroid cell with crucial functions in the process of thyroid hormone synthesis [1]. TPOab has been reported to be the most abundant thyroid autoantibody and the strongest predictor of ongoing autoimmunity [3, 4]. TPO-1 is a 933 amino acid (aa) long, transmembrane-spanning protein; therefore, reproducing correct folding in an *in vitro* system might be challenging. No single epitope has thus far been described for autoantibody interaction, but immunodominant regions have been defined in three regions across the TPO protein, from N to C-terminal: aa 210–225, aa 549–563, and aa 599–617 [5–9]. Autoantibodies are preferably measured in the liquid phase for the detection of conformation-dependent epitopes [10]. Reduced TPOab

binding following protein denaturation has previously been described [11] and can also occur in other autoimmune diseases, including type 1 diabetes [12, 13]. Previous studies have suggested that TPOab in healthy and clinically diagnosed patients is directed towards similar epitopes, but only patient-derived TPOab was found to interfere with enzyme activity and complement fixation processes [1, 14, 15].

Thyroglobulin is an intracellular protein expressed in the thyroid follicular cells and associated with thyroid hormone storage. The pathological role of TGab is less defined compared to TPOab, although the prevalence is comparable [4, 16].

AITD predominates in females with ratios being reported between 4-10/1 (female/male). [2]. The reported prevalence of TPOab in the general population differs substantially between studies and countries [17], including Australia (TPOab female 15.0 %, male 6.6 %) [18], Norway (TPOab female 13.9 %, male 2.8 %) [19], US (TPOab female 17.0 %, male 8.7 %) [4], China (TPOab female 11.7 %, male 4.2 %) [20]. This discrepancy could be explained by natural differences between geographical regions caused by genetic associations of human leukocyte antigen (HLA) and environmental factors, similar to other common pediatric autoimmune diseases, type 1 diabetes, and celiac disease [21, 22]. An additional explanation for the discrepancy in the reported prevalence of TPOab could be the variation in sensitivity and specificity of antibody detection assays, together with the absence of adequate international reference standards for cut-off determination [23].

This study aimed to develop and validate a novel TPOab radiobinding assay (RBA) and determine the prevalence of TPOab and TGab in the Swedish population.

Methods

Study populations

All experiments including human participants were ethically approved prior to sample collection. The control study groups included (demographics, ▶ **Table 1**):

a) **Blood donors** (n = 476; n = 475 included to assess TPOab diagnostic validity and prevalence). Serum samples were collected at the blood-donation center in Malmö, Sweden, during the spring of 2019. Blood samples were donated anonymously; only age and gender were reported.
b) **School children** (n = 297; n = 295 included to assess TGab diagnostic validity and prevalence) [24]. Serum samples from 13-year-old schoolchildren were collected in Jämtland county, Sweden, between April 2009 to January 2011, on separate occasions; April, October, and November 2009, May, November and December 2010, and January 2011.

Two patient cohorts were included for the purpose of assay validation (a and b were also used during validation):

▶ **Table 1** Demographics of the study cohort.

	n	Born	Gender (female, %)
Blood donors	476	1947–2000	n = 193 (40.6 %)
Schoolchildren	297	1996	n = 157 (52.9 %)

1) **Graves' disease patients**. Samples were donated at the clinic at 6 weeks (n = 27) and 6 months (n = 20) following diagnosis.
2) **Thyroid autoantibody-positive children** (n = 124, n = 109 TGab diagnostic validity). This group of children from the Diabetes Prediction in Skåne Study (DiPiS) has been described previously [25]. In short, TPOab and/or TGab-positive children at 10 years of age were asked to leave a confirmatory sample between 11–16 years of age. Samples were previously analyzed using radioimmunoassay (RIA) (RSR Limited, art. no. RS-TP/100 and RS-TG/100 respectively) and electrochemiluminescence (ECL) (Roche, Anti-TPO REF 190,06368590). The analyses were performed according to the manufacturer's instructions [25].

Thyroid peroxidase autoantibody determination using radio-binding assay

The principle of the RBA [26] is based on the interaction between blood-derived TPO autoantibodies and ^{35}S-radiolabeled TPO-antigen. Overnight incubation enables the formation of autoantibody-antigen complexes. Precipitation of these complexes is mediated using Protein A Sepharose. A higher concentration of measured TPO autoantibodies is correlated to the amount of precipitated radiolabeled antigen.

The cDNA was constructed to include the amino acid methionine (added for the intitiation of protein synthesis), followed by TPO aa 204–785. A standard RBA was developed, including coupled *in vitro* transcription-translation (ITT). TPO cDNA constructs were subcloned into a pTNT vector (Thermo Fisher Scientific). Radiolabeled TPO-antigens were expressed using rabbit reticulocyte lysate (Promega Corporation) and ^{35}S-methionine (Promega Corporation) at 30 °C, 90 min ITT-reaction. Unlabeled methionine was removed from incorporated antigens through size-restricted filtration using Nap-5 Columns (GE Healthcare).

Serum samples (2.5 µl) in duplicates were incubated in 96-well analytical plates (Nunc V96 MicroWell, Nunc A/S) with 60 µL radiolabeled TPO-antigens (diluted to 400 cpm/µL in assay-buffer; 150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.4), 0,15 % *v*/v polysorbate 20, 0,1 % *w*/*v* bovine serum albumin) at 4 °C overnight. Filtration plates (MultiScreen HTS-DV Plates, Millipore) were blocked with 2.5 % milk solution overnight. The next day, Protein A Sepharose was diluted to 20 % in assay buffer and then added to filtration plates with 50 µL of the overnight incubated antigen-antibody mixture. Incubation was for 1 h at 4 °C before unbound antigens were removed by vacuum-washing (405LS Microplate Strip Washer, Biotek Instruments, Inc.). The addition of scintillation liquid, 50 µL per well, was followed by analysis in luminescence BetaCounter (1450 MicroBeta TriLux β-counter, PerkinElmer). TPO autoantibody levels were expressed as in-house arbitrary units per milliliter (U/mL). A positive serum sample was diluted in nine steps (1000 U/mL, 500 U/mL, 250 U/mL, 125 U/mL, 63.5 U/mL, 31.25 U/mL, 15.63 U/mL, 7.81 U/mL, and 3.91 U/mL) and used for conversion of counts per minute to U/mL. The TPO RBA intra-assay CVs, as a measure of precision, and inter-assay, as a measure of reproducibility were 5.6 and 5.5 %, respectively.

Thyroglobulin electrochemiluminescence autoantibody determination

TG autoantibodies were analyzed using YHLO iFlash 1800 Chemiluminescence Immunoassay Analyzer (C89003G). The principle of the assay is a sandwich complex formation of 1) TGab in a patient sample, 2) microbeads coated with thyroglobulin antigen, and 3) anti-TG acridinium-ester labeled conjugates.

Following magnetic-based separation of unbound material, pretrigger (hydrogen peroxide solution) and trigger solutions (sodium hydroxide solution) were added to the mixture. The resulting chemiluminecent signal was detected as relative light units (RLUs). Conversion to International Units Per Milliliter (IU/mL) was automatically calculated by a 3-point standard curve; the amount of antigen in the sample was proportional to the detected signal of relative light units.

Two (low control, high control) control samples were included in each run; the inter-assay as a measure for reproducibility was 5.1 %. The TgAb assay had a calibration range of up to 1000 IU/mL, and levels higher than 1000 IU/mL were reported as 1000 IU/mL.

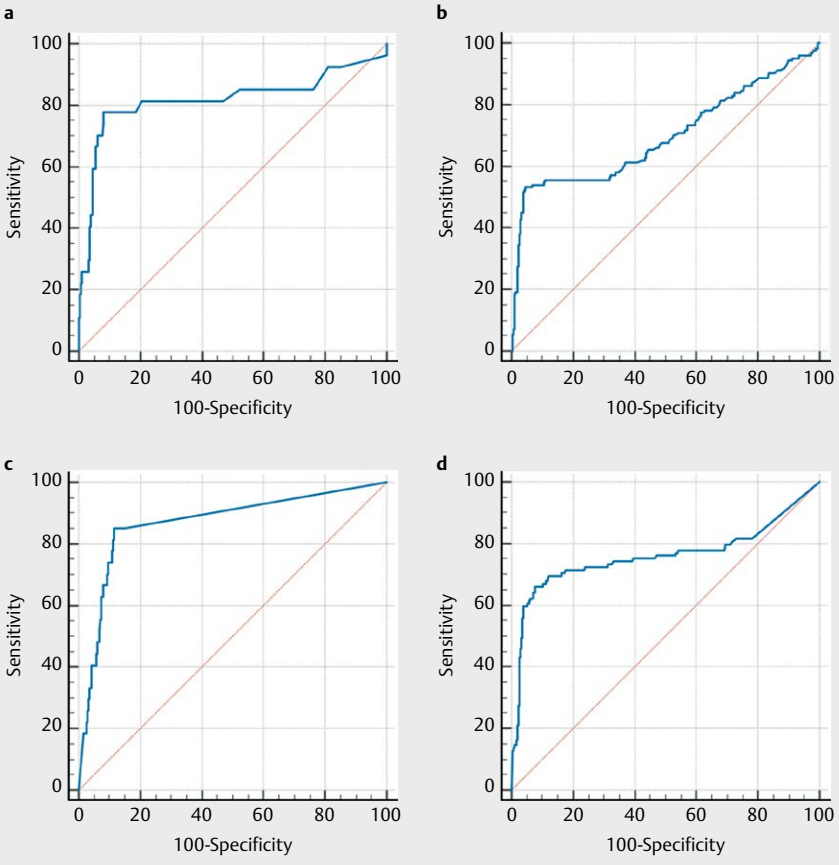
Statistical analysis

Autoantibody levels were found to be non-normally distributed. Nonparametric test, two-tailed Mann-Whitney U, were used for comparison of group differences of continuous variables. Comparison between group frequencies was assessed using the χ^2 test or Fisher's exact test. Correlations were assessed using Spearman Rho. The receiver operator characteristic (ROC) analysis was used to determine the area under the ROC curve (AUC) for the TPO and the TG autoantibody assays. A p-value ≤ 0.05 was considered statistically significant. All statistical analyses were conducted using GraphPad Prism (Prism 9.1.0, La Jolla, CA) and MedCalc (MedCalc Software Ltd.: https://www.medcalc.org/features/roccurves.php).

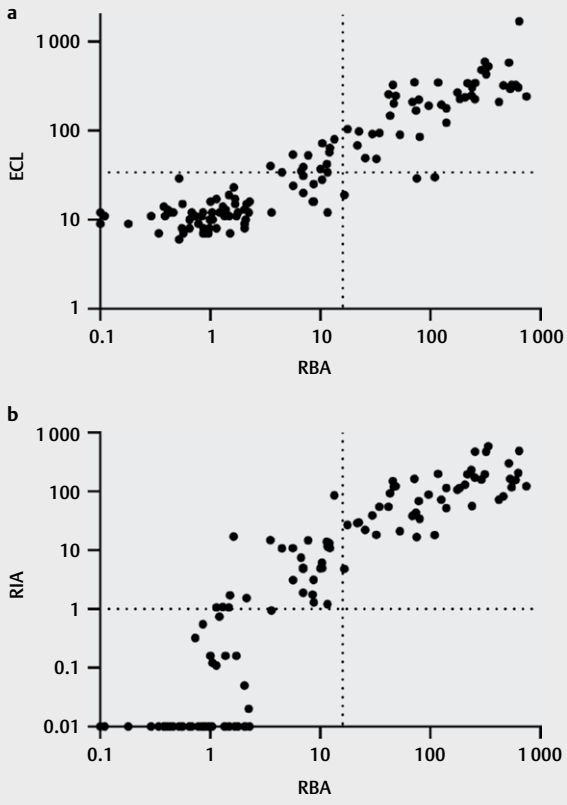
Results

Diagnostic validity

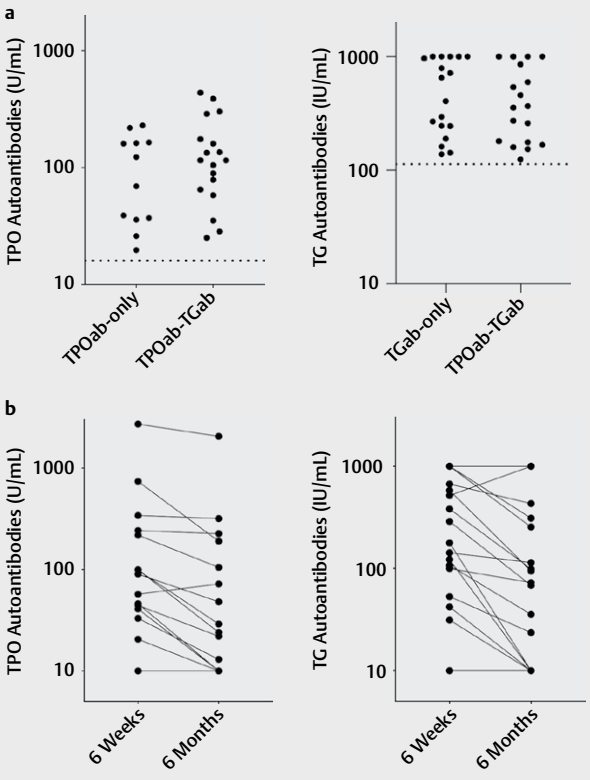
TPOAb in the 27 Graves' patients and 475 blood donors analyzed by a ROC curve resulted in an AUC value of 0.82 (0.78–0.85,



▶ **Fig. 1** Receiver Operator Characteristic analysis was used to determine the Area Under the Curve (AUC) for **a**) Thyroid peroxidase autoantibodies in the group of patients with Graves' disease (n = 27) compared to adult blood donors (n = 475), to a value of 0.82 (0.78–0.85, p < 0.0001), with a sensitivity of 77.8 % and a specificity of 91.8 %. **b**) Thyroid peroxidase autoantibodies in the group of autoimmune thyroid disease (AITD) autoantibody-positive children in prospective follow-up for increased genetic risk of type 1 diabetes (n = 124) compared to 13-year-old schoolchildren (n = 297), to a value of 0.70 (0.65–0.74, p < 0.0001), with a sensitivity of 53.2 % and a specificity of 95.3 %. **c**) Thyroglobulin autoantibodies in the group of patients with Graves' disease (n = 27) compared to adult blood donors (n = 476), to a value of 0.87 (0.83–0.90, p < 0.0001), with a sensitivity of 85.2 % and a specificity of 88.5 %. **d**) Thyroglobulin autoantibodies in AITD autoantibody-positive children in prospective follow-up for increased genetic risk of type 1 diabetes (n = 109) compared to 13-year-old schoolchildren (n = 295), to a value of 0.76 (0.72–0.80, p < 0.0001), with a sensitivity of 66.1 % and a specificity of 92.2 %.



▶ **Fig. 2** Correlations were assessed in a combined group of Graves' diseases patients (n = 27) and thyroid autoantibody-positive children (n = 124) and could be demonstrated for thyroid peroxidase radiobinding assay (RBA) in comparison to **a**) Electrochemiluminescence assay (Spearman's correlation coefficient ρ = 0.90 (0.85–0.93), p < 0.0001)). A total of 16 individuals diverged between RBA compared to ECL, with 3/124 (2.5 %) being RBA-only positive and 13/124 (10.5 %) being ECL-only positive. **b**) Radioimmunoassay (Spearman's correlation coefficient ρ = 0.93 (0.90–0.95), p < 0.0001)). Compared to RBA, 31/124 (25 %) individuals were RIA-only positive, and none were RBA-only positive.



▶ **Fig. 3** **a**) Median AITD autoantibody levels, TPOab and TGab were comparable between single-positive and double-positive individuals; TPOab single-positive 96.2 U/mL- double-positive 115.4 U/mL, TGab single-positive 527.5 IU/mL double-positive 360.5 IU/mL, p = 0.4908 and p = 0.5594 respectively. **b**) Thyroid autoantibody levels were demonstrated to decrease in the group of Graves' disease patients (n = 20) from 6 weeks compared to 6 months following diagnosis (TPOab p = 0.004 and TGab p = 0.0043).

p < 0.0001), with a sensitivity of 77.8 % and a specificity of 91.8 % (▶ **Fig. 1a**). The ROC analysis, including 124 thyroid autoantibody-positive children compared to 297 13-year-old schoolchildren, resulted in an AUC value of 0.70 (0.65–0.74, p < 0.0001). The sensitivity of the assay was 53.2 %, and the specificity was 95.3 % (▶ **Fig. 1b**).

TGab was determined using ECL. ROC analysis, including 27 Graves' patients compared to 476 blood donors, resulted in an AUC value of 0.87 (0.83–0.90, p < 0.0001), with a sensitivity of 85.2 % and a specificity of 88.5 % (▶ **Fig. 1c**). ROC analysis, including 109 thyroid autoantibody-positive children compared to 295 13-year-old schoolchildren, resulted in an AUC value of 0.76 (0.72–0.80, p < 0.0001), with a sensitivity of 66.1 % and a specificity of 92.2 % (▶ **Fig. 1d**).

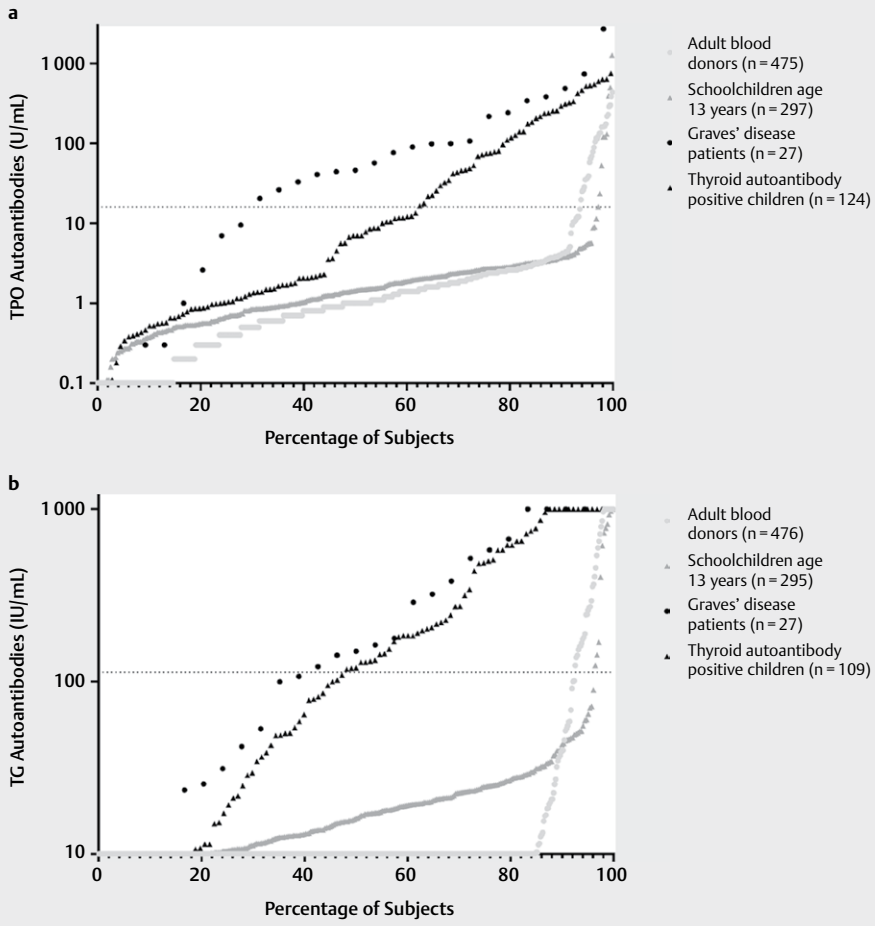
Radiobinding TPOab assay in comparison to standardized electrochemiluminescence and radioimmunoassay

A strong correlation could be demonstrated for TPOab, in Graves' patients (n = 27) and thyroid antibody-positive children (n = 124), in RBA compared to ECL (Spearman's correlation coefficient ρ = 0.90 (0.85–0.93), p < 0.0001, ▶ **Fig. 2a**) and compared to RIA (Spearman's correlation coefficient ρ = 0.93 (0.90–0.95), p < 0.0001, ▶ **Fig. 2b**). A total of 16 individuals deviated from RBA to ECL, with 3/124 (2.5 %) being RBA-only positive and 13/124 (10.5 %) being ECL-only positive. In comparison to RBA, 31/124 (25 %) individuals were RIA-only positive.

Thyroid autoantibody prevalence

The prevalence of TPOab was 6.3 % (30/475) in the adult group of blood donors and 3.0 % (9/297) in the group of 13-year-old schoolchildren, respectively, using a cut-off level of ≥16 U/mL (▶ **Fig. 3a**). TPOab was more frequent in the group of females (6.8 %, 24/351) compared to males (3.6 %, 15/421, p = 0.0386).

The prevalence of TGab was 7.6 % (36/476) in the adult group of blood donors and 3.7 % (11/295) in the group of 13-year-old



▶ **Fig. 4** Quantile-Quantile plots showing the distribution of **a**) Thyroid peroxidase autoantibodies, with a cut-off of ≥ 16 U/mL. The prevalence of TPOab was 6.3 % (30/475) in the group of adult blood donors and 3.0 % (9/297) in the group the 13-year-old schoolchildren, **b**) Thyroglobulin autoantibodies, with a cut-off of ≥114 IU/mL. The prevalence of TGab was 7.6 % (36/476) in the group of adult blood donors and 3.7 % (11/295) in the group the 13-year-old schoolchildren.

schoolchildren, respectively, using a cut-off level of ≥114 IU/mL (▶ **Fig. 3b**). TGab was more frequent in the group of females (8.0 %, 28/349) compared to males (4.5 %, 19/421, p = 0.0428).

In the group of adult blood donors, 10.1 % (48/476) were TPOab and/or TGab positive. In the group of TPOab-positive individuals, 60.0 % (18/30) were also TGab-positive, and in the group of TGab-positive individuals, 50.0 % (18/36) were also TPOab-positive. Single positivity was 2.5 % (12/476) and 3.8 % (18/476) for TPOab and TGab, respectively. TPOab and TGab double-positivity was 3.8 % (18/476).

In the group of adult blood donors, there was no difference in median autoantibody levels between TPOab and TGab single-positive compared to double-positive individuals (TPOab single-positive 96.2 U/mL, double-positive 115.4 U/mL; TGab single-positive 527.5 IU/mL, double-positive 360.5 IU/mL, p = 0.4908 and p = 0.5594 respectively, ▶ **Fig. 4a**).

Patients with Graves' disease (n = 20) were longitudinally analyzed for TPOab and TGab at 6 weeks and 6 months following diagnosis. A general reduction in thyroid autoantibody levels could be demonstrated between the sampling times (TPOab p = 0.004 and TGab p = 0.0043, ▶ **Fig. 4b**).

Discussion

Here we report the development and validation of RBA for TPO autoantibodies. The reported prevalence of TPO and TG autoantibodies in schoolchildren (3.0 %, 3.7 %) was in agreement with the reported prevalence among schoolchildren from Finland (2.6 %, 3.4 %) [27] and Sweden [28] (TPOab 2.8 %). Thyroid autoantibody prevalence varies substantially between studies. While geographical differences might occur and potentially explain part of the fluctuation, the methodological determination of autoantibodies is also speculated to contribute.

There is a lack of standardization for the detection of TPOabs and TGabs. Different laboratory methods result in a vast span of reference intervals for autoantibody measurements. The reference interval in the same group of patients has been reported to be between 0.2 to 10.0 IU/mL for TPOab and 0.53 to 14.23 IU/mL for TGab, depending on the method used for autoantibody detection [29, 30]. One explanation may be that the methods differ substantially in how they measure the presence of autoantibodies; furthermore, methods that use labels for the detection of autoantibodies may obscure antibody-antigen binding sites. Here, we present a novel RBA with a

higher sensitivity in adults compared to children. The RBA assay showed a strong correlation to both ECL and RIA, with a few patients classified as RBA-only negative and all high positive TPO samples identified using RBA. An expanded analysis in larger cohorts is warranted to further establish the cut-off level for positivity.

The current study reported a higher prevalence of thyroid autoantibodies in females compared to males, which is in line with previous studies [4, 17–20]. Two different explanatory mechanisms may be considered to understand this disparity between males and females. First, the time point of seroconversion in the early stages of autoimmune pathogenesis, and second, the timepoint in the progression from autoimmunity to clinical onset of disease. Of interest, although the TPOab frequency is lower among males, a 20-year longitudinal follow-up study reported that the prognostic value for hypothyroidism, if found positive for anti-thyroid antibodies, was increased in males compared to females (odds ratio: males 25, females 8) [31].

The progression from seroconversion to clinical onset of AITD is suggested to be slow, and not all antibody-positive individuals develop disease. Previous studies reported seroconversion as early as 2 years of age [25]. Our study adds to this understanding with an increased prevalence of TPOab and TGab from adolescence to adulthood.

Previous studies reported higher median thyroid autoantibody levels among TPOab/TGab double- compared to single-positive individuals, suggesting a mechanism of enhanced thyroid antigen leakage [32]. In contrast, antibody levels in our study were comparable between single and double-positive individuals. Further research is warranted to investigate the pathogenic mechanisms of AITD, with a focus on clinical outcomes in relation to individuals with single and double TPOab/TGab positivity.

One strength of the study is that longitudinal studies of thyroid autoantibody levels in the group of clinically diagnosed patients with Graves' disease are not common. To our knowledge, this study is novel in determining autoantibody levels in the interval between 6 weeks and 6 months following diagnosis. The reduction of autoantibodies during this period is of particular interest, and further studies are warranted to determine the clinical and long-term implications.

Weaknesses of the study include the missing determination of HLA-genotypes and clinical characteristics of adult blood donors to solidify the suggested group distinctions. Associations to HLA risk alleles in AITD is less defined in patients and controls compared to many other autoimmune diseases [33]. However, it cannot be excluded that specific HLA genotypes are related to seroconversion and autoimmune progression to clinical disease, rather than development of autoantibodies.

The sensitivity for the novel TPOab assay was adequate but could be improved further. The harmonization of TPOabs between assay platforms is a recognized issue. Two main explanations were outlined firstly, the selection of reference populations and secondly, the procedures for antigen preparation. A weakness in our study, and a potential way to improve the assay, was that the control-groups were not selected according to the standardized NACB criteria with the inclusion of individuals under the age of 30 years, only males, and exclusion of subjects with a family history of thyroid disease [34]. An international standardization program, as present for T1D [35], would be needed, as it is currently a wide dispersion in TPOab between assays and techniques. Other ways of improving the assays, including ours, could entail analyses in expanded and multi-diverse populations, as well as in-depth analysis of epitope recognition and assessment of autoantibody affinity.

Finally, we report an increased prevalence of thyroid autoantibodies from adolescence to adulthood, suggesting the importance of future studies aiming to determine the age of seroconversion.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgment

This article is part of the DiaUnion collaborative study, co-financed by EU Interreg ÖKS, Capital Region of Denmark, Region Skåne, Lund University, Steno Diabetes Center Copenhagen, and the Novo Nordisk Foundation (NNF20SA0066296).

Fundref Information

Region Skåne — http://dx.doi.org/10.13039/501100009780;
Region Hovedstaden — http://dx.doi.org/10.13039/501100005275;
European Regional Development Fund — http://dx.doi.org/10.13039/501100008530;
Steno Diabetes Center Copenhagen — http://dx.doi.org/10.13039/100018562;
Novo Nordisk Fonden — http://dx.doi.org/10.13039/501100009708;
NNF20SA0066296;
Lunds Universitet — http://dx.doi.org/10.13039/501100003252

Conflict of Interest

The authors declare that they have no conflict of interest.

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