



Lower proportion of intra-thyroidal B lymphocytes CD20⁺ associated to methimazole and lack of influence of iodide on lymphocyte subpopulations in Graves' disease

Ana Carolina Barros Silva^a, Ingrid Iara Damas^b, Camila Aparecida Moma^a, Icleia Siqueira Barreto^b, Denise Engelbrecht Zantut-Wittmann^{a,*}

^a Endocrinology Division, Department of Internal Medicine, School of Medical Sciences, University of Campinas, Campinas, São Paulo, Brazil

^b Department of Pathology, School of Medical Sciences, University of Campinas, Campinas, São Paulo, Brazil

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ABSTRACT

Graves' disease (GD), an autoimmune thyroid disease, is one of the main autoimmune diseases in the general population. It is known that the pathophysiology of this disease may be related to immunological mechanisms dysregulation. These mechanisms can be influenced by GD therapies, such as iodide or antithyroid drugs (ATD). **Objective:** Verify relation between clinical, biochemical and treatment modalities used prior to surgery and histopathological characteristics observed in total thyroidectomy products from patients previously diagnosed with Graves' disease. Furthermore, these data were related to composition of lymphocytic infiltrate in terms of proportions of lymphocytes CD4⁺, CD8⁺, CD25⁺ and CD20⁺. We aim to contribute to the understanding of the evolution pattern of GD, whose pathophysiology is not yet completely understood.

Methods: Cross-sectional study assessing thyroidectomy products for the presence of lymphocytic infiltrate, as well as the proportion and intensity of CD4⁺, CD8⁺, CD25⁺ and CD20⁺ markers. We selected 50 patients who underwent total or partial thyroidectomy in a tertiary service between 1996 and 2013 due to GD with histopathological confirmation. The control group (non-autoimmune disease group) consisted of 12 patients with histopathological data compatible with normal perilesional thyroid parenchyma. The intensity of lymphocytic infiltrate and immunohistochemical expression of the markers CD4⁺ (helper T lymphocytes), CD8⁺ (cytotoxic T lymphocytes), CD25⁺ (regulatory T lymphocytes) and CD20⁺ (B lymphocytes) were retrospectively evaluated and relationship with ultrasound, laboratory and clinical data was assessed.

Results: No differences were found in intensity, presence of lymphoid follicles, and expression of CD4⁺/CD8⁺/CD25⁺ in patients with GD who did or did not use ATD or iodide. In the group that did not use ATD, a higher proportion of CD20⁺ expression was found. The GD group was associated with hyperplastic epithelium and the control group was associated with simple epithelium. There was no difference in ultrasound thyroid volume between the groups. In GD patients with mild lymphocytic infiltrate, higher free thyroxine (FT4) levels were observed than those in patients with no infiltrate or moderate infiltrate.

Conclusion: We found a lower proportion of intrathyroidal CD20⁺ B lymphocytes in patients under use of methimazole. However, no difference was observed in intrathyroidal lymphocyte subpopulations related to the short-term use of iodide. The understanding of thyroid autoimmunity, as well as identifying points of pharmacological modulation, are very important for advancement and improvement in treatments for these diseases.

1. Introduction

Graves' disease (GD), an autoimmune thyroid disease (AITD), is characterized by thyroid infiltration of B and T lymphocytes and

production of autoantibodies directed to thyroid. GD presents circulating autoantibodies, such as TSH receptor autoantibodies (TRAb), thyroperoxidase autoantibodies (TPOAb) and thyroglobulin autoantibodies (TGAb) (Bogusławska et al., 2022). However, the immunological

* Corresponding author. Endocrinology Division, Department of Internal Medicine, Faculty of Medical Sciences, University of Campinas, Rua Tessalia Vieira de Camargo, 126, Cidade Universitária, Campinas, São Paulo, 13083-887, Brazil.

E-mail address: zantutw@unicamp.br (D.E. Zantut-Wittmann).

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mechanisms involved in the pathogenesis of AITDs are not completely understood.

Recent studies point to changes in the immune system that play key roles, such as the escape of autoreactive T cells from anergy, deletion and apoptosis processes that occur in the thymus; reduced activity of regulatory T lymphocytes ($CD25^{+}$), resulting in failure to limit the effector immune response by $CD4^{+}$ and $CD8^{+}$ T lymphocytes against self-antigen, molecular mimicry of infectious agents (*Yersinia enterocolitica*, *Borrelia*) and thyroid antigens; local secretion of cytokines (interferon-gamma, interferon-alpha) by non-thyroid-specific immune cells, that have stimulatory effect on thyroid-specific T and B lymphocytes; abnormal expression of MHC class II molecules (HLA-DR, DQ and DP) by thyrocytes, allowing them to present antigens to autoreactive T cells; and activation of autoreactive T lymphocytes, which infiltrate the thyroid (WernerIngar's et al., 2021). These lymphocytes can develop a Th1 response, inducing the death of thyroid cells, leading to hypothyroidism and Hashimoto's thyroiditis (HT), or a Th2 response, stimulating B cells to produce autoantibodies (Synoracki et al., 2016).

GD is mediated by autoantibodies and often, but not always, lymphocytic infiltration is present, but not as intense in HT. The lymphocytic infiltrate consists mainly of $CD4^{+}$ and $CD8^{+}$ T lymphocytes, as well as $CD20^{+}$ B lymphocytes, and ectopic germinal centers. These are secondary lymphoid follicles, which allow affinity maturation of autoreactive B lymphocytes, leading to the production of autoantibodies. Initially, interaction between $CD4^{+}$ T lymphocytes and autoreactive $CD20^{+}$ B lymphocytes is necessary to produce TSHR autoantibodies (Theofilopoulos et al., 2017).

The initial treatment for GD is anti-thyroid drugs (ATD), and numerous studies have documented their effects on various immune system levels.

The knowledge we have about immunomodulatory effects of ATD is based mainly on "in vitro" evidence that shows they may have direct effects on intrathyroidal T cells as increased Fas-Ligand expression by thyrocytes (Mitsiades et al., 2000) and reduced expression of transcription factors by T lymphocytes (Humar et al., 2008), as well as "in vivo" evidence for reduced HLA Class II (Zantut-Wittmann et al., 2001) expression by thyrocytes, increased numbers of circulatory suppressor T cells and decreased activated T cells (Totterman et al., 1987), and reduced plasmatic levels of cytokines produced by B lymphocytes (Pedro et al., 2010) in GD patients after treatment with methimazole.

The formation of free radicals, important in T cell responsiveness and complement-mediated thyrocyte injury, is inhibited by methimazole (WernerIngar's et al., 2021). As said above, these drugs can reduce the expression of histocompatibility complex (MHC) class II (HLA-DR) molecules in thyrocytes, which are important for the initiation and maintenance of Graves' disease (Zantut-Wittmann et al., 2001). Anti-thyroid drugs also influence the immune system by inducing the expression of the Fas ligand (FasL) in thyroid cells, which lead to Fas activation in lymphocytes and, consequently, Fas-induced apoptosis of these cells (Mitsiades et al., 2000). However, the mechanism of immunomodulatory action of these drugs is not yet completely understood.

Iodide is frequently used in the preoperative period of total thyroidectomy to control severe thyrotoxicosis. This drug has a suppressive effect on the production of thyroid hormones through the Wolff Chaikoff effect, in which iodide organification is inhibited due to a reduction in expression and activity of NIS (sodium-iodide co-transporter) (Bogusławska et al., 2022). Furthermore, its role in reducing intra-thyroid vascularization, resulting from the reduction in VEGF levels in the gland, is well described (Piantanida, 2017). However, little is known about the effect of iodide on different immune system levels involved in the development of GD.

As there is still much to be understood about the pathophysiology of GD and action mechanisms of anti-thyroid drugs, and aiming of contributing to this knowledge, we assessed the lymphocyte infiltrates composition of thyroid glands of GD patients in terms of $CD4^{+}$ lymphocytes (T helper), $CD8^{+}$ (cytotoxic T lymphocytes), $CD25^{+}$

(regulatory T lymphocytes) and $CD20^{+}$ (B lymphocytes), and the relationship with clinical characteristics, thyroid function and morphology, and treatment modalities.

2. Method

2.1. Study design

A cross-sectional study was carried out evaluating surgical thyroid pieces for the presence and intensity of lymphocytic infiltrate, in addition to immunohistochemical analysis for cellular identification markers $CD4^{+}$, $CD8^{+}$, $CD25^{+}$ and $CD20^{+}$.

Thyroid tissues and thyroidectomy products were studied, and the expression pattern of these markers was evaluated by two physicians, one of them being a thyroid pathologist (ACBS and ISB, respectively). Relationship between this pattern and age at the time of surgery, sex, goiter US volume, presence of antithyroid antibodies, use of pre-surgical antithyroid drugs or iodide and smoking was analyzed. The study was approved by the local Human Research Ethics Committee under number CAAE 58324322.1.0000.5404.

2.2. Graves' disease patients and control group without autoimmune thyroid disease

Fifty GD patients who had total or partial thyroidectomy and whose histopathological diagnoses were compatible with GD were selected. These patients underwent surgery in a tertiary service between 1996 and 2013. Forty-four of these patients were treated with ATD (Methimazole: 38 patients; propylthiouracil: 6 patients) and were in use of these drugs in the previous period next to surgery. Pharmacological treatment had an average duration of 41,93 months ($SD \pm 35,19$). The minimum period of treatment was 0,33 month (10 days) and the maximum was 120 months (10 years). Despite pharmacological treatment, all of them had active Graves' disease, evidenced by suppressed TSH (27 patients) or by impossibility of discontinuing ATD without persistence or recurrence of hyperthyroidism (17 patients). Three of these patients were referred to the service after years of treatment and they were in use of ATD for 10 years, and 5 patients used ATD for less than 1 year (10, 30, 90, 120, 180 days). Other patients had at least 1 year taking the medication. Six patients were not in use of ATDs at the moment of the surgery. All of them have used iodide in pre-operative period. The reasons for suspension of ATDs were drug induced hepatitis ($n = 1$), severe allergic reaction ($n = 2$), neutropenia ($n = 1$), not known ($n = 2$).

Control group was composed by 12 patients that did not have any kind of autoimmune thyroid pathology and had negative TPOAb and TGAb results. These patients were referred to total thyroidectomy due to cervical compressive symptoms ($n = 5$), or cervical cancer ($n = 7$). Surgeries were performed between 1999 and 2012 in a tertiary hospital. Thyroids pieces went through anatomopathological analysis by pathologist with thyroid expertise, who excluded the presence of histopathological alterations compatible with Graves' Disease or Hashimoto Thyroiditis. In those with thyroid cancer diagnosis, we examined parts of the slides with only normal thyroid tissue. Thus, the control group did not consist of patients without thyroid disease, as these individuals rarely undergo total thyroidectomy, but rather patients with cervical pathologies that did not involve thyroid autoimmunity, and with normal thyroid areas.

2.3. Histopathological analysis

After the selection of cases, microscope slides were made from paraffin blocks of thyroid tissue samples derived from thyroidectomy. These slides were stained with Hematoxylin-Eosin and later with immunohistochemical markers.

The diagnoses of normal perilesional thyroid and GD were based on the histological examination of the surgical specimen, analyzed by a

pathologist specialized in the field. Exclusion criteria were insufficient data in medical record or cases in which only frozen section biopsy or cytological analysis was performed.

The predominant type of follicular epithelium in each slide was classified as cubic, simple or hyperplastic. The intensity of lymphocyte infiltrate was classified as none, mild, moderate, or intense. The size of the follicles (small, large, or varied), the presence of vacuoles in the colloid, and the presence of lymphoid follicles were assessed.

2.4. Biochemical assessment of autoimmunity and thyroid function

Serum levels of TSH, free thyroxine (FT4), TPOAb and TgAb were analyzed. TSH levels were measured using electrochemiluminescence (EIA) – sandwich technique, used in an Elecsys TSH immunoassay analyzer (Roche), considering reference values of 0.4–4.5UI/ml. T4L levels were measured using EIA – competition principle used in an Elecsys FT4 immunoassay analyzer (Roche), considering reference values of 0.9–1.8 ng/ml. TPOAb and TgAb antibodies were measured by EIA, a competition principle used in an Elecsys Anti-TPO immunoassay analyzer (Roche), considering reference values below 35 IU/ml and 115 IU/ml, respectively.).

2.5. Cervical ultrasound

Data from the last cervical ultrasound performed before surgery were collected through medical record review. The assessments were performed at the institutional Radiology Service using a linear multiple frequency US device with a 10–12 MHz transducer for morphological analysis and 4.5–7 MHz for Doppler analysis. The total thyroid volume was determined in milliliters (ml) through the product of the longitudinal, transversal, and anteroposterior measurements, multiplying by the constant 0.524, analyzing volumes of right, left and isthmus lobes. The normal thyroid gland volume was considered to be 6–15 ml (10–11 ± 3–4 ml).

2.6. Immunohistochemical CD4⁺, CD8⁺, CD25⁺ and CD20⁺ EXPRESSION

A prospective review of histology was carried out by a pathologist experienced in thyroid lesions; followed by demarcation of areas of interest to be studied using the conventional technique for analysis of immunohistochemical expression.

Sections stained with hematoxylin and eosin (HE) were evaluated by selecting representative paraffin tissue blocks for IHC studies. Sections were dewaxed and rehydrated in an increasing alcohol gradient. Antigen retrieval was performed in 10 mM citrate buffer, pH 6, in a pressure cooker for 10 min, reaching room temperature. This was followed by blocking endogenous peroxidase with 0.3% H₂O₂ and nonspecific binding sites with 3% BSA. The tissue was incubated overnight with anti-CD4, anti-CD8, anti-CD25⁺ or anti-CD20⁺ antibodies at 4 °C. The negative control was performed by replacing the antibodies with saline. The reaction was visualized using a streptavidin-biotin-immunoperoxidase system (Dako) with diaminobenzidine as chromogen. Counterstaining was performed with hematoxylin.

2.7. Evaluation of immunohistochemical reactions

Qualitative and semiquantitative evaluation of the expression of CD4⁺, CD8⁺, CD25⁺ and CD20⁺ was performed. To analyze the positivity of immunohistochemical reactions, the Allred scoring system was used, in which the proportion of cells reactive to the antibody and the intensity of the reaction are evaluated. In this score, proportion relates to the number of cells expressing the marker, and intensity is related to the antigen concentration in each of those cells. The sum of the proportion with the intensity generated a number that was converted into a qualitative variable (negative or positive).

For proportion, the classification system used was: 0 = negative; 1 = >0 to 1/100; 2 = > 1/100 to 1/10; 3 = 1/10 to 1/3; 4 = 1/3 to 2/3 and 5 = 2/3 to 1. For intensity, the classification system used was: 0 = negative; 1 = weak; 2 = intermediate; 3 = strong. Reactions whose sum of proportion and intensity classifications was greater than or equal to 3 were considered positive. Colloid reactions without positivity in membrane or cytoplasm were considered negative. All parameters were evaluated by a thyroid pathologist.

The immunohistochemical reaction was not completely adequate in some cases, and they were excluded from statistical analysis, which led to some differences between total number described and number of patients represented in the tables.

2.8. Statistical analysis

To describe the sample profile according to the variables under study, frequency tables (absolute and percentage) of the categorical variables and descriptive measures (mean, standard deviation, minimum, median and maximum) of the quantitative variables were created.

Comparison between groups was performed using the chi-square/Fisher's exact test for categorical variables and the Mann-Whitney test for numerical variables, followed by Dunn's test, if necessary. The significance level adopted for the study was 5%.

The statistical tests were carried out by the institution's statistical professionals, using the SAS System for Windows (Statistical Analysis System), version 9.4 and R version 4.1.3 programs.

3. Results

3.1. Comparisons between patients with Graves' disease and euthyroid individuals

Patients with GD were younger (GD: 39.7 ± 11.0; CG: 51.6 ± 14.0; p0.0038) and had lower serum TSH concentrations than euthyroid individuals (GD: 1.3 ± 2.7; CG 1.5 ± 1.1; p 0.0122). There was a higher percentage of detectable TPOAb in individuals with GD (GD: 24–61.5%; CG 0–0.0%; p 0.0314). There was no difference in prevalence between the sexes, and the frequency of smoking was similar between the 2 groups (Data not shown- Supplementary material).

Regarding histopathological characteristics, hyperplastic epithelium was more common in GD and simple epithelium in euthyroid individuals (p 0,0030). Absence of lymphocytic infiltrate was associated with euthyroid individuals (p 0.0370); in GD there was a predominance of mild and moderate lymphocytic infiltrate and absence of infiltrate in 36% of cases. There was no difference in frequency of lymphoid follicles between the GD and euthyroid groups (Data not shown- Supplementary material).

In the analysis of composition of lymphocytic infiltrate in relation to CD4⁺ helper T lymphocytes and CD8⁺ cytotoxic T lymphocytes, a higher CD4⁺ expression positivity was observed in the GD than in the normal thyroid group, in which there was an absence of CD4⁺ T lymphocytes. GD was associated with high CD4⁺ staining intensity. In the GD population, most of the slides were positive for CD8⁺, with euthyroid group being associated with negative CD8⁺ expression (Table 1).

Groups did not differ in relation to proportion, intensity, and positivity of CD25⁺ marker expression.

GD was associated with high proportion and strong intensity expression of CD20⁺ marker (B lymphocytes), as well as the positivity of this marker. The normal thyroid group was associated with lower levels of proportion and intensity of this marker, as well as the negativity of its expression (Table 2).

We compared each of Graves' disease subgroups related to the use of ATD to the control group in terms of infiltrate composition by lymphocytes subtypes. Both the GD groups, with or without ATD, in comparison to control group, had significative higher positivity of CD4⁺, CD8⁺, and CD20⁺ markers (Data not shown, supplementary material).

Table 1

Composition of Lymphocyte Infiltrate - CD4⁺ (helper T lymphocytes), CD8⁺ (cytotoxic T lymphocytes) in Graves' disease and normal thyroid.

			Graves' disease (n=50)	Euthyroids (n=12)	p- value
CD4	Proportion	1	41 (83.7%)	12 (100.0%)	0.3366
		2	8 (16.3%)	0 (0.0%)	
	Intensity	1	22 (44.9%)	10 (83.3%)	0.0169
		2	27 (55.1%)	2 (16.7%)	
	Total	P	28 (57.1%)	2 (16.7%)	0.0119
		N	21 (42.9%)	10 (83.3%)	
CD8	Proportion	1	34 (68.0%)	12 (100.0%)	0.0263
		2	16 (32.0%)	0 (0.0%)	
	Intensity	1	19 (38.0%)	10 (83.3%)	0.0047
		2	31 (62.0%)	2 (16.7%)	
	Total	P	31 (62.0%)	2 (16.7%)	0.0047
		N	19 (38.0%)	10 (83.3%)	

Caption: Proportion: 1 = Low (0, 1, 2)/2 = High (3, 4).
0 = Negative/1 = >0 to 1/100/2 = > 1/100 to 1/10/3 = 1/10 to 1/3/4 = 1/3 to 2/3
Intensity: Weak (0, 1, 2)/2 = Strong (3).
0: Negative/1: Weak/2: Intermediate/3: Strong.
Total: P = positive/N = negative.
CD4⁺: 1 GD patient excluded due to missing information.

Table 2

Composition of the Lymphocyte Infiltrate - CD25⁺ regulatory T lymphocytes and CD20⁺ B lymphocytes.

			Graves' disease (n=50)	Euthyroids (n=12)	p- value
CD25	Proportion	1	44 (93,6%)	9 (81,8%)	0.2370
		2	3 (6,4%)	1 (18,2%)	
	Intensity	1	25 (53.2%)	8 (72.7%)	0.4982
		2	22 (46.8%)	3 (27.3%)	
	Total	P	34 (72.3%)	6 (54.5%)	0.2901
		N	13 (27.7%)	5 (45.5%)	
CD20	Proportion	1	25 (53.2%)	10 (90.9%)	0.0368
		2	22 (46.8%)	1 (9.1%)	
	Intensity	1	22 (46.8%)	9 (81.8%)	0.0475
		2	25 (53.2%)	2 (18.2%)	
	Total	P	29 (61.7%)	2 (20%)	0.0324
		N	18 (38.3%)	8 (80.0%)	

Caption: Proportion: 1 = Low (0, 1, 2)/2 = High (3, 4).
0 = Negative/1 = >0 to 1/100/2 = > 1/100 to 1/10/3 = 1/10 to 1/3/4 = 1/3 to 2/3
Intensity: 1 = Weak (0, 1, 2)/2 = Strong (3).
0: Negative/1: Weak/2: Intermediate/3: Strong.
Total: P = positive/N = negative.
CD25+: 3 GDand 1 or 2 CG patients excluded due to missing information.
CD20+: 3 GDand 1 or 2 CG patients excluded due to missing information.

In Fig. 1, some of the study findings are illustrated.

3.2. Comparisons between patients with Graves' disease regarding the use of anti-thyroid drugs and short-term of iodide

In GD population, made up of 50 patients, 44 patients (88%) used ATD (22 of them associated with iodide), 27 patients (54%) used iodide (6 used it exclusively, and 21 used it associated with ATD), and 5 patients (10.56%) had undergone radioiodine therapy before surgery.

Table 3 describes histopathological characteristics of patients with Graves' disease according to the use of anti-thyroid drugs (ATD) or iodide before surgery. Presence of lymphoid follicles, type of follicular epithelium, presence of vacuoles in the colloid, follicles size, as well as the intensity of the lymphocytic infiltrate did not differ between GD patients who used or did not use ATD or iodide.

Table 4 describes composition of infiltrate in terms of CD4⁺ and CD8⁺ lymphocytes in patients with Graves' disease according to the use of anti-thyroid drugs (ATD) or iodide before surgery. The positivity or

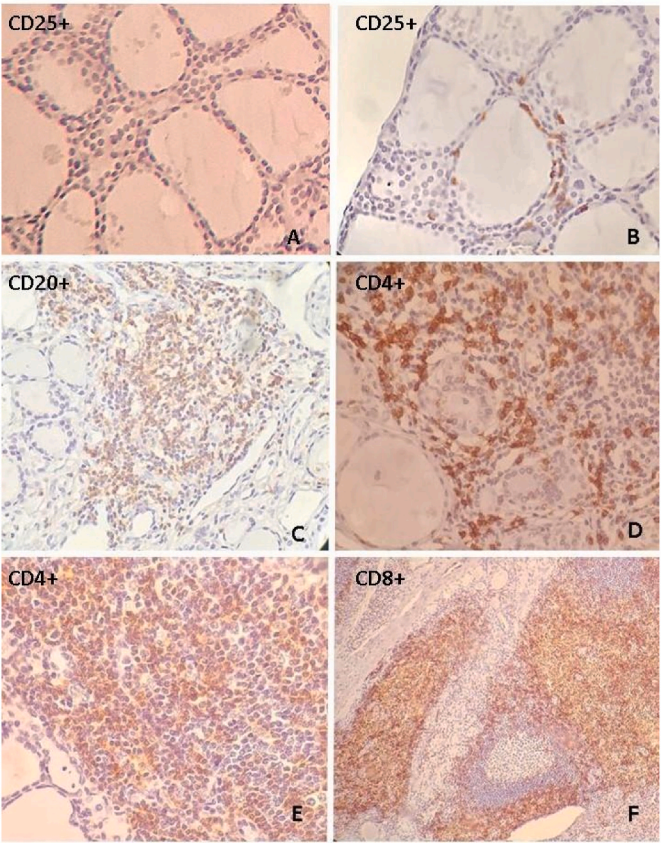


Fig. 1. Immunohistochemical expression in thyroid sections: A- CD25⁺ marker with no expression in normal thyroid (200x); B- CD25⁺ marker expression in Graves' disease with low-intensity lymphocytic infiltrate (Proportion 2 = 1/100 to 1/10; intensity 2 = moderate) (200x); C- CD20⁺ marker expression in Graves' disease with moderate-intensity lymphocytic infiltrate (proportion 4 = 1/3 to 2/3; intensity 1 = weak) (200x); D- CD4⁺ marker expression in Graves' disease with moderate intensity lymphocytic infiltrate (proportion 3 = 1/10 to 1/3; intensity 3 = strong) (400x); E- CD4⁺ marker expression in Graves' disease with moderate intensity lymphocytic infiltrate (proportion 4 = 1/3 to 2/3; intensity 2 = moderate) (400x); F- CD8⁺ marker expression in high-intensity lymphocytic infiltrate in Graves' disease (ratio 5 = 2/3 to 1; intensity 3 = strong). Lymphoid follicle formation (200x).

negativity of immunohistochemical staining for detection of CD4⁺ and CD8⁺, as well as their proportion in the infiltrate and its intensity did not differ between GD patients who used or did not use ATD or iodide.

Table 5 describes the composition of infiltrate in relation to CD25⁺ and CD20⁺ lymphocytes in patients with Graves' disease according to the use of anti-thyroid drugs (ATD) or iodide before surgery. It was observed that patients who did not use ATD had significantly higher proportion of CD20⁺ B lymphocytes than those who used it. The positivity or negativity of immunohistochemical reaction for detection of CD25⁺ and CD20⁺, as well as its intensity, did not differ between GD patients who used or did not use ATD or iodide.

4. Discussion

Many advances have been made in recent decades regarding the understanding of the pathogenesis of GD. Particularly in recent years, there has been much discussion about the possibility of modulating immunological mechanisms to control or even cure these diseases (Zhou et al., 2022). Despite these advances, there is still controversy about the action mechanisms of drugs that have long been used in treatment, such as ATDs (methimazole, propylthiouracil), whose effects on the immune system have been widely documented. In relation to other drugs used to

Table 3
Use of ATD and iodide x Histological Characteristics in Graves' disease.

		ATD			Iodide ^a		
		Without ATD (n=8)	With ATD (n=42)	p-value	With Iodide (n=27)	Without iodide (n=19)	p-value
Lymphoid Follicles	P	2 (25.0%)	1 (2.4%)	0.0653	2 (7.4%)	1 (5.6%)	1.0000
	A	6 (75.0%)	40 (97.6%)		25 (92.6%)	17 (94.4%)	
Follicular epithelium	2	3 (42.9%)	19 (48.7%)	1.0000	10 (41.7%)	10 (55.6%)	0.4426
	3	4 (57.1%)	18 (46.2%)		13 (54.2%)	8 (44.4%)	
Colloid (vacuoles)	P	5 (71.4%)	31 (79.5%)	0.6361	19 (79.2%)	15 (83.3%)	1.0000
	A	2 (28.6%)	8 (20.5%)		5 (20.8%)	3 (16.7%)	
Follicular size	1	4 (57.1%)	17 (43.6%)	0.2339	12 (50.0%)	8 (44.4%)	1.0000
	2	2 (28.6%)	5 (12.8%)		3 (12.5%)	3 (16.7%)	
Infiltration intensity	3	1 (14.3%)	17 (43.6%)	0.7912	9 (37.5%)	7 (38.9%)	0.3740
	0	2 (25.0%)	16 (38.1%)		10 (37.0%)	7 (36.8%)	
	1	4 (50.0%)	15 (35.7%)		12 (44.4%)	6 (31.6%)	
	2	1 (12.5%)	8 (19.0%)		2 (7.4%)	5 (26.3%)	
	3	1 (12.5%)	3 (7.1%)		3 (11.1%)	1 (5.3%)	

Captions: ATD = anti-thyroid drug; Lymphoid Follicles: P = present/A = absent; Follicular epithelium: 1 = cubic/2 = simple/3 = hyperplastic; Vacuoles: A = absent/P = present; Follicular size: 1 = large/2 = small/3 = varied; Infiltrate intensity: 0 = absent/1 = mild/2 = moderate/3 = intense.

^a Four patients excluded due to missing information.

Table 4
Use of ATD or Iodide and expression of CD4⁺ and CD8^a markers in patients with Graves Disease.

		ATD		p-value	Iodide ^a		p-value
		Without ATD (n=8)	With ATD (n=42)		With iodide (n=27)	Without iodide (n=19)	
CD4	Proportion	0	2 (28.6%)	0.4209	11 (40.7%)	9 (50.0%)	0.0985
		1	1 (14.3%)		2 (7.4%)	1 (5.6%)	
		2	0 (0.0%)		1 (3.7%)	4 (22.2%)	
		3	2 (28.6%)		9 (33.3%)	1 (5.6%)	
		4	2 (28.6%)		4 (14.8%)	3 (16.7%)	
	Intensity	0	2 (28.6%)	-	11 (40.7%)	9 (50.0%)	-
		1	1 (14.3%)		1 (3.7%)	0 (0.0%)	
		2	3 (42.9%)		10 (37.0%)	7 (38.9%)	
		3	1 (14.3%)		5 (18.5%)	2 (11.1%)	
		P	5 (71.4%)		16 (59.3%)	9 (50.0%)	
CD8	Proportion	N	2 (28.6%)	0.6830	11 (40.7%)	9 (50.0%)	-
		0	2 (25.0%)		10 (37.0%)	8 (42.1%)	
		1	0 (0.0%)		1 (3.7%)	0 (0.0%)	
		2	0 (0.0%)		1 (3.7%)	1 (5.3%)	
		3	3 (37.5%)		7 (25.9%)	3 (15.8%)	
	Intensity	4	3 (37.5%)	0.5398	8 (29.6%)	7 (36.8%)	0.2855
		0	2 (25.0%)		10 (37.0%)	8 (42.1%)	
		2	3 (37.5%)		8 (29.6%)	2 (10.5%)	
		3	3 (37.5%)		9 (33.3%)	9 (47.4%)	
		P	6 (75.0%)		17 (63.0%)	1 (57.9%)	
Total		N	2 (25.0%)	0.6933	10 (37.0%)	8 (42.1%)	0.7287
			17 (40.5%)				

Captions: ATD = anti-thyroid drug; Proportion: 0 = Negative/1 = >0 to 1/100/2 = > 1/100 to 1/10/3 = 1/10 to 1/3/4 = 1/3 to 2/3; Intensity: 0: Negative/1: Weak/2: Intermediate/3: Strong; Total: P = positive/N = negative.

^a Four patients excluded due to missing information.

control GD, such as iodide and beta-blockers, little is known about how they act, or even if they act, on the lymphocyte population (Barin et al., 2005).

In comparison between Graves' and non-autoimmune disease group, it was observed greater expression of CD4⁺, CD8⁺ and CD20⁺ in Graves' group, which is compatible with disease activity. These findings are in line with previous studies that have described the presence of T CD4⁺ and CD8⁺ and B lymphocytes in intrathyroidal infiltrate of Graves' Disease (Nakashima et al., 1996; Duh et al., 1990; Misaki et al., 1985), however, there was no comparison with non-autoimmune disease individuals, as performed in the present study.

In the present study, the use of ATDs and short-term iodide in patients with Graves' disease did not influence the intensity, presence of lymphoid follicles and composition of intra-thyroidal lymphocytic infiltrate, except for patients treated with methimazole who had a lower proportion of CD20⁺ B lymphocytes when compared to patients who did not use the drug. The finding of reduced proportion of CD20⁺ marker intrathyroidal expression after ATD treatment have not been reported in previous studies.

Although the inhibitory effect of antithyroid drugs on the production of thyroid hormones is well established through inhibition of thyroperoxidase (TPO), reduction of DIT + MIT and DIT + DIT coupling and, in case of PTU, inhibition of peripheral deiodination of T4 into T3, much has been discovered about its immunomodulatory action, which is evidenced by a reduction in TRAb levels and often remission of GD with long-term treatment with ATDs. Evidence confirms that this effect may be due to its direct action on the immune system, regardless of its ability to alter thyroid hormone levels (WernerIngbar's et al., 2021).

There is evidence that changes in circulatory T cell subtypes occur within the first seven days of treatment with methimazole 20 mg/day, with an increased number of suppressor T cells and decreased activated T cells, as observed by Totterman (Karlsson and Tötterman, 1988). The author also found a significant reduction of expression of 4F2+ (activation marker) by B lymphocytes (CD19⁺ cells) in peripheral blood after methimazole treatment, when compared to control, but there was not an analysis of intrathyroidal lymphocytes infiltrate, and CD20⁺ marker was not evaluated. An indirect evidence of B lymphocytes suppression by methimazole was the reduced plasmatic levels of cytokines produced by

Table 5
Use of ATD or Iodide and expression of CD20⁺ and CD25⁺ in patients with Graves' Disease.

		ATD		p-value	Iodide ^a		p-value
		With ATD (n=44)	Without ATD (n=6)		With iodide (n=27)	Without iodide (n=19)	
CD25	Proportion	0	13 (30.2%)	0.1740	8 (32.0%)	5 (26.3%)	-
		2	11 (25.6%)		8 (32.0%)	4 (21.1%)	
		3	17 (39.5%)		9 (36.0%)	8 (42.1%)	
		4	2 (4.7%)		0 (0.0%)	2 (10.5%)	
	Intensity	0	13 (30.2%)	0.4311	8 (32.0%)	5 (26.3%)	0.6500
		1	12 (27.9%)		8 (32.0%)	5 (26.3%)	
		2	13 (30.2%)		8 (32.0%)	6 (31.6%)	
		3	5 (11.6%)		1 (4.0%)	3 (15.8%)	
	Total	P	29 (69.0%)	0.3027	17 (68.0%)	5 (27.8%)	0.7662
		N	13 (31.0%)		8 (32.0%)	5 (27.8%)	
CD20	Proportion	0	17 (41.5%)	0.0384	11 (44.0%)	7 (38.9%)	0.2006
		3	7 (17.1%)		1 (4.0%)	5 (27.8%)	
		4	8 (19.5%)		4 (16.0%)	2 (11.1%)	
		5	9 (22.0%)		9 (36.0%)	4 (22.2%)	
	Intensity	0	17 (41.5%)	- ^b	11 (44.0%)	7 (38.9%)	-
		1	2 (4.9%)		2 (8.0%)	0 (0.0%)	
		2	12 (29.3%)		7 (28.0%)	7 (38.9%)	
		3	10 (24.4%)		5 (20.0%)	4 (22.2%)	
	Total	P	24 (58.5%)	0.3839	14 (56.0%)	11 (61.1%)	0.7375
		N	17 (41.5%)		11 (44.0%)	7 (38.9%)	

Captions: ATD = anti-thyroid drug; Proportion: 0 = Negative/1 = >0 to 1/100/2 = > 1/100 to 1/10/3 = 1/10 to 1/3/4 = 1/3 to 2/3/5 = 2/3 to 1; Intensity: 0: Negative/1: Weak/2: Intermediate/3: Strong; Total: P = positive/N = negative.

^a Four patients excluded due to missing information.

^b Test was not possible due to small number of patients in each category. When intensity was divided in two categories (weak: 0,1; strong: 2,3), a p-value = 0,5701 was obtained.

B cells (IL-1, IL-2, IL-5, IL-6 and TNF-alpha) observed by Takei (2010) (Pedro et al., 2010). Also, we applied the Allred scoring system to analyze thyroid sample slides after immunohistochemistry, and we highlight that this scoring system is not usually used in the study of autoimmune thyroid diseases but is commonly used in oncology. This characteristic is in line with the finding in the present study of a higher proportion of CD20⁺ in patients who were not treated with methimazole.

The CD20⁺ marker has been studied as a possible target for new therapies for treatment of Graves' Disease and Graves' Ophthalmopathy. Studies carried out with rituximab, an anti-CD20⁺ antibody, demonstrated a reduction in levels of circulating and intrathyroidal CD20⁺ lymphocytes, through complement-mediated pathways or induction of apoptosis. This effect leads to an improvement in the clinical picture by reducing the production of anti-TSH receptor antibodies, or by favoring the production of non-stimulating TRAb to greater extent than the stimulating TRAb (Dalan and Leow, 2012).

CD25⁺ lymphocytes negatively regulate the immune response by four mechanisms: release of inhibitory cytokines, deprivation of stimulatory cytokines, inhibition of antigen-presenting cells, and direct killing of T cells. Increasing evidence shows that Treg cells are diminished or functionally impaired in GD. Thus, the role of ATDs in increasing the population of CD25⁺ lymphocytes becomes essential for controlling the disease (Dalan and Leow, 2012).

When comparing patients with GD that used ATD with those who did not, there is no difference in relation to proportion, intensity, or positivity of CD25⁺ marker. Although GD patients were in use of ATD, disease was not under control at the time of surgery, which was indicated because of the failure of pharmacological treatment. A possible explanation would be that the failure in the treatment with methimazole could be due to its ineffectiveness in increasing the activity of regulatory T lymphocytes (CD25⁺), which shows that this effect may play a key role in the induction of GD remission by ATDs. No differences were observed in the intensity of lymphocytic infiltrate or positivity of CD4⁺ and CD8⁺ markers between GD patients that were or not treated with ATD, probably because of the lack of Treg lymphocytes regulatory effect on these cells populations.

Lack of effectiveness of methimazole treatment prior to surgery

could also be observed when comparing each of the subgroups of GD patients (With ATD X Without ATD) with control group in terms of CD4⁺, CD8⁺, CD20⁺ and CD25⁺ markers expression. In this sense, both of GD subgroups had higher positivity of CD4⁺, CD8⁺ and CD20⁺ markers than control group, and no difference in CD25⁺ positivity. We would expect that in ATD subgroup there would be no difference when compared to the non-autoimmune disease group in terms of infiltrate lymphocyte subtypes composition. However, it did not occur, and we believe that it is due to the ineffectiveness of methimazole immunosuppression in this group of patients with active disease even with ATD treatment.

Although the primary objective of the use of iodide prior to total thyroidectomy is the reduction of thyroid vascularization and consequent prevention of intraoperative hemorrhage (Piantanida, 2017), little is known about the acute effects of this compound on thyroid autoimmunity. In literature, there is a description of an early peak of thyroid autoantibodies in populations that started iodine supplementation in the diet, followed by reduction and stabilization of this amount in the long term, which suggests that iodide has a transient stimulatory role on the immune system (Ruggeri and Trimarchi, 2021). This increase in the production of thyroid autoantibodies was associated with an increase in the iodination of thyroglobulin and consequent increase in its antigenicity, leading to greater reactivity of T lymphocytes to thyroid antigens¹¹. During longitudinal follow-up of these populations, an increase in the prevalence of subclinical hypothyroidism in young women was observed, but there was no increase in the incidence of Graves' disease (Ruggeri and Trimarchi, 2021). In the present study, no differences were found in the intensity, presence of lymphoid follicles and composition of the lymphocytic infiltrate between GD patients who used and those who did not use iodide before surgery, which suggests that the short period of use, which varied from 2 to 10 days, was not enough for detecting differences in pattern and composition of infiltrate induced by this compound. Another hypothesis would be that iodide would not really influence lymphocyte distribution.

In relation to histopathological findings, the type of prevalent follicular epithelium occurred as expected. In the GD, follicular cell hyperplasia was compatible with the lack of effectiveness of anti-thyroid drug to control the disease in these patients, as it is indicative of

excessive stimulation of the cells by the binding of TRAb to TSHR.

In GD, thyroid cells themselves act as antigen-presenting cells to autoreactive T and B lymphocytes, and T lymphocytes have a primarily auxiliary role (Th2 response), stimulating TSHR-reactive B lymphocytes to produce TRAb (Dalan and Leow, 2012). In the present study, most patients with GD had mild or moderate infiltrates, and 36% of them did not have infiltrates. A possible explanation for this finding was the inclusion of 88% of patients who used ATD and, although it did not induce disease remission, possibly contributed to the reduction of glandular inflammation. Furthermore, some patients had undergone RIT before surgery, which may have reduced the intensity of lymphocytic infiltration.

A possible limitation in this study was the sample size of GD group, particularly the number of patients without ATD, which could have led to insufficient statistical power to detect differences between groups that received or did not receive a certain treatment prior to surgery. The proportion and intensity assessments of each marker were carried out separately, in different sections, which prevented comparative visualization of the distribution of each marker and direct comparison between them. Furthermore, the inclusion of five patients who had previously undergone RIT may have contributed to attenuating possible differences between the lymphocytic infiltrates of treated and untreated patients. Another point that deserves attention is the use of proportion and intensity scales based on the observer's perception, although the analysis of all slides by the same evaluator may have contributed to greater accuracy of the found results. Also, we could not analyze TRAb serum level differences between GD patients that received or not treatment with ATD or iodide because of the lack of data regarding the dosage of this antibody prior to surgery, as this exam was not largely available in our service until the mid-2000s.

The inclusion of a non-autoimmune disease group, as a control group, with analyzes of normal thyroid tissue was a strength of the study and allowed its comparison to the thyroid affected by autoimmune inflammation. The fact that there was a small number of normal thyroids reflects the difficulty in obtaining these samples. We also had a relevant number of thyroid samples derived from a homogeneous group of patients with active (despite pharmacological treatment) and histopathological confirmed Graves' Disease, which enabled this *in vivo* observational study. Another strong point of the study was the use of the Allred scoring system, which is not commonly used in autoimmune thyroid disease studies. Even with this different approach, our findings were in line with current knowledge on thyroid autoimmunity.

In conclusion, this study sought to contribute to the documentation and understanding of the differences in characteristics and composition of lymphocytic infiltrates, types of follicular epithelium, TSH and FT4 levels and thyroid volume between patients with GD and normal thyroid. It was possible to observe the differences in follicular epithelium types between them, and the immunosuppressive effect of thyroxine on lymphocytic infiltrate in the GD group. We also sought to contribute to the documentation and understanding of immunological effects of ATDs and iodide on the lymphocyte populations of patients with GD, having found a lower proportion of CD20⁺ B lymphocytes in the population under the use of methimazole, indicating lower number of cells expressing the marker, and no influence of short-term iodide in the intrathyroidal lymphocyte distribution.

Further studies understanding thyroid autoimmunity, as well as identifying points subject to pharmacological modulation, are necessary and of great importance for the advancement and improvement of treatments offered for these diseases.

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CRediT authorship contribution statement

Ana Carolina Barros Silva: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ingrid Iara Damas:** Validation, Methodology. **Camila Aparecida Moma:** Validation, Methodology, Investigation. **Icleia Siqueira Barreto:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Data curation, Conceptualization. **Denise Engelbrecht Zantut-Wittmann:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Denise Engelbrecht Zantut Wittmann reports equipment, drugs, or supplies and statistical analysis were provided by State University of Campinas. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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