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**Thyroid Autoimmunity:
Exploring the Role of Th17-associated
Cytokines and Pathomorphological
Mechanisms Involved in the Pathogenesis
of Hashimoto's Thyroiditis
and Graves' Disease**

Summary of the Doctoral Thesis for obtaining
a doctoral degree (*Ph.D.*)

Sector – Clinical Medicine
Sub-Sector – Internal Medicine

Rīga, 2021



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Abbreviations

AITD	autoimmune thyroid diseases
ANA	antinuclear antibodies
FT3	free triiodothyronine
FT4	free thyroxine
GD	Graves' disease
GM-CSF	granulocyte macrophage colony-stimulating factor
GO	Graves' orbitopathy
GPx	glutathione peroxidase
HT	Hashimoto's thyroiditis
IFN	interferon
IHC	immunohistochemistry
IL	interleukin
ROR	retinoic acid receptor-related orphan receptor
SELENO	selenoprotein
TEM	transmission electron microscopy
Tg	thyroglobulin
TGF	transforming growth factor
Th	T helper
Tj	tight junctions
TNF	tumour necrosis factor
TPO	thyroperoxidase
Treg	T regulatory
TSH	thyroid stimulating hormone
TSHR	thyroid stimulating hormone receptor
tTG-IgA	tissue-transglutaminase IgA autoantibodies
ZO	<i>zonula occludens</i>

Introduction

Autoimmune thyroid disease (AITD), predominantly presenting as Graves' disease (GD) or Hashimoto's thyroiditis (HT), is the most frequent autoimmune disease with increasing prevalence and incidence in recent decades. In both autoimmune disorders, the complex interactions among various environmental and endogenous factors trigger thyroid autoimmunity in predisposed individuals, leading to enhanced thyroid autoantigen presentation and impairment of immune tolerance (Ruggeri, Giuffrida and Campenni, 2018). However, the immunological aspects of AITD pathogenesis are not yet fully understood.

Cytokines produced by inflammatory and thyroid follicular cells have pivotal roles in the induction and modulation of cellular and humoral immune responses. Although both diseases belong to AITD, they are different in pathogenic mechanisms and the presentation of the dysregulated immune system. HT development is associated predominantly with the T helper (Th) 1 immune response, which triggers cellular immune reactions and causes thyroid cell apoptosis with subsequent hypothyroidism via upregulation of interferon (IFN)- γ , interleukin (IL)-1 β , IL-2, and tumour necrosis factor (TNF)- α expression. In GD, the upregulation of IL-4, IL-5, IL-6, and IL-13 expression is a result of the Th2-driven humoral immune response, which causes the secretion of autoantibodies recognising thyroid-stimulating hormone receptor (anti-TSHR) and contributes to thyroid cell hypertrophy, eventually leading to hyperthyroidism (Ramos-Leví and Marazuela, 2016).

The understanding of the Th1 and Th2 cell dichotomy, which is essential for autoimmune disorders, shifted with the discovery of Th17 lymphocytes. Th17 lymphocytes mainly secrete IL-17 and IL-22. IL-17 is a crucial pro-inflammatory cytokine mediating chronic and autoimmune inflammation and neutrophil recruitment. Supporting evidence suggesting the contribution of Th17 cells and

IL-17 to AITD, particularly HT, has been produced in the last decade (Figueroa-Vega et al., 2010; Peng et al., 2013; Konca Degertekin et al., 2016; Esfahanian et al., 2017). It has been proposed that AITD results from Th1/Th2 dichotomy and Th17/T regulatory (Treg) cell imbalances (González-Amaro and Marazuela, 2016). However, the roles of Th17 cells in the initiation and progression of AITD remain unclear.

The initial development of Th17 cells from naïve Th cells is induced by transforming growth factor (TGF)- β in the presence of IL-6 and IL-21, while IL-23 and IL-1 β are essential for both the complete maturation and generation of pathogenic Th17 lymphocytes (Chung et al., 2009; Stadhoudersab, Lubberts and Hendriks, 2018). The understanding that Th17 cells are heterogeneous and exhibit under certain conditions different Th17 cell phenotypes – pathogenic and non-pathogenic has only recently been proposed. The role of interleukins promoting the differentiation of pathogenic Th17 cells has not been thoroughly evaluated in HT and GD pathogenesis.

Tight junctions (Tj), called also occluding junctions, are composed of a complex, interwoven network of membrane proteins and glycoproteins. Tj maintain cell polarity and establish the barrier sealing the paracellular space between neighbouring thyroid cells. Impairment of follicular tightness through modifications in the expression of junction proteins could be significant in the development of AITD. Many interleukins were found to modulate Tj proteins such *zonula occludens* (ZO)-1, occludin, and claudin proteins causing downregulation of these molecules (Huppert et al., 2010; Pérez et al., 2014); however, the role of IL-17 in the maintenance of Tj integrity in AITD patients has not yet been evaluated.

Currently, a role for selenium deficiency in the development of AITD is suggested along with genetic predisposition and environmental triggers. Selenium in the form of selenocysteine is present in specific selenoproteins,

25 of which have been identified in humans. Selenoproteins protect against oxidative stress, regulate the function of the immune system, and production of active thyroid hormone (Rayman, 2012). Selenium deficiency can impair the differentiation of CD4+ T cells, leading to cellular and humoral response dysfunction. In addition, selenium leads to changes in the levels of IL-6, TGF- β , and IL-23 and may further contribute to impaired differentiation of Th17 cells (Nettleford and Prabhu, 2018). Very little information is currently available on the selenium content in soils in Latvia or the selenium status in AITD. To date, the association between selenium and Th17-related cytokines has been insufficiently explored, mostly in experimental models.

Aim of the research

To explore the role of Th17 cells in the pathogenesis of HT and GD by the use of morphology methods and xMAP technology, and correlating these data with the selenium status.

To conduct the research, the following **objectives** were proposed:

1. To characterise the Th17 immune response in AITD by measuring the plasma concentrations of Th17- and Treg-associated cytokines – IL-17, IL-22, IL-23, IL-6, and IL-10 in the Latvian patients with newly diagnosed, treatment-naïve GD or HT compared to healthy subjects without autoimmune disorders.
2. To estimate the distribution and levels of immunoexpression of IL-17, IL-23, and IL-1 β within thyroid tissue of patients with HT and GD; to compare these indices with the control group.
3. To study the relationship between IL-17 and pathogenic Th17-promoting cytokines in AITD patients.

4. To investigate the integrity of the thyroid follicle by studying immunoexpression of cellular Tj – ZO-1 and claudin-1 proteins coupled to IL-17A and CD68 detection; to compare the expression levels in the AITD patients and controls.
5. To assess the selenium status in patients with newly diagnosed, treatment-naïve GD or HT to determine whether the selenium level is reduced in AITD patients compared to healthy subjects.
6. To explore the potential associations between measured Th17 cytokines and selenium levels.

Scientific hypotheses of the given research

1. Th17 cells and their cytokines have an important role in the pathogenesis of AITD, thus changing the traditional paradigm of Th1/Th2 dichotomy.
2. The cytokine-regulated disruption of Tj enabling the migration of antigen-presenting cells across thyroid follicular epithelium is essential in the pathogenesis of thyroid autoimmunity.
3. Selenium has an important role in the development of AITD; its status is lowered in AITD patients compared to healthy subjects.

The novelty of the research

In this research, for the first time, the involvement of Th17 cell cytokines in the pathogenesis of HT and GD was explored in association with selenium status and assessment of the integrity of the thyroid follicle. Levels of Th17 cytokines were analysed in both plasma and thyroid tissue of AITD patients by the use of immunohistochemistry, immunofluorescence, immunogold labelling, and xMAP technology. Simultaneous assessment of various Th17-associated cytokines provided new information on the complex cytokine pattern that characterises AITD. To our knowledge, the expression of IL-23 in the thyroid

tissues of GD patients has not been studied before. Associations between the levels of Th17-related cytokines and hyperthyroidism severity in GD patients observed in the study create a theoretical background for further studies of thyroid autoimmunity. Although GD induced by thyroid-stimulating autoantibodies is considered to be a Th2-mediated disease, current findings have suggested Th17-related cytokines may play a role in GD pathogenesis. Remarkably, the assessment of selenium status in Latvian patients with AITD has not been established before.

Ethical aspects

The research was conducted according to the Declaration of Helsinki and was approved by the Research Ethics Committee of Rīga Stradiņš University on June 25, 2014, No E-9 (2). All patients and volunteers included in the study signed a written informed consent form to participate in the study.

1 Material and methods

The prospective study was carried out at Rīga East Clinical University Hospital. Thyroid tissue blocks of thyroidectomy samples along with the histopathology reports were obtained from the Pathology Centre of Rīga East Clinical University Hospital. All morphological studies were conducted at the Joint Laboratory of Electron Microscopy, Institute of Anatomy and Anthropology. The analysis of plasma cytokine levels was performed at the Department of Human Physiology and Biochemistry, whereas selenium concentrations were determined at the Scientific Laboratory of Biochemistry, Rīga Stradiņš University.

1.1 The first study. Investigation of the integrity of the thyroid follicle by studying immunoexpression of cellular Tj –ZO-1 and claudin-1 proteins coupled to IL-17A and CD68 detection in patients with AITD

The study is described in the manuscript “Heterogeneity of tissue IL-17 and tight junction proteins expression demonstrated in patients with autoimmune thyroid diseases” by Tatjana Zaķe, Sandra Skuja, Ieva Kalere, Ilze Konrade, and Valerija Groma. *Medicine (Baltimore)*, 2018 Jun; 97(25): e11211, doi: 10.1097/MD.00000000000011211. PMID: 29924048.

1.1.1 Patients’ characteristics

Twenty-five adult AITD patients presented with HT (n = 18) and GD (n = 7) following thyroidectomy were enrolled in this study. Ten age and gender-matched subjects presented with colloid goitre without autoimmune component were recruited and used as controls. The mean age of AITD patients and controls was 49.68 ± 11.01 years (range, 28–68 years; 23 females) and 47.90 ± 11.29 years (range, 33–71 years; 9 females), respectively.

AITD was confirmed by the clinical diagnostic criteria of HT and GD, complemented by results of thyroid biochemistry, ultrasound imaging, and histopathology findings. Controls (n = 10) presented with colloid goitre were euthyroid and displayed the absence of thyroid antibodies.

1.1.2 Routine histopathology

Thyroid tissue blocks of thirty-five thyroidectomy samples (equal to the number of patients enrolled in the study) along with the histopathology reports were obtained from the Pathology Centre of Riga East Clinical University Hospital, Latvia between January 2013 and December 2014. All available specimens were evaluated; the diagnosis was confirmed by analysing the slides stained routinely with haematoxylin and eosin. A single representative paraffin-embedded thyroid tissue block was chosen for immunohistochemical study.

1.1.3 Immunohistochemical reactions

Thyroid tissues were fixed in formalin and embedded in paraffin blocks; thereafter 4–5 µm thick sections were cut from blocks and collected on slides. Immunohistochemical reactions were performed manually on deparaffinised thyroid tissue sections using the following primary antibodies: polyclonal rabbit anti-IL-17A (Biorbyt, Cambridge, UK, dilution 1:300); polyclonal rabbit anti-ZO-1 (Biorbyt, Cambridge, UK, dilution 1:200), and polyclonal rabbit anti-claudin-1 (Biorbyt, Cambridge, UK, dilution 1:100) antibodies. Additionally, macrophages were visualised by monoclonal mouse anti-CD68 antibody (DacoCytomation, Glostrup, Denmark, clone PG-M1, dilution 1:100).

The results of immunohistochemical reactions were visualised by use of a highly sensitive HRP Polymer system – HiDef Detection™ system manufactured by CellMarque, Rocklin, CA, USA. The antigen sites were visualised by applying 3,3'-diaminobenzidine tetrahydrochloride

(DAB+Chromogen and DAB+Substrate buffer (CellMarque, Rocklin, CA, USA)) whereas nuclei were stained with Mayer's haematoxylin. Negative immunohistochemical controls were performed either by omitting the primary antibody or substituting it with buffer solution. Tissue sections were analysed applying both low and high power magnification from $\times 100$ up to $\times 400$. Microphotographs were taken with a Leitz DMRB microscope equipped with a digital camera DFC 450C.

1.1.4 Assessment of results of immunohistochemical reactions

Cells were considered immunopositive when labelled by the aforementioned antibodies revealed brown reaction products. IL-17, claudin-1, and ZO-1 positive structures displayed either cytoplasmic or membranous reaction patterns. Levels of immunopositivity for IL-17A and claudin-1 were assessed semiquantitatively distinguishing the following grades: negative (0), weak (1), moderate (2), or strong (3), when cells were positive at 0–5, 6–25, 26–50, or $> 50\%$, respectively; whereas, for ZO-1 – negative (0), very weak or discontinuous (1), weak (2), and strong or intact (3) following Abd El Atti and Shash (2012) recommendations. CD68 positivity was defined as the percentage of CD68 positive thyroid follicles determined by counting the total number of thyroid follicles with CD68 positive cells in 15 representative fields at high magnification ($\times 400$).

1.1.5 Immunofluorescence assay

To visualise IL-17A more clearly, an immunofluorescence assay of IL-17A was performed. It was performed using goat anti-mouse IgG-FITC: sc-2010 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, dilution 1:300); sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, Invitrogen, UK, dilution 1:3000) and finally

embedded in Prolong Gold with DAPI (Thermo Fisher Scientific, Invitrogen, UK). Digital images were captured using a confocal microscope Eclipse Ti-E (Nikon).

1.1.6 Statistical methods

Immunohistochemical results were expressed as medians and interquartile range. Mann-Whitney test was applied to examine differences between two groups and Kruskal-Wallis H test – for more than two study groups. To assess the potential correlations between estimated variables Spearman rank correlation test was carried out. A two-tailed significance level of 0.05 was applied. Statistical testing was performed using SPSS (version 20.0).

1.1.7 Transmission electron microscopy

To better assess the ultrastructural appearance of Tj, 5 thyroid tissue specimens obtained during thyroidectomy were fixed in 2.5% glutaraldehyde and further processed for conventional TEM – the gold standard technique for visualisation of cell-to-cell junctions. After primary fixation in 2.5% glutaraldehyde, the specimens were rinsed and postfixed with 1% OsO₄, stained en bloc with uranyl acetate, dehydrated with ascending concentrations of ethanol, infiltrated, and embedded in Epon resin, which was subsequently polymerized at 62 °C for 24 hr. Semithin sections of 1 µm thickness were cut and stained with 1% toluidine blue. Thereafter, ultrathin sections with a thickness of 60 nm were cut with an ultramicrotome (LKB Ultratome V), mounted on 200-mesh copper grids, and double stained with 2% uranyl acetate in 70% methanol for 10 min, followed by lead citrate for 4 min. The specimens were imaged with a JEM 1011 JEOL (Japan) transmission electron microscope operating at 100 kV. Thyroid tissue cells were examined at ×3000–×30000 magnification.

1.2 The second study. Investigation of the association between IL-17 and pathogenic Th17-promoting cytokines in AITD by studying the immunoexpression patterns of IL-17, IL-23, and IL-1 β within thyroid tissue

The study is described in the manuscript “Upregulated tissue expression of T helper (Th) 17 pathogenic interleukin (IL)-23 and IL-1 β in Hashimoto's thyroiditis but not in Graves' disease” by Tatjana Zaķe, Sandra Skuja, Ieva Kalere, Ilze Konrade, and Valerija Groma. *Endocrine Journal*. 2019, 66(5): 423–430. doi: 10.1507/endocrj.EJ18-0396. PMID: 30814438.

1.2.1 Patients' characteristics

Twenty-nine adult patients diagnosed with AITD (21 cases of HT and 8 of GD) who underwent thyroidectomy at Rīga East Clinical University Hospital, Latvia, between January 2014 and December 2016 were enrolled in this study. Eighteen age- and gender-matched patients diagnosed with colloid goitres, displaying normal thyroid hormone levels and an absence of thyroid antibodies were used as controls. The mean age of AITD patients and controls was 49.52 years (range, 28–68 years; 27 females) and 51.78 years (range, 33–72 years; 16 females), respectively.

1.2.2 Routine histopathology

Forty-seven paraffin-embedded thyroid tissue blocks from 47 patients along with the histopathology reports were obtained from the Pathology Centre of Rīga East Clinical University Hospital, Latvia. The diagnosis was confirmed by analysing the slides stained routinely with haematoxylin and eosin.

1.2.3 Immunohistochemical reactions

The sections cut from thyroid tissue blocks were deparaffinised, endogenous peroxidase activity was blocked with 0.1% H₂O₂ in methanol and heat-induced antigen retrieval was performed. The sections were incubated at 4°C overnight with the following primary antibodies: polyclonal rabbit anti-IL-17A (Biorbyt, Cambridge, UK, 1:300); polyclonal rabbit anti-IL-23 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, 1:100); and polyclonal rabbit anti-IL-1 β (Santa Cruz Biotechnology, Inc., 1:50) antibodies. The results of the immunohistochemistry (IHC) reactions were visualised by HRP HiDef Polymer Detection™ System (CellMarque, Rocklin, CA, USA). The sections were then counterstained with haematoxylin, washed, dehydrated and coverslipped.

IHC controls included substitution of the primary antibody with buffer solution. Assessment of results of immunohistochemical reactions

The cells labelled by the aforementioned antibodies displayed a brown cytoplasmic staining pattern and were considered immunopositive. The levels of immunopositivity for each antibody were expressed in a semiquantitative manner and graded as negative (0), weak (1), moderate (2) or strong (3), when thyroid follicular cells were positive at 0–5, 6–25, 26–50, or > 50%, respectively.

1.2.4 Immunofluorescence assay

Thyroid tissue specimens obtained from five HT patients during thyroidectomy were fixed in aldehyde and further processed for immunofluorescent labelling and confocal microscopy. Immunofluorescence staining of IL-1 β , IL-17A, and IL-23 was performed using goat anti-mouse IgG-FITC: sc-2010 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, 1:300) as the secondary antibody; the samples were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, Invitrogen, UK,

1:3000) and then embedded in Prolong Gold with DAPI (Thermo Fisher Scientific, Invitrogen, UK). Digital photos were taken using a confocal microscope Eclipse Ti-E (Nikon, Tokyo, Japan).

1.2.5 Statistical methods

The results of immunohistochemical reactions are shown as medians and interquartile range. Nonparametric statistics were used, i.e., the Mann-Whitney test, to assess differences between two independent groups. The Spearman rank correlation test was used to describe correlations. A significance level of $p = 0.05$ was applied. Statistical analyses were performed by using IBM SPSS software, version 23.

1.2.6 Electron microscopy immunogold labelling

For the better exploration of the localisation of IL-17, IL-23, and IL-1 β at the cellular level, electron microscopy immunogold labelling was performed. The specimens obtained during thyroidectomy were fixed in 1.5% glutaraldehyde and 4% paraformaldehyde and further processed and embedded in Epon resin. For immunogold labelling, pretreatment of the ultrathin sections mounted to nickel grids using 0.01 M citrate buffer in a warming chamber (15 min, 98 °C) was applied. Afterwards, incubation with primary antibodies: polyclonal rabbit anti-IL-17A (Biorbyt, Cambridge, UK, 1:300); polyclonal rabbit anti-IL-23 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, 1:100); and polyclonal rabbit anti-IL-1 β (Santa Cruz Biotechnology, Inc., 1:50) antibodies were used (overnight at 4 °C). The immunogold labelling was performed by goat anti-rabbit IgG conjugated to 10-nm gold particles (1:100; Polysciences Europe GmbH, Germany). Finally, sections were stained with 2% uranyl acetate and imaged with a JEM 1011 JEOL (Japan) at $\times 15000$ – $\times 50000$ magnification.

1.3 The third study. Assessment of plasma levels of Th17-associated cytokines and selenium status in AITD

The study is described in the manuscript “Plasma levels of Th17-associated cytokines and selenium status in autoimmune thyroid diseases” by Tatjana Zaķe, Ieva Kalere, Sabine Upmale-Engela, Simons Svirskis, Gita Gersone, Andrejs Skesters, Valerija Groma, and Ilze Konrade. *Immunity, Inflammation and Disease*, 2021;1–12. doi.org/10.1002/iid3.433.

1.3.1 Patients’ characteristics

Eleven adult patients with untreated newly diagnosed hyperthyroid GD and 41 patients with untreated new-onset HT were recruited into this prospective study between January and November 2020 at the Department of Endocrinology, Rīga East University Hospital, Latvia. Thirty-three out of 41 HT patients were euthyroid (median TSH – 1.90 (1.00–2.62) μ IU/mL), and eight out of the 41 patients had mild subclinical hypothyroidism (median TSH – 5.90 (5.41–7.35) μ IU/mL). The median age was 36 (27–50.5) years for the HT patients, 41 (29–62) years for the GD patients, and 30 (27–42.25) years for the control group ($p = 0.139$).

The control group consisted of 26 age- and sex-matched healthy subjects without any autoimmune disease and with normal thyroid function, who were negative for anti-thyroperoxidase (TPO), anti-thyroglobulin (Tg), antinuclear antibodies (ANAs), and anti-tissue-transglutaminase IgA (tTG-IgA) autoantibodies.

The exclusion criteria for the study included (1) pregnancy; (2) presence of malignancy, active infection, chronic inflammatory or other autoimmune diseases; (3) use of selenium-containing commercial supplements; (4) vegetarian or vegan diet; (5) use of medications affecting the thyroid and immune function (amiodarone, corticosteroids, nonsteroidal anti-inflammatory drugs,

antidepressants, or anticonvulsants); and (6) significant renal or hepatic impairment.

1.3.2 Blood sample collection

Peripheral blood samples were collected from all 78 patients and volunteers after overnight fasting early in the morning. Thereafter, the samples were centrifuged at 1500 rpm for 10 minutes and stored at -80°C until analysis. Venous blood samples were collected from hyperthyroid and hypothyroid patients prior to therapy with methimazole or levothyroxine, respectively.

1.3.3 Thyroid function and antibody tests

The levels of serum-free thyroxine (FT4), free triiodothyronine (FT3) and TSH as well as anti-TPO and anti-Tg autoantibodies were measured by chemiluminescence immunoassay (Siemens, Malvern, PA, USA) performed on an Advia Centaur XP (Siemens) analyser. The normal values were as follows: FT4, 0.7–1.48 ng/dL; FT3, 0.2–0.44 ng/dL; and TSH, 0.35–4.94 $\mu\text{IU/mL}$; anti-TPO, 0–5.61 IU/mL; anti-Tg, 0–40 U/mL. The levels of anti-TSHR, ANAs and tTG-IgA autoantibodies were measured by ELISA (Pharmacia Diagnostics Freiburg, Germany) according to the manufacturer's instructions (reference range: anti-TSHR, 0–1.58 IU/L; ANA, reference range would be “negative”; tTG-IgA, 0–10.0 U/mL).

1.3.4 Detection of cytokines

Cytokine patterns analysed in the current study included the following groups: Th17-related cytokines (IL-17a and IL-22), Th17-promoting cytokines (IL-23 and IL-6), and a Treg-associated cytokine (IL-10). EDTA plasma immunological markers were detected by xMAP technology (Magpix system;

Luminex Corporation, USA). All tests were performed in accordance with the manufacturer's instructions (Cat#: HTH17MAG-14K; Kit Lot#: 3323752; Milliplex). The limit of detection for each cytokine was as follows: IL-17a, 0.009 pg/mL; IL-22, 0.021 ng/mL; IL-23, 0.098 ng/mL; IL-6, 1.7 pg/mL; and IL-10, 0.3 pg/mL.

1.3.5 Selenium assay

The plasma selenium concentration was determined fluorometrically by using the fluorescence spectrophotometer "Cary Eclipse" (Varian, Inc., Netherlands) (Alfthan, 1984). Inter-laboratory quality control was conducted by employing two standards – a selenium AAS solution (Aldrich, USA, Cat# 24, 792-8) and Seronorm TE Serum Level I (Sero AS, Cat# 201 405, Norway) – for the Seronorm™ Trace Elements-Controls Programme. External quality assessment services were provided by Labquality Oy (Finland). The lower detection limit for selenium was 4 µg/L.

1.3.6 Statistical methods

The normal distribution of data was confirmed by D'Agostino and Pearson, Anderson–Darling, and Shapiro–Wilk normality tests. Homogeneity of variances was tested using Brown–Forsythe and Bartlett's tests. Since dispersion did not correspond to a normal distribution in most cases, data were analysed by the nonparametric Kruskal-Wallis H-test followed by the two-stage step-up method of Benjamini, Krieger, and Yekutieli as a post hoc procedure, and the results are displayed as the median and interquartile range. Depending on the data distribution, both parametric Pearson's analysis and nonparametric Spearman's correlation analysis were performed to determine the relationships of studied parameters. A $p < 0.05$ was considered statistically significant for all statistical tests. All graphical images and statistical analyses were performed

using GraphPad Prism 9.0 for MacOS software (GraphPad Software, San Diego, CA, USA).

1.4 The fourth study. Immunological mechanisms of AITD: a shift in the traditional Th1/Th2 paradigm

Despite the significant advancement in the understanding of AITD pathogenesis in the last decade, the exact immunological mechanisms responsible for the disease development have not been thoroughly understood. The fourth study tailored as a review describes the aetiology and pathogenesis of AITD and addresses an important and novel issue on recent data regarding the role of Th17 and Treg lymphocytes in thyroid autoimmunity. In addition, the impact and proposed mechanisms of the predominant environmental factors triggering the autoimmune response to the thyroid are discussed in a review article (Publication 4) and presented in the Literature review of the Thesis.

2 Results

2.1 The first study. Investigation of the integrity of the thyroid follicle by studying immunoeexpression of cellular Tj –ZO-1 and claudin-1 proteins coupled to IL-17A and CD68 detection in patients with AITD

2.1.1 Histopathological features of AITD

The presence of widespread mild-to-extensive inflammatory infiltration of the thyroid gland by lymphocytes, macrophages, and plasma cells was confirmed in AITD by using microscopic examination of slides processed for routine histopathology. Intraepithelial lymphocytes were also seen.

In HT patients, diffuse and extensive inflammatory infiltration (7; 39%), formation of lymphoid follicles (6; 33%), or rarely mild inflammation without formation of lymphoid follicles (5; 28%) was demonstrated. Lymphoplasmacytic infiltration in the interfollicular area separating and distorting thyroid follicles along with interfollicular fibrosis and deposition of accumulated collagenous fibres was demonstrated in some HT patients. Still, extensive destruction of thyroid parenchyma with minimal residual follicles was revealed in other HT patients. Simultaneously, the vast majority of GD patients presented with mild inflammatory cell infiltration and diffuse hyperplasia of the follicular cells.

2.1.2 Assessment of immunohistochemical expression of tested antigens

The results describing the immunohistochemical expression of the antigens tested in the given study and found in AITD and colloid goitre are summarised in Table 2.1.

**Patterns of immunohistochemical expression of tested antigens
in patients with AITD and colloid goitre**

	IL-17A median (IQR)	ZO-1 median (IQR)	Claudin-1 median (IQR)	CD68 median (IQR)
Hashimoto's thyroiditis	2.4 (1.8; 2.8)	0.5 (0.5; 1)	1.5 (1; 2.2)	23.5 (8.9; 38.3)
Graves' disease	1.4 (1; 2.4)	2 (1.5; 2)	1.2 (1; 1.4)	10.8 (5.4; 13.2)
Colloid goitre	0.8 (0.6; 1)	2 (1.5; 2.5)	1.6 (1.2; 2.0)	5.6 (3.1; 8.7)

* Data are shown as a median and interquartile range. Abbreviations: IL, interleukin; ZO, *zonula occludens*; IQR, interquartile range.

In HT patients, the expression of IL-17A was detected both in the inflammatory lymphocytic infiltrates and follicular epithelial cells and confirmed by conventional immunohistochemistry and immunofluorescence (Figure 2.1).

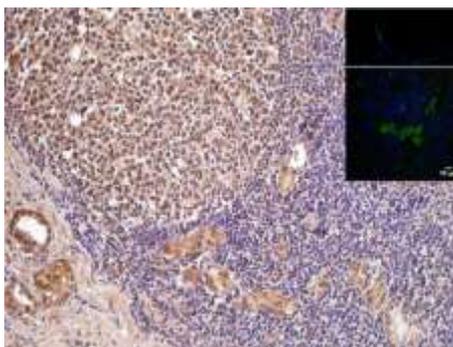


Figure 2.1 IL-17A positivity demonstrated in the remnants of thyroid follicles and lymphoid follicles as well as in some inflammatory cells in HT patient, ×200. Confocal microscopy, representative images of IL-17A-positive thyrocytes and lymphocytes: green staining shows IL-17A-specific staining, blue staining shows nuclei, inserts, ×1000

Notably, thyroid epithelial cells located close to lymphocytes or surrounded by inflammatory cells had a strongly marked IL-17A immunopositivity. Moreover, these thyroid follicles revealed atrophic changes with the destruction of epithelial cells and nuclear polymorphism. The highest

expression level of IL-17A in the follicular epithelial cells was observed in HT patients; among 18 samples, 11 tissue samples presented with IL-17A staining estimated as grade 2 to 3.

In contrast, the thyroid tissues obtained from ordinary colloid goitre and GD patients demonstrated mostly the weak or almost negative expression of IL-17A. Only in 1 tissue sample of GD and 1 sample of colloid goitre the grade of IL-17A positivity was ≥ 3 and ≥ 2 , respectively.

In HT and GD patients, the expression level of IL-17A was significantly higher when compared to colloid goitre ($p < 0.001$; $p = 0.007$, respectively) (Figure 2.2).

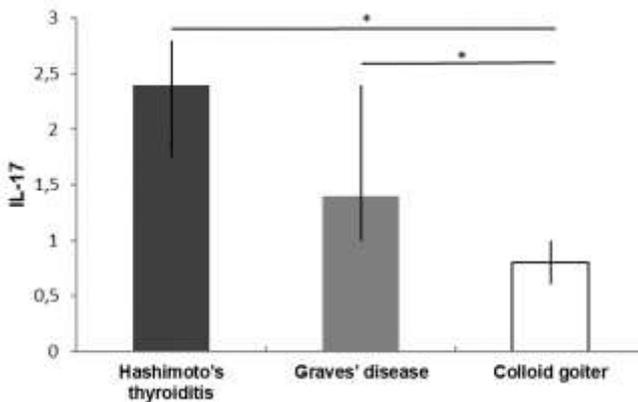


Figure 2.2 Comparison of immunohistochemical results between HT, GD, and colloid goitre patients

* A bar chart represents expression levels of IL-17A in the thyroid tissue samples of patients with AITD and colloid goitre. Comparisons were made by Mann–Whitney U test. Data are shown as median (interquartile range); * $p < 0.05$.

When studying the occurrence and localisation of Tj proteins, and more specifically claudin-1 by the use of immunohistochemistry, it was found displaying a circumferential and membranous pattern related to the thyroid epithelial cells (Figure 2.3).

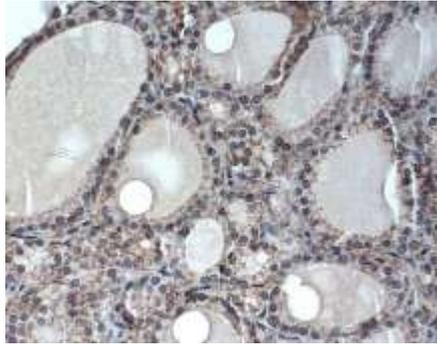


Figure 2.3 Moderate circumferential membranous claudin-1 immunostaining revealed in a thyroid tissue sample of a patient with GD, ×400

The expression of claudin-1 was greatly varying from being almost nil in 1/3 of HT cases (6; 33%), but still being heavily expressed in others (5; 28%). There were no significant differences found in claudin-1 expression levels between patients with AITD and colloid goitre (Figure 2.4). However, the number of claudin-1 positive follicles in HT patients was significantly higher than that demonstrated in colloid goitre ($p = 0.03$ and data not shown).

Predominantly negative and very weak or discontinuous ZO-1 expression patterns were demonstrated in the samples of patients presented with HT. In 10 HT cases (56%), the ZO-1 staining score was confirmed as negative or up to 1, whereas in the GD group, only one specimen (14%) showed such a staining score.

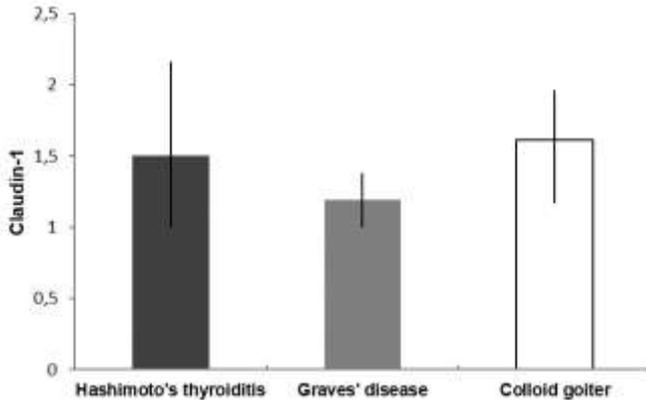


Figure 2.4 Comparison of immunohistochemical results between HT, GD, and colloid goitre patients

* A bar chart depicts claudin-1 expression levels in the thyroid tissue samples of patients with AITD and colloid goitre. Comparisons were made by Mann–Whitney U test. Data are shown as median (interquartile range).

A significant reduction of ZO-1 expression level in the follicular epithelial cells was confirmed in the samples of patients presented with HT when compared to the samples of patients presented with colloid goitre (Figure 2.5) and GD) ($p < 0.001$ for both, Figure 2.6). ZO-1 immunopositivity did not differ significantly between these two last groups of patients ($p = 0.47$).

Both intra- and extrafollicular localisation of CD68 positive macrophages was confirmed in all tissue samples. The cells were irregularly shaped and displayed a vacuolated cytoplasmic appearance being arranged either in clusters (Figure 2.7) or individually distributed within the thyroid follicles.

Large clusters of intrafollicular CD68 positive cells were found exclusively in HT patients, whereas occasional CD68 positive cells were demonstrated in samples obtained from patients presented with GD and colloid goitre. Lymphocytes, along with macrophages, were also seen within the follicles.

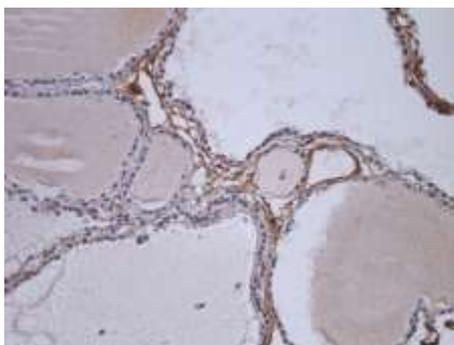


Figure 2.5 The thyroid tissue sample of a patient with colloid goitre evidencing weak to moderate and moderate positive ZO-1 expression in the thyrocytes and vascular endothelium, respectively, ×250

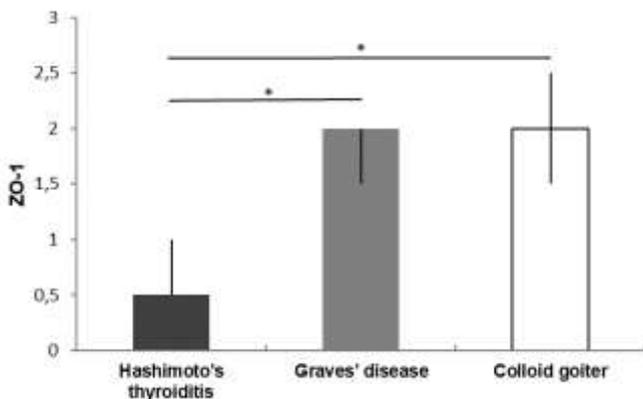


Figure 2.6 Comparison of immunohistochemical results between HT, GD, and colloid goitre patients

* A bar chart depicts immunopositivity levels of ZO-1 in the thyroid tissue samples of patients with AITD and colloid goitre. Comparisons were made by Mann–Whitney U test. Data are shown as median (interquartile range); * $p < 0.05$.

A significantly higher number of thyroid follicles with CD68 positive cells was found in HT patients than that in patients with GD or colloid goitre ($p = 0.05$ and $p = 0.001$, respectively) (Figure 2.8). CD68 positivity observed

in GD patients was higher compared to colloid goitre but the difference was not significant ($p = 0.06$).

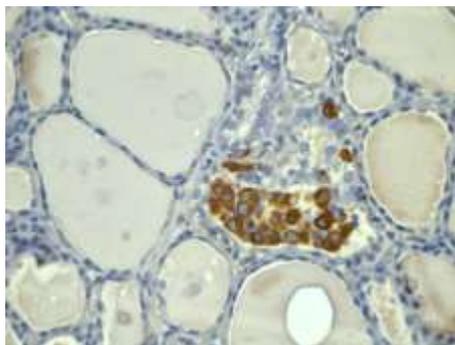


Figure 2.7 The cluster of CD68 positive cells demonstrated within thyroid follicle in a tissue sample obtained from a patient with HT, $\times 400$

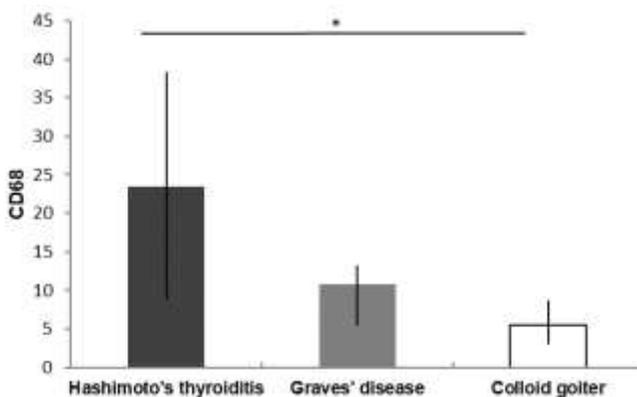


Figure 2.8 Comparison of immunohistochemical results between HT, GD, and colloid goitre patients

* A bar chart depicts the number of CD68 positive thyroid follicles in the thyroid tissue samples of patients with AITD and colloid goitre. Comparisons were made by Mann–Whitney U test. Data are shown as median (interquartile range); * $p < 0.05$.

2.1.3 Ultrastructural examination of intercellular junctions of thyroid epithelial cells

Significant changes of intercellular junctions, in general, and Tj, in particular, were confirmed in the tissue samples of patients presented with HT when examined by the use of electron microscopy. Notably, the ultrastructural examination was found supportive of formerly reported IHC data. Ultrastructurally, signs of the thyrocytes' damage were confirmed. The integrity of the cellular plasma membrane was impaired; the intercellular junctions, including those presented by Tj and localised at the apical aspect of the lateral surface, were altered. Both condensed and electron-lucent cytoplasm were noticed (Figure 2.9). A reduction in the number of cytoplasmic organelles and the appearance of lipid inclusions were often confirmed. Lymphocytic infiltration gradually led to the destruction of thyrocytes, evidenced ultrastructurally.

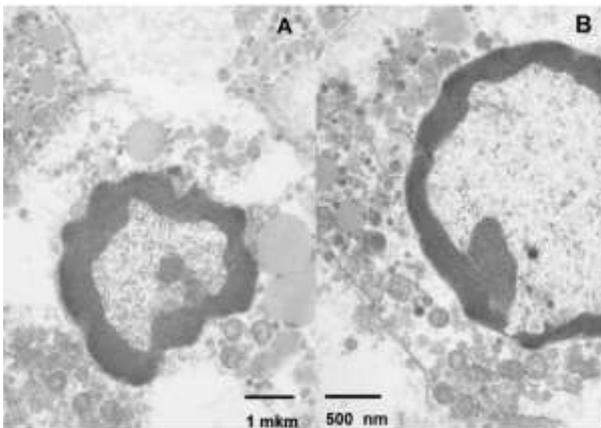


Figure 2.9 **Electron micrograph demonstrating damage of thyrocytes**

* Typical signs of cell apoptosis are visible: chromatin condensation and margination in the nuclei of thyroid epithelial cells; the electron-lucent appearance of the cell cytoplasm, the presence of scanty organelles. Scale bars: A – 1 mkm; B – 500 nm.

2.1.4 Analysis of relationship between thyroid expression of IL-17A and intrafollicular CD68 in HT patients

In HT patients, the expression of IL-17A in thyroid epithelial cells was positively correlated with the immunopositivity of intrafollicular CD68 macrophages ($r = 0.631$, $p = 0.005$). When analysing associations between IL-17A and Tj proteins expression, no significant correlations between IL-17A and ZO-1 or claudin-1 were found in HT patients ($r = 0.267$, $p = 0.28$ and $r = 0.194$, $p = 0.44$, respectively). No significant correlations between IL-17A and ZO-1 and claudin-1 as well as CD68 were found in GD patients ($r = -0.199$, $p = 0.67$ and $r = 0.734$, $p = 0.06$, and $r = 0.631$, $p = 0.13$, respectively).

2.2 The second study. Investigation of the association between IL-17 and pathogenic Th17-promoting cytokines in AITD by studying the immunoexpression patterns of IL-17, IL-23, and IL-1 β within thyroid tissue

2.2.1 Immunohistochemical analysis of Th17-associated interleukins in patients with AITD and colloid goitre

In HT patients, IL-17 immunopositivity was confirmed in thyroid epithelial cells and inflammatory infiltrates (Figure 2.10). The level of immunoexpression was mostly estimated as moderate (13/21 samples revealed the staining grade ≥ 2). Additionally, in HT patients, the impaired integrity and destruction of follicular cells were frequently revealed in the IL-17-positive thyroid follicles.

In contrast to HT, predominantly weak and negative IL-17 expression was demonstrated in the samples of patients presented with GD and colloid goitres, respectively. The expression level of IL-17 in the thyrocytes was significantly higher in HT and GD patients than that in patients with colloid goitres ($p < 0.001$ for both) (Figure 2.11). Moreover, higher IL-17 immunoreactivity was

demonstrated in the thyroid tissue of patients presented with HT when compared to GD, but the difference was not significant ($p = 0.069$).

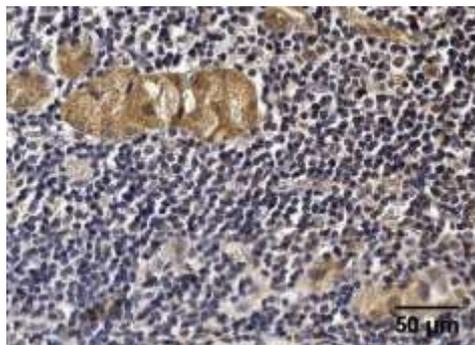


Figure 2.10 **IL-17-positive thyroid follicles and inflammatory cells evidenced in a thyroid tissue sample of a patient with HT, ×400**

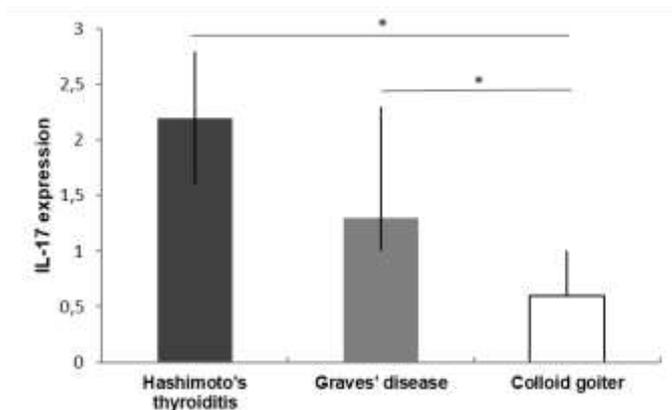


Figure 2.11 **Comparison of IHC results of IL-17 in patients with AITD and colloid goitre**

* A bar chart depicts immunopositivity levels of IL-17 confirmed in the samples of patients with AITD and colloid goitre. Comparisons were made by using the Mann–Whitney U test. Data are shown as the median (interquartile range), * $p < 0.05$.

Similar to IL-17, the expression of IL-23 (Figures 2.12 and 2.13) and IL-1 β (Figure 2.14) was confirmed both by conventional immunohistochemistry and immunofluorescence in the abundant inflammatory infiltrates characteristic of HT.

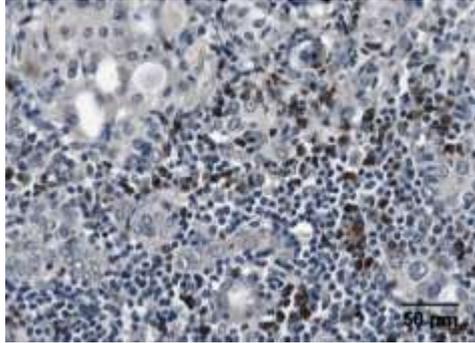


Figure 2.12 Destruction of thyroid follicles. Lymphocytes infiltrating into the surrounding connective tissue; Hürthle cells. IL-23 positive cells evident within the inflammatory infiltrate in a thyroid tissue sample of a patient with HT, $\times 400$

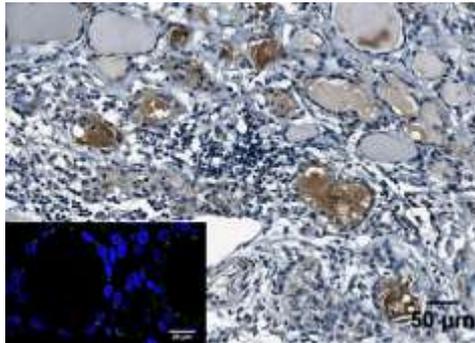


Figure 2.13 IL-23 positivity demonstrated in the follicular epithelial cells in a thyroid tissue sample of a patient with HT, $\times 200$. Confocal microscopy, a representative image of IL-23 positive thyrocytes: green staining shows IL-23-specific staining, blue staining shows the cells' nuclei, insert, $\times 1000$

In contrast to HT, predominantly weak IL-23 (Figure 2.15) and IL-1 β (Figure 2.16) thyrocytic immunostaining was demonstrated in the tissues obtained from GD patients.

Similarly, a vast majority of patients with colloid goitres presented with weak or negative expression of IL-23 and IL-1 β confirmed in the thyroid tissue samples.

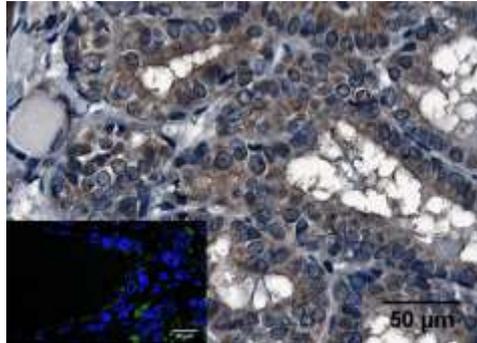


Figure 2.14 Strong cytoplasmic expression of IL-1 β demonstrated in the thyrocytes in a thyroid tissue sample of a patient with HT, $\times 400$. Confocal microscopy, a representative image of IL-1 β -positive thyrocytes: IL-1 β -specific staining (green), the cells' nuclei (blue), insert, $\times 1000$

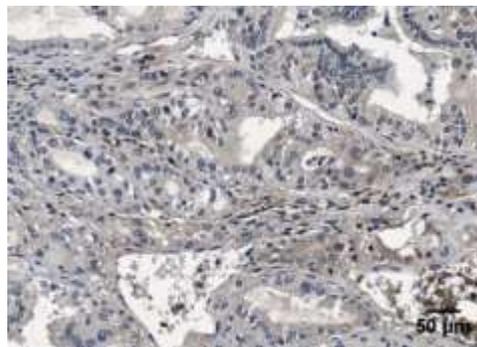


Figure 2.15 Weak IL-23 immunoreactivity revealed in a thyroid tissue sample of a patient with GD, $\times 250$

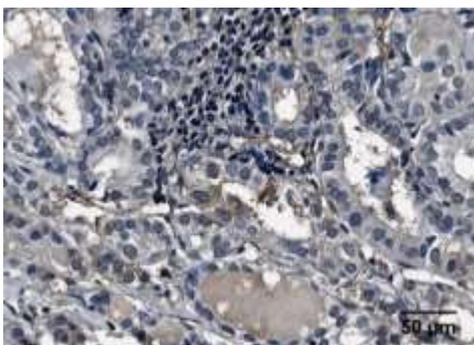


Figure 2.16 Occasional IL-1 β positivity revealed in a thyroid tissue sample of a patient with GD, $\times 400$

The expression of IL-23 was significantly increased in HT patients when compared to both GD and colloid goitre patients ($p = 0.043$ and $p < 0.001$, respectively) (Figure 2.17). No difference was found between the expression level of IL-23 in patients with GD and colloid goitres ($p = 0.324$).

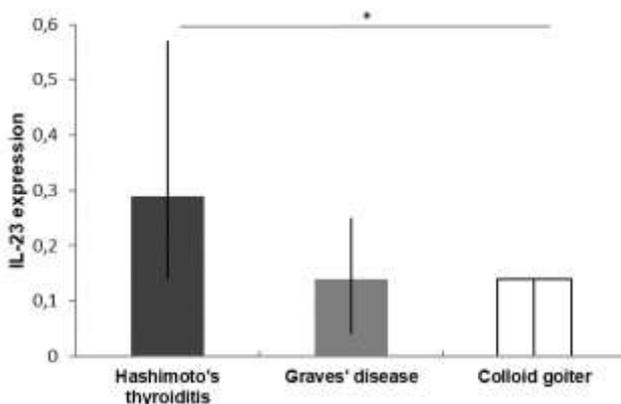


Figure 2.17 Comparison of IHC results of IL-23 in patients with AITD and colloid goitre

* A bar chart depicts immunopositivity levels of IL-23 confirmed in the thyroid tissue samples of patients with AITD and colloid goitre. Comparisons were made by using the Mann–Whitney U test. Data are shown as the median (interquartile range),

* $p < 0.05$.

The highest immunopositivity of IL-1 β was observed in the samples of patients with HT. Furthermore, the IL-1 β immunopositivity was significantly higher than that in colloid goitre and GD patients ($p < 0.001$ for both) (Figure 2.18). Similar to IL-23, the expression level of IL-1 β did not differ significantly between patients with GD and colloid goitres ($p = 0.144$).

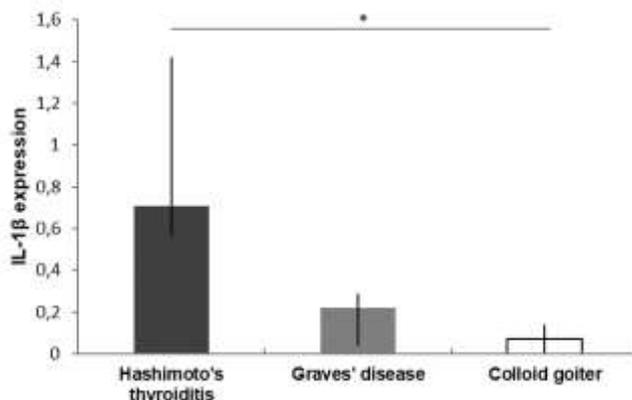


Figure 2.18 **Comparison of IHC results of IL-1 β in patients with AITD and colloid goitre**

* A bar chart depicts immunopositivity levels of IL-1 β confirmed in the thyroid tissue samples of patients with AITD and colloid goitre. Comparisons were made by using the Mann–Whitney U test. Data are shown as the median (interquartile range),
* $p < 0.05$.

Apart from thyrocytes and the thyroid tissue infiltrating lymphocytes, the expression of IL-23 and IL-1 β was observed in dendritic cells demonstrating S100 positivity. Heavily decorated and irregularly shaped S100 positive cells were observed in the inflammatory infiltrates.

The semiquantitative data describing the IHC expression of the interleukins used in this study are summarised in Table 2.2.

Table 2.2

**Patterns of immunohistochemical expression of tested interleukins
in patients with AITD and colloid goitre**

	IL-17 median (IQR)	IL-23 median (IQR)	IL-1β median (IQR)
Hashimoto's thyroiditis	2.2 (1.6; 2.8)	0.29 (0.14; 0.57)	0.71 (0.57; 1.42)
Graves' disease	1.3 (1; 2.3)	0.14 (0.04; 0.25)	0.22 (0.04; 0.29)
Colloid goitre	0.6 (0.6; 1)	0.14 (0.0; 0.14)	0.07 (0.0; 0.14)

* Data are shown as a median and interquartile range. Abbreviations: IL, interleukin; IQR, interquartile range.

2.2.2 Immunogold labelling of Th17-associated interleukins in HT patients

Precise intracellular localisation of IL-23, IL-1 β , and IL-17 was determined by the use of TEM after immunogold labelling. At the ultrastructural level, the presence of gold particles was evidenced in the cytoplasm of thyroid follicular cells in HT patients (Figure 2.19). Gold labelling was confirmed in the close vicinity to the nuclear envelope, swollen mitochondria, and the rough endoplasmic reticulum.

2.2.3 Analysis of relationships between IL-17 and Th17-promoting cytokines

A moderate positive correlation between IL-17 and both IL-23 and IL-1 β expression was found in HT patients ($r = 0.574$, $p = 0.007$ and $r = 0.461$, $p = 0.036$, respectively). Furthermore, in HT patients, a positive correlation ($r = 0.545$, $p = 0.011$) between IL-23 and IL-1 β immunopositivity was confirmed.

In the GD group, the expression of IL-17 was positively correlated with that for IL-1 β ($r = 0.817$, $p = 0.013$). However, no significant relationship between IL-17 and IL-23 ($r = 0.680$, $p = 0.063$) or IL-23 and IL-1 β immunoreactivity ($r = 0.662$, $p = 0.074$) was observed in this patient group.

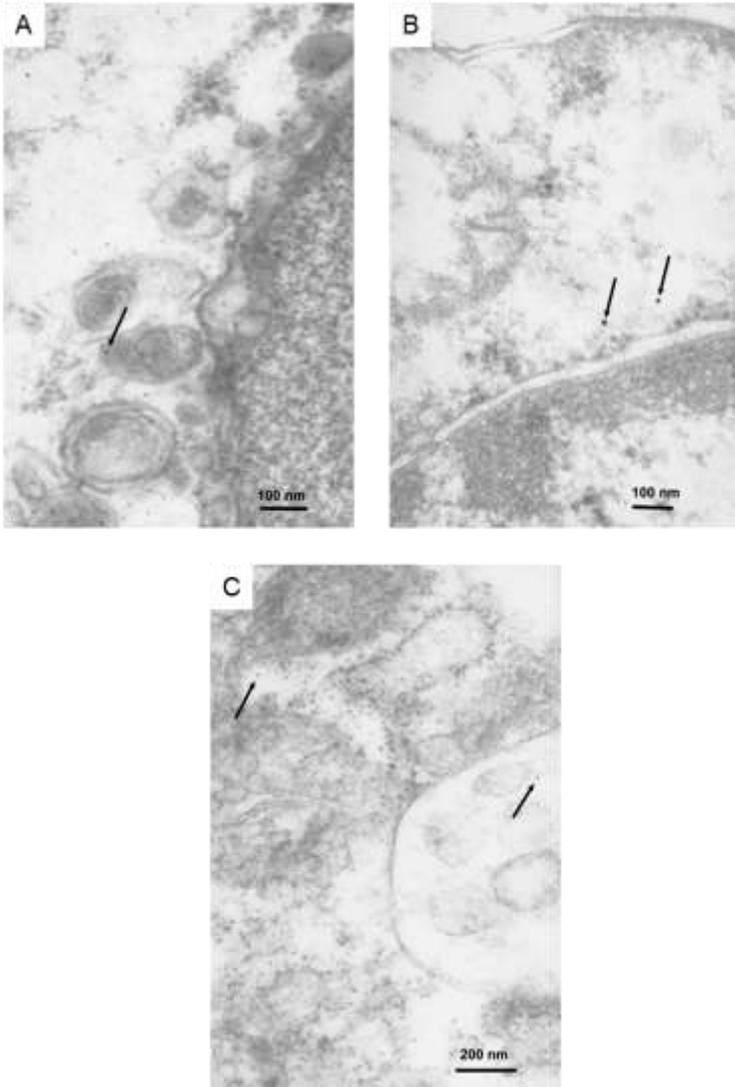


Figure 2.19 TEM. Immunogold labelled IL-17, $\times 60000$ (A), IL-23, $\times 50000$ (B), and IL-1 β , $\times 40000$ (C) detected in the cytoplasm of thyroid follicular cells in HT patient

* Black arrows indicate gold nanoparticles. Scale bars:
A and B – 100 nm; C – 200 nm.

2.3 The third study. Assessment of plasma levels of Th17-associated cytokines and selenium status in AITD

2.3.1 Demographic and biochemical characteristics of the participants

The demographic and biochemical characteristics of the participants are presented in Table 2.3. HT patients were stratified into two groups according to their serum TSH level: euthyroid ($0.354 \leq \text{TSH} \leq 4.94 \mu\text{IU/mL}$; $n = 33$) and hypothyroid ($4.94 < \text{TSH} < 10 \mu\text{IU/mL}$; $n = 8$) patients.

Table 2.3

Demographic and biochemical characteristics of the participants

	HT patients (n = 41)	GD patients (n = 11)	Controls (n = 26)	P
Sex (female/male)	39/2	9/2	21/5	0.152
Age (years)	36 (27–50.5)	41 (29–62)	30 (27–42.25)	0.139
TSH ($\mu\text{IU/mL}$)	2.16 (1.42–3.77)	0.0003 (0.0000–0.0004)	1.11 (0.96–1.67)	< 0.001
FT4 (ng/dL)	0.91 (0.85–0.98)	1.97 (1.76–2.55)	0.94 (0.91–1.07)	< 0.001
FT3 (ng/dL)	0.32 (0.29–0.35)	1.21 (1.04–2.47)	0.32 (0.30–0.35)	< 0.001
Anti-TPO (IU/mL)	202.99 (75.80–421.40)	121.04 (3.74–929.28)	0.46 (0.25–0.78)	< 0.001
Anti-Tg (U/mL)	0.00 (0.00–51.02)	5.96 (0.00–43.46)	< 20	–
Anti-TSHR (IU/L)	< 1.58	9.18 (4.05–19.68)	< 1.58	–

* Data are presented as the median (interquartile range). The Kruskal–Wallis test was used to calculate the p-value for comparisons among three groups for non-normally distributed data. Abbreviations: FT3, triiodothyronine; FT4, thyroxine; GD, Graves' disease; HT, Hashimoto's thyroiditis; Tg, thyroglobulin; TPO, thyroid peroxidase; TSH, thyroid-stimulating hormone; TSHR, thyroid-stimulating hormone receptor. $p < 0.05$: statistically significant.

2.3.2 Plasma levels of Th17 cytokines in patients with AITD and controls

No significant differences in IL-17a, IL-22, IL-23, IL-6, or IL-10 levels were found among the patients with HT, patients with GD, and controls (all $p > 0.05$) (Figure 2.20). Interleukin levels also did not differ between the euthyroid and hypothyroid HT patients (all $p > 0.05$).

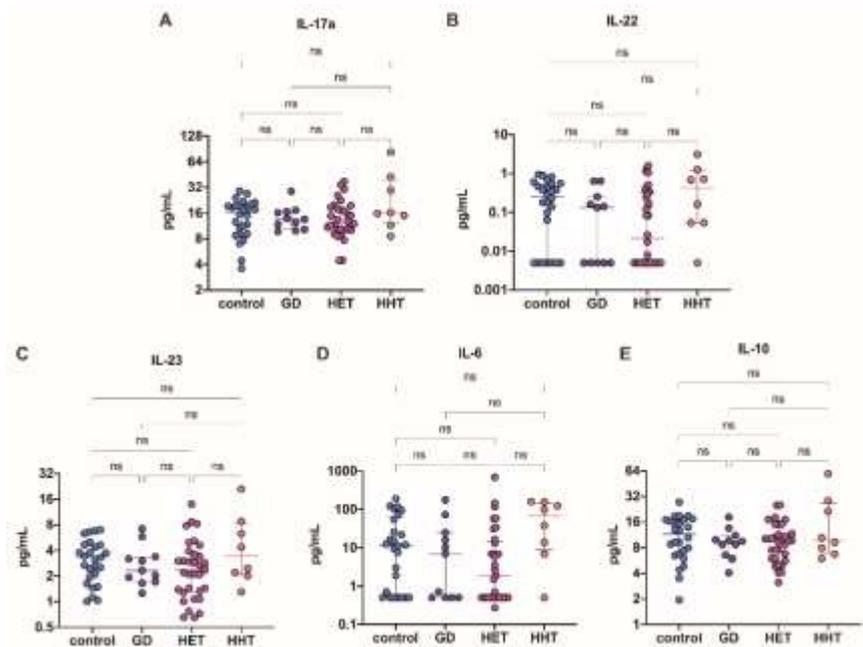


Figure 2.20 Comparisons of plasma IL-17a, IL-22, IL-23, IL-6, and IL-10 levels among patients with HET (n = 33), HHT (n = 8), or GD (n = 11) and healthy subjects (n = 26)

* The dot plots represent the quantified data for IL-17a (A), IL-22 (B), IL-23 (C), IL-6 (D), and IL-10 (E) plasma levels. (A-E) Each dot represents a single data point. Abbreviations: ns, not significant; GD, Graves' disease; HET, Hashimoto's thyroiditis, euthyroidism; HHT, Hashimoto's thyroiditis, hypothyroidism.

The plasma levels of cytokines are summarised in Table 2.4.

Table 2.4

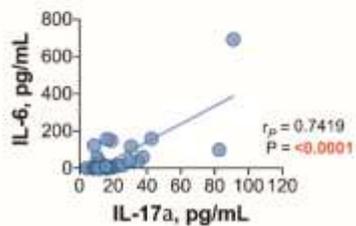
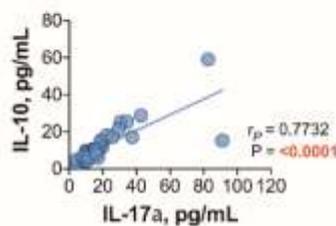
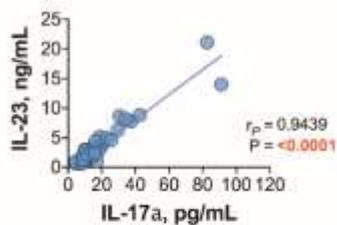
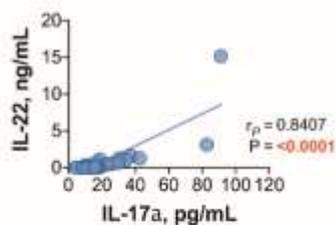
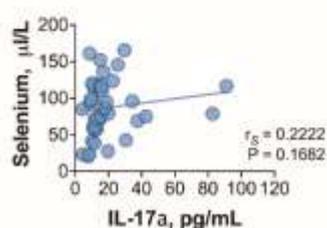
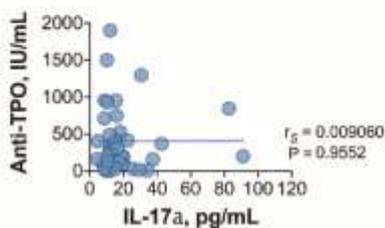
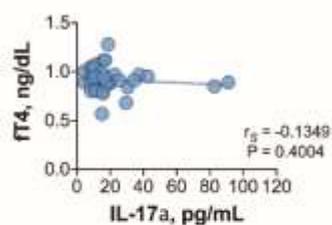
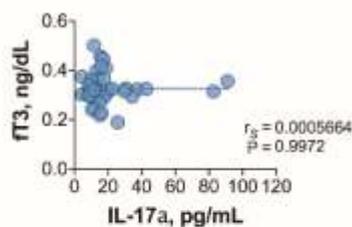
Plasma levels of interleukins in the study groups

	HT patients (n = 41)		GD patients (n = 11)	Controls (n = 26)	P
	Euthyroid HT (n = 33)	Hypothyroid HT (n = 8)			
IL-17a (pg/mL)	14.83 (10.37–19.75)		13.53 (10.33–16.72)	16.43 (9.14–19.75)	0.906
	12.95 (10.18–19.18)	15.99 (12.38–39.37)			0.606
IL-22 (ng/mL)	0.08 (0.00–0.47)		0.13 (0.00–0.25)	0.25 (0.00–0.54)	0.393
	0.026 (0.000–0.351)	0.421 (0.054–1.138)			0.180
IL-23 (ng/mL)	2.45 (1.35–4.49)		2.31 (1.70–3.36)	3.23 (1.86–4.64)	0.653
	2.37 (1.30–3.76)	3.42 (2.06–8.18)			0.408
IL-6 (pg/mL)	5.88 (0.00–36.24)		6.92 (0.00–24.90)	11.61 (0.00–59.50)	0.682
	1.90 (0.00–14.93)	68.62 (8.67–148.25)			0.059
IL-10 (pg/mL)	9.70 (6.35–15.21)		9.50 (6.54–11.11)	11.62 (6.70–17.04)	0.658
	9.70 (5.96–13.47)	9.83 (7.03–26.90)			0.585

* Data are presented as the median (interquartile range). The Kruskal–Wallis test was used to calculate the p-value for comparisons among three groups for non-normally distributed data. Abbreviations: GD, Graves' disease; HT, Hashimoto's thyroiditis. p < 0.05: statistically significant.

In HT patients, the plasma levels of IL-17a were positively correlated with the levels of IL-22, IL-23, IL-6, and IL-10 (Figure 2.21A). Additionally, IL-22 was correlated with IL-6, IL-23, and IL-10 (Figure 2.21B).

Similarly, in GD patients, positive associations between IL-17a and the levels of IL-22, IL-23, and IL-10 were found; however, IL-17a was not correlated with IL-6 (Figure 2.22A). There were positive correlations between the levels of IL-22 and those of IL-23, IL-6, and IL-10 in patients with GD as well (Figure 2.22B).

A

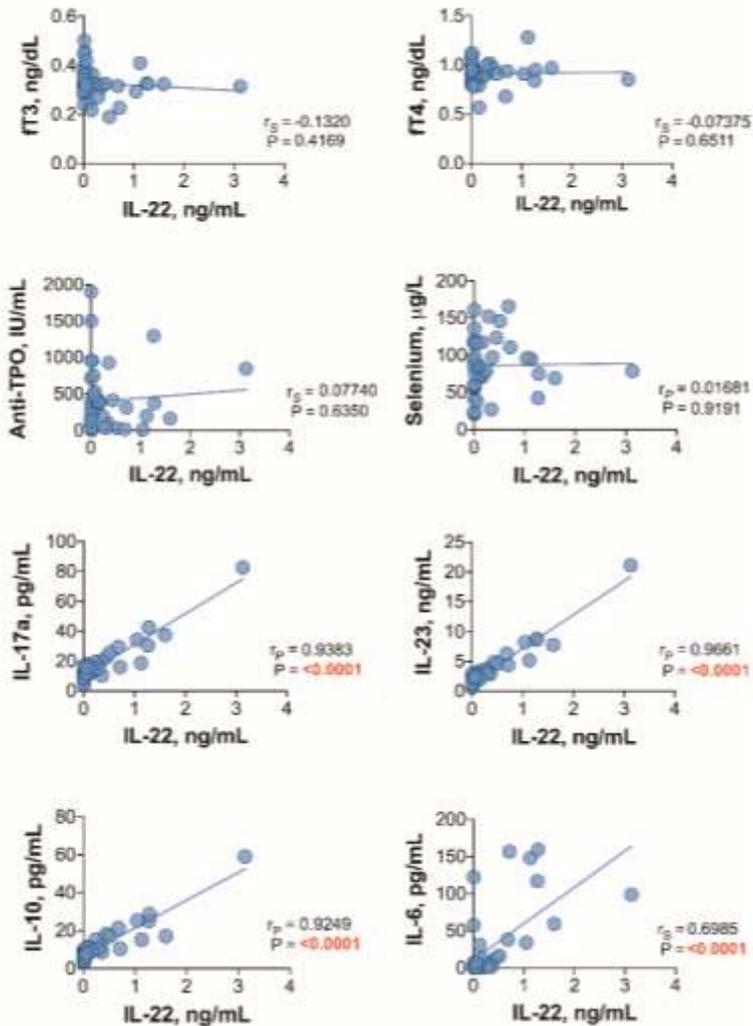
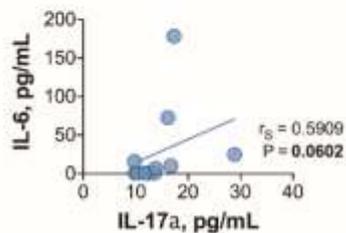
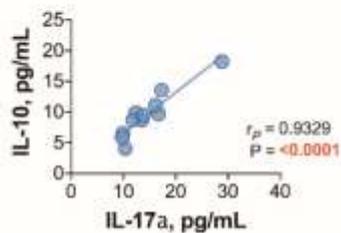
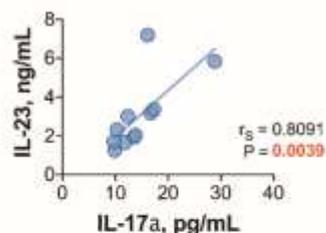
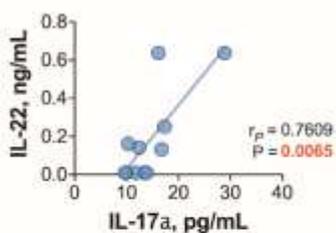
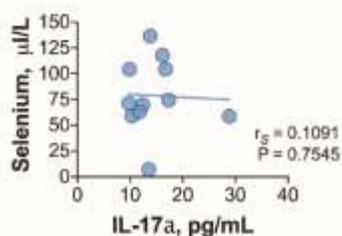
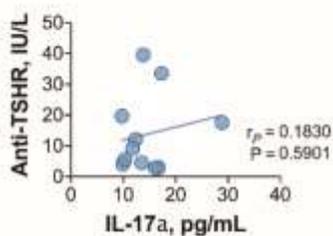
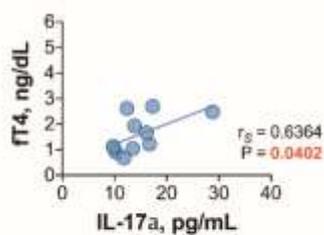
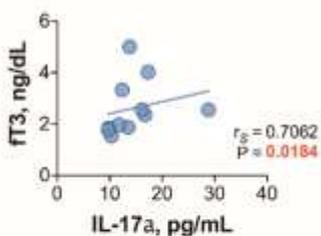
B

Figure 2.21 Scatter plots demonstrating the relationships between IL-17a (A) or IL-22 (B) and the studied biomarkers in the HT patient group

A



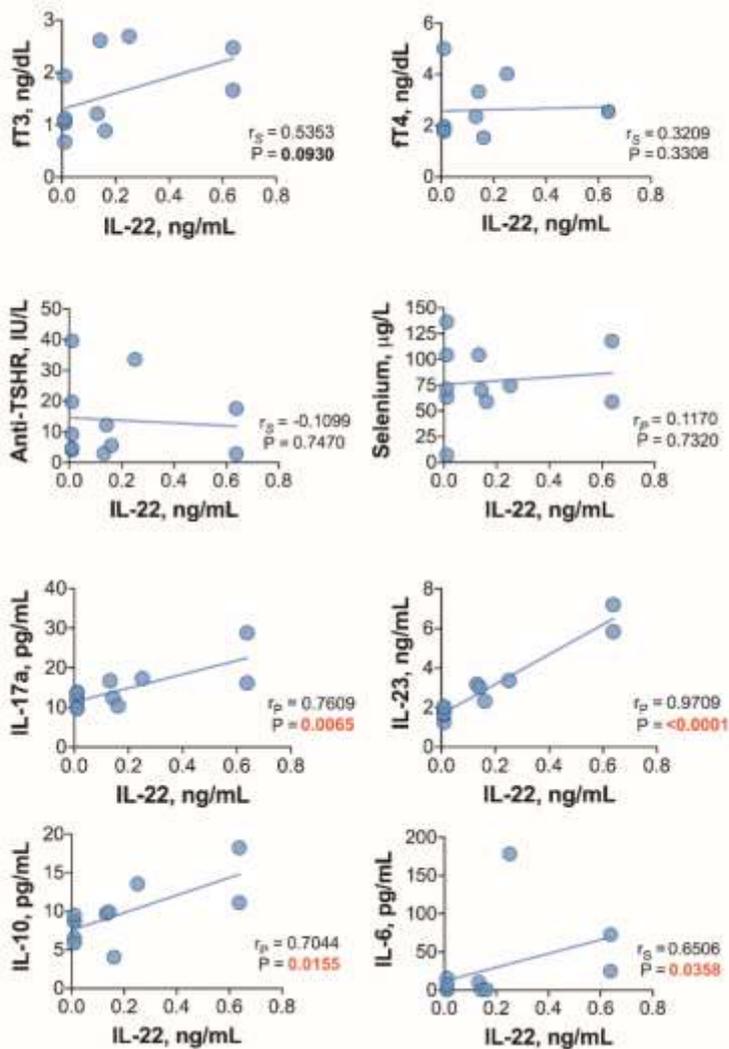
B

Figure 2.22 Scatter plots demonstrating the relationships between IL-17a (A) or IL-22 (B) and the studied biomarkers in the GD patient group

Positive relationships between different interleukins were revealed in the control group and both euthyroid HT patients and hypothyroid HT patients. Variance-covariance matrices with Spearman's rank correlation coefficients and p values representing the association level between studied cytokines in the patient groups are given in Figure 2.23.

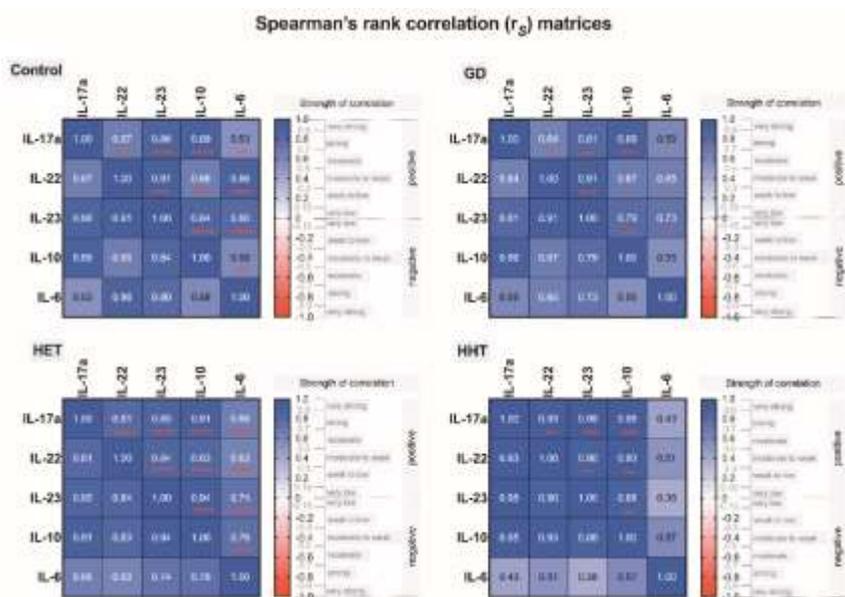


Figure 2.23 Variance-covariance matrices with Spearman's rank correlation coefficients (as values shown in small squares) representing the association level between studied cytokines in patient groups

* Abbreviations: GD, Graves' disease; HET, Hashimoto's thyroiditis, euthyroidism; HHT, Hashimoto's thyroiditis, hypothyroidism.

*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

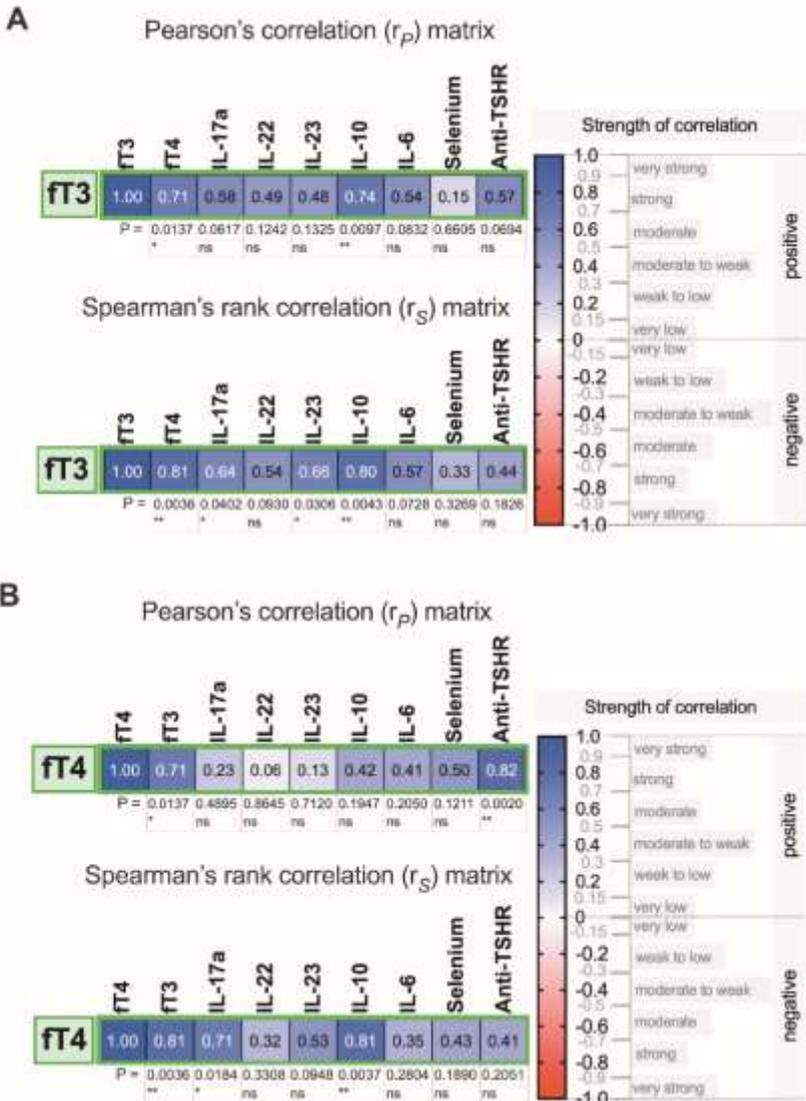


Figure 2.24 Associations of ft3 (A) or ft4 (B) with the studied biomarkers in the GD patient group

* Abbreviations: ft3, triiodothyronine; ft4, thyroxine; GD, Graves' disease; TSHR, thyroid-stimulating hormone receptor. ns, not significant; *, $p < 0.05$; **, $p < 0.01$.

Moreover, no significant correlations were found between the levels of interleukins and thyroid autoantibodies, TSH, FT3, or FT4 in HT patients (all $p > 0.05$). However, in newly diagnosed GD patients, the levels of FT3 were positively correlated with IL-17, IL-23, and IL-10 (Figure 2.24A), while FT4 was positively correlated with IL-17 and IL-10 levels (Figure 2.24B). In addition, anti-TSHR autoantibody titres were not correlated with the levels of interleukins in the GD group (all $p > 0.05$).

2.3.3 Plasma selenium levels in patients with AITD and controls

The median plasma selenium levels were 85.03 (60.09–116.60) $\mu\text{g/L}$ for HT patients, 71.33 (59.05–104.42) $\mu\text{g/L}$ for GD patients and 80.35 (67.15–113.67) $\mu\text{g/L}$ for control participants (Table 2.5).

Table 2.5

Plasma selenium levels in the study groups

	HT patients (n = 40)		GD patients (n = 11)	Controls (n = 21)	p
	Euthyroid HT (n = 32)	Hypothyroid HT (n = 8)			
Se ($\mu\text{g/L}$)	85.03 (60.09–116.60)		71.33 (59.05–104.42)	80.35 (67.15–113.67)	0.763
	85.03 (60.09–116.56)	94.78 (47.46–117.02)			0.886

* Data are presented as median (interquartile range).

The plasma selenium distribution in the patient groups is presented in Figure 2.25A. The levels of selenium did not differ significantly among the studied groups (Figure 2.25B).

Interestingly, a negative correlation between plasma selenium levels and anti-TPO autoantibody titres was found in patients with HT ($r = -0.376$, $p = 0.02$) (Figure 2.26). In addition, significantly lower selenium levels were observed in HT patients with higher anti-TPO levels (≥ 400 IU/mL) than in those

with lower autoantibody titres (< 400 IU/mL) (72.75 (52.10–88.43) vs 89.04 (65.53–117.51) µg/L, respectively; p = 0.05).

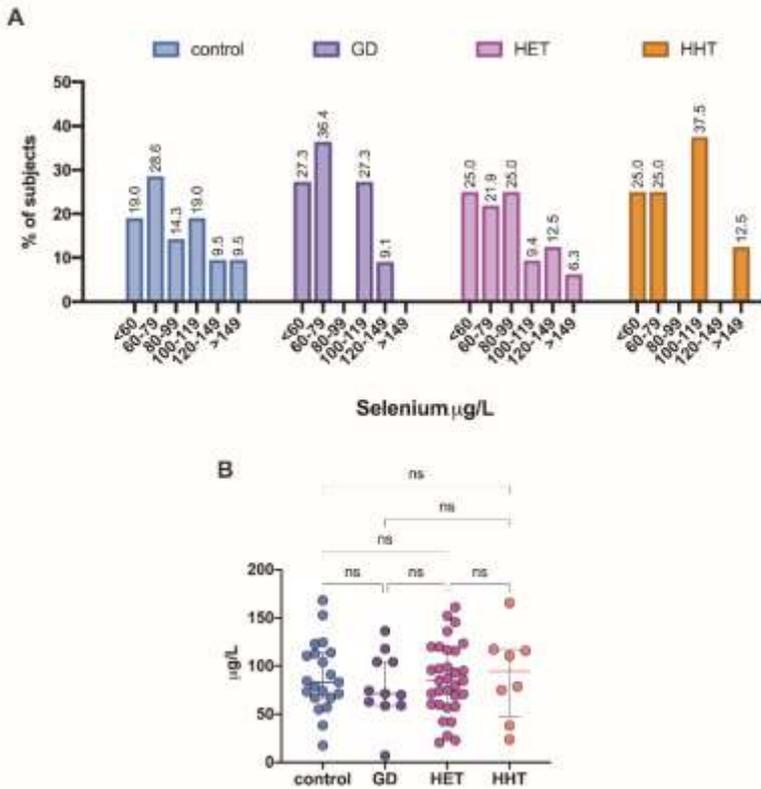


Figure 2.25 **Histograms showing the frequency distribution of plasma selenium (A) and comparison of selenium levels (B) in patient groups**

* (B) Each dot represents a single data point. GD, Graves' disease; HET, Hashimoto's thyroiditis, euthyroidism; HHT, Hashimoto's thyroiditis, hypothyroidism; ns, not significant.

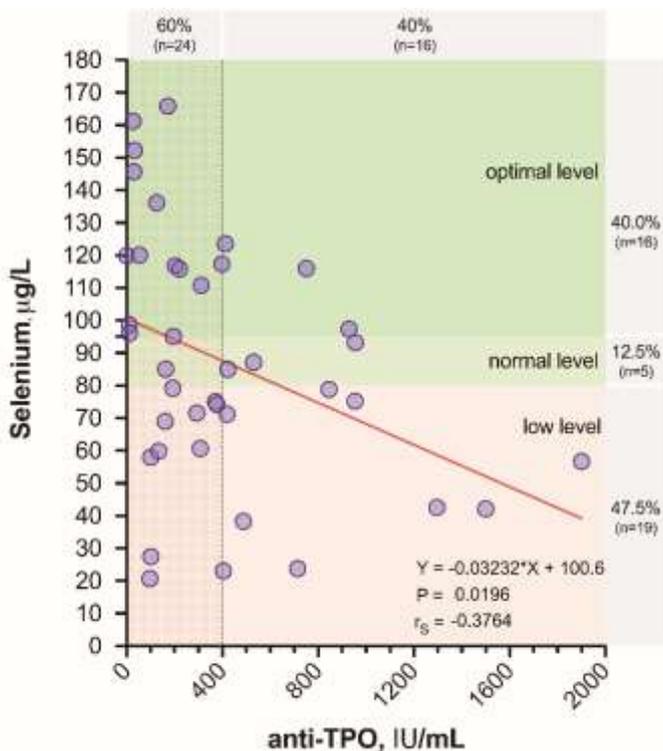


Figure 2.26 **Scatter plots demonstrating the correlation between selenium and anti-TPO autoantibody levels in HT patients**

* Plasma selenium level below 80 µg/L was defined as low, 80–95 µg/L as normal, and the level above 95 µg/L as optimal. Abbreviations: HT, Hashimoto's thyroiditis; TPO, thyroid peroxidase.

When the associations between plasma selenium and cytokines were analysed, no significant correlations were found between selenium levels and the plasma levels of IL-17a, IL-22, IL-23, IL-6, and IL-10 for all study groups (all $p > 0.05$). The levels of selenium failed to correlate with other characteristics, such as age, titres of anti-Tg and anti-TSHR, TSH, FT3, and FT4 (all $p > 0.05$).

3 Discussion

3.1 Heterogeneity of tissue IL-17 and tight junction proteins expression demonstrated in patients with AITD (the first study)

The cytokine-regulated disruption of Tj is thought to be essential in the initiation and/or development of several diseases. However, the role of IL-17 in maintaining Tj integrity in AITD has not yet been evaluated. We aimed to explore the integrity of the thyroid follicle by studying the immunoexpression of cellular Tj – ZO-1 and claudin-1 proteins coupled to IL-17A and CD68 detection in AITD patients compared to controls.

Although Th1-driven autoimmune response has long been considered dominant for HT development, recent studies have suggested evident participation of Th17 cells in AITD, particularly, in HT pathogenesis. In this study, higher expression levels of tissue IL-17A, assessed immunohistochemically, are identified in the thyroid tissue samples of HT and GD patients when compared to those with colloid goitre. Similarly, Li et al. (2013) have detected increased levels of thyroid tissue infiltrating Th17 cells as well as higher serum levels of IL-17 in HT patients when compared to controls and patients with other thyroid pathologies. Additionally, an association between thyroid IL-17 expression and fibrosis evidenced within the gland stroma was demonstrated in patients suffering from HT (Li et al., 2013). Other authors have demonstrated increased levels of IL-22 and IL-17 in the thyroid tissue and peripheral blood of HT patients, as well as high levels of Th17 differentiation affecting cytokines, such as IL-6, IL-15, and IL-23 (Figuerola-Vega et al., 2010). Finally, Konca Degertekin et al. (2016) have found that HT patients displaying normal thyroid function presented with higher circulating levels of IL-17 and IL-23 when compared to HT patients suffering from hypothyroidism, thus suggesting that hypothyroidism may have an inhibitory effect on cytokine

production. Overall, these findings provide strong supportive evidence of Th17 cell involvement in the development of HT.

Participation of Th17 lymphocytes and their produced cytokines in GD pathogenesis is less understood. However, other authors have previously reported on significantly enhanced levels of both Th22 and Th17 cells measured in peripheral blood, paralleled by higher detectable concentrations of IL-22 and IL-17 in the blood plasma in Chinese patients with newly diagnosed GD when compared to controls (Peng et al., 2013). Similarly, a difference in IL-17A expression between GD and colloid goitre is proven in the given study. Still, it seems the association between Th17 lymphocytes and the development of GD is less obvious than that of HT.

IL-17A positive cells were found within inflammatory cell infiltrates invading the thyroid gland. In addition, a marked expression of IL-17 in the thyroid follicular cells surrounded by lymphocytes was demonstrated in the given study. To the best of our knowledge, few studies have previously reported on various cell types including epithelial and smooth muscle cells, Paneth cells, neutrophilic leukocytes, and mast cells, natural killer cells, as well as $\gamma\delta$ and $\alpha\beta$ T lymphocytes, expressing IL-17 from A to F (Singh et al., 2014). Furthermore, the ability of thyroid follicular cells, expressing MHC class II molecules co-localised with B7.1 antigens and interacting with CD4+ T cells, to function as an antigen presenting cell, initiating and/or maintaining the autoimmune process, was demonstrated (Weetman, 1994). Therefore, thyrocytes may have a much larger role to play in the pathogenesis of AITD than initially thought.

A vast majority of intrafollicular CD68 positive cells were observed in patients with HT, thus pointing to their migration to the lumen of the thyroid follicle. In addition, a strong positive correlation between CD68 and IL-17A immunopositivity was confirmed in this study. Although we did not find any significant correlation between the IL-17 and Tj proteins expression,

accumulation of macrophages in the follicular lumen can be interpreted as an impairment of the thyroid epithelium integrity. Previous studies have explored antibody- and complement-dependent cytotoxicity issues demonstrating anti-TPO autoantibody caused damage of cultured thyrocytes (Rebuffat et al., 2008). Moreover, a pivotal role of monocytes and their Fc γ RI in antibody-dependent cytotoxicity mediated by thyroid antibodies was proven. Therefore, the further exploration of the role of intrafollicular macrophages in HT pathogenesis remains of great interest.

The pass for anti-TPO autoantibodies, attempting to get access to the molecule expressed on the apical pole of thyrocyte, facing colloid and non-accessible to immune cells, is still poorly understood. Some studies have assumed that TPO might be translocated by lateral diffusion to the basolateral pole of the thyrocytes and recognised by macrophages and/or dendritic cells (Kuliawat, Lisanti and Arvan, 1995). Modifications of Tj proteins, enabling migration of antigen presenting cells across the epithelium of thyroid follicle, have been proposed to explain the impairment of epithelial integrity (Rebuffat et al., 2013).

In this study, we evaluated the immunoreactivity of Tj ZO-1 and claudin-1 proteins in the thyroid tissues obtained from patients with AITD and colloid goitre. A significant reduction in ZO-1 expression demonstrated in our study in HT patients reflects the impaired integrity of the thyroid epithelium in HT. In contrast, a higher number of claudin-1 positive follicles was demonstrated in HT when compared to colloid goitre. The aforementioned differences in claudin-1 and ZO-1 expression observed in HT and GD patients are in agreement with a study describing underexpression of ZO-1 in thyroid tissues of HT patients, although claudin-1 appeared to be more strongly expressed (Rebuffat et al., 2013). We speculate that increased claudin-1 immunoreactivity

demonstrated in the samples of patients presented with HT might be interpreted as cell survival and/or compensatory mechanism.

Several paracrine factors, including cytokines and growth factors, are known to affect Tj and regulate the epithelial barrier function (Steed, Balda and Matter, 2010). IL-17A was shown to downregulate the expression of occludin, thus leading to disruption of the haematoencephalic and testicular barrier (Huppert et al., 2010; Pérez et al., 2014). However, whether the elevated IL-17 in HT affects Tj integrity and, thereby, alters the barrier function of thyroid follicular cells is unknown. A marked negative effect of the IL-1 α on the Tj and loss of the epithelial cell integrity have been previously explored in cultured thyroid cells (Nilsson et al., 1998). Previous studies have suggested that the organisation and localisation of Tj proteins – ZO-1, claudin-1, and JAM-A protein in the thyroid tissue of patients suffering from AITD are modified by pro-inflammatory cytokine IL-1 β , resulting in damage of the follicular epithelium (Rebuffat et al., 2013). Zhang et al. (2016) have explored signalling pathway that links IL-17 with Tj in salivary gland cell culture and postulated that IL-17 could directly modulate the Tj protein expression and distribution through the NF- κ B signalling pathway. In this study, a relationship between IL-17 and disruption of Tj was analysed, but no significant association was found.

A few limitations should be considered when interpreting our data, including a small number of thyroid tissue samples, semiquantitative estimation of immunoexpression, and the lack of data regarding the expression of Treg cytokines since nowadays both T cell types should be investigated as important players in AITD pathogenesis. The given retrospective study was based on analysis of archived paraffin-embedded tissues. Further prospective studies based on a larger number of samples and methods estimating causal effect between IL-17 and Tj disruption are required.

3.2 Upregulated tissue expression of Th17 pathogenic IL-23 and IL-1 β in HT but not in GD (the second study)

Although Th17 cells have become associated with autoimmune inflammation leading to disease over the past decade, the understanding that Th17 cells are heterogeneous and exhibit under certain conditions different Th17 cell phenotypes – pathogenic and non-pathogenic has only recently been proposed (Stadhouders, Lubberts and Hendriks, 2018). These pathogenic lymphocytes can secrete both common and pathogenic molecules, such as IL-17A/F, IL-21, IL-22, IL-26, IFN- γ , TNF- α , and GM-CSF, thereby driving autoimmune inflammation. IL-17 promotes neutrophil recruitment and activation, targeting immune and nonimmune cells, such as fibroblasts, epithelial and endothelial cells, thus triggering the release of cytokines, chemokines, and inflammatory mediators (Park et al., 2005). Previous studies have provided supportive evidence of Th17 and IL-17 participation in the pathogenesis of HT (Li et al., 2013; Esfahanian et al., 2017), whereas their contributions to GD development remain poorly understood. Notably, an increased number of CD4+IL-17+ lymphocytes in the peripheral blood of paediatric patients with untreated HT but not in GD patients was demonstrated by Bossowski et al. (2012), whereas a difference in IL-17 levels in the peripheral blood and thyroid tissues was not confirmed in the study conducted by Qin et al. (2012) comparing GD patients and controls. In our study, the expression of IL-17 in thyrocytes was higher in both HT and GD patients than in patients with colloid goitres, which may support a role for Th17 in AITD.

Further studies unravelling factors required for Th17 differentiation could be of interest. CD4+ T lymphocytes expressing the retinoic acid receptor (RAR)-related orphan receptor (ROR) γ t transcription factor, encoded by gene *Rorc*, are shown to be implicated in the development of autoimmune inflammatory disorders (Capone and Volpe, 2020). Furthermore, recently, RORC is shown

to play a pivotal role in directing the Th17 lineage and modulating the polarisation of Th22 cells (Peng et al., 2021). In this context, the recognition of factors regulating the transcription of *Il17a* or *Rorc* by interacting with ROR γ t or by binding their specific DNA regions may add clarity to this issue.

IL-23, synthesised by macrophages and dendritic cells, is another essential cytokine maintaining Th17 cell differentiation, suppressing IL-10 secretion, and facilitating IL-22 and GM-CSF production; it belongs to the IL-12 family implicated in various autoimmune and inflammatory diseases (Codarri et al., 2011). Moreover, IL-23 was also found to be an important survival factor for Th17 lymphocytes (Langowski et al. 2006). Notably, Th0 cells upon exposure to IL-23, IL-1 β , and IL-6 can generate highly pathogenic Th17 lymphocytes (Chung et al., 2009; Ghoreschi et al., 2010).

Interestingly, Ruggeri et al. (2014a) have described increased levels of serum IL-23 in untreated and euthyroid HT patients compared to controls, thus representing early pathogenetic events characteristic of HT. Recently, the enhanced expression of IL-23 in the thyroid tissue of patients with HT when compared to controls was demonstrated by another research group (Zheng et al., 2018). Additionally, these authors have demonstrated the accumulation of reactive oxygen species and the inhibition of autophagy in thyrocytes induced by increased IL-23. In the present study, we found that the immunoexpression of IL-23 in the thyroid follicular cells of HT patients was significantly higher than that in both GD and colloid goitre patients, which may further result in the increased generation of Th17 lymphocytes and production of IL-17. Furthermore, a positive correlation between IL-17 and IL-23 as well as IL-17 and IL-1 β expression was observed in HT patients, supporting a role for Th17 cells in HT development.

To the best of our knowledge, the expression of IL-23 in the thyroid tissues of GD patients has not been studied before. Only two studies have previously explored the levels of IL-23 measured in the serum of GD patients (Figueroa-Vega et al., 2010; Jia et al., 2015). Increased serum levels of IL-23 have been reported in the study conducted by Jia et al. (2015), whereas Figueroa-Vega et al. (2010) have not observed any significant differences in the IL-23 levels in GD patients compared to controls. In this study, no difference between IL-23 immunopositivity within thyroid tissues in GD and colloid goitre patients was found, suggesting that pathogenic Th17-promoting cytokines might be less involved in GD pathogenesis. However, further studies based on a larger number of thyroid tissue samples and appropriate methods to confirm these data are needed. Although no significant relationship between IL-17 and IL-23 expression was observed in GD patients, IL-23 tended to correlate with IL-17 immunopositivity ($p = 0.063$). Therefore, the results of the given study, as well as conclusions regarding the role of Th17 cells in GD pathogenesis, must be interpreted with caution due to a low number of recruited and analysed GD patients.

Interestingly, an increase in IL-17 and ROR γ t mRNA expression in IL-23-stimulated GD patient cultured cells when compared to those from controls and cultures without IL-23 was shown by Zheng et al. (2018). Similar results were demonstrated in euthyroid GD patients, indicating an independent effect of thyroid hormone levels (Zheng, Ye and Liu, 2013). Increased tissue levels of IL-23 have not yet been demonstrated in GD; few studies have focused on the role of the IL-23 receptor in these patients. Furthermore, IL-23 receptor gene polymorphisms have been strongly associated with GD, especially with Graves' orbitopathy (GO) (Huber et al., 2008).

IL-1 β , produced primarily by macrophages and dendritic cells, exhibits different immunomodulatory and inflammatory activities. In AITD, IL-1 β impairs the barrier function of thyroid follicular cells, altering Tj proteins by stimulating IL-6 production (Rebuffat et al., 2013). IL-1 β has been shown to induce Fas expression, promoting thyroid cell death as well (Paolieri et al., 1999). In the current study, we found enhanced levels of IL-1 β expression in the thyroid tissue of patients with HT compared to patients with colloid goitre and GD. Similar to IL-23, the IL-1 β expression did not differ significantly when analysing the thyroid tissues of patients presented with GD and colloid goitre; however, the expression of IL-17 was upregulated in the GD group. Taking into account that IL-17 is a common Th17 molecule secreted by both cell phenotypes and that IL-23/IL-1 β stimulates a shift towards a pathogenic Th17 phenotype (Stadhoudersab, Lubberts and Hendriks, 2018) our findings are suggestive of a lesser role of pathogenic Th17 cells in the development of GD compared to HT. In this context, supportive evidence has been provided in a recent study performed by Vitales-Noyola et al. (2017) demonstrating the elevation of Th17 pathogenic cells in the peripheral blood of GD (especially in those with GO) but not HT patients. However, when analysing the absolute number of pathogenic cells, no significant differences between HT, GD, and controls were found.

Similar to the first study, the second study has some methodological limitations. Firstly, only archived paraffin-embedded thyroid tissues were investigated. Secondly, due to the limited availability of the tissue samples, a small number of thyroid tissue specimens obtained from patients presented with GD were analysed. Therefore, the results of GD group analyses must be concluded and interpreted with caution.

3.3 Plasma levels of Th17-associated cytokines and selenium status in AITD (the third study)

Cytokines, along with T cell receptors, transcription factors, and the intestinal microbiota, are important factors regulating the Th17/Treg cell balance. Th17 cells have been implicated in the development of autoimmunity, whereas Treg cells producing IL-10, IL-35, and TGF- β maintain self-tolerance and protect against thyroid autoimmunity. Moreover, IL-10 is a principal anti-inflammatory cytokine that has been shown to inhibit Th17 cell-mediated inflammation (Chaudhry et al., 2011). Furthermore, current evidence suggests the Th17 lineage is plastic and heterogeneous (González-Amaro and Marazuela, 2016). Another pro-inflammatory cytokine contributing to thyroid autoimmunity is IL-23. Produced by antigen-presenting cells such as macrophages and dendritic cells, IL-23 is crucial for the differentiation of pathogenic Th17 lymphocytes (Lee et al., 2017). Furthermore, it has been found to stimulate the survival of highly pathogenic Th17 cells.

In this study, we did not find any significant differences in the plasma levels of either IL-17a or IL-23 among euthyroid and hypothyroid patients with HT, GD, and controls, although both interleukins were positively correlated in the HT and GD groups. Only limited data are available for serum IL-23 and IL-22 levels in AITD patients. Shi et al. (2010) demonstrated upregulated mRNA expression of IL-17 and the Th17-related transcription factor ROR γ t in peripheral blood mononuclear cells along with elevated plasma levels of IL-17 and IL-23 in newly diagnosed HT patients. More recently, Konca Degertekin and colleagues (2016) demonstrated lower serum levels of IL-17 and IL-23 in hypothyroid HT patients than in euthyroid patients, thus suggesting that hypothyroidism has an inhibitory impact on Th17 cytokine responses. However, no differences in IL-17 or IL-23 levels were found between hypothyroid HT patients and healthy subjects. Elevated levels of serum IL-23 were found

in untreated euthyroid HT patients compared to controls, reflecting the early course of thyroiditis, in a study conducted by Ruggeri et al. (2014a). Jia et al. (2015) observed increased serum levels of IL-23 in GD patients. Previously, we demonstrated thyrocyte-related IL-23 overexpression in HT patients, while no difference was found between GD and colloid goitre patients, suggesting that IL-23 may be less implicated in GD pathogenesis than in HT pathogenesis (Zağcı et al., 2019).

IL-22 produced by Th17 cells can also be secreted by another novel subset of Th cells – CCR⁺IL-17⁻IL-22⁺ (Th22) cells. We failed to demonstrate any differences in IL-22 levels among the three groups of subjects enrolled in this study. However, Ruggeri and collaborators (2014b) found significantly increased serum levels of IL-22 in untreated euthyroid HT patients compared to patients with nodular goitre or healthy subjects. In another study, drug-naïve HT patients suffering from overt or subclinical hypothyroidism exhibited elevated serum levels of IL-17, while no differences were found in IL-22 or IL-23 levels between the HT patients and healthy individuals (Esfahanian et al., 2017). Song et al. (2014) reported that peripheral blood Th22 cell levels, serum IL-22 levels and IL-22 mRNA expression were elevated in GD patients compared to controls, suggesting that IL-22 may participate in the initiation of GD. Similar to the results confirmed in the present study, significant differences in the Th22 profile were not found in HT patients.

In this study, positive correlations between the levels of IL-22, IL-17a, IL-23, and thyroid hormones were found in newly diagnosed GD patients. However, no correlations between the levels of interleukins and thyroid-specific autoantibodies were found. These results suggest that Th17 cytokines may have a stimulatory effect on the severity of hyperthyroidism independent of autoantibody levels. No such correlation was found in the HT group since a vast majority of the recruited HT patients presented as euthyroid. Due to the limited

availability of treatment-naïve patients, only a small number of GD patients was recruited. Therefore, the results of GD group analyses must be concluded and interpreted with caution because of the small sample size in this group.

Among the non-genetic factors involved in the pathogenesis of thyroid autoimmunity, high iodine intake and a low selenium status appear to be the most important. Previous studies have suggested the immunomodulatory and protective role of selenium and selenoproteins. It has been suggested that selenium deficiency presents with inhibition of selenoproteins and altered secretion of IL-10, IL-12p40, and IFN- γ , leading to Th1/Th2 imbalance by shifting the balance towards Th1 responses (Sun et al., 2018). Additionally, GPx1-deficient Th lymphocytes demonstrate a shift towards Th1 cells and attenuate the differentiation of Th cells into the Th17 lineage (Won et al., 2010). In another study, selenium supplementation exerted protective effects against oxidative stress and peroxide-induced cell damage in human thyrocytes and fibroblasts (Ruggeri et al., 2020). However, more data from clinical studies are needed for understanding the relationship between selenium supplementation, immune response, and antioxidant effects.

Other authors have explored immunological changes following selenium administration in HT patients. However, no significant differences in the production of Th1- or Th2-related cytokines (IFN- γ , TNF- α , IL-2, IL-4, IL-5, IL-6, IL-10, and IL-13) before and 3 months after the administration of 200 μ g sodium selenite were found (Karanikas et al., 2008). In the current study, we did not find any correlations between the plasma levels of selenium and interleukins within the patient groups, indicating that adequate selenium intake does not skew the differentiation of CD4+ T cells towards the Th1 or Th2 lineage and that Th1/Th2 differentiation is largely determined by the pattern of secreted cytokines, transcription factors, and other environmental signals.

In this study, selenium concentrations did not differ significantly among the patients with HT, patients with GD, and controls. In a population-based Danish study, Bülow Pedersen et al. (2013) explored serum selenium levels in patients with newly diagnosed AITD. The authors found significantly lower selenium levels in patients with GD than in controls; however, no difference in serum selenium levels between HT patients and healthy subjects was observed. Other authors have demonstrated lower serum selenium levels in GD patients with moderate-to-severe GO with adequate selenium intake than in GD patients without GO, suggesting that selenium deficiency may be an independent provoking factor for GO (Khong et al., 2014).

In 2014, a multicentre, cross-sectional study was conducted in Austria, Greece, Romania, and Italy to assess the selenium status of euthyroid patients with thyroid autoimmunity and those with non-autoimmune thyroid diseases (Krassas et al., 2014). The study confirmed the presence of a strong positive correlation between the plasma levels of selenium and SELENOP, indicating a less-than-optimal selenium status in these European countries. Additionally, significantly lower plasma selenium levels were found in patients with GD or HT than in those with non-autoimmune thyroid disease (75.1 ± 27.2 , 84.6 ± 42.2 and 95.9 ± 34.7 $\mu\text{g/L}$, respectively; $p < 0.001$ and $p < 0.05$, respectively), suggesting that selenium supplementation might be beneficial in the case of thyroid autoimmunity. Federige et al. (2017) also explored serum selenium and SELENOP levels in AITD patients. Similar to the current study, no difference in serum selenium levels was found among HT patients, HT+LT4 patients, GD patients with or without GO, and healthy individuals, although SELENOP levels were lower in the GO and HT patients than in the control subjects.

Various plasma or whole-blood selenium cut-offs have been proposed by different authors to reflect selenium sufficiency. The activity of GPx3 in human serum is used as a marker of selenium sufficiency. It has been shown that the

plasma selenium level needed to optimise platelet GPx activity is 100–115 µg/L, as proposed by Alftan et al. (1991), or 95 µg/L according to Thomson et al. (1993), whereas a plasma selenium range of 88–142 µg/L is associated with the maximal SELENOP concentration (Hurst et al., 2013). A selenium level below 80 µg/L is considered to indicate an inadequate selenium status (Neve et al., 1991; Van Dael and Deelstra, 1993). The aforementioned cut-offs suggest that 35% of the HT patients and 36.4% of the GD patients enrolled in the study had plasma selenium levels necessary for saturation of GPx, while 47.5% of the HT patients and 63.3% of the GD patients had a poor selenium status. The frequency of selenium deficiency in the control group was 47.6%.

A few limitations should be considered when interpreting our data. Although simultaneous assessment of various cytokines provided more information on the complex cytokine pattern that characterises AITD, a small number of AITD patients may lead to a possible loss of statistical significance. Due to the lack of data regarding the levels of other Treg cytokines, it will be of interest to further pursue studies of Th17 and Treg cells as important players in AITD pathogenesis.

Conclusions

1. Overall, the pathogenesis of thyroid autoimmunity and the involvement of the IL-17/IL-23 axis in the development of these immune-mediated disorders remains unravelled. In our research, no significant differences in the plasma levels of Th17-associated cytokines were found among the patients with AITD and control subjects. However, plasma levels of Th17-associated cytokines were positively correlated with the severity of hyperthyroidism, independent of autoantibody levels, suggesting their possible role in GD pathogenesis.
2. Analysing the immunoexpression patterns of Th17-associated cytokines in thyroid tissue, the expression level of IL-17 in the thyrocytes was found significantly higher in the HT and GD patients than in patients with colloid goitres; in the HT group, it simultaneously correlated with IL-23 and IL-1 β immunopositivity within thyroid tissue. Furthermore, increased expression of IL-23 and IL-1 β , cytokines that promote pathogenic Th17 cell differentiation, was observed in the HT group, suggesting that pathogenic Th17-promoting cytokines may play a role in HT pathogenesis.
3. A positive correlation between plasma levels of Th17-associated cytokines detected in the HT and GD patients is not strong enough to suggest their possible role in AITD pathogenesis; it mainly reflects the involvement of these interleukins in the differentiation of Th17 cells. Further experimental studies to investigate the role and regulatory effects of Th17-related cytokines in the pathogenesis of AITD are required.
4. In the HT patients, strong overexpression of IL-17A evidenced in the thyroid epithelial cells was associated with the presence of intrafollicular CD68 positive cells, suggesting that the role of affected thyrocytes and antigen-presenting cells in thyroid autoimmunity might extend far deeper than initially thought. The changes in molecules of thyrocyte junctional complexes

highlighting impairment of the thyroid follicle integrity were observed in the HT patients, but no significant association with IL-17 was found.

5. The selenium status of the Latvian patients with newly diagnosed GD or HT is at a suboptimal level, however, no difference in selenium levels was observed between the AITD patients and controls. Among all HT and GD patients enrolled in the study, one-third was characterised by plasma levels of selenium sufficient for saturation of glutathione peroxidase, while 47.5% and 63.3% of HT and GD patients, respectively – by low levels.
6. In HT patients, plasma levels of selenium were negatively correlated with anti-TPO autoantibody titres, thus supporting the immunomodulatory role of selenium in AITD. Moreover, HT patients with higher anti-TPO autoantibody levels had lower levels of selenium, suggesting that these patients might benefit from selenium supplementation.

Publications

Articles in international peer-reviewed journals

1. **Zake, T.**, Kalere, I., Upmale-Engela, S., Svirskis, S., Gersone, G., Skesters, A., Groma, V., Konrade, I. 2021. Plasma levels of Th17-associated cytokines and selenium status in autoimmune thyroid diseases. *Immunity, Inflammation and Disease*. doi: 10.1002/iid3.433. Epub ahead of print. PMID: 33943012.
2. **Zake, T.**, Skuja, S., Kalere, I., Konrade, I., Groma, V. 2019. Upregulated tissue expression of Th17 pathogenic IL-23 and IL-1 β in Hashimoto's thyroiditis but not in Graves' disease. *Endocrine Journal*. 66(5): 423–430. doi: 10.1507/endocrj.EJ18-0396. PMID: 30814438.
3. **Zake, T.**, Skuja, S., Lejnicks, A., Groma, V., Konrāde, I. 2019. Immunological mechanisms of autoimmune thyroid diseases: a shift in the traditional Th1/Th2 paradigm. *Proceedings of the Latvian Academy of Sciences. Section B. Natural, Exact, and Applied Sciences*. 73(2), 67–77. doi: 10.2478/prolas-2019-0012.
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1. **Zake, T.**, Groma, V., Konrāde, I., Skuja, S. Vairogdziedzera folikulocītu blīvo savienojumu proteīna klauđina-1 imūnēkspresijas atšķirības pacientiem ar Hasimoto tireoidītu un Greivsa slimību. *Zinātniskie raksti: 2015. gada medicīnas nozares pētnieciskā darba publikācijas: Medicīnas bāzes zinātnes.* / Rīgas Stradiņa universitāte. – Rīga: Rīgas Stradiņa universitāte, 2016. – 273.–281.

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1. **Zake, T.**, Kalere, I., Upmale-Engela, S., Gersone, G., Skesters, A., Groma, V., Konrade, I. The pathogenic role of Th17 cell pathway in autoimmune thyroid diseases. *Rīga Stradiņš University International Research Conference on Medical and Health Care Sciences "Knowledge for Use in Practice"*, 24–26 March, 2021: Abstracts. Rīga: RSU, i-xl p., 175 p. (Oral presentation).
2. Kalere, I., **Zake, T.**, Upmale-Engela, S., Strele, I., Skesters, A., Konrade, I. Plasma selenium status in newly diagnosed Graves' disease and Hashimoto's thyroiditis patients: case-control study. *Rīga Stradiņš University International Research Conference on Medical and Health Care Sciences "Knowledge for Use in Practice"*, 24–26 March, 2021: Abstracts. Rīga: RSU, i-xl p., 168 p. (Poster presentation).

3. Kalere, I., **Zake, T.**, Upmale-Engela, S., Cgojeva-Uzulniece, F., Skesters, A., Konrade, I. Analysis of selenium intake score and its affecting factors in young adults. *Rīga Stradiņš University International Research Conference on Medical and Health Care Sciences "Knowledge for Use in Practice"*, 24–26 March, 2021: Abstracts. Rīga: RSU, i-xl p., 158 p. (Poster presentation).
4. **Zake, T.**, Upmale-Engela, S., Kalere, I., Skesters, A., Konrade, I. Selenium status in Latvian patients with autoimmune thyroid diseases. *The 3rd International Conference "Nutrition and Health"*, Riga, Latvia, December 9–11, 2020, Book of Abstracts. University of Latvia Press, 2020, 92 p. (Poster presentation).
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7. **Zake, T.**, Skuja, S., Kalere, I., Konrade, I., Groma, V. Th17 cells and impairment of the thyroid follicle integrity in the pathogenesis of thyroid autoimmunity: Morphological assessment. Rīga Stradiņš University. (2019). *International Conference on Medical and Health Care Sciences Knowledge for Use in Practice* (Rīga, 1–3 April 2019): Abstracts. Rīga: RSU, XLVII p., 580 p. (Oral presentation).
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Abstracts and presentations in local conferences

1. **Zaķe, T.**, Skuja, S., Kalere, I., Konrāde, I., Groma, V. Ar Th17 diferenciāciju saistīta citokīna IL-23 imūnepsresijas atšķirības pacientiem ar autoimūnām vairogdziedzera slimībām. *Rīgas Stradiņa universitātes 2017. gada zinātniskā konference*: Tēzes. – Rīga: RSU, 2017. – 197. lpp. (Poster presentation).
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4. **Zaķe, T.**, Groma, V., Skuja, S., Kleina, R. Intrafolikulāro makrofāgu īpatnību izpēte pacientiem ar Hašimoto tireoidītu. *Rīgas Stradiņa universitātes 2014. gada zinātniskā konference*: Tēzes. – Rīga: RSU, 2014. – 54. lpp. (Oral presentation).

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