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**Thyroid Autoimmunity:
Exploring the Role of Th 17-associated
Cytokines and Pathomorphological
Mechanisms Involved
in the Pathogenesis
of Hashimoto's Thyroiditis
and Graves' Disease**

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

Sector – Clinical Medicine
Sub-Sector – Internal Medicine

Rīga, 2021

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Abstract

The prevalence and incidence of autoimmune thyroid diseases (AITD), presenting as Graves' disease (GD) or Hashimoto's thyroiditis (HT), has increased significantly in recent decades. It is crucial to identify immunological and pathomorphological factors involved in thyroid autoimmunity. Classically, HT has long been considered as a T helper (Th)1-mediated disease, while a Th2-driven autoimmune response is dominant for GD. Recently, Th17 cells have been established to play a role in the pathogenesis of AITD, however, their contribution to the initiation and progression of AITD remains unclear. Furthermore, selenium deficiency can impair the differentiation of Th cells, leading to dysfunction of cellular and humoral response.

The aim of this thesis was to explore the role of Th17 cells in the pathogenesis of HT and GD by the use of different morphology methods and xMAP technology, and correlating these data with the selenium status.

The initial study included 29 adult patients with AITD who underwent thyroidectomy, whereas subsequent clinical research project involved 52 patients with newly diagnosed, treatment-naïve AITD, as well as 26 healthy subjects served as controls. The plasma levels of Th17-associated cytokines – interleukin (IL)-17, IL-22, IL-23, IL-6, and IL-10 and the distribution and levels of immunoexpression IL-17, IL-23, and IL-1 β within thyroid tissue were measured to characterize Th17 immune response in AITD. The integrity of the thyroid follicle by studying immunoexpression of cellular tight junctions – *zonula occludens*-1 and claudin-1 proteins, coupled to IL-17 and CD68, was explored. In addition, the selenium status was assessed.

No significant differences in the plasma levels of Th17-associated cytokines were found among the patients with AITD and control subjects. However, the expression level of IL-17 in the thyrocytes was significantly higher in the HT and GD patients than in controls, simultaneously correlating with IL-23 and IL-1 β immunopositivity in the HT group. Plasma Th17-associated cytokines' levels were positively correlated with the severity of hyperthyroidism, independent of autoantibody levels, thus suggesting their possible role in GD pathogenesis.

The changes in molecules of thyrocyte junctional complexes highlighting impairment of the integrity of thyroid follicle in HT were observed, but no significant association with IL-17 was found. Although no difference in selenium levels was observed between the AITD patients and controls, the results of the given research suggest the selenium status of the Latvian patients with newly diagnosed GD or HT is at a suboptimal level. Plasma selenium levels were

negatively correlated with anti-thyroperoxidase (TPO) autoantibody titres in the HT patients, 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.

Essential information deepening our knowledge about thyroid autoimmunity was obtained conducting this research, however, further experimental studies exploring the role and regulatory effects of Th17-related cytokines in the pathogenesis of AITD are required. More data from clinical studies are needed for a better understanding of the relationship between selenium supplementation and immune response.

Keywords: autoimmune thyroid diseases; Th17 cells; selenium; tight junctions.

Anotācija

Vairogdziedzera autoimunitāte: ar Th17-asociēto citokīnu un patomorfoloģisko mehānismu izpēte Hašimoto tireoidīta un Greivsa slimības patoģenēzē

Pēdējo dekāžu laikā ievērojami pieaugusi tādu autoimūno vairogdziedzera slimību (AIVS) kā Greivsa slimība (GS) un Hašimoto tireoidīts (HT) saslimstība un izplatība. Ir būtiski identificēt imunoloģiskos un patomorfoloģiskos faktorus, kas iesaistīti vairogdziedzera autoimunitātes izcelsmē. Lai gan 1. un 2. tipa T līdzētājšūnu (Th) izmainīta aktivitāte AIVS patoģenēzē uzskatāma par pierādītu, nesen veiktie pētījumi liecina arī par iespējamu Th17 šūnu lomu AIVS attīstībā. Turklāt Th šūnu diferenciāciju var ietekmēt selēna deficīts, veicinot šūnu un humorālās imūnatbildes disfunkciju.

Šī promocijas darba mērķis bija izpētīt Th17 šūnu nozīmi HT un GS patoģenēzē, lietojot dažādas morfoloģiskās diagnostikas metodes un xMAP tehnoloģiju, kā arī korelēt iegūtos datus ar selēna statusu.

Sākotnēji pētījumā tika iekļauti 29 pieauguši pacienti ar AIVS, kuriem veikta tireoidektomija, savukārt pētījuma klīniskā daļa ietvēra 52 pacientus ar pirmreizēji diagnosticētu, iepriekš neārstētu AIVS, kā arī 26 klīniski veselas pētāmās personas, kuras iekļautas kontroles grupā. Lai izvērtētu ar Th17 saistītu imūnatbildi AIVS ietvaros, tika mērīts ar Th17 saistīto citokīnu – interleikīna (IL)-17, IL-22, IL-23, IL-6 un IL-10 – līmenis plazmā, kā arī IL-17, IL-23 un IL-1β imūnekspresijas izplatība un līmenis vairogdziedzera audos. Vairogdziedzera folikulu integritāte tika pētīta, analizējot blīvo šūnu savienojumu jeb slēgējūglu proteīnu *zonula occludens-1* un kladīna-1 imūnekspresiju un korelējot iegūtos datus ar IL-17 un CD68 ekspresiju. Papildus tam tika noteikts selēna statuss plazmā.

Starp AIVS un kontroles grupas pacientiem statistiski ticamas ar Th17 saistīto citokīnu plazmas līmeņa atšķirības netika konstatētas. Tomēr IL-17 ekspresijas līmenis tireocītos bija ievērojami augstāks pacientiem ar HT un GS nekā kontroles grupas pētāmajām personām, vienlaicīgi korelējot ar IL-23 un IL-1β imūnpozitivitāti HT grupā. Tika noteikta statistiski nozīmīga pozitīva korelācija starp ar Th17 saistīto citokīnu līmeni plazmā un hipertireozes smaguma pakāpi, kas bija neatkarīga no autoantivielu līmeņa, tādējādi norādot uz to iespējamo lomu GS patoģenēzē. HT pacientu vairogdziedzera audos tika novērotas tireocītu slēgējūglu molekulārās pārmaiņas, uzsverot vairogdziedzera folikulu integritātes bojājumu, taču to saistība ar IL-17 netika konstatēta.

Nozīmīgai vairogdziedzera enzīmu daļai, kura nodrošina gan antioksidatīvo un pretiekaisuma funkciju, gan aktivē vairogdziedzera hormonus mērķa audos, funkcionālai aktivitātei nepieciešams pietiekams daudzums selēna, tādēļ selēna deficīts tiek saistīts ar AIVS

patoģenēzi un pat terapiju. Lai gan mūsu pētījumā starp AIVS pacientiem un kontroles grupu netika noteiktas statistiski nozīmīgas atšķirības plazmas selēna līmenī, rezultāti pierāda, ka selēna statuss plazmā pirmreizēji diagnosticētiem GS un HT pacientiem Latvijā ir suboptimāls. Analizējot selēna līmeni plazmā HT pacientiem, iegūta statistiska nozīmīga negatīva asociācija ar autoantivielu pret tireoperoksidāzi (TPO) titru, tādējādi liecinot par selēna imūnmodulējošo lomu AIVS patoģenēzē. Turklāt HT pacientiem ar augstāku autoantivielu pret TPO līmeni tika novērots zemāks selēna līmenis plazmā, kas liek domāt, ka selēna papildterapija šiem pacientiem var sniegt ieguvumus.

Veicot šo pētījumu, tika iegūta nozīmīga informācija, kas padziļina mūsu zināšanas par vairogdziedzera autoimunitātes patoģenēzi, kā arī ar Th17 saistīto citokīnu lomu un regulatoro ietekmi, tomēr būtu nepieciešami turpmāki pētījumi, lai vēl precīzāk izpētītu Th17 šūnu lomu AIVS patoģenēzē. Pētījums apstiprina arī nepietiekamu selēna līmeni plazmā AVS pacientiem, tomēr, lai izprastu saistību starp selēna papildterapiju un imūnatbildes reakciju, nepieciešams iegūt vairāk klīnisko pētījumu datu.

Atslēgvārdi: zinātnes nozare – medicīna, apakšnozare – internā medicīna; autoimūnas vairogdziedzera slimības; Th17 šūnas; selēns; slēģjūgles.

Table of content

Abstract	2
Anotācija	4
Abbreviations	9
Introduction	10
Topicality of the research	10
Aim of the research	12
Scientific hypotheses of the given research	13
The novelty of the research	13
Ethical aspects	13
1 Literature review	14
1.1 Epidemiology and general characteristics of AITD	14
1.2 Genetic susceptibility to AITD	15
1.3 Protection against thyroid autoimmunity	16
1.4 Pathogenesis of AITD	18
1.5 Th17 cells and thyroid autoimmunity	20
1.5.1 Interleukin-17	22
1.5.2 Interleukin-22	22
1.6 Differentiation of Th17 cells	23
1.7 Thyroid epithelial integrity	25
1.8 Environmental factors	26
1.8.1 Iodine intake	26
1.8.2 Selenium status	28
2 Material and methods	31
2.1 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	31
2.1.1 Patients' characteristics	31
2.1.2 Routine histopathology	32
2.1.3 Immunohistochemical reactions applying a polymer-based visualisation system	32
2.1.4 Assessment of results of immunohistochemical reactions	33
2.1.5 Immunofluorescence assay	33
2.1.6 Statistical methods applied for assessment of results of immunohistochemical reactions	34
2.1.7 Transmission electron microscopy	34
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	34
2.2.1 Patients' characteristics.....	34
2.2.2 Routine histopathology	35
2.2.3 Immunohistochemical reactions applying a polymer-based visualisation system	35
2.2.4 Assessment of results of immunohistochemical reactions	36
2.2.5 Immunofluorescence assay	36
2.2.6 Statistical methods applied for assessment of results of immunohistochemical reactions	37
2.2.7 Electron microscopy immunogold labelling for identifying the localisation of IL-17, IL-23, and IL-1 β	37

2.3	The third study. Assessment of plasma levels of Th17-associated cytokines and selenium status in AITD .	38
2.3.1	Patients' characteristics	38
2.3.2	Blood sample collection	39
2.3.3	Thyroid function and antibody tests applying chemiluminescence immunoassay and ELISA	39
2.3.4	Detection of cytokines applying xMAP technology	39
2.3.5	Assessment of plasma selenium concentration applying fluorescence spectrophotometry	39
2.3.6	Statistical methods applied for assessment of results of thyroid function, levels of autoantibodies, plasma cytokines, and selenium concentrations ..	40
2.4	The fourth study. Immunological mechanisms of AITD: a shift in the traditional Th1/Th2 paradigm	40
3	Results	41
3.1	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	41
3.1.1	Histopathological features of AITD	41
3.1.2	Assessment of immunohistochemical expression of tested antigens in patients with AITD and colloid goitre	42
3.1.3	Ultrastructural examination of intercellular junctions of thyroid epithelial cells	50
3.1.4	Analysis of relationship between thyroid expression of IL-17A and intrafollicular CD68 in HT patients	51
3.2	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	51
3.2.1	Immunohistochemical analysis of Th17-associated interleukins in patients with AITD and colloid goitre	51
3.2.2	Immunogold labelling of Th17-associated interleukins in HT patients	57
3.2.3	Analysis of relationships between IL-17 and Th17-promoting cytokines IL-23 and IL-1 β estimated in the thyroid tissue by the use of immunohistochemistry	59
3.3	The third study. Assessment of plasma levels of Th17-associated cytokines and selenium status in AITD	59
3.3.1	Demographic and biochemical characteristics of the participants	59
3.3.2	Plasma levels of Th17 cytokines in patients with AITD and controls	61
3.3.3	Plasma selenium levels in patients with AITD and controls	69
4	Discussion	72
4.1	Heterogeneity of tissue IL-17 and tight junction proteins expression demonstrated in patients with AITD (the first study)	72
4.2	Upregulated tissue expression of Th17 pathogenic IL-23 and IL-1 β in HT but not in GD (the second study)	74
4.3	Plasma levels of Th17-associated cytokines and selenium status in AITD (the third study)	77
	Conclusions	81
	Publications	83
	Articles in international peer-reviewed journals	83
	Articles in local peer-reviewed journals	83
	Abstracts and presentations in international conferences	83
	Abstracts and presentations in local conferences	85
	References	86

Acknowledgements	95
Supplements	96
Publication 1	97
Publication 2	103
Publication 3	111
Publication 4	123
Ethical permission	134

Abbreviations

AITD	autoimmune thyroid diseases
ANA	antinuclear antibodies
Breg	regulatory B cells
CTLA-4	cytotoxic T lymphocyte antigen-4
FT3	free triiodothyronine
FT4	free thyroxine
GD	Graves' disease
GM-CSF	granulocyte macrophage colony-stimulating factor
GO	Graves' orbitopathy
GPx	glutathione peroxidase
HLA	human leukocyte antigen
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

Topicality of the research

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. GD is the most common cause of hyperthyroidism worldwide, whereas HT remains the most common cause of hypothyroidism in iodine-sufficient populations. 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 Campennì, 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. Acting on immune and non-immune cells such as epithelial cells, endothelial cells, fibroblasts, and osteoclasts, IL-17 promotes the expression of various cytokines (IL-1 β , TNF- α , IL-6, IL-8, granulocyte macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor), chemokines (CXCL1, CXCL5, and CCL2), and metalloproteinases by cells (Song, Gao and Qian, 2014). 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. Moreover, this newly discovered T cell subset is responsible for the initiation and development of chronic inflammatory responses in autoimmune diseases, such as psoriasis, rheumatoid arthritis, juvenile idiopathic arthritis, systemic lupus erythematosus, and inflammatory bowel disease (Yasuda, Takeuchi and Hirota, 2019).

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. Continuing studies have highlighted a broad spectrum of cytokines able to affect the crucial function – barrier function of endothelial and epithelial cells by influencing the structural and functional characteristics of the Tj. 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. Relatively low selenium levels have been found in patients with AITD, particularly those with Graves' orbitopathy (GO) (Khong et al., 2014). 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. 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). The low selenium content that is characteristically found in soils in most European countries and especially in the eastern and southern parts of the Baltic Sea

region (Ermakov, 1992) explains the development of mild to moderate selenium deficiencies. 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 (Supplement 5). All patients and volunteers included in the study signed a written informed consent form to participate in the study.

1 Literature review

Epidemiology and general characteristics of AITD

AITD present with two pathogenetically different diseases: HT or GD. These two are clinically diverse and taken together make thyroid condition the commonest autoimmune disease. However, there is a significant geographic variation in the worldwide reported incidence and prevalence of HT and GD. AITD prevalence is about 5% in the overall population, whereas the prevalence of thyroid antibodies is estimated to be 10–20% of all women (Hollowell et al., 2002; Pyzik et al., 2015). The worldwide incidence of HT is reported to be 0.3–1.5 cases per 1,000 persons per year (Latina et al., 2013). Furthermore, HT is the most common cause of primary hypothyroidism in iodine-sufficient regions. Like most autoimmune disorders, AITD exhibit a strong female bias in prevalence: HT affects women five to ten times (Pyzik et al., 2015), GD – four to five times more often than men (Carlé et al., 2011). It has been also known that the prevalence of thyroid autoimmunity increases with the age reaching the highest number of cases between 45–65 years in HT and between 33–50 years in GD (Cooper and Stroehla, 2003; Davies et al., 2020).

The prevalence and incidence of autoimmune diseases, including AITD, have increased significantly over the last decades. AITD have become a public health problem worldwide, including Latvia. It was revealed that 10.7% of the 739 pregnant women participating nationwide had elevated anti-thyroperoxidase (TPO) autoantibody levels suggesting pre-existing thyroid autoimmunity (Konrade et al., 2015). Furthermore, when analysing data concerning thyroid medicinal product use trends from the Latvian National Health Service and State Agency of Medicines, there is a steady increase in the number of people using thyroxine and thiamazole: thyroid therapy defined daily dose per 1,000 inhabitants has increased from 10.23 in 2009 to 17.40 in 2017, thereby extremely increasing disease burden on health, health costs, education, society, and family life (Anonymous, 2018).

AITD are characterised by the presence of circulating antibodies against the thyroid autoantigens and infiltration of the thyroid gland by autoreactive lymphocytes (Tomer, 2014). Interestingly, although anti-TPO and anti-thyroglobulin (Tg) autoantibodies are detected in 90–95% and 70–80% of AITD patients, respectively, the prevalence of both antibodies in patients with non-thyroid immune diseases is reported to be 10–15% (Carvalho et al., 2013). HT results predominantly from the cellular immune response, which involves Th1 and Th17 cells and leads to cell death of the thyrocytes with subsequent hypothyroidism, whereas GD mainly promotes Th2-related humoral autoimmunity causing the production of TSH receptor antibodies and growth of the thyrocytes leading to the development of hyperthyroidism

(Morshed et al., 2012). However, both thyroid disorders may coexist in the same individual causing mixed Th1/Th2/Th17 immune responses.

Various environmental and endogenous factors may act together to trigger thyroid autoimmunity in persons with predisposing genetic variants causing elevated thyroid autoantigen presentation and a loss of immune tolerance. Despite numerous studies undertaken and knowledge accumulated on the aetiology and pathogenesis of AITD in the last decade, the exact immunological mechanisms responsible for the disease development have not been thoroughly understood.

Genetic susceptibility to AITD

The role of genetic background in the aetiology of AITD is highlighted by numerous epidemiological studies showing that genetic factors contribute to about 70–80% of the development of thyroid autoimmunity (Tomer and Davies, 2003; Hansen et al., 2006). The familial clustering of AITD was confirmed by several studies 50 years ago. It has been shown later that almost 50% of the siblings of GD patients have thyroid autoantibodies and that 33% of those with HT or GD develop AITD themselves (Villanueva, et al., 2003). Increased familial clustering of AITD has been reported in German study, indicating a 32-fold enhanced risk for developing HT in children and a 21-fold enhanced risk in siblings of patients with HT (Dittmar et al., 2011).

During the last decades, some twin studies have examined the aetiology of several phenotypes related to AITD and have provided new data on the genetic contribution to AITD. A strong influence of genetic factors on the development of AITD has been demonstrated by twin studies as well – the heritability of GD and thyroid autoantibody (TPO and Tg) formation has been reported to be 79% and 73%, respectively (Brix et al., 2001; Hansen et al., 2006). A study of Hashimoto's hypothyroidism showed a 55% and 0% concordance in Danish monozygotic and dizygotic twins, respectively, indicating an essential role of environmental factors in the disease development (Brix et al., 2000). Thus, twin studies have shown that 21–27% of the total phenotypic variance in AITD can be explained by environmental factors.

Therefore, it is critical to investigate and identify the possible interplay of environmental and genetic factors involved in thyroid autoimmunity to propose individualized AITD management strategies since most environmental factors may be modified. However, the mechanisms by which the environmental factor triggers thyroid autoimmunity in individuals with known susceptibility loci remain unclear. The main known susceptibility genes for AITD can be classified as either immunoregulatory or thyroid-specific genes (Effraimidis and Wiersinga, 2014). TSHR and Tg were recognised as thyroid-specific genes, whereas the human

leukocyte antigen (HLA), PTPN22, cytotoxic T lymphocyte antigen-4 (CTLA-4), FOXP3, FCRL3, CD25, CD40, and other genes – as immune-modulating playing a critical role in the development of the effective immune response including self-tolerance, and cell-mediated and humoral immunity. However, single nucleotide polymorphisms in these genes, which are not specific for AITD were recognised as risk factors for other autoimmune diseases (Lee et al., 2015). Tg is one of the main thyroid autoantigens assumed as a major susceptibility gene for AITD (Tomer et al., 2002). Whereas in GD patients, a consistent association with polymorphisms of the TSHR gene was reported (Xiong et al., 2016).

The occurrence of both AITD is associated with specific variants of the HLA class II genes. They are vital for regulating immune activity by determining the specificity of antigen binding and the initiation of the immune response. HLA controls cytokine production and modulates the immune response by cytokine genes on haplotypes as well. Thus, polymorphisms in the HLA genes may lead to HT or GD via regulating the Th1/Th2 and possibly Th17 pathways. HLA-DQA1 and HLA-DR3 were shown to be associated with GD, while polymorphisms in HLA-DR3, DR4, DR5, DQ7, DQB1, DQA1, DQW7 correlated with the incidence of HT (Badenhoop et al., 1990; Zeitlin et al., 2008).

Numerous susceptibility genes and loci for AITD have been detected by genome-wide association studies during the last decade. However, these studies involve mainly single genetic factors, which might have a minor effect on the disease development and only the simultaneous presence of certain genetic and environmental factors altogether may lead to the clinical manifestation of thyroid autoimmunity. Therefore, genome-wide association studies should focus on the simultaneous investigation into multiple genetic and environmental factors and interactions between them.

Protection against thyroid autoimmunity

Mechanisms of the central and peripheral tolerance provide a proper regulation of the immune system, thus protecting against thyroid autoimmunity. Maturation of T helper CD4⁺ lymphocytes occurs in the thymus via processes of positive and negative selection. The central immune tolerance refers to apoptosis of autoreactive T cells in the thymus during foetal life. Those T cells that escape negative selection are prevented from triggering autoimmunity by mechanisms of peripheral tolerance where Treg play a crucial role (Shevach, 2006; Weetman, 2010). Their protective role has been also demonstrated in several animal models of autoimmune diseases (Morris et al., 2009).

Treg cells derive from the thymus as a subpopulation of fully differentiated T cells (thymus-derived Treg), naive Th0 cells in the peripheral tissues (peripheral differentiated Treg) or develop *in vitro* conditions (iTregs) (Shevach and Thornton, 2014). The first properly characterised Treg cell subset exhibits the expression of the transcription factor forkhead box protein 3 (Foxp3) and high constitutive levels of α chain of the IL-2 receptor (CD25). Foxp3 contributes to the maintenance of self-tolerance by promoting the development of Treg while inhibiting the differentiation of Th17 cells (González-Amaro and Marazuela, 2016). Foxp3 mutations in humans can cause an allotropic syndrome of multi-organ autoimmunity such as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome. Several studies evidenced a defective function of Foxp3⁺ Treg cells in patients with HT and GD (Glick et al., 2013).

Treg cells have been shown to suppress target cells by direct cell-to-cell interaction or secretion of inhibitory cytokines such as TGF- β , IL-10, and IL-35. IL-10 induces suppression of pathogenic Th17 cell responses and maintains Treg suppressive activity and expression of Foxp3; TGF- β regulates differentiation, proliferation, and survival of lymphocytes, while the exact role of IL-35 requires further investigation (Chaudhry et al., 2011; Huber et al., 2011).

Another subset of Treg cells recently described demonstrates a constitutive expression of CD69 but does not express Foxp3 or CD25. These cells exert their immunosuppressive activity mainly through the production of IL-10 and TGF- β (Han et al., 2009). Interestingly, increased levels of CD69⁺ Treg cells were found in both peripheral blood and thyroid tissue of AITD patients. However, these lymphocytes were dysfunctional and unable to effectively down-modulate the autoimmune process and tissue damage, thus suggesting their role in the pathogenesis of autoimmune diseases (Rodríguez-Muñoz et al., 2016). Finally, Foxp3- IL-10 secreting type 1 regulatory (Tr1) cells have been shown to inhibit T cell activity by different mechanisms such as inhibitory cell-cell interactions via CTLA-4 or programmed cell death protein-1 (PD-1) and secretion of granzyme B and perforin (Roncarolo et al., 2014).

The decreased frequency and/or a defective function of Treg in patients with HT or GD was demonstrated in several studies (Li et al., 2016), thus highlighting the essence of results previously observed in animal models. Although the number of Treg cells reported is variable due to the use of different detection markers and estimations related to disease stages, the majority of studies suggest that the reduction or functional defects of Treg are tightly associated with the development of thyroid autoimmunity. Recently, a possible role of regulatory B cells (Breg) in the maintenance of peripheral tolerance and inhibition of immune responses to specific self-antigens via release of IL-10, TGF- β , Fas ligand, and TNF-related apoptosis inducing ligand has been established as well (Kristensen et al., 2015). Thus, both

Treg and Breg cells may act as inhibitors of inflammatory and autoimmune responses to prevent autoimmunity.

In certain individuals, however, autoreactive T lymphocytes escape from the control of these immunoregulatory mechanisms and may lead to loss of the peripheral self-tolerance with subsequent development of autoimmune disease via activation, proliferation, and differentiation. Reduction of peripheral self-tolerance occurs due to enhanced Th17 cell response as compared with induced regulatory Th10 cell response (Papp et al., 2017).

Pathogenesis of AITD

Thyroid autoimmunity appears as a result of the breakdown or reduction of self-tolerance to the three major thyroid proteins – TPO, Tg, and TSHR occurring in the central or peripheral organs of the immune system (Orgiazzi, 2012). This causes the infiltration of the thyroid gland by T and B lymphocytes followed by the production of AITD specific antibodies, induction of the thyrocyte apoptosis, and the gland destruction in HT. Although traditionally the pathogenesis of HT is due to T cell-mediated autoimmunity and GD is a result of humoral autoimmunity, cellular and humoral immune responses are closely connected and cross-linked (Ramos-Leví and Marazuela, 2016). Despite comprehensive research in the last decades, the exact mechanisms of initiation and progression of thyroid autoimmunity have not been completely established.

The genetic background, self-antigens, and various environmental triggers altogether determine peculiarities of the autoimmune response in AITD and cause the enhanced thyroid antigen presentation and reduction of immune tolerance (Antonelli et al., 2015). Antigen-presenting cells, and especially dendritic cells colonizing the thyroid gland, present specific thyroid antigens to T lymphocytes via the MHC complex in the lymph nodes. It results in activation and proliferation of autoreactive T and B lymphocytes, and production of different cytokines by immune and thyroid cells. Dendritic cells may be functionally subdivided into two groups – immunogenic or tolerogenic – depending on the activation of autoreactive T-cells or Treg, respectively (Maldonado and von Andrian, 2010). Moreover, several studies have revealed increased levels of dendritic cells within inflammatory infiltrates of the thyroid in both GD and HT patients (Roura-Mir et al., 2005; Mao et al., 2011). Once activated, T helper (CD4+) cells can be differentiated into at least four main functional subsets according to the production of specific cytokines – Th1, Th2, Th17, and Treg. The development of the Th1 population is stimulated in the presence of either IL-12 or IFN- γ through transcription of T-bet, whereas differentiation of Th2 cells is driven by IL-4 via master transcription factor GATA3 (Peck and Mellins, 2010).

Classically, HT has long been considered as a Th1-mediated disease, while a Th2-driven autoimmune response is dominant for GD development. However, this classification has altered due to the description of Th17 cells, suggesting evident participation of these cells in AITD, and particularly HT pathogenesis. In HT, increased Th1 activity caused by upregulation of pro-inflammatory cytokines such as IL-2, IL-1 β , IFN- γ , TNF- α leads to cell-mediated immunity and thyrocyte death in apoptotic pathways. Thus, autoreactive Th1 cells recruit CD8⁺ cytotoxic T cells, which cause thyrocyte apoptosis by the action of granzyme and perforin. In addition, autoreactive T lymphocytes induce antibody production by B cells leading to necrosis of thyroid cells by complement system activation. Finally, thyrocytes can undergo caspase-mediated apoptotic death in a paracrine way by expression of the CD95/Fas death receptor (Figure 1.1) (Fröhlich and Wahl, 2017). Although the contribution of anti-TPO autoantibodies to the destruction of the thyroid gland compared to cytotoxicity is minor, autoantibodies have shown pathogenic effects including complement activation and induction of oxidative stress, indicating that HT is also a Th2-mediated disease. Moreover, it has been revealed previously that anti-TPO autoantibodies can cause damage to cultured thyroid cells performed upon binding to TPO located on the apical cell membrane via both antibody-dependent cytotoxicity and complement-dependent cytotoxicity (Rebuffat et al., 2008).

In GD, there are increased levels of Th2 released cytokines IL-4, IL-5, IL-6, IL-10, IL-13, which mainly mediate humoral response by stimulating the production of TSHR antibodies, growth of the thyrocytes, and apoptosis of the thyroid gland infiltrating lymphocytes via downregulation of Fas/Fas ligand and upregulation of the anti-apoptotic molecule Bcl-2 (Salmaso et al., 2002). TSHR antibodies act stimulatory by increasing iodide metabolism via cAMP/PKA and promoting thyroid cell proliferation (Figure 1.1). Antibodies to TPO and, less frequently, to Tg have been identified in most GD patients too. Furthermore, TPO and Tg autoantibodies may comprise immunoglobulin (Ig) G4 as well as IgG1 subclasses, suggesting the participation of both Th2 and Th1 cytokines in GD.

Figure 1.1 Pathogenesis of autoimmune thyroid diseases

* Adapted from Fröhlich and Wahl, 2017. Abbreviations: APC, antigen-presenting cells; cAMP, cyclic adenosine monophosphate; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; PI3K, phosphatidylinositol 3,4,5, triphosphate kinase; PKA, protein kinase.

During the last decade, a role of a new subset of Th cells, designated Th17, has been studied in the pathogenesis of thyroid autoimmunity, thus changing the traditional paradigm of the Th1/Th2 dichotomy. Several research groups have shown that IL-17A, the prominent effector cytokine of Th17, can induce the release of different pro-inflammatory cytokines responsible for the induction and development of chronic inflammatory responses in many autoimmune diseases, including AITD.

Th17 cells and thyroid autoimmunity

The discovery of a third Th subset has introduced a new understanding of the classical Th1 and Th2 dichotomy. Th17 cells and their hallmark cytokine IL-17 were identified to play an important role in the pathogenesis of different inflammatory and autoimmune diseases, which were previously classified as Th1-dependent pathologies. An altered balance between Th17 and Treg cells as well as Treg cell dysfunction has been found in autoimmune diseases (Papp et al., 2017).

Previous studies have shown a significant increase of Th17 cells and IL-17, revealed in the peripheral blood and thyroid tissues of AITD patients when compared to control subjects, suggesting their contribution to the thyroid autoimmunity. Li and colleagues revealed an increased number of thyroid tissue infiltrating Th17 cells and serum levels of IL-17 in patients with HT compared to the thyroid cancers, nodular goitres, or healthy controls. Additionally, a strong association between IL-17 expression and stromal fibrosis was observed in HT patients (Li et al., 2017). Furthermore, Figueroa-Vega et al. evidenced enhanced levels of Th17 cells and IL-17 mRNA in the peripheral blood and thyroid tissue infiltrating inflammatory cells in patients with HT along with higher levels of IL-6, IL-15, and IL-23, which are involved in the Th17 cell differentiation (Figueroa-Vega et al., 2010). Later, Konca Degertekin and colleagues found that the hypothyroid patients had lower levels of IL-17 and IL-23 compared to euthyroid HT patients, suggesting that hypothyroidism itself has an inhibitory effect on Th17 cytokine responses (Konca Degertekin et al., 2016). Participation of Th17 cells and their cytokines in the pathogenesis of GD is less evidenced. Previous studies showed an enhanced number of CD4+IL-17+ cells in the peripheral blood of children with untreated HT but not in GD patients (Bossowski et al. 2012). Similarly, IL-17 levels in the peripheral blood and thyroid tissues did not differ significantly between adult patients with GD and controls (Qin Q et al., 2012).

GO, a serious and most frequent extrathyroidal complication of GD, manifests as orbital inflammation and expansion of adipose tissue and extraocular muscles. Several studies have investigated the involvement of Th17 cells in the pathogenesis and disease activity of GO. Serum IL-17 levels were found to be significantly increased in GO patients compared to controls. Patients with active GO had higher serum concentrations of IL-17 compared to inactive GO patients. Furthermore, IL-17 levels were correlated with the clinical activity score of GO, thus supporting the role of IL-17 in the pathogenesis and progression of GO (Kim et al., 2012). Fibrosis and adipogenesis are the two dominant pathophysiologic orbital processes involved in the pathogenesis of GO. Recently, Fang and colleagues have reported enhanced levels of pathogenic IFN- γ and IL-22-producing Th17 cells in active GO patients compared to inactive GO patients and controls (Fang et al., 2017). Additionally, a positive correlation between pathogenic Th17 cells and the clinical activity score of GO was observed, indicating that the number of these cells might serve as an indicator of GO severity and activity. The authors have also demonstrated that Th17 lymphocytes can promote the production of pro-inflammatory cytokine in both CD90+ (favour fibrosis) and CD90- (favour adipose hyperplasia) orbital fibroblasts and stimulate expression of a costimulatory molecule on orbital CD34 positive fibroblasts. Moreover, both orbital fibroblast subsets can directly induce naive

T cell differentiation toward the Th17 phenotype via prostaglandin E2 production (Fang et al., 2017).

Interleukin-17

Th17 cells are mainly characterised by the production of IL-17A and IL-17F responsible for the development of inflammation, particularly in autoimmune diseases. They can act on epithelial cells, fibroblasts, and macrophages inducing the release of pro-inflammatory mediators such as cytokines (IL-6, IL-8, TNF- α , IL-1 β , GM-CSF, G-CSF), chemokines (CXCL1, CXCL8, CCL2, CCL7, CCL20), and metalloproteinases (Fossiez, 1996; Song, Gao and Qian, 2014). IL-17 regulates the differentiation of immune cells within the germinal centre of the lymphoid follicle and the production of autoantibody. It has been found that IL-17 stimulates neutrophil recruitment by inducing CXCL8 by both immune and non-immune cells. In addition, IL-17 can regulate the expression of pro-inflammatory genes via activation of the NF- κ B signalling pathway (Chung et al., 2009). IL-17 plays a crucial role in host defence against bacterial and fungal infections at epithelial surfaces by inducing the expression of antimicrobial peptides, such as β -defensins and S100 proteins (Song, Gao and Qian, 2014).

Interleukin-22

IL-22 produced by Th17 cells can also be secreted by another novel subset of Th cells – CCR⁺IL-17⁺IL-22⁺ or Th22 cells acting at mucosal surfaces. Importantly, Th22 lymphocytes produce mainly IL-22, IL-13, and TNF α , but not IL-17. Differentiation of these cells requires IL-6 and TNF α , whereas IL-22 secretion depends on transcription factor aryl hydrocarbon receptor. Although IL-22 receptor (IL-22R) is expressed in the skin, gastrointestinal tract, liver, lungs, and kidney, but not on cells of the hematopoietic lineage, it has been shown that IL-22 binding protein, a structural homolog of IL-22R1, is highly expressed by dendritic cells as well as T and B cells (Nagalakshmi et al., 2004). Acting in synergy with IL-17, IL-22 maintains barrier function and host defence against pathogens by inducing the expression of antimicrobial peptides at mucosal surfaces. Moreover, IL-22 can induce innate immunity and has an important role in wound healing and injured epithelium repair, promoting both proliferation and antiapoptotic gene expression within cells (Kolls and Khader, 2010; Sonnenberg et al., 2011). Finally, IL-22 has been shown to stimulate the expression of IL-6 and MMP-1, indicating that it can exert pro-inflammatory effects as well.

Differentiation of Th17 cells

As mentioned above, different cytokines and transcription factors are required for regulating the differentiation of naïve Th lymphocyte into a Th1, Th2, Th17, or Treg cell subset. In contrast to convincing experimental data highlighting Th17 cell development, only a few human studies performed in the past have established the cytokine pattern involved in the generation of Th17 cells (Stritesky et al., 2008; Peck and Mellins, 2010).

The cytokine profile necessary for driving the differentiation of Th17 lymphocytes is similar in both humans and mice. TGF- β along with the pro-inflammatory cytokines such as IL-1 β , IL-6, IL-21, and IL-23 have been found to have an essential role in the development of Th17 cells. It has been evidenced that Th17 lineage is directed by their master transcription factor retinoic acid receptor-related orphan nuclear receptor gamma (ROR γ), which is induced by IL-6 and IL-23 via signal transducer and activator of transcription 3 (STAT3). Interestingly, TGF- β is important for the differentiation of both Th17 and induced Treg cells. It has been shown that high doses of TGF- β induce expression of Foxp3, thus promoting Treg differentiation, while low doses of TGF- β in combination with IL-6 promote Th17 polarisation (Stadhoudersab, Lubberts and Hendriks, 2018).

In mice, the combination of IL-6, IL-1 β , and IL-23 is sufficient for the differentiation of Th17 cells, indicating TGF- β independent signalling of Th17 development. Th17 cells were also found colonising the gut of TGF- β signalling-deficient mice (Qin et al., 2009). In contrast to mice observations, TGF- β seems to be indispensable for the differentiation of Th17 cells in man. Thus, TGF- β in the presence of IL-6 and IL-21 induces the initial differentiation of Th17 from naïve Th cells, while IL-23 and IL-1 β are essential for both complete maturation and stabilisation of the Th17 population (naïve Th cells do not express IL-1 and IL-23 receptor) (Stritesky et al., 2008). Although IL-23 is not required for the induction of Th17 development, it is a crucial factor for the stabilisation and generation of the pathogenic phenotype of Th17 cells via STAT4 (Croxford et al., 2012). IL-23, produced by dendritic cells and macrophages, can maintain Th17 cell differentiation by suppressing IL-10 secretion and promoting IL-22 and GM-CSF production.

Figure 1.2 **Differentiation of Th17 subsets and production of cytokines**

* Adapted from Stadhoudersab, Lubberts and Hendriks, 2018.

Although the Th17 subset was identified more than a decade ago, it has become clear only recently that Th17 cells are heterogeneous and not uniform in function exhibiting under certain cytokine exposure two different cellular phenotypes: pathogenic and non-pathogenic (Figure 1.2) (Stadhoudersab, Lubberts and Hendriks, 2018). Th17 cells differentiated in the presence of TGF-β1 and IL-6 co-produce IL-17 and IL-10. These cells are assigned to the non-pathogenic subset of the Th17-lineage, as they do not induce tissue inflammation and may inhibit an autoimmune inflammation.

By contrast, Th cells upon exposure to IL-23, IL-1β, and IL-6 can generate highly pathogenic Th17 lymphocytes, which drive the autoimmune inflammation (Ghoreschi et al., 2010). These pathogenic lymphocytes can secrete both common and pathogenic sets of molecules, such as IL-17A/F, IL-21, IL-22, IL-26, IFN-γ, TNF, and GM-CSF (Stadhoudersab, Lubberts and Hendriks, 2018). Enhanced levels of IL-23 were observed in the peripheral blood and within thyroid tissues of HT patients, but it remains unclear whether the IL-23 is involved in the pathogenesis of GD.

Cytokines play a crucial role in modulating an immune response in both AITD. Cytokines produced by the activated T cells may drive the autoimmune response skewing toward the Th17 phenotype and away from Th1 or Th2 phenotypes. Therefore, evaluation of T cell subsets linked to certain cytokine production in AITD could help identify the main immunological mechanisms involved in thyroid autoimmunity, thus deepening our knowledge about disease outcome and treatment strategies (Fasching et al., 2017).

Th17 cells exhibit various therapeutic targets, and treatment strategies include blockade of the differentiation of Th17 cells, inhibition of Th17 neutralising cytokines, and Th17-specific transcription factors (Maddur et al., 2012). Furthermore, several IL-17 neutralising monoclonal antibodies such as secukinumab, ixekizumab, and brodalumab have been developed in recent years demonstrating impressive therapeutic effects in patients suffering from psoriasis (McInnes et al., 2015). Further experimental and clinical studies are needed to better clarify the role of different Th subsets in AITD.

Thyroid epithelial integrity

Tj, called also occluding junctions, are composed of a complex, interwoven network of membrane proteins and glycoproteins. These are distinguished at the apical part of the lateral membranous surface of polarised endothelial and epithelial cells. Tj maintain cell polarity and establish the barrier sealing the paracellular space between neighbouring thyrocytes (Figure 1.3).

Numerous studies have demonstrated that the claudin family is the key component of Tj, fully performing paracellular barrier function in the absence of occludin (Schulzke et al., 2005). From the currently known 27 claudins expressed in humans, claudin-1 is considered to be the most important member responsible for epithelial barrier integrity (González-Mariscal et al., 2003). Furthermore, claudin-1 deficiency appeared to cause vital alterations of barrier function leading to the death of claudin-1 deficient mice (Furuse et al., 2002). Simultaneously, an essential role of interactions of ZO-1 protein and claudin for the formation of Tj assembly have been recognised. Numerous studies with ZO-1 and/or ZO-2 knockdown cells demonstrated disruption in both claudin localisation and paracellular permeability. Moreover, in the absence of ZO-1, these cells have been shown to exhibit polarity but fail to form Tj (Umeda et al., 2006).

Continuing studies have highlighted a broad spectrum of cytokines able to affect the crucial function – barrier function of endothelial and epithelial cells by influencing the structural and functional characteristics of the Tj. Many interleukins were found to modulate Tj proteins such 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.

Figure 1.3 **Tight junctions preventing inappropriate release of thyroglobulin to the circulation and contributing to the restricted localisation of the TSHR and TPO to the basolateral and apical domains of the cell surface**

* Adapted from Förster, 2008.

Environmental factors

An increased iodine intake appears as the most important among environmental factors studied and suggested as ones influencing the development of AITD. Other suggested risk factors include selenium deficiency, smoking, alcohol, stressful life events, infections, several drugs, exposure to chemical compounds. Prevention of iodine deficiency disorders by food iodine fortification has been successfully implemented in many countries in the past century. However, other preventive interventions for AITD may be complicated or even impracticable. It would be extremely important to recognise which preventive interventions can be taken by subjects with a risk to develop AITD. Excess iodine intake and a relative selenium deficiency appear to be the predominant environmental factors triggering the autoimmune response to the thyroid.

Iodine intake

Iodine intake contributes to thyroid function and the development of thyroid gland disorders. The recommended daily allowance of iodine for adults is 150 µg, increasing to 250 µg in pregnancy and lactation. It is well known that iodine-sufficient populations compared to those that are iodine-deficient appear to have higher incidences of thyroid-specific

antibodies and autoimmune hypothyroidism. Data from epidemiological studies show that due to reduced synthesis of thyroid hormone severe iodine deficiency may develop revealing the increased prevalence of goitre and hypothyroidism, whereas chronic mild-to-moderate iodine deficiency may cause a toxic nodular goitre and hyperthyroidism (Zimmermann and Boelaert, 2015).

By contrast, chronic excessive iodine intake or the rise in usual intake, especially after the implementation of mandatory food iodine fortification (e.g., universal salt iodisation) may lead to the increased prevalence of thyroid autoimmunity and subclinical hypothyroidism in parallel with a decrease in rates of iodine deficiency-related thyroid disorders (Teng et al., 2006). Thus, the prevalence of anti-TPO autoantibodies in the Danish population before and 4–5 years after cautious mandatory iodisation of salt aiming to adjust iodine intake to a low recommended level was 14.3% and 23.8%, respectively (Bülow Pedersen et al., 2011). Furthermore, the rise in the frequency of thyroid antibodies was most pronounced in women aged 18–45. Five to 7 years after iodine fortification, the overall incidence rate of overt hypothyroidism increased as well reaching 47.2/100.000 per year compared to 38.3/100.000 per year at baseline (Bülow Pedersen et al., 2007). In the Pescopagano survey in Italy, the introduction of the salt iodisation programme 15 years later resulted in the increased prevalence of anti-TPO autoantibodies (12.6% vs 19.5%) and HT rates (3.5% vs 14.5) as well (Aghini Lombardi et al., 2013).

However, it has been also shown that the optimisation of nutritional iodine intake may result in a lowering of the incidence of autoimmune thyroiditis. After the implementation of mandatory fortification of salt with iodine in Brazil in 1956, adjustments of the iodine concentration in salt have been performed in 2003 and 2013 by decreasing the recommended iodine concentration to 20–60 and 15–45 mg per kg of salt, respectively. A cross-sectional study conducted in 2013 demonstrated a trend toward a decreased prevalence of HT and a decrease in urinary iodine concentration to optimal levels (Miranda et al., 2015). However, it is unclear what will be the impact of a new continuing reduction in salt iodine concentration on thyroid function. The first systematic review and meta-analysis regarding the effect of excess iodine intake on thyroid disease prevalence in different populations has been published recently, indicating that although universal salt iodisation decreased goitre rates, chronic excess iodine intake from water or poorly monitored salt is a risk factor for hypothyroidism in free-living populations (Katagiri et al., 2017). Therefore, accurate monitoring of iodine concentration both in salt and water is required for the prevention of thyroid disorders.

The mechanisms by which increased chronic intake of dietary iodine contributes to thyroid autoimmunity are not completely understood. In susceptible individuals, iodine excess induces thyroid gland infiltration by lymphocytes liberating cytokines and chemokines, thus leading to the generation of thyroid antibodies. Moreover, high iodine intake enhances Th17 and inhibits Treg cell differentiation, while it triggers an abnormal expression of TNF-related apoptosis-inducing ligand in thyrocytes thereby inducing cell death of thyroid cells (Yu et al., 2011). It has been shown recently that excess iodine promotes apoptosis of thyroid follicular epithelial cells seen in HT by inhibiting autophagy activity. This process is mediated through the downregulation of TGF- β 1 and activation of the Akt/mTOR signalling pathway. In addition, excess iodine increases the production of reactive oxygen species contributing to oxidative stress and thyrocyte apoptosis (Xu et al., 2016). Excessive iodine may also alter the conformation of Tg and promote its immunogenicity by increasing the affinity of Tg determinants for the T cell receptor (Carayanniotis, 2011). Furthermore, chronic high iodine exposure may induce autoantigen presentation activity of thyrocytes and dendritic cells as well as activate autoreactive T cells. Finally, excess iodine-induced hypothyroidism in HT may be due to a pathologically persistent inhibitory Wolff-Chaikoff effect on thyroid hormone synthesis (Markou et al., 2001).

Selenium status

Selenium status is an important parameter of general population health, and it has been hypothesised that nutritional selenium deficiency may trigger the initiation or progression of thyroid autoimmunity. Various effects of selenium are recognised ranging from antioxidant and anti-inflammatory to the production of active thyroid hormone (Rayman, 2012). Amino acid selenocysteine is a major form of the thyroid selenium presented either by enzymes such as glutathione peroxidases (GPx), thioredoxin reductases, iodothyronine deiodinases, or proteins without enzymatic function known as selenoproteins (SELENO) P, M, S (Valea and Georgescu, 2018). Enzyme proteins have an important role in protecting thyrocytes from oxidative damage and, also, in thyroid hormone metabolism. GPx provide thyroid antioxidant protection against free radicals and oxidative stress, catalysing the detoxification of hydrogen peroxide and organic hydroperoxides. The biomarkers of selenium status and function available for human studies include GPx3 (10%–25% of plasma selenium), SELENOP (20%–70% of plasma selenium), and GPx1, which can be assayed in erythrocytes, lymphocytes as well as tissue biopsy specimens (Combs, 2015). GPx3 is also secreted at the apical pole of the thyrocyte where it degrades excess hydrogen peroxide that has not been used by TPO, whereas SELENOP, the major selenoprotein in plasma, is crucial for the specific distribution and

transport of selenium to several target tissues. Studies in many countries have shown the effects of geographic variation in the selenium contents in food and plasma selenium concentrations in different populations around the world (Winther et al., 2020).

Selenium deficiency has been recognised in several pathological thyroid conditions including nodular goitre, thyroid cancer, and AITD. Serum selenium levels are lower in newly diagnosed GD patients compared to healthy subjects (Bülow Pedersen et al., 2013). Lower selenium levels were evidenced in GD subjects compared to those without this disease, suggesting that relative selenium deficiency may be an independent risk factor contributing to the development of orbitopathy in GD patients (Khong et al., 2014). It is also well known that selenium supplementation is recommended to prevent the progression of mild GO. Selenium levels are lower in patients with HT as well. A cross-sectional study involving more than six thousand participants from two provinces in China that had high genetic, environmental, lifestyle as well as iodine status similarities, except for very different soil-selenium concentrations, has revealed an increased prevalence of HT but not GD in the province with lower selenium intake (Wu et al., 2015).

Selenium deficiency can impair the differentiation of CD4⁺ T cells, leading to dysfunction of cellular and humoral response. 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). In inflammatory bowel disease treated with selenium supplementation, macrophages secrete Δ 12-prostaglandin J2 and 15d-prostaglandin J2, which activate peroxisome proliferator-activated receptor γ , thus inhibiting the differentiation of both Th1 cells and Th17 cells and leading to the development of Treg lymphocytes (Nettleford and Prabhu, 2018).

Analysis of selenium status in HT and GD patients suggests that supplementation of this trace element may be beneficial in these patients. It has been observed that selenium supplementation decreases anti-TPO autoantibody levels more effectively in individuals with lower baseline levels (Toulis et al., 2010). Furthermore, regions with deficient, higher than adequate, or high iodine intake may have more need for selenium through its role within selenoproteins protecting the thyroid gland from oxidative damage as well as increasing immune tolerance (Hu and Rayman, 2017). It has been also demonstrated that selenium supplementation can upregulate Treg cells that were reduced by excess iodine intake (Xue et al., 2015).

Reduction of circulating thyroid autoantibody levels by selenium supplementation in individuals with HT was evidenced almost two decades ago. Over the last years, numerous prospective clinical trials were performed to examine whether selenium supplementation either

with sodium selenite or selenomethionine might have beneficial effects for the treatment of HT. A meta-analysis published in 2016 showed that selenium supplementation effectively reduces anti-TPO autoantibody levels at 3, 6, and 12 months in levothyroxine-treated HT patients. Importantly, in untreated patients, there was a decrease in anti-TPO autoantibody levels after 3 months, but not after 6 or 12 months of supplementation (Wichman et al., 2016). Another meta-analysis study was published evaluating several types of outcomes, except for anti-TPO autoantibody levels (Winther et al., 2017). It demonstrated that TSH and ultrasound finding improvement with selenium has a very low to low level of evidence, while the quality of life improvement has a low to moderate level of evidence. In another study, selenium treatment resulted in reduction of anti-TPO autoantibody titres by reducing oxidative stress markers in euthyroid HT patients (Tian et al., 2020).

Although a role for selenium supplementation in the treatment of HT is still discrepant, the correction of a selenium deficiency may have other health benefits. It is highly important to assess patients' selenium status before and during selenium administration as well as to identify and correct selenium deficiency in these patients. However, it remains unclear how selenium supplementation may affect HT in patients with higher baseline selenium intake. Further randomised controlled trials are required before selenium supplementation can be routinely suggested by clinicians in patients with HT.

2 Material and methods

The research was carried out by the use of clinical patients' examination methods, morphological methods as well as laboratory biochemical and molecular tests. Upon enrollment, data on a disease duration, course, medication, family's history was collected. A broad spectrum of morphological methods, including immunohistochemistry, immunofluorescence, and transmission electron microscopy, both conventional and immunogold labelling was used to conduct the research. Finally, chemiluminescence immunoassay and ELISA assay, xMAP technology, and fluorescence spectrophotometry were applied to test thyroid function and the levels of autoantibodies, plasma cytokines, and selenium concentrations.

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.

2.1 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 Zake, Sandra Skuja, Ieva Kalere, Ilze Konrade, and Valerija Groma. *Medicine* (Baltimore), 2018 Jun; 97(25): e11211, doi: 10.1097/MD.00000000000011211. PMID: 29924048; PMCID: PMC6024462.

2.1.1 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 were confirmed by the clinical diagnostic criteria of HT and GD, complemented by results of thyroid biochemistry, ultrasound imaging, and histopathology findings. Patients with GD presented with thyroid hormone levels above the laboratory reference range, positive anti-TSHR antibodies and diffuse goitre proved by ultrasound examination. Anti-TSHR antibodies were negative, whereas anti-TPO and/or Tg autoantibodies were found at high titres in all HT patients, and thyroid ultrasound examination showed signs of chronic autoimmune thyroiditis. Controls (n = 10) presented with colloid goitre were euthyroid and displayed the absence of thyroid antibodies.

2.1.2 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.

2.1.3 Immunohistochemical reactions applying a polymer-based visualisation system

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), which recognises and labels lysosomal membranes and shuttle vesicles travelling between lysosomes and the plasma membrane of these cells. PG-M1 reacts strongly with a fixative-resistant epitope of the macrophage-restricted intracytoplasmic molecule.

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. Finally, the stained

sections were mounted in Roti® Histokitt (Carl Roth, Karlsruhe, Germany). Negative immunohistochemical controls were performed either by omitting the primary antibody or substituting it with buffer solution. Two certified pathologists with no access to the clinical records and histopathological data contributed to the verification of the immunohistochemistry results. 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.

2.1.4 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 ($\times 400$).

2.1.5 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).

2.1.6 Statistical methods applied for assessment of results of immunohistochemical reactions

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).

2.1.7 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 postfixated 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 h. 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.

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-1β within thyroid tissue

The study is described in the manuscript “Upregulated tissue expression of T helper (Th) 17 pathogenic interleukin (IL)-23 and IL-1β in Hashimoto's thyroiditis but not in Graves' disease” by Tatjana Zake, Sandra Skuja, Ieva Kalere, Ilze Konrade, and Valerija Groma. Endocrine Journal. 2019 May 28;66(5): 423–430. doi: 10.1507/endocrj.EJ18-0396. PMID: 30814438.

2.2.1 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.

AITD was confirmed by the clinical manifestations of HT and GD, complemented by thyroid biochemical tests, ultrasound imaging, and histopathological examination. At the time of GD diagnosis, the patients presented with hyperthyroidism, positive anti-TSH receptor antibodies, and diffuse goitre on ultrasound examination. HT patients demonstrated positive antibodies to TPO and/or Tg, negativity for anti-TSH receptor antibodies, and features of chronic autoimmune thyroiditis on ultrasound examination.

Large thyroid nodules with or without obstructive symptoms and suspicion of malignancy were used as indications for surgery in HT patients. In the GD group, large goitres and the need for definitive treatment due to multiple relapses following medical treatment were used as main indications for thyroidectomy. In turn, patients with colloid goitres underwent thyroidectomy due to large goitres and/or nodules, cosmetic reasons, and suspicion of malignancy. Patients with thyroid malignancies confirmed on pathology were excluded.

2.2.2 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 between January 2014 and December 2016. 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.

2.2.3 Immunohistochemical reactions applying a polymer-based visualisation system

Initially, 4–5 µm thick sections were cut from formalin-fixed and paraffin-embedded thyroid tissue samples and mounted on slides; consecutive sections were used as negative controls for the immunohistochemical reactions. The sections were deparaffinised, endogenous peroxidase activity was blocked with 0.1% H₂O₂ in methanol and heat-induced antigen retrieval was accomplished in 10 mmol/L citrate buffer, pH 6.0. Thereafter, the sections were successively 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). For visualisation of the antigen sites, 3,3'-diaminobenzidine tetrahydrochloride (DAB+Chromogen and DAB+Substrate buffer (CellMarque, Rocklin, CA, USA)) was used. The thyroid tissue sections were then counterstained with haematoxylin, washed, dehydrated and coverslipped.

IHC controls included substitution of the primary antibody with buffer solution. The assessment of immunostaining was performed by two independent observers blinded to the clinicopathological data. Tissue specimens were analysed at both medium and high power magnification ($\times 200$ to $\times 400$) using a DMRB light microscope (Leica, Wetzlar, Germany) equipped with a DFC 450C digital camera. The selected slides were scanned and digitised using a Glissando Slide Scanner (Objective Imaging Ltd., Cambridge, UK), and the images were captured using Aperio ImageScope software (Leica Biosystems, version 12.2.2.5015).

2.2.4 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.

2.2.5 Immunofluorescence assay

Additionally, to provide clear visual details about the cellular distribution and localisation of the cytokines, 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).

2.2.6 Statistical methods applied for assessment of results of immunohistochemical reactions

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.

2.2.7 Electron microscopy immunogold labelling for identifying the localisation of IL-17, IL-23, and IL-1 β

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 by the use of five thyroid tissue specimens. For this purpose, thyroid tissue specimens obtained during thyroidectomy were fixed in 1.5% glutaraldehyde and 4% paraformaldehyde and further processed and embedded in Epon resin. Ultrathin sections with a thickness of 60 nm were cut with an ultramicrotome (LKB Ultratome V), mounted to 200-mesh nickel grids. 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 following Kandere-Grzybowska and co-authors (2003) recommendations. Free aldehyde groups were blocked using glycine in PBS for 30 min. Nonspecific sticking of the immunoreagents was prevented by the incubation of sections in blocking buffer (5% BSA, 5% normal goat serum, 0.1% cold water fish gelatin in PBS; pH 7.6) for 1h at room temperature. Afterward, 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 incubation 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). To remove unbound gold conjugate, sections were washed with PBS (3 $\times$ 15 min), then tri-distilled water, and dried for 30 min at room temperature. Finally, sections were stained with 2% uranyl acetate. The specimens were imaged with a JEM 1011 JEOL (Japan) TEM operating at 100 kV. Immunogold labelling was examined at $\times 15000$ – 50000 magnification. Cellular structures were considered to be immunogold labelled when they were in direct contact with at least one colloidal gold particle and contained within a profile in which there were at least two gold particles, as recommended for the brain samples by Brorson (2004).

2.3 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 Zake, 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.

2.3.1 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 diagnosis of AITD was confirmed by evaluating the clinical manifestations of GD and HT, complemented by biochemical thyroid tests and ultrasound imaging. 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-TPO, anti-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.

The study protocol was conducted according to the Declaration of Helsinki and approved by the Rīga East Hospital Research Committee and the Central Medical Ethics Committee, Latvia (Decision No. 01-29.1/5033). All patients and volunteers provided written informed consent before enrolment and blood sample collection.

2.3.2 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.

2.3.3 Thyroid function and antibody tests applying chemiluminescence immunoassay and ELISA

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).

2.3.4 Detection of cytokines applying xMAP technology

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.

2.3.5 Assessment of plasma selenium concentration applying fluorescence spectrophotometry

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.

2.3.6 Statistical methods applied for assessment of results of thyroid function, levels of autoantibodies, plasma cytokines, and selenium concentrations

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).

2.4 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.

3 Results

3.1 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

3.1.1 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%) (Figure 3.1) 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 (Figure 3.2 and 3.3). Simultaneously, the vast majority of GD patients presented with mild inflammatory cell infiltration and diffuse hyperplasia of the follicular cells.

Figure 3.1 Histologic sections of HT demonstrating thyroid follicles without colloid surrounded by lymphocytes affecting their integrity and leading to the formation of the pink Hurtle cells. Haematoxylin and eosin, ×200

Figure 3.2 Histologic sections of HT demonstrating exaggregated lymphoid hyperplasia. At the top of the micrograph, remnants of thyroid follicles lack any colloid; they are completely surrounded by lymphocytes. Hurtle cells are evident. Haematoxylin and eosin, $\times 200$

Figure 3.3 Histologic sections of HT demonstrating lymphoid infiltrates with germinal centers and Hurtle cells, which are follicular epithelial cells with abundant, eosinophilic, granular cytoplasm. Haematoxylin and eosin, $\times 100$

3.1.2 Assessment of immunohistochemical expression of tested antigens in patients with AITD and colloid goitre

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 3.1.

Table 3.1

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 3.4). Notably, thyroid epithelial cells located close to lymphocytes or surrounded by inflammatory cells had a strongly marked IL-17A immunopositivity. Thyroid follicles revealed atrophic changes with the destruction of epithelial cells and nuclear polymorphism (Figure 3.5).

Figure 3.4 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

In contrast, weak or negative expression of IL-17A was demonstrated in the thyroid tissues obtained from ordinary colloid goitre and GD patients. Notably, in a single tissue sample of GD and colloid goitre, the grade of IL-17A positivity was ≥ 3 and ≥ 2 , respectively. The highest expression level of IL-17A in the follicular epithelial cells was observed in HT; among 18 samples, 11 tissue samples presented with IL-17A staining estimated as grade 2 to 3. Furthermore, 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 3.6).

Figure 3.5 **Destructive IL-17A positive thyroid follicles surrounded by inflammatory cells in a thyroid tissue sample of a patient with HT, ×400**

Figure 3.6 **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 (Figures 3.7 and 3.8).

Figure 3.7 Moderate circumferential membranous claudin-1 immunostaining revealed in a thyroid tissue sample of a patient with GD, ×400

Figure 3.8 Strong membranous claudin-1 expression in the thyroid follicular cells demonstrated in a thyroid tissue sample of a patient with HT, ×250

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 3.9). 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).

Figure 3.9 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).

Regarding the second Tj protein – ZO-1 in the thyroid tissue samples studied, 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%) demonstrated such a staining score.

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 3.10) and GD (Figure 3.11) ($p < 0.001$ for both, Figure 3.12). Simultaneously, ZO-1 immunopositivity was found to be not significantly different when the specimens of the last two aforementioned groups were compared ($p = 0.47$).

Figure 3.10 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 3.11 Weak ZO-1 immunoreactivity was confirmed in a thyroid tissue sample of a patient with GD, ×250

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 3.13) or individually distributed within the thyroid follicles (Figure 3.14).

Figure 3.12 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); * $p < 0.05$.

Figure 3.13 The cluster of CD68 positive cells demonstrated within thyroid follicle in a tissue sample obtained from a patient with HT, $\times 400$

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.

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 3.15). CD68 positivity observed in GD patients was higher compared to colloid goitre but the difference was not significant ($p = 0.06$).

Figure 3.14 Occasional intrafollicular CD68 positive cells visible within the lumens of thyroid follicles in a sample obtained from a patient with HT, $\times 250$

Figure 3.15 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); * $p < 0.05$.

3.1.3 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 3.16). 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. In the final stage of the process, thyrocytes were destroyed by apoptosis, which presumably might be initiated by T cells through two pathways: by perforins secreted by lymphocytes into the junctions between lymphocytes and thyrocytes and causing lysis of the cells, by granzymes B activating the caspase cascade leading to apoptosis, and, finally, by lymphocytes stimulating death receptors on the cell surface.

Figure 3.16 **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.

3.1.4 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 ($r = 0.631$, $p = 0.005$) (Figure 3.17). 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). In contrast, no significant correlations between IL-17A, Tj – ZO-1 and claudin-1 ($r = -0.199$, $p = 0.67$ and $r = 0.734$, $p = 0.06$, respectively) or CD68 ($r = 0.631$, $p = 0.13$) were found in GD patients.

Figure 3.17 A scatter plot demonstrating the presence of a moderate positive correlation established between CD68 and IL-17A immunopositivity in HT patients

3.2 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

3.2.1 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 3.18). 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.

Figure 3.18 **IL-17-positive thyroid follicles and inflammatory cells evidenced in a thyroid tissue sample of a patient with HT, ×400**

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. Notably, 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 3.19). 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 3.19 **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 3.20 and 3.21) and IL-1 β (Figure 3.22) was confirmed both by conventional IHC and IF in the abundant inflammatory infiltrates characteristic of HT.

Figure 3.20 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 3.21 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, $\times 1000$

Figure 3.22 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, $\times 1000$

In contrast to HT, predominantly weak IL-23 (Figure 3.23) and IL-1 β (Figure 3.24) thyrocytic immunostaining was demonstrated in the tissues obtained from GD patients.

Notably, 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 3.25).

Figure 3.23 Weak IL-23 immunoreactivity revealed in a thyroid tissue sample of a patient with GD, $\times 250$

Figure 3.24 Occasional IL-1 β positivity revealed in a thyroid tissue sample of a patient with GD, $\times 400$

Figure 3.25 Almost nil IL-1 β expression demonstrated in a thyroid tissue sample of a patient with colloid goitre, $\times 250$

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 3.26). No difference was found between the expression level of IL-23 in patients with GD and colloid goitres ($p = 0.324$).

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 3.27). 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 3.26 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), * $p < 0.05$.

Figure 3.27 Comparison of IHC results of IL-1β in patients with AITD and colloid goitre

* A bar chart depicts immunopositivity levels of IL-1β 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-1β was observed in dendritic cells demonstrating S100 positivity. Heavily decorated and irregularly shaped S100 positive cells were observed in the inflammatory infiltrates (Figure 3.28).

Figure 3.28 **Dendritic cells observed within the inflammatory infiltrate in a thyroid tissue sample of a patient with HT, ×400**

The semiquantitative data describing IHC expression of the interleukins used in this study are summarised in Table 3.2.

Table 3.2

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.

3.2.2 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 3.29). Gold labelling was confirmed in the close vicinity to the nuclear envelope, swollen mitochondria, and the rough endoplasmic reticulum.

Figure 3.29 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.

3.2.3 Analysis of relationships between IL-17 and Th17-promoting cytokines IL-23 and IL-1 β estimated in the thyroid tissue by the use of immunohistochemistry

When analysing the association 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) (Figures 3.30A and 3.30B). Furthermore, in HT patients, a positive correlation ($r = 0.545$, $p = 0.011$) between IL-23 and IL-1 β immunopositivity was confirmed (Figure 3.30C).

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.

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

3.3.1 Demographic and biochemical characteristics of the participants

The demographic and biochemical characteristics of the participants are presented in Table 3.3. 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 3.3

Demographic and biochemical characteristics of the participants

	HT patients (n = 41)	GD patients (n = 11)	Controls (n = 26)	<i>p</i>
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.

Figure 3.30 Scatter plots demonstrating the relationships between IHC expression levels of IL-17 and Th17-promoting cytokines IL-23 and IL-1 β

* In the HT group, IL-17A was positively correlated with IL-23 (A) and IL-1 β (B) immunoreactivity. Furthermore, IL-23 was positively correlated with IL-1 β immunopositivity (C). Correlations were determined by the Spearman rank correlation test.

3.3.2 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 3.31). Interleukin levels also did not differ between the euthyroid and hypothyroid HT patients (all $p > 0.05$).

Figure 3.31 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 (D) 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 3.4.

Table 3.4

Plasma levels of interleukins in the study groups

	HT patients (n = 41)		GD patients (n = 11)	Controls (n = 26)	<i>p</i>
	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 3.32A). Additionally, IL-22 was correlated with IL-6, IL-23, and IL-10 (Figure 3.32B).

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 3.33A). 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 3.33B).

A

B

Figure 3.32 Scatter plots demonstrating the relationships between IL-17a (A) or IL-22 (B) and the studied biomarkers in the HT patient group

A

Figure 3.33 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 3.34.

Figure 3.34 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. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

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 3.35A), while FT4 was positively correlated with IL-17 and IL-10 levels (Figure 3.35B). In addition, anti-TSHR autoantibody titres were not correlated with the levels of interleukins in the GD group (all $p > 0.05$).

Figure 3.35 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; *, $p < 0.05$; **, $p < 0.01$.

3.3.3 Plasma selenium levels in patients with AITD and controls

The median plasma selenium levels were 85.03 (60.09–116.60) µg/L for HT patients, 71.33 (59.05–104.42) µg/L for GD patients and 80.35 (67.15–113.67) µg/L for control participants (Table 3.5).

Table 3.5

Plasma selenium levels in the study groups

	HT patients (n = 40)		GD patients (n = 11)	Controls (n = 21)	<i>p</i>
	Euthyroid HT (n = 32)	Hypothyroid HT (n = 8)			
Se (µg/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 3.36A. The levels of selenium did not differ significantly among the studied groups (Figure 3.36B).

Similarly, no differences in selenium levels were observed between euthyroid HT patients and HT patients with subclinical hypothyroidism (Table 3.5). 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 3.37). 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 3.36 **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.

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 $p > 0.05$).

Figure 3.37 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.

4 Discussion

4.1 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 (Figueroa-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 agree 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 study was retrospective in nature and 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.

4.2 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. Furthermore, upregulation of IL-37, exerting a protective role against oxidative stress and inflammation by inhibiting the effects of Th1/Th17-mediated response, was demonstrated in HT subjects (Ruggeri et al., 2018). 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 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. Considering 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. Furthermore, when suggesting the role of Th17 in the development and progression of GO, more prospective studies analysing this patient group are needed. Finally, although we did not aim to analyse the expression levels of other interleukins, it would be important to further investigate the role of different phenotypes of Th17 and their cytokine profiles in thyroid autoimmunity.

4.3 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. In contrast, Figueroa-Vega et al. (2010) did not find any significant differences in IL-23 levels between GD patients and healthy subjects. 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 (Zake 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. Enhanced levels of peripheral blood Th17 and Th22 cells along with elevated levels of plasma IL-17 and IL-22 were reported in Chinese patients with newly diagnosed GD by Peng et al. (2013). Moreover, these cells and cytokines were positively correlated with the plasma levels of anti-TSHR autoantibodies.

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 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 $\mu\text{g/L}$, as proposed by Alfthan et al. (1991), or 95 $\mu\text{g/L}$ according to Thomson et al. (1993), whereas a plasma selenium range of 88–142 $\mu\text{g/L}$ is associated with the maximal SELENOP concentration (Hurst et al., 2013). A selenium level below 80 $\mu\text{g/L}$ 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. **Zaķe, T.**, Kalere, I., Upmale-Engela, S., Svirskis, S., Gersone, G., Šķesters, A., Groma, V., Konrāde, 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. **Zaķe, T.**, Skuja, S., Kalere, I., Konrāde, I., Groma, V. 2019. Upregulated tissue expression of Th17 pathogenic IL-23 and IL-1β in Hashimoto's thyroiditis but not in Graves' disease. *Endocrine Journal*. 66(5): 423–430. doi: 10.1507/endocrj.EJ18-0396. PMID: 30814438.
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1. **Zaķe, T.**, Groma, V., Konrāde, I., Skuja, S. Vairogdziedera folikuloģitū blīvo savienojumu proteīna klaidina-1 imūnspresijas atšķirības pacientiem ar Hashimoto 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. **Zaķe, T.**, Kalere I., Upmale-Engela, S., Gersone, G., Šķesters, A., Groma, V., Konrāde, 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., **Zaķe, T.**, Upmale-Engela, S., Strēle, I., Šķesters, A., Konrāde, 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., **Zaķe T.**, Upmale-Engela, S., Cgojeva-Uzulniece, F., Šķesters, A., Konrāde, 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)
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6. **Zaķe, T.**, Kalere, I., Upmale-Engela, S., Gersone, G., Konrāde, I. Plasma levels of Th17 associated cytokines in patients with autoimmune thyroid disease. 22nd European Congress of Endocrinology, Online, September 5–9, 2020. Endocrine Abstracts (2020) 70 AEP909 DOI: 10.1530/endoabs.70.AEP909. (Poster presentation)
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9. Kalere, I., Konrāde, I., **Zaķe, T.**, Strēle, I. Selenium-rich food consumption in Latvia from 2000 to 2016. *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., 495 p. (Poster presentation)
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11. **Zaķe, T.**, Skuja, S., Kalere, I., Konrāde, I., Groma, V. The increased immunohistochemical expression of IL-23 in Hashimoto's thyroiditis but not in Graves' disease patients. *19th European Congress of Endocrinology*, Lisbon, Portugal, May 20–23, 2017. Endocrine Abstracts (2017) 49 GP209 DOI:10.1530/endoabs.49.GP209. (Poster presentation)
12. **Zaķe, T.**, Skuja, S., Kalere, I., Konrāde, I., Groma, V. Comparison of immunohistochemical expression of IL-1β and IL-23 in autoimmune thyroid disease. *40th Annual Meeting of the European Thyroid Association*, Belgrade, Serbia, September 9–12, 2017. Eur Thyroid J 2017;6(suppl 1):78 p. P2-01-59. (Poster presentation)
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19. **Zaķe, T.**, Groma, V., Skuja, S., Kleina, R. Morphologic peculiarities in the thyroid gland of patients with Hashimoto's thyroiditis. *Baltic Morphology VII Scientific Conference "Morphological sciences in the experimental and clinical medicine"* (Rīga, Latvia, Nov.7-9, 2013): Abstract Book / Rīga Stradiņš University. – Rīga, 2013. 65 p. (Poster presentation)

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1. **Zaķe, T.**, Skuja, S., Kalere, I., Konrāde, I., Groma, V. Ar Th17 diferenciāciju saistīta citokīna IL-23 imūnekspresijas 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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Supplements

Heterogeneity of tissue IL-17 and tight junction proteins expression demonstrated in patients with autoimmune thyroid diseases

Tatjana Zake, MD^{a,b,*}, Sandra Skuja, PhD^a, Ieva Kalere, MD^b, Ilze Konrade, MD, PhD^b, Valerija Groma, MD, PhD^a

Abstract

Th17 cells together with their hallmark cytokine interleukin (IL)-17 were identified as crucial contributing factors in the pathogenesis of thyroid autoimmunity. The cytokine-regulated tight junction (TJ) disruption is thought to be essential in the initiation and/or development of several diseases. Still, the role of IL-17 maintaining TJ integrity in autoimmune thyroid diseases (AITDs) has not yet been evaluated. We aimed to investigate integrity of the thyroid follicle by studying immunoreactivity of cellular TJ – zonula occludens (ZO)-1 and claudin-1 proteins coupled to IL-17A and CD68 detection in AITD patients compared with controls.

Thirty-five adult patients undergoing thyroidectomy and presenting 18 cases of Hashimoto thyroiditis (HT), 7 of Graves' disease (GD) as well as 10 subjects of colloid goiter without autoimmune component served as controls were enrolled in this study. An immunohistochemical analysis including IL-17A, ZO-1, claudin-1, and CD68 detection was performed in each case. The correlation of IL-17A with TJ and CD68 in patients with AITD was also analyzed.

Apart from inflammatory cells, we evidenced a stronger expression level of IL-17A in the thyroid follicular cells in HT patients when compared with GD or colloid goiter. A significant reduction of ZO-1 immunoreactivity was observed in the thyrocytes in HT patients, whereas no significant differences were found in claudin-1 expression in HT and GD compared with colloid goiter patients. A significantly higher number of thyroid follicles with CD68-positive cells was found in HT patients than that in patients with GD or colloid goiter. In HT patients, the expression of IL-17A in the follicular cells was positively correlated with CD68 immunopositivity, whereas no association with claudin-1 or ZO-1 expression was found. GD patients did not reveal any significant correlation of IL-17A with TJ and CD68.

Strong overexpression of IL-17A observed in the thyroid epithelial cells is associated with the presence of intrafollicular CD68-positive cells in HT patients. We evidenced the changes in molecules of thyrocyte junctional complexes highlighting impairment of the thyroid follicle integrity in HT, but no association with IL-17A was found.

Abbreviations: AITD = autoimmune thyroid diseases, GD = Graves' disease, HT = Hashimoto thyroiditis, IL = interleukin, T helper = Th, TJ = tight junctions, TPO = thyroid peroxidase, TSHR = thyroid-stimulating hormone receptor, ZO = zonula occludens.

Keywords: Graves' disease, Hashimoto thyroiditis, interleukin-17, tight junction protein

1. Introduction

The prevalence and incidence of autoimmune diseases including autoimmune thyroid diseases (AITD) has increased significantly over the last decades.^[1] Thyroid autoimmunity occurs as a result

of immune system impairment leading to the breakdown of self-tolerance to the 3 major thyroid proteins and inflammatory cell, predominantly lymphocytic infiltration of the gland.^[2] It is critical to identify immunological and pathomorphological aspects involved in thyroid autoimmunity. AITD comprise 2 clinically and pathogenetically diverse phenotypes: Hashimoto thyroiditis (HT) and Graves' disease (GD). GD induced by thyroid-stimulating autoantibodies to thyroid stimulating hormone receptor leading to hyperthyroidism is considered to be a T helper type (Th)2-related disease, whereas in HT Th1-driven autoimmune response characterized by the lymphocytic infiltration results in apoptosis of thyrocytes and subsequent hypothyroidism.^[3,4] However, an evident humoral autoimmune response in HT patients and diversity of T cell mediated response and inflammatory infiltration in GD is also demonstrated.^[4,5]

Recently, Th17 cells and their hallmark cytokine (interleukin, IL)-17 have been recognized as crucial contributors to the pathogenesis of thyroid autoimmunity.^[6–8] Production of IL-17A and IL-17F is characteristically attributed to Th17 cells. These ILs can act on a broad range of cells, including epithelial cells, fibroblasts, and macrophages inducing the release of proinflammatory tissue mediators such as interleukins IL-1 β , IL-6, and IL-8, growth and hematopoiesis stimulating factors – tumor necrosis factor- α (TNF- α), granulocyte-macrophage colony-stimulating

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factor, and granulocyte colony-stimulating factor (G-CSF), and tissue components degrading enzymes metalloproteinases.^[10–12] Early studies demonstrated that IL-17 stimulates production of other cytokines such as IL-6, IL-8, and G-CSF in nonimmune cells such as connective tissue fibroblasts and epithelial cells via activation of the nuclear factor- κ B (NF- κ B) transcription factor.^[13,14] In addition, IL-17 demonstrates strong synergic action being combined with other cytokines, such as IL-1 β and TNF α .^[15,16] Previous studies have shown that patients suffering from AITD have enhanced levels of IL-17A and Th17 lymphocytes revealed in blood and colonizing thyroid tissue as well as a marked in vitro differentiation of Th17 cells; however, the expression level of IL-17A in the thyroid epithelial cells assessed by immunohistochemistry has not been addressed thoroughly before.

Tight junctions (Tj), also called occluding junctions, are composed of a complex, interwoven network of membrane proteins and glycoproteins. These are distinguished at the apical part of the lateral membranous surface of polarized endothelial and epithelial cells. Tj maintain cell polarity and establish the barrier sealing the paracellular space between neighboring thyrocytes. Numerous studies have demonstrated that claudin family is the key component of Tj, fully performing paracellular barrier function in the absence of occludin.^[17] From the currently known 27 claudins expressed in humans, claudin-1 is considered to be the most important member responsible for the epithelial barrier integrity.^[18] Furthermore, claudin-1 deficiency appeared to cause vital alterations of barrier function leading to the death of claudin-1 deficient mice.^[19] Simultaneously, an essential role of interactions of zonula occludens (ZO)-1 protein and claudin for formation of Tj assembly have been recognized. Numerous studies with ZO-1 and/or ZO-2 knockdown cells demonstrated disruption in both claudin localization and paracellular permeability. Moreover, in the absence of ZO-1, these cells have been shown to exhibit polarity but fail to form Tj.^[20]

Continuing studies have highlighted a broad spectrum of cytokines able to affect the crucial function – barrier function of endothelial and epithelial cells by influencing the structural and functional characteristics of the Tj. Many ILs were found to modulate Tj proteins such ZO-1, occludin, and claudin proteins causing downregulation of these molecules^[21,22]; however, the role of IL-17 in the maintenance of Tj integrity in AITD patients has not yet been evaluated. This study investigates integrity of the thyroid follicle by studying immunorexpression of cellular Tj – ZO-1 and claudin-1 proteins coupled to IL-17A and CD68 detection in AITD patients compared with controls. The correlation of IL-17A with Tj and CD68 in patients with AITD was also assessed, thus making an attempt to determine the possible relationship between IL-17 and Tj integrity.

2. Methods

2.1. Patients

Twenty-five adult AITD patients presenting 18 cases of HT and 7 of GD were enrolled in this study. Ten age and gender-matched cases of colloid goiter without autoimmune component, which served as a control group, were also recruited. 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 were confirmed by the clinical diagnostic criteria of HT and GD, complemented by results of thyroid biochemistry, ultrasound imaging, and histopathology findings. Patients with GD had thyroid hormone levels above the reference range, positive anti-thyroid stimulating hormone receptor (anti-TSHR) antibodies, and

diffuse goiter proved by ultrasound examination. Anti-TSHR antibodies were negative, whereas antibodies to thyroid peroxidase (anti-TPO) and/or thyroglobulin were found at high titers in all HT patients, and thyroid ultrasound examination showed signs of chronic autoimmune thyroiditis. Ten patients with colloid goiter were used as a control group. They were euthyroid and displayed the absence of thyroid antibodies. The current study was approved by the Riga Stradins University Ethics Committee, and all patients gave informed consent.

Thyroid tissue blocks of 35 thyroidectomy samples (equal to the number of patients enrolled in the study) along with the histopathology reports were obtained from Pathology Center of Riga East Clinical University Hospital, Latvia, between January 2013 and December 2014. All available specimens were analyzed, and a single representative paraffin-embedded block was chosen for immunohistochemical study.

2.2. Immunohistochemistry

Thyroid tissues were formalin-fixed and embedded in paraffin blocks; thereafter, 4 to 5 μ m thick sections were cut from blocks, collected and fixed on slides. The diagnosis was confirmed analyzing the slides stained routinely with hematoxylin and eosin. Immunohistochemical reactions were performed manually on deparaffinized 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. In addition, macrophages were visualized by monoclonal mouse anti-CD68 antibody (DacoCytomation, Glostrup, Denmark, clone PG-M1, dilution 1:100), which recognizes and labels lysosomal membranes and shuttle vesicles travelling between lysosomes and the plasma membrane of these cells. PG-M1 reacts strongly with a fixative-resistant epitope of macrophage-restricted intracytoplasmic molecule.

The results of immunohistochemical reactions were visualized by use of highly sensitive HRP Polymer system – HiDef Detection system manufactured by CellMarque, Rocklin, CA. The antigen sites were visualized applying 3,3'-diaminobenzidine tetrahydrochloride [DAB+Chromogen and DAB+Substrate buffer (CellMarque, Rocklin, CA)], whereas nuclei were stained with Mayer hematoxylin. Finally, the stained sections were mounted in Roti-Histokitt (Carl Roth, Karlsruhe, Germany). Negative immunohistochemical controls were performed either by omitting of the primary antibody or substitution of it with buffer solution. Two certified pathologists with no access to the clinical records and histopathological data contributed to the verification of the immunohistochemistry results. Tissue sections were analyzed applying both low and high power magnification from $\times 100$ up to $\times 400$. Microphotographs were taken with a Leitz DMRB microscope (Leica, Wetzlar, Germany) equipped with a digital camera Leica DFC 450C.

Immunofluorescence staining of IL-17A was performed using goat anti-mouse IgG-FITC: sc-2010 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, 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 confocal microscope Eclipse Ti-E (Nikon).

Cells were considered immunopositive when labeled by the aforementioned antibodies revealed brown reaction products. IL-

17, claudin-1, and ZO-1 positive structures displayed either cytoplasmic or membranous reaction pattern.

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% to 5%, 6% to 25%, 26% to 50%, or >50%, respectively, whereas, for ZO-1 – negative (0), very weak or discontinuous (1), weak (2), and strong or intact (3) following the recommendations of Abd El Atti and Shash.⁽²³⁾

CD68 positivity was defined as the percentage of CD68-positive thyroid follicles determined by counting total number of thyroid follicles with CD68-positive cells in 15 representative fields at high magnification ($\times 400$).

2.3. Statistical analysis

Immunohistochemical results were expressed as medians and interquartile range. Mann–Whitney test was applied to examine differences between 2 groups and Kruskal–Wallis H test – for more than 2 study groups. To assess the potential correlations between estimated variables, Spearman rank correlation test was carried out. A 2-tailed significance level of .05 was applied. Statistical testing was performed using SPSS (version 20.0, IBM, Armonk, NY).

3. Results

Microscopic examination of slides processed for routine histopathology revealed widespread mild-to-extensive inflammatory infiltration of the thyroid gland by lymphocytes, macrophages, and plasma cells in AITD. Intraepithelial lymphocytes were also seen. HT patients demonstrated extensive diffuse inflammatory infiltration (7; 39%), lymphoid follicles formation (6; 33%), or rarely mild inflammation of the thyroid gland without lymphoid follicle formation (5; 28%). Some HT patients revealed a marked stromal fibrosis qualified as interfollicular presented with the deposition of increasingly accumulated collagenous fibers and lymphoplasmacytic infiltration in the interfollicular area, thus separating and distorting the thyroid follicles. Still, some tissue blocks obtained from HT patients revealed extensive destruction of thyroid parenchyma with minimal residual follicles. Simultaneously, the vast majority of GD patients presented with mild inflammatory cell infiltration and diffuse hyperplasia of the follicular cells.

The results describing immunohistochemical expression of the antigens tested in the given study and applied to the study groups are summarized in Table 1. In HT patients, the expression of IL-17A was detected both in the inflammatory lymphocytic infiltrates and follicular epithelial cells confirmed by immunohistochemistry and immunofluorescence (Fig. 1A). 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

Figure 1. Immunohistochemistry. (A) IL-17A positivity demonstrated in the remnants of thyroid follicles and lymphoid follicles as well as in some inflammatory cells in HT patient ($\times 200$). Confocal microscopy, representative images of IL-17A positive thyrocytes, and lymphocytes: green staining shows IL-17A specific staining, blue staining shows nuclei, inserts ($\times 1000$). (B) Destructive IL-17A positive thyroid follicles surrounded by inflammatory cells in HT patient ($\times 400$). (C) Moderately circumferential membranous claudin-1 immunostaining revealed in GD patient ($\times 400$). (D) Strong membranous claudin-1 expression in the thyroid follicular cells in HT patient ($\times 250$). (E) Weak ZO-1 immunoreactivity in GD patient ($\times 250$). (F) Colloid goiter evidencing weak to moderate and moderate positive ZO-1 expression in the thyrocytes and vascular endothelium, respectively ($\times 250$). (G) Cluster of CD68-positive cells demonstrated within the thyroid follicle in HT patient ($\times 400$). (H) Occasional intralobular CD68-positive cells individually distributed within the lumen of thyroid follicles in HT patient ($\times 250$). GD = Graves' disease, HT = Hashimoto thyroiditis, IL = interleukin, ZO = zonula occludens.

Table 1
Immunohistochemical expression patterns of tested antigens in patients with autoimmune thyroid diseases and colloid goiter.

	IL-17A median (IQR)	ZO-1 median (IQR)	Claudin-1 median (IQR)	CD68 median (IQR)
Hashimoto 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 goiter	0.8 (0.6–1)	2 (1.5–2.5)	1.6 (1.2–2.0)	5.6 (3.1–8.7)

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changes with destruction of epithelial cells and nuclear polymorphism (Fig. 1B). By contrast, the tissues obtained from ordinary colloid goiter and GD patients demonstrated mostly the weak or almost negative expression of IL-17A. Only in 1 case of GD and 1 case of colloid goiter the grade of IL-17A positivity was ≥ 3 and ≥ 2 , respectively. The highest expression level of IL-17A in the follicular epithelial cells was observed in HT patients: 11 out of 18 cases demonstrated IL-17A staining grade of 2 to 3. Furthermore, in HT and GD patients, it was significantly higher than colloid goiter ($P < .001$; $P = .007$, respectively) (Fig. 2A).

When studying the occurrence and localization of T_j proteins, and more specifically claudin-1 by the use of immunohistochemistry, we found that it displayed a circumferential and

17, claudin-1, and ZO-1 positive structures displayed either cytoplasmic or membranous reaction pattern.

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% to 5%, 6% to 25%, 26% to 50%, or >50%, respectively, whereas, for ZO-1 – negative (0), very weak or discontinuous (1), weak (2), and strong or intact (3) following the recommendations of Abd El Atti and Shash.^[2,3]

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Microscopic examination of slides processed for routine histopathology revealed widespread mild-to-extensive inflammatory infiltration of the thyroid gland by lymphocytes, macrophages, and plasma cells in AITD. Intraepithelial lymphocytes were also seen. HT patients demonstrated extensive diffuse inflammatory infiltration (7; 39%), lymphoid follicles formation (6; 33%), or rarely mild inflammation of the thyroid gland without lymphoid follicle formation (5; 28%). Some HT patients revealed a marked stromal fibrosis qualified as interfollicular presented with the deposition of increasingly accumulated collagenous fibers and lymphoplasmacytic infiltration in the interfollicular area, thus separating and distorting the thyroid follicles. Still, some tissue blocks obtained from HT patients revealed extensive destruction of thyroid parenchyma with minimal residual follicles. Simultaneously, the vast majority of GD patients presented with mild inflammatory cell infiltration and diffuse hyperplasia of the follicular cells.

The results describing immunohistochemical expression of the antigens tested in the given study and applied to the study groups are summarized in Table 1. In HT patients, the expression of IL-17A was detected both in the inflammatory lymphocytic infiltrates and follicular epithelial cells confirmed by immunohistochemistry and immunofluorescence (Fig. 1A). 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

Figure 1. Immunohistochemistry. (A) IL-17A positivity demonstrated in the remnants of thyroid follicles and lymphoid follicles as well as in some inflammatory cells in HT patient ($\times 200$). Confocal microscopy, representative images of IL-17A positive thyrocytes, and lymphocytes: green staining shows IL-17A specific staining, blue staining shows nuclei, inserts ($\times 1000$). (B) Destructive IL-17A positive thyroid follicles surrounded by inflammatory cells in HT patient ($\times 400$). (C) Moderate circumferential membranous claudin-1 immunostaining revealed in GD patient ($\times 400$). (D) Strong membranous claudin-1 expression in the thyroid follicular cells in HT patient ($\times 250$). (E) Weak ZO-1 immunoreactivity in GD patient ($\times 250$). (F) Colloid goiter evidencing weak to moderate and moderate positive ZO-1 expression in the thyrocytes and vascular endothelium, respectively ($\times 250$). (G) Cluster of CD68-positive cells demonstrated within the thyroid follicle in HT patient ($\times 400$). (H) Occasional intralobular CD68-positive cells individually distributed within the lumen of thyroid follicles in HT patient ($\times 250$). GD = Graves' disease, HT = Hashimoto thyroiditis, IL = interleukin, ZO = zonula occludens.

Table 1
Immunohistochemical expression patterns of tested antigens in patients with autoimmune thyroid diseases and colloid goiter.

	IL-17A median (IQR)	ZO-1 median (IQR)	Claudin-1 median (IQR)	CD68 median (IQR)
Hashimoto 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 goiter	0.8 (0.6–1)	2 (1.5–2.5)	1.6 (1.2–2.0)	5.6 (3.1–8.7)

IQR = interquartile range, ZO = zonula occludens.

changes with destruction of epithelial cells and nuclear polymorphism (Fig. 1B). By contrast, the tissues obtained from ordinary colloid goiter and GD patients demonstrated mostly the weak or almost negative expression of IL-17A. Only in 1 case of GD and 1 case of colloid goiter the grade of IL-17A positivity was ≥ 3 and ≥ 2 , respectively. The highest expression level of IL-17A in the follicular epithelial cells was observed in HT patients: 11 out of 18 cases demonstrated IL-17A staining grade of 2 to 3. Furthermore, in HT and GD patients, it was significantly higher than colloid goiter ($P < .001$; $P = .007$, respectively) (Fig. 2A).

When studying the occurrence and localization of Tj proteins, and more specifically claudin-1 by the use of immunohistochemistry, we found that it displayed a circumferential and

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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

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Abstract. T helper (Th) 17 cells and interleukin (IL)-17 play a significant role in the pathogenesis of autoimmune thyroid disease (AITD). However, it has recently become clear that Th17 cells are more heterogeneous and exhibit two different phenotypes, whereas IL-23 and IL-1 β are crucial for the generation of pathogenic Th17 lymphocytes. We aimed to investigate the association between IL-17 and Th17-promoting cytokines in AITD by studying the immunorepression patterns of IL-17, IL-23, and IL-1 β in thyroid tissue. Following thyroidectomy, 29 patients with AITD (21 cases of Hashimoto's thyroiditis (HT) and 8 cases of Graves' disease (GD)) and 18 patients with colloid goiter, as controls, were enrolled in this study, and immunohistochemistry was performed. The expression level of IL-17 in thyrocytes was significantly higher in HT and GD patients than in colloid goiter patients. Immunopositivity for both IL-23 and IL-1 β was significantly increased in HT patients compared to GD and colloid goiter patients. However, no difference was found between IL-23 or IL-1 β expression in patients with GD and colloid goiter. A positive correlation between IL-17 and IL-23 as well as IL-17 and IL-1 β expression was observed in HT patients ($r = 0.574$, $p = 0.007$ and $r = 0.461$, $p = 0.036$, respectively). In the GD group, IL-17 was positively correlated with IL-1 β ($r = 0.817$, $p = 0.013$) but not with IL-23 expression. We found increased IL-23 and IL-1 β expression in the HT group but not in the GD group. Furthermore, both interleukins were correlated with IL-17 immunopositivity in thyroid tissue, suggesting that pathogenic Th17-promoting cytokines may play a role in HT pathogenesis.

Key words: Hashimoto's thyroiditis, Graves' disease, Interleukin-17, Interleukin-23, Interleukin-1 β

AUTOIMMUNE THYROID DISEASES (AITD), with their main clinically opposite manifestations as hypothyroidism in Hashimoto's thyroiditis (HT) and hyperthyroidism in Graves' disease (GD), are the most common autoimmune disorders [1]. Although both diseases belong to AITD, they are different in pathogenic mechanisms and the presentation of the dysregulated immune system. The absence of cytotoxicity and structural damage of the follicle and the prevalence of humoral autoimmune response against TSH receptor are the main features distinguishing GD from HT. In the case of HT, cytotoxic T lymphocytes induce programmed cell death in thyrocytes by the release of perforin and granzymes, subsequently leading to the development of hypothyroidism. Additionally, thyroid follicular cells can undergo caspase-mediated apoptosis by expressing

Fas/Fas ligands [2]. Although the contribution of anti-thyroid peroxidase (TPO) antibodies to thyroid follicular destruction compared to T cell attack and cytotoxic effects is minor, antibodies have shown several pathogenic effects, such as complement activation and the inhibition of enzymatic activity [3]. Furthermore, higher oxidant parameters and lower antioxidant parameters have been found in euthyroid treated-naïve HT patients, indicating that the presence of TPO antibodies is the main independent risk factor for developing oxidative stress, irrespective of thyroid function [4].

Two classical thyroid autoimmunity phenotypes can be distinguished by a balance between T helper (Th) 1- and Th2-mediated immune activity. Thus, HT predominantly promotes the Th1 immune response, characterized by the upregulation of pro-inflammatory cytokines, such as interleukin (IL)-2, IL-1 β , interferon (IFN)- γ , tumor necrosis factor (TNF)- α , leading to cell-mediated immunity and thyrocyte death in apoptotic pathways. In GD, there are increased levels of Th2-released cytokines, such as IL-4, IL-5, IL-6, IL-10, and IL-13; these cytokines promote the humoral response, stimulating the pro-

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duction of TSH receptor antibodies and enhancing the survival and growth of thyroid cells and apoptosis of infiltrating lymphocytes either by the downregulation of Fas/Fas ligand or the upregulation of the anti-apoptotic molecule Bcl-2 [5]. The fact that both thyroid disorders may coexist in the same individual is indicative of the presence of mixed Th1/Th2 and probably Th17 responses in both HT and GD patients.

A growing body of evidence suggests that IL-17A, the prominent effector cytokine of Th17, can induce the release of different pro-inflammatory cytokines and chemokines responsible for the induction and development of chronic inflammatory responses in many autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, psoriasis, juvenile idiopathic arthritis, and inflammatory bowel disease [6–8]. Th17 cells also play an essential role in the pathogenesis of AITD.

Various cytokines and lineage-associated transcription factors are essential for the regulation of human Th0 lymphocyte differentiation into Th1, Th2, Th17, Th9, Th22, follicular T helper cells or regulatory T cell subsets. Thus, the development of the Th1 population is induced by IL-12 or IFN- γ , whereas IL-4 initiates the differentiation of the humoral response promoting Th2 cells [9]. Continuing studies have highlighted a spectrum of transcription factors and cytokines that can induce and maintain Th17 cell differentiation in both mouse models and humans. The combination of transforming growth factor (TGF)- β and pro-inflammatory cytokines, such as IL-1 β , IL-6, IL-21, and IL-23, has been found to play a crucial role in humans. It has been shown that TGF- β in the presence of IL-6 and IL-21 promotes the initial differentiation of Th17 cells by the activation of RAR-related orphan receptor (ROR) C, while IL-23 and IL-1 β are important for both the full maturation and stabilization of the Th17 phenotype [10, 11]. Interestingly, although IL-23 is not required for the induction of Th17 differentiation (naïve T cells do not express IL-23 receptor), IL-23-dependent signaling is crucial for the generation of highly pathogenic Th17 cells *via* signal transducer and activator of transcription (STAT) 4 [12]. It has recently been reported that IL-23 levels are enhanced in the peripheral blood [13] and thyroid tissues [14] of HT patients; however, it is still unknown whether IL-23 is involved in the pathogenesis of GD. IL-1 β is another pro-inflammatory cytokine that participates in AITD [15] and is essential for the conversion of classical Th17 lymphocytes into the pathogenic Th17 subset [16]. Remarkably, IL-1 β synergizes with both IL-23 and IL-6 to enhance the expression of interferon regulatory factor 4, which in combination with STAT3 further induces RORC during Th17 development [11].

Several studies have demonstrated a significant

increase of Th17 lymphocytes and IL-17 in the peripheral blood and/or thyroid tissues of AITD patients, suggesting their contributions to thyroid autoimmunity. The overexpression of IL-17 within thyroid tissues from HT and GD patients compared to controls was also revealed in our previous study [17]. However, the role of interleukins in promoting the differentiation of Th17 cells has not been thoroughly evaluated in HT and GD pathogenesis. In the current study, we aimed to investigate the association between IL-17 and pathogenic Th17-promoting cytokines in AITD by studying the immunoreexpression patterns of IL-17, IL-23, and IL-1 β in thyroid tissue.

Materials and Methods

Patients

Twenty-nine adult patients with AITD (21 cases of HT and 8 of GD) who underwent thyroidectomy at Riga East Clinical University Hospital, Latvia, between January 2014 and December 2016 were enrolled in this study. Eighteen age- and gender-matched patients with colloid goiters displaying normal thyroid function served as the control group. 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. AITD was confirmed by the clinical manifestations of HT and GD, complemented by thyroid biochemical tests, ultrasound imaging, and histopathological examination. At the time of GD diagnosis, the patients had hyperthyroidism, positive anti-TSH receptor antibodies, and diffuse goiter on ultrasound examination. HT patients demonstrated positive antibodies to TPO and/or thyroglobulin, negativity for anti-TSH receptor antibodies, and features of chronic autoimmune thyroiditis on ultrasound examination. The eighteen patients with colloid goiters displaying normal thyroid hormone levels and an absence of thyroid antibodies were used as controls. The indications for surgery in HT patients were large thyroid nodules with or without obstructive symptoms and suspicion of malignancy. The main indications for thyroidectomy in the GD group were large goiters and the need for definitive treatment due to multiple relapses following medical treatment. Patients with colloid goiters underwent thyroidectomy due to large goiters and/or nodules, cosmetic reasons and suspicion of malignancy. Patients with thyroid malignancies confirmed on pathology were excluded. The current study was approved by the Riga Stradins University Ethics Committee, and written informed consent was obtained from all patients.

Forty-seven paraffin-embedded thyroid tissue blocks from 47 patients along with their histopathology reports

were obtained from the Pathology Center of Riga East Clinical University Hospital and used for immunohistochemical study.

Immunohistochemistry

Initially, 4–5 μ m thick sections were cut from formalin-fixed and paraffin-embedded thyroid tissue samples and mounted on slides; consecutive sections were used as negative controls for the immunohistochemical reactions. The sections were deparaffinized, endogenous peroxidase activity was blocked with 0.1% H₂O₂ in methanol and heat-induced antigen retrieval was accomplished in 10 mmol/L citrate buffer, pH 6.0. Thereafter, the sections were successively 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 visualized by HRP HiDef Polymer Detection™ System (CellMarque, Rocklin, CA, USA). For visualization of the antigen sites, 3,3'-diaminobenzidine tetrahydrochloride (DAB + Chromogen and DAB + Substrate buffer (CellMarque, Rocklin, CA, USA)) was used. The thyroid tissue sections were then counterstained with hematoxylin, washed, dehydrated and coverslipped.

IHC controls included substitution of the primary antibody with buffer solution. The assessment of immunostaining was performed by two independent observers blinded to the clinicopathological data. Tissue specimens were analyzed at both medium and high power magnification ($\times 200$ to $\times 400$) using a DMRB light microscope (Leica, Wetzlar, Germany) equipped with a DFC 450C digital camera. The slides were scanned and digitized using a Glissando Slide Scanner (Objective Imaging Ltd., Cambridge, UK), and the images were captured using Aperio ImageScope software (Leica Biosystems, version 12.2.2.5015).

The cells labeled 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.

Additionally, to provide clear visual details about the cellular distribution and localization of the cytokines, thyroid tissue specimens obtained from five HT patients during thyroidectomy were fixed in aldehyde and further processed for immunofluorescent labeling and confocal

microscopy. Immunofluorescence (IF) 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, 1:3,000) 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).

Statistical analysis

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

Results

The semiquantitative data describing IHC expression of the antigens used in this study are summarized in Table 1. IL-17 immunopositivity was observed in the thyrocytes and inflammatory infiltrates of HT patients (Fig. 1A), and the level of expression was assessed mostly as moderate (13/21 patients showed staining grade ≥ 2). Additionally, the impaired integrity and destruction of follicular cells was frequently revealed in the IL-17-positive thyroid follicles of HT patients. In contrast to HT, patients with GD and colloid goiters showed predominantly weak and negative IL-17 expression, respectively. The expression level of IL-17 in the thyrocytes was significantly higher in HT and GD patients than in patients with colloid goiters ($p < 0.001$ for both) (Fig. 2A). Moreover, HT patients demonstrated higher IL-17 immunoreactivity than did GD patients, but the difference was not significant ($p = 0.069$).

Similarly, the expression of IL-23 (Fig. 1B, 1C) and IL-1 β (Fig. 1D) was detected in the abundant inflammatory infiltrates characteristic of HT and thyrocytes, as confirmed by IHC and IF. Additionally, IL-23, IL-1 β , and IL-17 were localized in the cytoplasm of thyroid follicular cells in HT patients by transmission electron microscopy after immunogold labeling (Fig. S1). In contrast, tissues obtained from GD patients demonstrated weak IL-23 (Fig. 1E) and IL-1 β (Fig. 1F) thyrocytic immunostaining predominantly. Notably, a vast majority of patients with colloid goiters revealed the occasional or negative expression of IL-23 and IL-1 β (Fig. 1G). The expression of IL-23 was significantly increased in HT

Table 1 Immunohistochemical analysis of tested interleukins in patients with autoimmune thyroid disease and colloid goiter.

	IL-17	IL-23	IL-1 β
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 goiter	0.6 (0.6; 1)	0.14 (0.0; 0.14)	0.07 (0.0; 0.14)

Data are shown as median and interquartile range of the immunohistochemical findings. Interleukin (IL).

patients when compared to both GD and colloid goiter patients ($p = 0.043$ and $p < 0.001$, respectively) (Fig. 2B). No difference was found between the expression level of IL-23 in patients with GD and colloid goiters ($p = 0.324$).

The highest immunopositivity of IL-1 β was observed in patients with HT. Furthermore, the IL-1 β immunopositivity was significantly higher than that in colloid goiter and GD patients ($p < 0.001$ for both) (Fig. 2C). Similar to IL-23, the expression level of IL-1 β did not differ significantly between patients with GD and colloid goiters ($p = 0.144$).

Apart from thyrocytes and infiltrating lymphocytes, the expression of IL-23 and IL-1 β was observed in dendritic cells demonstrating S100 positivity. The localization of S100 showed a brown granular immunostaining pattern in the cytoplasm and/or nuclei of dendritic cells (Fig. 1H).

When the association between IL-17 and Th17-promoting cytokines was analyzed, 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) (Fig. 3A, 3B). Furthermore, HT patients showed a positive correlation between IL-23 and IL-1 β immunopositivity ($r = 0.545$, $p = 0.011$) (Fig. 3C). In the GD group, the expression of IL-17 was positively correlated with IL-1 β immunopositivity ($r = 0.817$, $p = 0.013$). 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.

Discussion

IL-17A/F, IL-21, IL-22 and granulocyte macrophage colony-stimulating factor (GM-CSF) represent the cytokine profile produced by a CD4 $^{+}$ T cell subset known as Th17. Recently, Th17 cells have been recognized as important contributors to the pathogenesis of Th1-dependent autoimmune diseases [18], and HT is now considered to be both a Th1- and Th17-mediated disease, while the role of Th17 cells in GD development and progress has not yet been clearly established.

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 [11]. 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 [19]. Previous studies have provided supportive evidence of Th17 and IL-17 participation in the pathogenesis of HT [20, 21], whereas their contributions to GD development remain poorly understood. Thus, an increased number of CD4 $^{+}$ IL-17 $^{+}$ lymphocytes in the peripheral blood of pediatric patients with untreated HT but not in GD patients was demonstrated by Bossowski *et al.* [22], whereas Qin *et al.* [23] did not find a significant difference in IL-17 levels in the peripheral blood and thyroid tissues of GD patients compared to controls. In our study, the expression level of IL-17 in thyrocytes was higher in both HT and GD patients than in patients with colloid goiters, which may support a role for Th17 in AITD.

In contrast to the presence of convincing data regarding the cytokine combinations required for Th17 differentiation in mice, several studies have been performed recently to establish the cytokine profile, which can generate human Th17 cells expressing RORC. IL-23 has been shown to play an essential role in maintaining Th17 cell differentiation, suppressing IL-10 secretion and facilitating IL-22 and GM-CSF production [24]. Moreover, IL-23 was also found to be an important survival factor for Th17 lymphocytes [25]. Th0 cells upon exposure to IL-23, IL-1 β , and IL-6 can generate highly pathogenic Th17 lymphocytes [10, 26]. IL-23, synthesized by macrophages and dendritic cells, is a member of the IL-12 family implicated in various autoimmune and inflammatory diseases.

Fig. 1 (A) HT patient evidencing IL-17-positive thyroid follicles and inflammatory cells ($\times 400$). (B) IL-23-positive cells within the inflammatory follicle in HT patients ($\times 400$). (C) IL-23 positivity demonstrated in the follicular epithelial cells of HT patients ($\times 200$). Confocal microscopy, representative image of IL-23 positive thyrocytes: green staining shows IL-23-specific staining, blue staining shows nuclei, insert ($\times 1,000$). (D) Strong cytoplasmic expression of IL-1 β demonstrated in the thyrocytes of HT patients ($\times 400$). Confocal microscopy, representative image of IL-1 β -positive thyrocytes: IL-1 β -specific staining (green), nuclei (blue), insert ($\times 1,000$). (E) Weak IL-23 immunoreactivity in GD patients ($\times 250$). (F) Occasional IL-1 β positivity revealed in GD patients ($\times 400$). (G) Colloid goiter showing almost nil IL-1 β expression ($\times 250$). (H) Dendritic cells observed within inflammatory infiltrate in HT patients ($\times 400$). Graves' disease (GD), Hashimoto's thyroiditis (HT), interleukin (IL).

6

Zake et al.

Fig. 2 Comparison of IHC results of IL-17 (A), IL-23 (B) and IL-1β (C) in patients with autoimmune thyroid disease (Hashimoto's thyroiditis, $n = 21$ and Graves' disease, $n = 8$) and colloid goiter ($n = 18$). Comparisons were made by using the Mann-Whitney U test. Data are shown as the median (interquartile range), * = $p < 0.05$. Interleukin (IL).

Fig. 3 Relationship between IHC expression levels of cytokines. In the HT group, IL-17A was positively correlated with IL-23 (A) and IL-1β (B) immunoreactivity. Furthermore, IL-23 was positively correlated with IL-1β immunopositivity (C). Correlations were determined by the Spearman rank correlation test. IHC, immunohistochemistry; IL, interleukin; HT, Hashimoto's thyroiditis.

Interestingly, Ruggeri *et al.* described increased levels of serum IL-23 in untreated and euthyroid HT patients compared to controls, thus representing early pathogenic events characteristic of HT [13]. Furthermore, the authors described IL-37 upregulation in HT. IL-37 exerts a protective role against oxidative stress and inflammation by inhibiting effects of Th1/Th17-mediated response [27]. Recently, another research group found the

enhanced expression of IL-23 in the thyroid tissue of patients with HT when compared to controls [14]. Additionally, these authors 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 immunoreactivity of IL-23 in the thyroid follicular cells of HT patients was significantly higher than that in both GD and colloid goiter

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 our knowledge, the expression of IL-23 in the thyroid tissues of GD patients has not been studied before. There are only two studies in which the levels of IL-23 were measured in the serum of GD patients [20, 28]. Increased serum levels of IL-23 have been reported in the study conducted by Jia *et al.* [28], whereas Figueroa-Vega *et al.* did not observe any significant differences in the IL-23 levels in GD patients compared to controls [20]. In our study, no difference between IL-23 immunopositivity within thyroid tissues in GD and colloid goiter 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 different 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, these results as well as conclusions regarding the role of Th17 cells in GD pathogenesis must be interpreted with caution because the absolute number of patients with GD was only 8. Interestingly, Zheng *et al.* showed an increase in IL-17 and ROR γ t mRNA expression in IL-23-stimulated cultured cells from GD patients when compared to those from controls and cultures without IL-23 [14]. Similar results were demonstrated in euthyroid GD patients, indicating an independent effect of thyroid hormone levels [29]. Increased tissue levels of IL-23 have not yet been reported in GD, and very few studies have focused on the role of IL-23 receptor in these patients. Furthermore, IL-23 receptor gene polymorphisms have been strongly associated with GD, especially with Graves' ophthalmopathy [30].

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 tight junction proteins by stimulating IL-6 production [15]. IL-1 β has been shown to induce Fas expression, promoting thyroid cell death as well [31]. 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 goiter and GD. Similar to IL-23, IL-1 β expression did not differ significantly between patients with GD and colloid goiter; 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 [11], our findings indicate that pathogenic Th17 cells may play a lesser role in the development of GD than in HT. In this context, supportive evidence has been provided in a recent study performed by Vitales-Noyola *et al.*, demonstrating the elevation of Th17 pathogenic cells in the peripheral blood of GD (especially in those with Graves' ophthalmopathy) but not in HT patients; however, when analyzing the absolute number of pathogenic cells, no significant differences between HT, GD, and controls were found [32].

This study has some methodological limitations associated with a retrospective nature of the study and the assessment of archived paraffin-embedded thyroid tissues. We included and studied a small number of thyroid tissue specimens obtained from patients with GD because of the limited availability of these samples. Therefore, the results of GD group analyses must be concluded and interpreted discreetly because of a small sample size in this group. Furthermore, when considering recent data on the role of Th17 in the development and progression of Graves' ophthalmopathy, more prospective studies analyzing this patient group are needed. Finally, although we did not aim to analyze the expression levels of other interleukins, it would be important to further investigate the role of different phenotypes of Th17 and their cytokine profiles in thyroid autoimmunity.

In conclusion, we found that both HT and GD patients had higher expression levels of IL-17, which simultaneously correlated with IL-23 and IL-1 β immunopositivity within thyroid tissue in the HT group. Furthermore, increased expression of IL-23 and IL-1 β , cytokines that to promote pathogenic Th17 cell differentiation, was observed in the HT but not in the GD group. Our data suggest that pathogenic Th17-promoting cytokines may play a role in HT pathogenesis. Further experimental studies to investigate the role and regulatory effects of Th17-related cytokines in the pathogenesis of AITD are required.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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ORIGINAL ARTICLE

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Plasma levels of Th17-associated cytokines and selenium status in autoimmune thyroid diseases

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Abstract

Introduction: The contribution of Th17 cytokines to autoimmune thyroid disease (AITD) is generally accepted. However, the roles of Th17 cells in the initiation and progression of Hashimoto's thyroiditis (HT) and Graves' disease (GD) remain unclear. Selenium deficiency, along with genetic predisposition and environmental factors, may have a role in thyroid autoimmunity.

Aim: We aimed to assess (1) the Th17 immune response by measuring plasma levels of Th17- and Treg-associated cytokines and (2) the selenium status in treatment-naïve Latvian patients with newly diagnosed GD or HT.

Methods: Eleven GD patients, 41 HT patients, and 26 healthy subjects were recruited for this study. Plasma levels of IL-17a, IL-22, IL-23, IL-6, and IL-10 were detected by xMAP technology, while selenium was detected fluorometrically.

Results and Conclusions: No significant differences in IL-17a, IL-22, IL-23, IL-6, or IL-10 levels were found among the HT patients, GD patients, and controls. In the HT patients, IL-17a levels were positively correlated with IL-22, IL-23, IL-6, and IL-10, while IL-22 was correlated with IL-6, IL-23, and IL-10. In the GD patients, IL-17a levels were positively correlated with IL-22, IL-23, and IL-10; IL-22 was positively correlated with IL-23, IL-6, and IL-10; FT3 was positively correlated with IL-17a, IL-23, and IL-10; and FT4 was positively correlated with IL-17a and IL-10 levels. Plasma selenium levels were negatively correlated with antithyroid peroxidase antibody titers in the HT patients. Although no difference in selenium levels was observed between the AITD patients and controls, the selenium status of the Latvian patients with GD or HT was at a suboptimal level.

KEYWORDS

autoimmune thyroid disease, selenium, T helper 17 cells

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1 | 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.¹ In HT development, enhanced secretion of interferon (IFN)- γ , interleukin (IL)-1 β , IL-2, and tumor necrosis factor (TNF)- α paralleled by thyroid cell apoptosis occurs due to the impaired T helper (Th)-1-based immune response, whereas in GD, upregulated production of IL-4, IL-5, IL-6, and IL-13, resulting from the Th2-driven humoral response, causes the secretion of autoantibodies recognizing thyroid-stimulating hormone receptor (anti-TSHR) and hypertrophic changes.²

The understanding of the Th1 and Th2 cell dichotomy, which is essential for autoimmune disorders, shifted with the discovery of Th17 lymphocytes. Supporting evidence suggesting the contribution of Th17 cells and pro-inflammatory IL-17 to AITD, particularly HT, has been produced in the last decade.^{3–6} It has been proposed that AITD results from Th1/Th2 dichotomy and Th17/T regulatory (Treg) cell imbalances.⁷ Th17 lymphocytes mainly secrete IL-17 and IL-22. IL-17 is a critical pro-inflammatory cytokine mediating chronic and autoimmune inflammation and neutrophil recruitment. Acting on immune and nonimmune cells such as epithelial cells, endothelial cells, fibroblasts, and osteoclasts, IL-17 promotes the expression of various cytokines (IL-1 β , TNF- α , IL-6, IL-8, granulocyte macrophage colony-stimulating factor [GM-CSF], and granulocyte colony-stimulating factor), chemokines (CXCL1, CXCL5, and CCL2), and metalloproteinases by cells.⁸ 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 vital for both the complete maturation and stabilization of the Th17 phenotype.^{9,10} To date, the association between selenium and Th17-related cytokines has been insufficiently explored, mostly in experimental models.

Currently, a role for selenium deficiency in the development of AITD is suggested along with genetic predisposition and environmental triggers. Selenium deficiency can impair the differentiation of CD4+ T cells, leading to cellular and humoral response dysfunction. 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.¹¹ Very little information is

currently available on the selenium content in soils in Latvia or the selenium status in AITD.

In this study, we aimed to assess (1) the Th17 immune response in AITD by measuring the plasma concentrations of Th17- and Treg-associated cytokines and (2) the selenium status in Latvian patients with newly diagnosed, treatment-naïve GD or HT to determine whether the selenium level is reduced in AITD patients compared to healthy subjects without autoimmune disorders.

2 | MATERIALS AND METHODS

2.1 | Patients

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, Riga East University Hospital, Latvia. Thirty-three out of 41 HT patients were euthyroid (median TSH, 1.90 [1.00–2.62] μ U/ml), and 8 out of the 41 patients had mild subclinical hypothyroidism (median TSH, 5.90 [5.41–7.35] μ U/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 ($p = .139$). The diagnosis of AITD was confirmed by evaluating the clinical manifestations of GD and HT, complemented by biochemical thyroid tests and ultrasound imaging. 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-thyroid peroxidase autoantibodies (anti-TPO), anti-thyroglobulin autoantibodies (anti-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 disease; (3) use of selenium-containing commercial supplements; (4) vegetarian or vegan diet; (5) use of medications affecting thyroid and immune function (amiodarone, corticosteroids, non-steroidal anti-inflammatory drugs, antidepressants, or anticonvulsants); and (6) significant renal or hepatic impairment.

The study protocol was conducted according to the Declaration of Helsinki and approved by the Riga East Hospital Research Committee and the Central Medical Ethics Committee, Latvia (Decision No. 01-29.1/5033). All patients and volunteers provided written informed consent before enrollment and blood sample collection.

2.2 | Blood collection

Peripheral blood samples were collected from all 86 patients and volunteers after overnight fasting early in the morning. Thereafter, the samples were centrifuged at 1500 rpm for 10 min and stored at -80°C until analysis. Venous blood samples were collected from hyperthyroid and hypothyroid patients before therapy with methimazole or levothyroxine, respectively.

2.3 | Thyroid function and antibody tests

The levels of serum-free thyroxine (FT4), free triiodothyronine (FT3), and thyroid-stimulating hormone (TSH), as well as anti-TPO and anti-Tg were measured by chemiluminescence immunoassay (Siemens) performed on an Advia Centaur XP (Siemens) analyzer. 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 (TG-IgA autoantibodies were measured by ELISA (Pharmacia Diagnostics Freiburg) according to the manufacturer's instructions (reference range: anti-TSHR, 0–1.58 IU/L; ANA, reference range would be "negative"; (TG-IgA, 0–10.0 U/ml).

2.4 | Cytokines detection

Cytokine patterns analyzed 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). 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.

2.5 | Selenium assay

The plasma selenium concentration was determined fluorometrically by using the fluorescence spectrophotometer "Cary Eclipse" (Varian, Inc.).¹² Interlaboratory quality control was conducted by employing two standards—a selenium AAS solution (Cat# 24, 792-8; Sigma-Aldrich) and Seronorm TE Serum Level I (Cat# 201 405; Sero AS)—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 $\mu\text{g/L}$.

2.6 | Statistical analysis

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. As dispersion did not correspond to a normal distribution in most cases, data were analyzed 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. $p < .05$ 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).

3 | RESULTS

The demographic and biochemical characteristics of the participants are presented in Table 1. 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. 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 $p > .05$) (Figure 1). Interleukin levels also did not differ between the euthyroid and hypothyroid HT patients (all $p > .05$). The plasma levels of cytokines are summarized in Table 2.

When the relationships among Th17-related cytokines were investigated, we found that 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 2A); additionally, IL-22 was correlated with IL-6, IL-23, and IL-10 (Figure 2B). 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 3A). 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 3B). Positive relationships between different interleukins were revealed in the control group and both euthyroid HT patients and hypothyroid HT patients.

TABLE 1 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	.152
Age (years)	36 (27–50.5)	41 (29–62)	30 (27–42.25)	.139
TSH (μ U/ml)	2.16 (1.42–3.77)	0.0003 (0.0000–0.0004)	1.11 (0.96–1.67)	<.001
FT4 (ng/dl)	0.91 (0.85–0.98)	1.97 (1.76–2.55)	0.94 (0.91–1.07)	<.001
FT3 (ng/dl)	0.32 (0.29–0.35)	1.21 (1.04–2.47)	0.32 (0.30–0.35)	<.001
Anti-TPO (IU/ml)	202.99 (75.80–421.40)	121.04 (3.74–929.28)	0.46 (0.25–0.78)	<.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	

Note: 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 < .05; statistically significant.

FIGURE 1 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 (A) IL-17a, (B) IL-22, (C) IL-23, (D) IL-6, and (E) IL-10 plasma levels. (A–E) Each dot represents a single data point. ns, not significant; GD, Graves' disease; HET, Hashimoto's thyroiditis, euthyroidism; HHT, Hashimoto's thyroiditis, hypothyroidism

TABLE 2 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)	.906
	12.95 (10.18–19.18)	15.99 (12.38–39.37)			.606
IL-22 (ng/ml)	0.08 (0.00–0.47)		0.13 (0.00–0.25)	0.25 (0.00–0.54)	.393
	0.026 (0.000–0.351)	0.421 (0.054–1.138)			.180
IL-23 (ng/ml)	2.45 (1.35–4.49)		2.31 (1.70–3.36)	3.23 (1.86–4.64)	.653
	2.37 (1.30–3.76)	3.42 (2.06–8.18)			.408
IL-6 (pg/ml)	5.88 (0.00–36.24)		6.92 (0.00–24.90)	11.61 (0.00–59.50)	.682
	1.90 (0.00–14.93)	68.62 (8.67–148.25)			.059
IL-10 (pg/ml)	9.70 (6.35–15.21)		9.50 (6.54–11.11)	11.62 (6.70–17.04)	.658
	9.70 (5.96–13.47)	9.83 (7.03–26.90)			.585

Note: 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 < .05: statistically significant.

FIGURE 2 Relationships between (A) IL-17a or (B) IL-22 and the studied biomarkers in the Hashimoto's thyroiditis patient group

FIGURE 3 Relationships between (A) IL-17a or (B) IL-22 and the studied biomarkers in the Graves' disease patient group

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 4.

Moreover, no significant correlations were found between the levels of interleukins and thyroid autoantibodies, TSH, FT3 or FT4 in HT patients (all $p > .05$). However, in newly diagnosed GD patients, the levels of FT3 were positively correlated with IL-17, IL-23, and IL-10 (Figure 5A), while FT4 was positively correlated with IL-17 and IL-10 levels (Figure 5B). In addition, anti-TSHR autoantibody titers were not correlated with the levels of interleukins in the GD group (all $p > .05$).

The median plasma selenium levels were 85.03 (60.09–116.60) μg/L for HT patients, 71.33 (59.05–104.42) μg/L for GD patients, and 80.35 (67.15–113.67) μg/L for control participants (Table 3). The plasma selenium distribution in the patient groups is presented in Figure 6A. The levels of selenium did not differ significantly among the studied groups (Figure 6B). Similarly, no differences in selenium levels were observed between euthyroid HT patients and HT patients with subclinical hypothyroidism (Table 3). Interestingly, we found that plasma selenium levels showed a negative correlation with anti-TPO autoantibody titers in

patients with HT ($r = -.376$; $p = .02$) (Figure 7). 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 titers (< 400 IU/ml) [72.75 [52.10–88.43] vs. 89.04 [65.53–117.51] μg/L, respectively; $p = .05$].

When the associations between plasma selenium and cytokines were analyzed, 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 groups (all $p > .05$). The levels of selenium failed to correlate with other characteristics, such as age, titers of anti-Tg and anti-TSHR, TSH, FT3, and FT4 (all $p > .05$).

4 | DISCUSSION

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

Spearman's rank correlation (r_s) matrices

FIGURE 4 Variance-covariance matrices with Spearman's rank correlation coefficients (values in small squares) representing the association level between studied cytokines in patient groups. GD, Graves' disease; HET, Hashimoto's thyroiditis, euthyroidism; HHT, Hashimoto's thyroiditis, hypothyroidism. * $p < .05$; ** $p < .01$; *** $p < .001$; **** $p < .0001$

anti-inflammatory cytokine that has been shown to inhibit Th17 cell-mediated inflammation.¹¹ Furthermore, current evidence suggests the Th17 lineage is plastic and heterogeneous.⁷ 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.¹⁴ Furthermore, it has been found to stimulate the survival of highly pathogenic Th17 cells.

Only limited data are available for serum IL-23 and IL-22 levels in AITD patients. Shi et al.¹⁵ demonstrated upregulated messenger RNA (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 et al.³ 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 with controls, reflecting the early course of thyroiditis, in a study conducted by Ruggeri et al.¹⁶ Jia et al.¹⁷ observed increased serum levels of IL-23 in GD patients. In contrast, Figueroa-Vega et al.⁴ did not find any significant differences in IL-23 levels between GD patients and healthy subjects. Previously, we demonstrated thyrocyte-related IL-23 overexpression in HT patients, while no difference was found between GD and colloid goiter patients, suggesting that IL-23 may be less implicated in GD pathogenesis than in HT pathogenesis.¹⁸

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 et al.¹⁹ found significantly increased serum levels of IL-22 in untreated euthyroid HT patients compared with patients with nodular goiter or healthy subjects. In another study, drug-naïve HT patients suffering from overt or sub-clinical hypothyroidism exhibited elevated serum levels of IL-17, while no differences were found in IL-22 or

FIGURE 5 Associations of (A) ft3 or (B) ft4 with the studied biomarkers in the GD patient group. ns, not significant; * $p < .05$; ** $p < .01$. ft3, triiodothyronine; ft4, thyroxine; GD, Graves' disease; TSHR, thyroid-stimulating hormone receptor

TABLE 3 Plasma levels of selenium 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)	.763
	85.03 (60.09–116.56)	94.78 (47.46–117.02)			.886

Note: Data are presented as the median (interquartile range).

FIGURE 6 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

IL-23 levels between the HT patients and healthy individuals. Song et al.²⁰ reported that peripheral blood Th22 cell levels, serum IL-22 levels, and IL-22 mRNA expression were elevated in GD patients compared with 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 as 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 nongenetic 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 toward Th1 responses.²¹ Additionally, glutathione peroxidase (GPx)1-deficient Th lymphocytes demonstrate a shift toward Th1 cells and attenuate the differentiation of Th cells into the Th17 lineage.²² In another study, selenium supplementation exerted protective effects against oxidative stress and peroxide-induced cell damage in human thyrocytes and fibroblasts.²³ However, more data from clinical studies are needed for understanding the relationship between selenium supplementation, immune

FIGURE 7 Correlation between selenium and anti-TPO autoantibody levels in patients with HT. 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. HT, Hashimoto's thyroiditis; TPO, thyroid peroxidase

response, and antioxidant effects. 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 toward 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 a population-based Danish study, Pedersen et al.²⁴ 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. 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.²⁵ The study confirmed the presence of a strong positive correlation between the plasma levels of selenium and selenoprotein P (SePP), 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, suggesting that selenium supplementation might be beneficial in the case of thyroid autoimmunity. Federige et al.²⁶ also explored serum selenium and SePP 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 SePP 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 optimize platelet GPx activity is 100–115 µg/L, as proposed by Alfthan et al.,²⁷ or 95 µg/L according to Thomson et al.,²⁸ whereas a plasma selenium range of 88–142 µg/L is associated with the maximal SePP1 concentration.²⁹ A selenium level below 80 µg/L is considered to indicate an inadequate selenium status.^{30,31} 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%.

5 | CONCLUSIONS

Despite the recent data on the involvement of the IL-17/IL-23 axis in the development of thyroid autoimmunity available in the literature, we did not find any significant differences in the plasma levels of Th17-associated cytokines between patients with AITD and control subjects. However, a positive correlation between Th17 ILs was detected in both HT patients and GD patients. Furthermore, Th17 cytokines were positively correlated with the severity of hyperthyroidism, suggesting their possible role in GD pathogenesis. 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 characterizes 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. Although no difference in selenium levels was observed between AITD patients and controls, the selenium status of Latvian patients with newly diagnosed GD or HT was at a suboptimal level. Interestingly, plasma selenium levels were negatively correlated with anti-TPO autoantibody titers in HT patients. Moreover, HT patients with higher anti-TPO levels had lower levels of selenium, suggesting that these patients might benefit from selenium supplementation.

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AUTHOR CONTRIBUTIONS

Ilze Konrade and Tatjana Zake designed the study protocol, Tatjana Zake, Sabine Upmale-Engela, Ieva Kalere, Gita Gersone, Andrejs Skesters collected the data and performed patient selection, Gita Gersone and Andrejs Skesters performed laboratory work, Tatjana Zake, Ieva Kalere, Sabine Upmale-Engela, Gita Gersone, Simons Svirsksis, Andrejs Skesters analyzed and interpreted the data, Tatjana Zake, Ieva Kalere, Valerija Groma, Ilze Konrade, Simons Svirsksis wrote and edited the manuscript, Ilze Konrade and Valerija Groma supervised the study. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

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

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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Review

IMMUNOLOGICAL MECHANISMS OF AUTOIMMUNE THYROID DISEASES: A SHIFT IN THE TRADITIONAL TH1/TH2 PARADIGM

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Autoimmune thyroid diseases (AITD) mainly include Hashimoto's thyroiditis (HT) and Graves' disease (GD), which are characterised by the presence of circulating antibodies against various thyroid autoantigens and infiltration of the thyroid gland by autoreactive lymphocytes. Despite the significant advancement in the knowledge of AITD pathogenesis in the last decade, the specific immunological mechanisms responsible for development of the disease are not thoroughly understood. Classically, HT has long been considered as a T helper (Th)1-mediated disease, while a Th2-driven autoimmune response is dominant for GD development. However, this classification has changed due to the description of Th17 lymphocytes, which suggested participation of these cells in AITD, particularly HT pathogenesis. Moreover, a shift in the balance between Th17 and T regulatory (Treg) cells has been observed in thyroid autoimmunity. We have observed overexpression of IL-17, the prominent effector cytokine of Th17, within thyroid tissues from HT and GD patients in our studies. The present review will focus on recent data regarding the role of Treg and Th17 lymphocytes in AITD pathogenesis. In addition, the impact and proposed mechanisms of the predominant environmental factors triggering the autoimmune response to the thyroid will be discussed.

Key words: Hashimoto's thyroiditis, Graves disease, T helper 17 cell, T regulatory cell, environmental factors.

INTRODUCTION

Autoimmune thyroid diseases (AITD) present with two pathogenetically different diseases: Hashimoto's thyroiditis (HT) and Graves' disease (GD). These two are clinically diverse and taken together make thyroid condition the commonest autoimmune disease. However, there is significant geographic variation in the worldwide reported incidence and prevalence of HT and GD. AITD prevalence is about 5% in the overall population, whereas the prevalence of thyroid antibodies is estimated to be 10–20% of all women (Hollowell *et al.*, 2002; Pyzik *et al.*, 2015). The worldwide incidence of HT is reported to be 0.3–1.5 cases per 1000 persons per year (Latina *et al.*, 2013). Furthermore, HT is the most common cause of primary hypothyroidism in

iodine-sufficient regions. Like most autoimmune disorders AITD exhibits a strong female bias in prevalence: HT affects women five to ten times more (Pyzik *et al.*, 2015), and GD four to five times more often than men (Carlé *et al.*, 2011). It is also known that the prevalence of thyroid autoimmunity increases with age reaching the highest number of cases between 45–65 years in HT and between 33–50 years in GD (Cooper and Stochla, 2003).

The prevalence and incidence of autoimmune diseases, including AITD, has increased significantly over the last decades. AITD has become a public health problem worldwide, including in Latvia. In our previous study of iodine deficiency during pregnancy, it was revealed that 10.7% of the 739 pregnant women participating nationwide had elevated

thyroperoxidase antibody (TPOAb) levels suggesting preexisting thyroid autoimmunity (Konrade *et al.*, 2015). Furthermore, data on thyroid medicinal product use trends from the Latvian National Health Service and State Agency of Medicines showed a steady increase in the number of people using thyroxine and thiamazole: thyroid therapy defined daily dose (DDD) per 1000 inhabitants increased from 10.23 in 2009 to 17.40 in 2017, thereby extremely increasing disease burden on health, health costs, education, society and family life (Anonymous, 2018).

AITD are characterised by the presence of circulating antibodies against thyroid autoantigens and infiltration of the thyroid gland by autoreactive lymphocytes (Tomer, 2014). Interestingly, although TPOAb and thyroglobulin antibodies (TgAb) are detected in 90–95% and 70–80% of AITD patients, respectively, the prevalence of both antibodies in patients with non-thyroid immune diseases is 10–15% (Carvalho *et al.*, 2013). HT results predominantly from the cellular immune response, which involves T helper (Th)1 and Th17 cells and leads to cell death of the thyrocytes with subsequent hypothyroidism, whereas GD mainly promotes Th2-related humoral autoimmunity causing production of TSH receptor (TSHR) antibodies and growth of the thyrocytes leading to the development of hyperthyroidism (Morshead *et al.*, 2012). However, both thyroid disorders may co-exist in the same individual causing mixed Th1/Th2/Th17 immune responses.

Various environmental and endogenous factors may act together to trigger thyroid autoimmunity in persons with predisposing genetic variants causing elevated thyroid autoantigen presentation and a loss of immune tolerance. Despite numerous studies undertaken and knowledge accumulated on the aetiology and pathogenesis of AITD in the last decade, the specific immunological mechanisms responsible for development of the disease are not thoroughly understood.

GENETIC SUSCEPTIBILITY

The role of genetic background in the aetiology of AITD is highlighted by numerous epidemiological studies showing that genetic factors contribute in about 70–80% cases the development of thyroid autoimmunity (Tomer and Davies, 2003; Hansen *et al.*, 2006). The familial clustering of AITD was confirmed by several studies 50 years ago. It has been shown later that almost 50% of the siblings of GD patients have thyroid autoantibodies and that 33% of those with HT or GD develop AITD themselves (Villanueva *et al.*, 2003). Increased familial clustering of AITD has been reported in German study indicating a 32-fold enhanced risk for developing HT in children and a 21-fold enhanced risk in siblings of patients with HT (Dittmar *et al.*, 2011).

During the last decades, a number of twin studies have examined the aetiology of several phenotypes related to AITD and have provided new data on the genetic contribution to AITD. A strong influence of genetic factors on the development of AITD has been demonstrated by twin studies as

well — the heritability of GD and thyroid autoantibody (TPOAb and TgAb) formation has been reported to be 79% and 73%, respectively (Brix *et al.*, 2001; Hansen *et al.*, 2006). A study of Hashimoto's hypothyroidism showed 55% and 0% concordance in Danish monozygotic and dizygotic twins, respectively, indicating an essential role of environmental factors in development of the disease (Brix *et al.*, 2000). Thus, twin studies have shown that 21–27% of the total phenotypic variance in AITD can be explained by environmental factors.

Therefore, it is critical to investigate and identify the possible interplay of environmental and genetic factors involved in thyroid autoimmunity to propose individualised AITD management strategies, since most environmental factors may be modified. However, the mechanisms by which the environmental factor trigger thyroid autoimmunity in individuals with known susceptibility loci remain unclear. The main known susceptibility genes for AITD can be classified as either immunoregulatory or thyroid-specific genes (Effraïmidis and Wiersinga, 2014). TSHR and Tg were recognised as thyroid-specific genes, whereas the human leukocyte antigen (HLA), PTPN22, CTLA-4, FOXP3, FCRL3, CD25, CD40, and other genes as immune-modulating, playing a critical role in the development of the effective immune response including self-tolerance, and cell-mediated and humoral immunity. However, single nucleotide polymorphisms in these genes, which are not specific for AITD were recognised as risk factors for other autoimmune diseases (Lee *et al.*, 2015). Tg is one of the main thyroid autoantigens assumed as a major susceptibility gene for AITD (Tomer *et al.*, 2002). Whereas in GD patients, a consistent association with polymorphisms of the TSHR gene was reported (Xiong *et al.*, 2016).

The occurrence of both AITD is associated with specific variants of the HLA class II genes. They are vital for regulating immune activity by determining the antigen binding specificity and the initiation of the immune response. HLA controls cytokine production and modulates the immune response by cytokine genes on haplotypes as well. Thus, polymorphisms in the HLA genes may lead to HT or GD via regulating the Th1/Th2 and possibly Th17 pathways. HLA-DQA1 and HLA-DR3 were shown to be associated with GD, while polymorphisms in HLA-DR3, DR4, DR5, DQ7, DQB1, DQA1, DQW7 were correlated with the incidence of HT (Zeitlin *et al.*, 2008; Badenhop *et al.*, 1990).

Numerous susceptibility genes and loci for AITD have been detected by genome-wide association studies (GWAS) during the last decade. However, these studies involve mainly single genetic factors, which might have a minor effect on the disease development and only the simultaneous presence of certain genetic and environmental factors altogether may lead to the clinical manifestation of the thyroid autoimmunity. Therefore, GWAS should focus on simultaneous investigation of multiple genetic and environmental factors and interactions between them.

PROTECTION AGAINST THYROID AUTOIMMUNITY

Mechanisms of central and peripheral tolerance provide proper regulation of the immune system thus protecting against thyroid autoimmunity. Maturation of T helper CD4+ lymphocytes occurs in the thymus via processes of positive and negative selection. Central immune tolerance refers to apoptosis of autoreactive T cells in the thymus during the foetal life. T cells that escape negative selection are prevented from triggering autoimmunity by mechanisms of peripheral tolerance where T regulatory cells (Treg) play a crucial role (Weetman, 2010; Shevach, 2006). Their protective role has been also demonstrated in several animal models of autoimmune diseases (Morris *et al.*, 2009).

Treg cells derive from the thymus as a subpopulation of fully differentiated T cells (thymus-derived Treg), naive Th0 cells in the peripheral tissues (peripheral differentiated Treg) or develop *in vitro* conditions (iTregs) (Shevach and Thornton, 2014). The first properly characterised Treg cell subset exhibits expression of the transcription factor forkhead box protein 3 (Foxp3) and high constitutive levels of γ chain of the IL-2 receptor (CD25). Foxp3 contributes to the maintenance of self-tolerance by promoting development of Treg while inhibiting the differentiation of Th17 cells (González-Amaro and Marazuela, 2016). Foxp3 mutations in humans can cause an allotropic syndrome of multi-organ autoimmunity such as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome. Several studies evidenced a defective function of Foxp3+ Treg cells in patients with HT and GD (Glick *et al.*, 2013).

Treg cells have been shown to suppress target cells by direct cell-to-cell interaction or secretion of inhibitory cytokines such as transforming growth factor β (TGF- β), interleukin (IL)-10, and IL-35. IL-10 induces suppression of pathogenic Th17 cell responses and maintains Treg suppressive activity and expression of Foxp3. TGF- β regulates differentiation, proliferation and survival of lymphocytes, while the exact role of IL-35 requires further investigation (Chaudhry *et al.*, 2011; Huber *et al.*, 2011).

Another subset of Treg cells recently described demonstrates constitutive expression of CD69 but does not express Foxp3 or CD25. These cells exert their immunosuppressive activity mainly through the production of IL-10 and TGF- β (Han *et al.*, 2009). Interestingly, increased levels of CD69+ Treg cells were found in both peripheral blood and thyroid tissue of AITD patients. However, these lymphocytes were dysfunctional and unable to effectively down-modulate the autoimmune process and tissue damage, thus suggesting their role in the pathogenesis of autoimmune diseases (Rodríguez-Muñoz *et al.*, 2016). Finally, Foxp3- IL-10 secreting type 1 regulatory (Tr1) cells have been shown to inhibit T cell activity by different mechanisms, such as inhibitory cell-cell interactions via cytotoxic T lymphocyte antigen-4 (CTLA-4) or programmed cell death protein-1 (PD-1) and secretion of granzyme B and perforin (Roncarolo *et al.*, 2014).

Several studies have found decreased frequency and/or a defective function of Treg in patients with HT or GD (Li *et al.*, 2016) and highlighted the essence of results previously observed in animal models. Although the number of Treg cells reported is variable due to use of different detection markers and estimations related to disease stages, the majority of studies suggest that the reduction or functional defects of Treg is tightly associated with the development of thyroid autoimmunity. Recently, a possible role of regulatory B cells (Breg) in the maintenance of peripheral tolerance and inhibition of immune responses to specific self-antigens via release of IL-10, TGF- β , Fas ligand, and tumour necrosis factor (TNF)-related apoptosis inducing ligand has been established as well (Kristensen *et al.*, 2015). Thus, both Treg and Breg cells may act as inhibitors of inflammatory and autoimmune responses to prevent autoimmunity. In certain individuals, however, autoreactive T lymphocytes escape from the control of these immunoregulatory mechanisms and may lead to loss of the peripheral self-tolerance with subsequent development of autoimmune disease via activation, proliferation and differentiation. Reduction of peripheral self-tolerance occurs due to enhanced Th17 cell response as compared with induced regulatory Th10 cell response (Papp *et al.*, 2017).

PATHOGENESIS OF AITD

Thyroid autoimmunity appears as a result of the breakdown or reduction of self-tolerance to the three major thyroid proteins — TPO, TG, and TSHR occurring in the central or peripheral organs of the immune system. This causes the infiltration of the thyroid gland by T and B lymphocytes followed by production of AITD specific antibodies, induction of the thyrocyte apoptosis and the gland destruction in HT. Although traditionally the pathogenesis of HT is due to T cell-mediated autoimmunity and GD is a result of humoral autoimmunity, cellular and humoral immune responses are closely connected and cross-linked (Ramos-Levi and Marazuela, 2016). Despite comprehensive research in the last decades, the exact mechanisms of initiation and progression of thyroid autoimmunity have not been completely established.

The genetic background, self-antigens, and various environmental triggers together determine features of the autoimmune response in AITD and cause the enhanced thyroid antigen presentation and reduction of immune tolerance. Antigen-presenting cells (APC), and especially dendritic cells colonising the thyroid gland, present specific thyroid antigens to T lymphocytes via the MHC complex in the lymph nodes. This results in activation and proliferation of autoreactive T and B lymphocytes, and production of different cytokines by immune and thyroid cells. In fact, dendritic cells may be functionally subdivided into two groups — immunogenic or tolerogenic — depending on the activation of autoreactive T-cells or Treg, respectively (Maldonado and von Andrian, 2010). Moreover, several studies have revealed increased levels of dendritic cells within inflammatory infiltrates of the thyroid in both GD and HT patients

(Roura-Mir *et al.*, 2005; Mao *et al.*, 2011). Once activated, T helper (CD4+) cells can be differentiated into at least four main functional subsets according to the production of specific cytokines — Th1, Th12, Th17, and Treg. The development of the Th1 population is stimulated in the presence of either IL-12 or interferon (IFN)- γ through transcription of T-bet, whereas differentiation of Th2 cells is driven by IL-4 via the master transcription factor GATA3 (Peck and Mellins, 2010).

Classically, HT has long been considered as a Th1-mediated disease, while a Th2-driven autoimmune response is dominant for GD development. However, this classification has changed due to the description of Th17 cell, which suggested an evident participation of these cells in AITD, and particularly HT pathogenesis. In HT, increased Th1 activity caused by upregulation of pro-inflammatory cytokines such as IL-2, IL-1b, IFN- γ , TNF- α leads to cell-mediated immunity and thyrocyte death in apoptotic pathways. Thus, autoreactive Th1 cells recruit CD8+ cytotoxic T cells, which cause thyrocyte apoptosis by action of granzyme and perforin. In addition, autoreactive T lymphocytes induce antibody production by B cells leading to necrosis of thyroid cells by complement system activation. Finally, thyrocytes can undergo caspase-mediated apoptotic death in a paracrine way by expression of the CD95/Fas death receptor (Fröhlich and Wahl, 2017). Although the contribution of anti-TPO antibodies to destruction of the thyroid gland compared to cytotoxicity is minor, autoantibodies have shown pathogenic effects including complement activation and induction of oxidative stress, indicating that HT is also a Th2-mediated disease. Moreover, it has been revealed that anti-TPO antibodies can cause damage of cultured thyroid cells performed upon binding to TPO located on the apical cell membrane via both antibody-dependent cytotoxicity and complement-dependent cytotoxicity (Rebuffat *et al.*, 2008).

In GD, there are increased levels of Th2 released cytokines IL-4, IL-5, IL-6, IL-10, IL-13, which mainly mediate humoral response by stimulating production of TSH receptor antibodies, growth of the thyrocytes and apoptosis of the thyroid gland infiltrating lymphocytes via downregulation of Fas/Fas ligand and upregulation of the anti-apoptotic molecule Bcl-2 (Berger, 2000; Salmasso *et al.*, 2002). Also, antibodies to TPO and, less frequently, to Tg have been identified in most GD patients. Furthermore, TPO and Tg autoantibodies may comprise immunoglobulin (Ig) G4 as well as IgG1 subclasses, suggesting the participation of both Th2 and Th1 cytokines in GD.

During the last decade, a role of a new subset of Th cells, designated Th17, has been studied in the pathogenesis of thyroid autoimmunity, thus changing the traditional paradigm of Th1/Th2 dichotomy. Several research groups have shown that IL-17A, the prominent effector cytokine of Th17, can induce the release of different pro-inflammatory cytokines responsible for induction and development of chronic inflammatory responses in many autoimmune diseases including AITD.

TH17 CELLS AND THYROID AUTOIMMUNITY

The discovery of a third Th subset has introduced a new understanding of the classical Th1 and Th2 dichotomy. Th17 cells and their hallmark cytokine IL-17 were found to play an important role in the pathogenesis of different inflammatory and autoimmune diseases, which were previously classified as Th1-dependent pathologies. An altered balance between Th17 and Treg cells as well as Treg cell dysfunction has been found in autoimmune diseases (Papp *et al.*, 2017). Previous studies have shown a significant increase of Th17 cells and IL-17, revealed in the peripheral blood and thyroid tissues of AITD patients when compared to control subjects, suggesting their contribution to thyroid autoimmunity. Li and colleagues revealed an increased number of thyroid tissue infiltrating Th17 cells and serum levels of IL-17 in patients with HT compared to the thyroid cancers, nodular goiters or healthy controls. Additionally, a strong association between IL-17 expression and stromal fibrosis was observed in HT patients (Li *et al.*, 2017). Furthermore, Figueroa-Vega *et al.* observed enhanced levels of Th17 cells and IL-17 mRNA in peripheral blood and thyroid tissue infiltrating inflammatory cells in patients with HT, along with higher levels of IL-6, IL-15, and IL-23, which are involved in the Th17 cell differentiation (Figueroa-Vega *et al.*, 2010). Later, Konca Degertekin and colleagues found that hypothyroid patients had lower levels of IL-17 and IL-23 than in euthyroid HT patients, suggesting that hypothyroidism itself has an inhibitory effect on Th17 cytokine responses (Konca Degertekin *et al.*, 2016). Less is known about participation of Th17 cells and their cytokines in the pathogenesis of GD. Previous studies showed an enhanced number of CD4+IL-17+ cells in the peripheral blood of children with untreated HT, but not in GD patients (Bosowski *et al.*, 2012). Similarly, IL-17 levels in the peripheral blood and thyroid tissues did not differ significantly between adult patients with GD and controls (Qin Q *et al.*, 2012). An increased expression of IL-17 within thyroid tissues from HT and GD patients was found also in our study (Zake *et al.*, 2018). We observed stronger immunorepression level of IL-17A in thyroid follicular cells in HT and GD patients when compared to patients with colloid goiter, thus suggesting that Th17 cells had an essential role in AITD pathogenesis ($p < 0.001$ and $p = 0.007$, respectively) (Fig. 1).

Graves' orbitopathy (GO), a serious and most frequent extrathyroidal complication of GD, manifests as orbital inflammation and expansion of adipose tissue and extraocular muscles. Several studies have investigated the involvement of Th17 cells in the pathogenesis and disease activity of GO. Serum IL-17 levels were found to be significantly higher in GO patients than in controls. Patients with active GO had higher serum concentrations of IL-17 compared to inactive GO patients. Furthermore, IL-17 levels were correlated with clinical activity score of GO, thus supporting the role of IL-17 in the pathogenesis and progression of GO (Kim *et al.*, 2012). Fibrosis and adipogenesis are the two dominant pathophysiologic orbital processes involved in the

Fig. 1. (A) IL-17 positivity demonstrated in the follicular epithelial cells in HT patient (x400). Confocal microscopy, representative image of IL-17 positive thyrocytes: green staining shows IL-17-specific staining, blue staining shows nuclei. Insert (x1000). (B) Weak IL-17 immunoreactivity in GD patient (x250). (C) Colloid goiter with almost nil IL-17 expression (x400).

pathogenesis of GO. Recently, Fang and colleagues reported enhanced levels of pathogenic IFN- γ and IL-22-producing Th17 cells in active GO patients, compared to inactive GO patients and controls (Fang *et al.*, 2017). Additionally, a positive correlation between pathogenic Th17 cells and clinical activity score of GO was observed, indicating that the number of these cells might serve as an indicator of GO severity and activity. The authors also demonstrated that Th17 lymphocytes can promote proinflammatory cytokine production in both CD90+ (favour fibrosis) and CD90- (favour adipose hyperplasia) orbital fibroblasts that are CD34+. Moreover, both orbital fibroblast subsets can directly induce naive T cell differentiation toward the Th17 phenotype via prostaglandin E2 production (Fang *et al.*, 2017).

Th17 cells are mainly characterised by the production of IL-17A and IL-17F responsible for the development of inflammation, particularly in autoimmune diseases. They can act on epithelial cells, fibroblasts, and macrophages, inducing the release of pro-inflammatory mediators such as cytokines (IL-6, IL-8, TNF- α , IL-1 β , GM-CSF, G-CSF), chemokines (CXCL1, CXCL8, CCL2, CCL7, CCL20), and metalloproteinases (Song *et al.*, 2014). IL-17 regulates differentiation of immune cells within the germinal centre of the lymphoid follicle and autoantibody production. It has been found that IL-17 stimulates neutrophil recruitment by inducing CXCL8 by both immune and non-immune cells.

As mentioned above, different cytokines and transcription factors are required for regulating naive Th lymphocyte differentiation into a Th1, Th2, Th17 or Treg cell subset (Fig. 2). In contrast to convincing experimental data highlighting Th17 cell development, only a few human studies performed in the past have established the cytokine pattern involved in the generation of Th17 cells (Peck and Mellins, 2010; Stritesky *et al.*, 2008). In fact, the cytokine profile necessary for driving the differentiation of Th17 lymphocytes is similar in both humans and mice. TGF- β , along with the proinflammatory cytokines such as IL-1 β , IL-6, IL-21, and IL-23, have been found to have an essential role in the development of Th17 cells. It has been found that Th17 lineage is directed by their master transcription factor

Fig. 2. Th17 subsets and their cytokines. Adapted from "A cellular and molecular view of T helper 17 cell plasticity in autoimmunity" by R. Stadhoudersab, E. Lubbers, R.W. Hendriks, *J. Autoimmun.*, 2018, 87, 1–15.

retinoic acid receptor-related orphan nuclear receptor gamma (ROR γ), which is induced by IL-6 and IL-23 via signal transducer and activator of transcription 3 (STAT3). Interestingly, TGF- β is important for the differentiation of both Th17 and induced Treg cells. It has been shown that high doses of TGF- β induce expression of Foxp3, thus promoting Treg differentiation, while low doses of TGF- β in combination with IL-6 promote Th17 polarisation (Stadhouders *et al.*, 2018). In mice, the combination of IL-6, IL-1 β , and IL-23 has been found to be sufficient for the differentiation of Th17 cells, indicating TGF- β independent signalling of Th17 development. Th17 cells were also found colonising the gut of TGF- β signalling-deficient mice (Qin *et al.*, 2009). In contrast to mice observations, TGF- β seems to be indispensable for the differentiation of Th17 cells in man. Thus, TGF- β in the presence of IL-6 and IL-21 induces the initial differentiation of Th17 from naive Th cells, while IL-23 and IL-1 β are essential for both complete maturation and stabilisation of the Th17 population (naive Th cells do not express IL-1 and IL-23 receptor) (Stritesky *et al.*, 2008). Although IL-23 is not required for the induction of Th17 development, it is a crucial factor for the stabilisation and generation of the pathogenic phenotype of Th17 cells via STAT4 (Croxford *et al.*, 2012). IL-23, produced by dendritic cells and macrophages, can maintain Th17 cell differentiation by suppressing IL-10 secretion and promoting IL-22 and GM-CSF production.

Although the Th17 subset was identified more than a decade ago, it has become clear only recently that Th17 cells are heterogeneous and not uniform in function, exhibiting under certain cytokine exposure two different cellular phenotypes: pathogenic and non-pathogenic (Stadhouders *et al.*, 2018). Th17 cells differentiated in the presence of TGF- β 1 and IL-6 co-produce IL-17 and IL-10. These cells are assigned to the non-pathogenic subset of the Th17-lineage, as they do not induce tissue inflammation and may inhibit an autoimmune inflammation. By contrast, Th cells upon exposure to IL-23, IL-1 β , and IL-6 can generate highly pathogenic Th17 lymphocytes, which drive the autoimmune inflammation (Ghoreschi *et al.*, 2010). These pathogenic lymphocytes can secrete both common and pathogenic sets of molecules, such as IL-17A/F, IL-21, IL-22, IL-26, IFN- γ , TNF, and GM-CSF (Stadhouders *et al.*, 2018). Enhanced levels of IL-23 were observed in the peripheral blood and within thyroid tissues of HT patients but it still remains unclear whether IL-23 is involved in the pathogenesis of GD. Recently, when studying the immun-expression of IL-23 and IL-1 β within thyroid tissue of AITD subjects, we found that expression of both interleukins was significantly increased in HT patients compared to GD and colloid goiter patients, but no difference was found between the expression level of interleukins in patients with GD and colloid goiter (unpublished data).

Cytokines play a crucial role in modulating immune response in both AITD. Cytokines produced by activated T cells may drive the autoimmune response skewing toward the Th17 phenotype and away from Th1 or Th2 phenotypes. Therefore, evaluation of T cell subsets linked to certain cytokine production in AITD could help to identify the main immunological mechanisms involved in thyroid autoimmunity thus deepening our knowledge about disease outcome and treatment strategies. Th17 cells exhibit various therapeutic targets, and treatment strategies include blockade of the differentiation of Th17 cells, inhibition of Th17 neutralising cytokines, and Th17-specific transcription factors (Maddur *et al.*, 2012). Furthermore, several IL-17 neutralising monoclonal antibodies, such as secukinumab, ixekizumab and brodalumab, have been developed in recent years, which have demonstrated impressive therapeutic effects in patients suffering from psoriasis (McInnes *et al.*, 2015). Further experimental and clinical studies are needed to better clarify the role of different Th subsets in AITD.

ENVIRONMENTAL FACTORS

Increased iodine intake appears as the most important among environmental factors studied and is suggested as influencing the development of AITD. Other suggested risk factors include selenium deficiency, smoking, alcohol, stressful life events, infections, several drugs, and exposure to chemical compounds. Prevention of iodine deficiency disorders by food iodine fortification has been successfully implemented in many countries in the past century. However, other preventive interventions for AITD may be complicated or even impracticable. It would be extremely im-

portant to recognise which preventive interventions can be taken by subjects with a risk to develop AITD. Excess iodine intake and a relative selenium deficiency appear to be the predominant environmental factors triggering the autoimmune response to the thyroid.

Iodine intake. Iodine intake contributes to thyroid function and the development of thyroid gland disorders. The recommended daily allowance of iodine for adults is 150 μ g, increasing to 250 μ g in pregnancy and lactation. It is well known that iodine-sufficient populations compared to those that are iodine-deficient appear to have higher incidences of thyroid-specific antibodies and autoimmune hypothyroidism. Data from epidemiological studies show that, due to reduced synthesis of thyroid hormone, severe iodine deficiency may develop revealing the increased prevalence of goiter and hypothyroidism, whereas chronic mild-to-moderate iodine deficiency may cause toxic nodular goiter and hyperthyroidism (Zimmermann and Boelaert, 2015). By contrast, chronic excessive iodine intake or a rise in usual intake, especially after the implementation of mandatory food iodine fortification (e.g. universal salt iodisation), may lead to the increased prevalence of thyroid autoimmunity and subclinical hypothyroidism in parallel with a decrease in rates of iodine deficiency-related thyroid disorders (Teng *et al.*, 2006). Thus, the prevalence of TPOAb in the Danish population before and 4–5 years after a cautious mandatory iodisation of salt aiming to adjust iodine intake to a low recommended level was 14.3% and 23.8%, respectively (Pedersen *et al.*, 2011). Furthermore, the rise in the frequency of thyroid antibodies was most pronounced in women aged 18–45. Five to seven years after iodine fortification, the overall incidence rate of overt hypothyroidism increased as well, reaching 47.2/100 000 per year compared to 38.3/100 000 per year at baseline (Pedersen *et al.*, 2007). In the Pescopagano survey in Italy, introduction of the salt iodisation programme 15 years later resulted in increased prevalence of TPOAb (12.6% vs 19.5%) and also HT rates (3.5% vs 14.5) (Aghini Lombardi *et al.*, 2013). However, it has been also shown that the optimisation of nutritional iodine intake may result in lowered incidence of autoimmune thyroiditis. After implementation of mandatory fortification of salt with iodine in Brazil in 1956, adjustments of the iodine concentration in salt were performed in 2003 and 2013 by decreasing the recommended iodine concentration to 20–60 and 15–45 mg per kg of salt, respectively. A cross-sectional study conducted in 2013 demonstrated a trend toward a decreased prevalence of HT and a decrease in urinary iodine concentration to optimal levels. (Miranda *et al.*, 2015). However, it is unclear what will be the impact of a new continuing reduction in salt iodine concentration on thyroid function. The first systematic review and meta-analysis regarding the effect of excess iodine intake on thyroid disease prevalence in different populations, which was published recently, indicated that although universal salt iodisation decreased goiter rates, chronic excess iodine intake from water or poorly monitored salt was a risk factor for hypothyroidism in free-living populations (Katagiri *et al.*, 2017). Therefore, accurate monitoring of io-

dine concentration both in salt and water is required for prevention of thyroid disorders.

The mechanisms by which increased chronic intake of dietary iodine contributes to thyroid autoimmunity are not completely understood. In susceptible individuals, iodine excess induces thyroid gland infiltration by lymphocytes liberating cytokines and chemokines, thus leading to generation of thyroid antibodies. Moreover, high iodine intake enhances Th17 and inhibits Treg cell differentiation, while it triggers an abnormal expression of TNF-related apoptosis-inducing ligand in thyrocytes, thereby inducing cell death of thyroid cells (Yu *et al.*, 2011). It has been shown recently that excess iodine promotes apoptosis of thyroid follicular epithelial cells seen in HT by inhibiting autophagy activity. This process is mediated through TGF- β 1 downregulation and activation of the Akt/mTOR signaling pathway. In addition, excess iodine increases production of reactive oxygen species contributes to oxidative stress and thyrocyte apoptosis (Xu *et al.*, 2016). Excessive iodine may also alter the conformation of TG and promote its immunogenicity by increasing the affinity of TG determinants for the T cell receptor (Carayanniotis, 2011). Furthermore, chronic high iodine exposure may induce autoantigen presentation activity of thyrocytes and dendritic cells as well as activate autoreactive T cells. Finally, excess iodine-induced hypothyroidism in HT may be due to a pathologically persistent inhibitory Wolff-Chaikoff effect on thyroid hormone synthesis (Markou *et al.*, 2001).

Selenium status. Selenium status is an important parameter of general population health, and it has been hypothesised that nutritional selenium deficiency may trigger the initiation or progression of thyroid autoimmunity. Various effects of selenium are recognised, ranging from antioxidant and anti-inflammatory to the production of active thyroid hormone (Rayman, 2012). The amino acid selenocysteine is a major form of thyroid selenium presented either by enzymes such as glutathione peroxidases (GPx), thioredoxin reductases, iodothyronine deiodinases or proteins without enzymatic function, known as selenoproteins (SELENO) P, M, S (Valea *et al.*, 2018). Enzyme proteins have an important role in protecting thyrocytes from oxidative damage and, also, in thyroid hormone metabolism. GPx provide thyroid antioxidant protection against free radicals and oxidative stress, catalysing the detoxification of hydrogen peroxide and organic hydroperoxides. The biomarkers of selenium status and function available for human studies include GPx3 (10–25% of plasma selenium), SELENOP (20–70% of plasma selenium), and GPx1, which can be assayed in erythrocytes, lymphocytes as well as tissue biopsy specimens (Combs, 2015). GPx3 is also secreted at the apical pole of the thyrocyte, where it degrades excess hydrogen peroxide that has not been used by thyroid peroxidase, whereas SELENOP, the major selenoprotein in plasma, is crucial for the specific distribution and transport of selenium to several target tissues. Studies in many countries have shown the effects of geographic variation in the selenium contents in food and plasma selenium concentrations in different populations around the world.

Selenium deficiency has been recognised in several pathological thyroid conditions including nodular goiter, thyroid cancer, and AITD. Serum selenium levels have been shown to be lower in newly diagnosed GD patients compared to healthy subjects (Bülow Pedersen *et al.*, 2013). Lower selenium levels were evidenced in GD subjects compared to those without this disease, suggesting that relative selenium deficiency may be an independent risk factor contributing to the development of orbitopathy in GD patients (Khong *et al.*, 2014). It is also well known that selenium supplementation is recommended to prevent progression of mild GO. Selenium levels have been shown to be lower in patients with HT as well. A recent cross-sectional study involving more than six thousand participants from two provinces in China that had high genetic, environmental, lifestyle as well as iodine status similarities, except for very different soil-selenium concentrations, revealed an increased prevalence of HT but not GD in the province with lower selenium intake (Wu *et al.*, 2015).

Analysis of selenium status in HT and GD patients suggests that supplementation of this trace element may be beneficial in these patients. It has been observed that selenium supplementation decreases TPOAb levels more effectively in individuals with a lower baseline level (Toulis *et al.*, 2010). Furthermore, regions with deficient, higher than adequate or high iodine intake may have more need for selenium through its role within selenoproteins, protecting the thyroid gland from oxidative damage as well as increasing immune tolerance (Hu and Rayman, 2017). It has been also demonstrated that selenium supplementation can upregulate Treg cells that have been reduced by excess iodine intake (Xue *et al.*, 2015).

Reduction of circulating thyroid autoantibody levels by selenium supplementation in individuals with HT was described almost two decades ago. Over the last years, numerous prospective clinical trials were performed to examine whether selenium supplementation either with sodium selenite or selenomethionine might have beneficial effects for the treatment of HT. A meta-analysis published in 2016 showed that selenium supplementation effectively reduced TPOAb levels at 3, 6, and 12 months in levothyroxine-treated HT patients. Importantly, in untreated patients, there was a decrease in TPOAb levels after 3 months, but not after 6 or 12 months of supplementation (Wichman *et al.*, 2016). Another meta-analysis study was published evaluating several types of the outcome, except for TPOAb levels (Winther *et al.*, 2017). It demonstrated that TSH and ultrasound finding improvement with selenium had a very low to low level of evidence, while the quality of life improvement had a low to moderate level of evidence. Although a role for selenium supplementation in the treatment of HT is still discrepant, the correction of a selenium deficiency may have other health benefits. It is highly important to assess patients' selenium status before and during selenium administration as well as to identify and correct selenium deficiency in these patients. However, it remains unclear how selenium supplementation may affect HT in patients with

higher baseline selenium intake. Further randomised controlled trials are required before selenium supplementation can be routinely suggested by clinicians in patients with HT.

Stress as a trigger. According to studies and observations, stress does not have any triggering role in HT. However, among the non-genetic factors involved in GD, one is stress. Despite several retrospective case-control studies, which found a higher frequency of stressful life events in the year preceding the primarily diagnosed GD, the role of stress as a trigger still remains controversial. A prospective study by Effraïmidis *et al.* with a five-year follow-up period did not detect any causal relationship between stressful life events and GD (Effraïmidis *et al.*, 2011). In contrast, a role of stress in triggering both the onset and the recurrences of hyperthyroidism in patients with GD was highlighted by continued follow-up for 5 years after antithyroid drug withdrawal (Vita *et al.*, 2014). Stress may affect the immune system both directly and indirectly through the activation of nervous and endocrine systems. Activation of the hypothalamic-pituitary-adrenal axis and the sympathoadrenal system occurs under exposure to stress causes enhanced secretion of glucocorticoids and catecholamines, which both have a similar immunomodulatory effect and are consistent with an imbalance in favour of Th2 cells. Hormones suppress the production of IL-2 by antigen-presenting cells and increase the secretion of IL-4 and IL-10 by Th2 cells, directing the immunity toward a Th2 response (Chrousos and Elenkov, 2006). In addition, the decrease in DHEA during stress can also stimulate a Th2-type immunity. Altogether, this may promote the development of GD, which is considered to be a Th2-dependent disease. However, recent studies provide evidence for the presence and role of Th17 cells in the pathogenesis of GD. Interestingly, it has been shown that female C57BL/6 mice under chronic exposure to stress demonstrate a shift toward proinflammatory Th1/Th17 versus Th2 responses (Harpaz *et al.*, 2013).

Immune-related thyroiditis. Drugs such as amiodarone, lithium, and biological agents can contribute to the manifestation of AITD via immune mediated effects or direct effects of drugs on the thyroid. Biological agents are widely used in the treatment of cancer, nonmalignant diseases, and several autoimmune disorders. Indeed, the reported incidence of *de novo* development of thyroid autoantibodies in hepatitis C patients treated with IFN varied in studies from 2% to 40% (Mandac *et al.*, 2006). IFN-related thyrotoxicosis in more than 50% of these patients was due to destructive thyroiditis, while in the remainder due to GD. Interestingly, a recent review showed that treatment with biological antirheumatic agents (TNF- α inhibitors and rituximab) did not seem to induce or worsen AITD in rheumatoid arthritis patients (Bliddal *et al.*, 2017). However, the monitoring of thyroid status is recommended due to the well-established association between rheumatoid arthritis and AITD. Similarly, in patients with inflammatory bowel disease, there was no change regarding thyroid autoantibodies after treatment with anti-TNF (Paschou *et al.*, 2018).

Immune checkpoint inhibitors (ICPI) (anti-CTLA4 and anti-PD1 therapy), which are widely used in cancer therapy, can also trigger thyroiditis. However, ICPI-related thyroid dysfunction is typically due to destructive thyroiditis, which manifests as thyrotoxicosis followed by the transition to hypothyroidism. The role of antibodies in the pathophysiology of ICPI-related thyroiditis remains unclear (Priyanka *et al.*, 2018; Chang *et al.*, 2019). The reported prevalence of TPOAb/TgAb positivity is relatively low, and only two cases of ICPI-related GD have been reported. Furthermore, the *de novo* development of thyroid autoantibodies might be due to humoral response to the exposure of antigens caused by a destructive thyroiditis. Therefore, routine monitoring of TPOAb/TgAb has not been demonstrated to be helpful (Chang *et al.*, 2019).

CONCLUSIONS

Although AITD present with two pathogenetically and clinically different pathologies, the cellular and humoral immune responses implicated in HT and GD are closely connected and cross-linked. Despite comprehensive research in the last decades, the specific immunological mechanisms of the initiation and progression of thyroid autoimmunity remain unclear. Numerous recent studies have highlighted an essential pro-inflammatory role of a new subset of the Th cells, designated Th17, in the pathogenesis of AITD, thus changing the traditional paradigm of Th1/Th2 dichotomy. It has been found that Th17-lineage is heterogeneous, exhibiting under certain cytokine exposure two different cellular phenotypes: pathogenic and non-pathogenic. On the other hand, Treg cells are responsible for the maintenance of self-tolerance, thus protecting against thyroid autoimmunity. An altered balance between Th17 cells and Treg as well as Treg cell dysfunction has been found in AITD. Future studies highlighting the role of Th17 in autoimmune inflammation are highly required. Evaluation of different T cell subsets linked to certain cytokine production in AITD could help identify the immune mechanisms involved in thyroid autoimmunity, thus deepening our knowledge about disease outcome and treatment strategies. Many environmental factors can influence the development of AITD, including increased iodine intake, selenium deficiency and stress. The mechanisms of the interaction between genetic and environmental factors also deserve further investigation, which will have a significant impact on the prevention of AITD. Therefore, it is critical to investigate the possible interplay of environmental and genetic factors as well as to identify the possible mechanisms and immunomodulating effects of environmental factors involved in thyroid autoimmunity, to propose individualised AITD management strategies, since most environmental factors may be modified.

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AUTOIMŪNO VAIROGDZIEDZERA SLIMĪBU IMUNOLOĢISKIE MEHĀNISMI: TRADICIONĀLĀS TH1/TH2 PARADIGMAS MAIŅA

Autoimūnās vairogdziedzera slimības ietver Hasimoto tireoidītu (HT) un Greivsa slimību (GS), kuras raksturo asinīs cirkulējošas antivielas pret dažādiem vairogdziedzera autoantigēniem un vairogdziedzera audu infiltrācija ar autoreaktīviem limfocītiem. Lai gan pēdējo desmit gadu laikā sasniegts neizmērojams progress vairogdziedzera autoimūno slimību patoģenēzes izpratnē, tomēr detalizēts slimību attīstības imunoloģiskais mehānisms joprojām pilnībā nav skaidrs. Pieņemts uzskatīt, ka HT attīstību nosaka 1. tipa T līdzētājšūnas (Th1), bet GS attīstās pa Th2 šūnu determinēto autoimūnās reakcijas patoģenēzes ceļu. Šo tradicionālo pieņēmumu mainījuši pierādījumi par Th17 šūnu lomu vairogdziedzera autoimūno slimību attīstībā, galvenokārt HT patoģenēzē. Jāuzsver, ka attīstoties autoimūnajam procesam, izzīd līdzsvaru regulēto T šūnu un Th17 limfocītu starpā. Savos iepriekš veiktajos pētījumos GS un HT pacientu vairogdziedzera audos esam pierādījuši pastiprinātu IL-17 ekspresiju, kas ir Th17 šūnu galvenais efektorais citokīns. Šajā literatūras apskatā galvenokārt analizēta regulēto T un Th17 šūnu loma autoimūno vairogdziedzera slimību patoģenēzē. Tāpat apskatīta svarīgāko ārvaldību faktoru nozīme un mehānismi autoimūno slimību izcelsmē, kas ir svarīgi arī Latvijas kontekstā.

Ethical permission

Veidlapa Nr. E-9 (2)

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vairorgdziedzera autoimunitātes ierosināšanā un to izraisītu
izmaiņu morfoloģiskā izpēte”.

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Pētījuma protokols: Izskatot augstāk minētā pētījuma pieteikuma materiālus (protokolu) ir redzams, ka pētījuma mērķis tiek sasniegts veicot pacientu medicīniskās dokumentācijas izpēti, ievācot anamnēzes datus un objektīvā stāvokļa izvērtē, vairorgdziedzera audu operācijas materiāla analīzi, iegūto datu apstrādi un analīzi, kā arī izsakot priekšlikumus. Personu (pacientu, dalībnieku) datu aizsardzība, brīvprātīga informēta piekrišana piedalīties pētījumā un konfidencialitāte tiek nodrošināta. Līdz ar to pieteikums atbilst pētījuma ētikas prasībām.

Izskaidrošanas formulārs: ir

Piekrišana piedalīties pētījumā: ir

Komitejas lēmums: piekrist pētījumam

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