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Childhood Thyroid and Islet Autoimmunity

Immunogenetics, Risk Factors and Prediction

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DEPARTMENT OF CLINICAL SCIENCES | LUND UNIVERSITY



Childhood Thyroid and Islet Autoimmunity

Immunogenetics, Risk Factors and Prediction

Berglind Jónsdóttir



LUND
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DOCTORAL DISSERTATION

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<p>Abstract</p> <p>Aims: The overall objective was to investigate the prevalence of thyroid autoimmunity (TA; autoantibodies against thyroid peroxidase (TPOAb) and/or thyroglobulin (TGAb)) and the association with <i>HLA-DQ</i> genotypes, islet autoantibodies and thyroid function in newly diagnosed type 1 diabetes (T1D) children and in children followed for their increased risk for T1D in the Diabetes Prediction in Skåne (DiPiS) study. Furthermore, we wanted to investigate perinatal factors in relation to development of TA in children at genetic risk of T1D and predictive markers for thyroid disease in T1D patients.</p> <p>Methods: Blood samples from 2,433 children diagnosed with T1D were analyzed for TA, glutamic acid decarboxylase 65 (GADA), all three variants of zinc transporter 8 (ZnT8A), insulin (IAA), insulinoma associated protein-2 (IA-2A), <i>HLA-DQA1-B1</i> genotypes, thyroid stimulating hormone (TSH) and free thyroxine. Information on thyroxine prescription was gathered, 5.1-9.5 years after T1D diagnosis (Study I, II). Above mentioned autoantibodies as well as <i>HLA-DQ</i> genotypes were additionally analyzed in 1,874 10-year-old children in the DiPiS study. Prospectively collected samples from 2 years of age were analyzed for TA and confirming samples at age 11-16 in children TA positive at 10 years of age. Information on perinatal events and autoimmune hereditary was related to thyroid autoimmunity at age 10 (Study III, IV).</p> <p>Results: TA was found in 12% at T1D diagnosis and in 6.9% of children followed in the DiPiS study, more common in girls. TA was found from 2 years, increasing with age. The haplotype, <i>HLA DQ5.1</i> was found negatively related to TA as well as thyroxine prescription. At T1D diagnosis, TPOAb and TGAb were found related to GADA and ZnT8A. TPOAb were associated to GADA, ZnT8A and IA-2A and TGAb to ZnT8A in children followed for their increased risk, these relations were dependent on male gender. Apart from prematurity, perinatal events did not affect the risk of TA at 10 years of age. Father with rheumatic or thyroid disease and sibling or first-degree relative with celiac disease increase risk for TA at 10 years. TA, GADA and abnormal TSH at T1D diagnosis were found predictive for later thyroxine prescription.</p> <p>Conclusions: TA was common in children and adolescents at T1D diagnosis as well as in children followed for their increased T1D risk, more often found in girls. However, the gender related association between TA and islet autoimmunity in the DiPiS study indicates increased risk for boys with dependent concomitant autoimmune disease. The association between TA and GADA and ZnT8A may due to shared immunogenetic risk or environmental triggers. The <i>HLA DQ</i> relation were overall weak and therefore not suitable for screening, while <i>HLA DQ 5.1</i> was found protective. Children positive for TA in the DiPiS study were more likely to have first degree relative with autoimmune disease while perinatal events were generally not related to TA. TA, GADA and abnormal TSH at T1D diagnosis predict later thyroxine prescription and these parameters are therefore suggested for screening of children at T1D diagnosis.</p>			
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Childhood Thyroid and Islet Autoimmunity

Immunogenetics, Risk Factors and Prediction

Berglind Jónsdóttir



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To my family

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List of papers

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals and are reprinted with kind permission from Springer Science, Oxford Academic, and S Karger AG.

- I. **B. Jonsdottir**, C. Andersson, A. Carlsson, A. Delli, G. Forsander, J. Ludvigsson, C. Marcus, U. Samuelsson, E. Örtqvist, Å. Lernmark, S-A Ivarsson, and H. Elding Larsson for the BDD study group. Thyroid autoimmunity in relation to islet autoantibodies and HLA-DQ genotypes in newly diagnosed type 1 diabetes children and adolescents. *Diabetologia* (2013) 56:1735–1742.
- II. **B. Jonsdottir**, C. Larsson, A. Carlsson, G. Forsander, S-A. Ivarsson, Å. Lernmark, J. Ludvigsson, C. Marcus, U. Samuelsson, E Örtqvist, and H. Elding Larsson for the BDD study group. Thyroid and islet autoantibodies predict autoimmune thyroid disease at type 1 diabetes diagnosis. *J Clin Endocrinol Metab* (2017) 102:1277–1285.
- III. **B. Jonsdottir**, C. Larsson, M. Lundgren, I Jönsson, Å Lernmark, and H. Elding Larsson for the DiPiS study group. Childhood thyroid autoimmunity and relation to islet autoantibodies in children at risk for type 1 diabetes. Manuscript.
- IV. **B. Jonsdottir**, M. Larsson, S. Wallengren, Å. Lernmark, I Jönsson, and H. Elding Larsson. Are perinatal events risk factors for childhood thyroid autoimmunity? *European Thyroid Journal* (2017) 19/9, Epub ahead of print.

Abbreviations

APC	antigen-presenting cell
APS	autoimmune polyendocrine syndrome
BDD	Better Diabetes Diagnosis
CI	confidence interval
DASP	Diabetes Autoantibody Standardization Program
DBS	dried blood spots
DiPiS	Diabetes Prediction in Skåne
FDR	first-degree relative
F:M	female to male ratio
FT3	free triiodothyronine
FT4	free thyroxine
GAD65	glutamic acid decarboxylase 65
GADA	glutamic acid decarboxylase 65 autoantibodies
HbA _{1c}	glycosylated hemoglobin
HLA	human leukocyte antigen
IAA	insulin autoantibodies
IA-2	insulinoma-associated protein 2
IA-2A	insulinoma-associated protein 2 autoantibodies
MHC	major histocompatibility complex
OR	odds ratio
PCR	polymerase chain reaction
PTPN22	protein tyrosine phosphatase N 22 gene
RBA	radioligand binding assay
SNP	single-nucleotide polymorphism
T1D	type 1 diabetes
TA	thyroid autoimmunity, positivity to TPOAb or TGAb or both
TG	thyroglobulin
TGAb	thyroglobulin autoantibodies
Th1	T helper 1
Th2	T helper 2
TPO	thyroid peroxidase
TPOAb	thyroid peroxidase autoantibodies
TSH	thyroid-stimulating hormone
TSHr	thyroid-stimulating hormone receptor
WHO	World Health Organization
ZnT8RA	arginine 325 zinc transporter 8 autoantibodies
ZnT8WA	tryptophan 325 zinc transporter 8 autoantibodies
ZnT8QA	glutamine 325 zinc transporter 8 autoantibodies
ZnT8A	zinc transporter 8 autoantibodies to any of the amino acid variants at position 325

Abstract

Aims: The overall objective was to investigate the prevalence of thyroid autoimmunity (TA; autoantibodies against thyroid peroxidase (TPOAb) and/or thyroglobulin (TGAb)) and the association with *HLA-DQ* genotypes, islet autoantibodies and thyroid function in newly diagnosed type 1 diabetes (T1D) children and in children followed for their increased risk for T1D in the Diabetes Prediction in Skåne (DiPiS) study. Furthermore, we wanted to investigate perinatal factors in relation to development of TA in children at genetic risk of T1D and predictive markers for thyroid disease in T1D patients.

Methods: Blood samples from 2,433 children diagnosed with T1D were analyzed for TPOAb, TGAb, glutamic acid decarboxylase 65 (GADA), all three variants of zinc transporter 8 (ZnT8A), insulin (IAA), insulinoma associated protein-2 (IA-2A), *HLA-DQA1-B1* genotypes, thyroid stimulating hormone (TSH) and free thyroxine. Information on thyroxine prescription was gathered from the National Board of Health and Welfare's Prescribed Drug Register, 5.1-9.5 years after T1D diagnosis (Study I and II). Above mentioned autoantibodies as well as *HLA-DQ* genotypes were additionally analyzed in 1,874 10-year-old children in the DiPiS study. Prospectively collected samples from 2 years of age were analyzed for TA and confirming samples at age 11-16 in children TA positive at age 10 (Study III). Information on perinatal events and autoimmune hereditary was gathered from prospectively collected questionnaires and related to thyroid autoimmunity at age 10 (Study IV).

Results: TA was found in 12% of children at T1D diagnosis and in 6.9% of children followed for their increased T1D risk, more common in girls. TA was found from 2 years, increasing in titers and incidence with age. The haplotype, *HLA DQ5.1* was found negatively related to TA as well as thyroxine prescription. At T1D diagnosis, TPOAb and TGAb were found related to GADA and ZnT8A. TPOAb were associated to GADA, ZnT8A and IA-2A and TGAb to ZnT8A in children followed for their increased risk, these relations were dependent on male gender. Apart from prematurity, perinatal events did not affect the risk of TA at 10 years of age. Father with rheumatic or thyroid disease and sibling or first-degree relative with celiac disease increased the risk for TA at 10 years. TA, GADA and abnormal TSH at T1D diagnosis were found predictive for later thyroxine prescription, although the predicted value differs in different age groups.

Conclusions: TA was common in children and adolescents at T1D diagnosis as well as in children followed for their increased risk of T1D and more often found in girls. However, the gender related association between TA and islet autoimmunity in the DiPiS study indicates increased risk for boys with dependent concomitant autoimmune disease. The association between TA and GADA and ZnT8A may due to shared immunogenetic risk or environmental triggers. The *HLA DQ* relation were

overall weak and therefore not suitable for screening, while *HLA DQ 5.1* was found protective. Children positive for TA in the DiPiS study were more likely to have first degree relative with autoimmune disease while perinatal events were generally not related to TA.

TA, GADA and abnormal TSH at T1D diagnosis predict later thyroxine prescription, a surrogate marker for autoimmune hypothyroid disease, and these parameters are therefore suggested for screening of children at T1D diagnosis.

Background

Introduction

Autoimmunity is defined as an immune response against self-antigens, representing a significant cause of disease and estimated to affect 5% of the population in developed countries, where the prevalence of several autoimmune conditions is increasing^{1,2}. The main endocrine autoimmune disorders impacting children are autoimmune thyroid disease and type 1 diabetes, both of which are organ specific; it appears that having one of these disorders increases the risk for the other in individuals with genetic susceptibility. Although the disease pathology is undeniably under genetic control, it is highly likely that other factors also play a significant role in the disease progression. Environmental factors that are either introduced or removed presumably participate in this context.

Autoimmune thyroid disease is commonly divided into two opposing clinical phenotypes: hyperthyroidism, as seen in Graves' disease, and hypothyroidism, as occurs in Hashimoto's thyroiditis; the latter is far more common in childhood and is the phenotype of interest in this thesis. The term diabetes also includes many diseases, all of which are characterized by high blood glucose, although the pathogenesis can vary. Type 1 diabetes is the most common form of diabetes in childhood and the only type discussed in this thesis. Type 1 diabetes is often considered to be a childhood disease, and Hashimoto's thyroiditis is regarded as a disorder that mainly affects older women, although the two diseases can occur in both genders and in combination throughout life, because no cure is available.

Here, I summarize the main aspects of Hashimoto's thyroiditis and type 1 diabetes in children, along with the present knowledge concerning thyroid and islet autoimmunity, and the prediction, pathogenesis, and etiology of both diseases. In addition, I report new findings concerning relationships between thyroid and islet autoimmunity, as well as genotypes in subjects in two different investigations: children followed in the Diabetes Prediction in Skåne (DiPiS) study due to an increased risk of type 1 diabetes, and children with newly diagnosed type 1 diabetes evaluated in the Better Diabetes Diagnosis (BDD) study. In the latter study, prediction of thyroid autoimmunity and development of Hashimoto's thyroiditis in children and adolescents will be described. Lastly, the impact of perinatal factors and heredity on autoimmune diseases for development of childhood thyroid autoimmunity in 10-year-old children followed in the DiPiS Study will be evaluated

Hashimoto's thyroiditis and type 1 diabetes in children and adolescents

Hashimoto's thyroiditis in children

When the immune system attacks the thyroid (as described below), the lymphatic cells infiltrate the gland, which results in destruction and fibrosis that in turn leads to impaired thyroid hormone production and consequently also hypothyroidism. There is no clear definition of autoimmune hypothyroidism, although two clinical forms of the condition have been described ³. Hashimoto's thyroiditis with lymphocytic infiltration and goiter, and an atrophic form without goiter. In this thesis, Hashimoto's thyroiditis refers to autoimmune thyroiditis, with or without goiter.

Thyroid autoimmunity precedes and predicts clinical disease, but with that said, not all individuals with positive thyroid autoantibodies develop disease. Furthermore, there are different stages of the disease: Hashimoto's thyroiditis with subclinical hypothyroidism characterized by high thyroid stimulating hormone (TSH) and normal free thyroxine (FT4), and Hashimoto's thyroiditis with overt hypothyroidism characterized by high TSH and low FT4. Due to this lack of a valid disease definition, the criteria for delineating thyroid disease vary between studies. In this thesis, thyroid autoimmunity is defined as the presence of detectable autoantibodies against thyroid peroxidase (TPO) and/or thyroglobulin (TG) and Hashimoto's thyroiditis, when additional morphological changes can be seen in the thyroid gland, and/or thyroid function is impaired.

Disease prevalence

Prevalence rates in children and adolescents vary depending on which of the following study criteria is applied: thyroid autoimmunity only, subclinical hypothyroidism, overt hypothyroidism, or thyroxine treatment. The prevalence of thyroid autoimmunity in children aged 5–18 years in the general population is reported to be 0.4–4.6% ⁴⁻⁶. In a recent assessment ⁷, the prevalence of TPOAb in healthy 12-year-olds in Sweden was reported to be 2.8%. In other investigations, the prevalence of subclinical hypothyroidism in children and adolescents was noted to be around 2% ^{8,9} whereas the prevalence of overt disease was found to be 0.135% based on thyroxine prescription and hospital records ¹⁰ and 0.8% based on thyroid function test ⁹. Female predominance in Hashimoto's thyroiditis is well known, although the female-to-male ratio (F:M) differs significantly between different age groups, being high in adults (10.3:1) compared to children and adolescents (4.1–6.7:1), and even lower in prepubertal children (1.6:1) ¹¹⁻¹⁴.

Clinical presentation

The presentation of Hashimoto's thyroiditis in children and adolescents varies, because thyroid function at presentation can differ, ranging from a transient hyperthyroid phase to overt hypothyroidism. Screening for Hashimoto's thyroiditis is suggested in children diagnosed with other autoimmune diseases or related syndromes, considering that some patients can present without goiter or symptoms.

Goiter is the most common presenting symptom, found in two thirds of patients with Hashimoto's thyroiditis^{13,15}. Other systemic manifestations and symptoms vary due to the broad actions of thyroid hormone on several organs and tissues. Symptoms are often non-specific, such as fatigue, lethargy or impaired school performance, dry skin, edema, obstipation, cold intolerance, and menstrual irregularities.

Unique for children and adolescents are the age-dependent maturation effects of thyroid hormone on linear growth and puberty, as well as on brain development in the very young. Poor linear growth with delay of bone maturation can be the first symptom of hypothyroidism and can compromise adult height if undiagnosed. Children can also present with pubertal delay or, in the case of longstanding hypothyroidism, precocious puberty^{16,17} in the absence of accelerated bone maturation and linear growth. Although rare, the disease does occur in infants, resulting in severe effects on psychomotor development and growth if left untreated^{18,19}, which is why suspicion of Hashimoto's thyroiditis is also important in the very young.

Additional symptoms of long-standing hypothyroidism can be pericardial effusion and pituitary hyperplasia that resolve after initiation of treatment^{20,21}.

Also, as mentioned, children and adolescents can present with a toxic phase (toxic thyroiditis) that entails a biochemical hyperthyroidism with related symptoms, which is believed to be due to unregulated release of thyroid hormones induced by inflammatory destruction of the thyroid cells²². This condition is self-limiting and is often followed by hypothyroidism²³.

Diagnosis of autoimmune thyroid disease

Thyroid function is evaluated by laboratory analysis of serum TSH and FT4. Elevated TSH in combination with low FT4 is found in overt hypothyroidism, whereas slightly elevated TSH and normal FT4 indicate subclinical hypothyroidism. Measurement of FT3 is not necessary when diagnosing or monitoring hypothyroidism. High TSH levels lead to increased conversion of FT4 to FT3 and the preferential secretion of FT3 by residual thyroid tissue²⁴. Test results indicating elevated TSH should lead to measurement of thyroid autoantibodies.

Autoantibodies to thyroid peroxidase and thyroglobulin (TPOAb and TGAb) are predictive of thyroid disease (described below) and can be found in serum long before thyroid dysfunction occurs. A clear majority of patients are positive for

thyroid autoantibodies at diagnosis, and titers are also noted to be related to thyroid cell damage²⁵.

Thyroid ultrasonography is the imaging technique of choice for diagnosis of Hashimoto's thyroiditis in children and adolescents, because the thyroid gland is easy to locate due to its superficial position, and the procedure is neither time consuming nor painful. The highly indicative pattern in Hashimoto's thyroiditis is seen as decreased echogenicity in the thyroid as a result of reduced reflection of the ultrasound beam caused by lymphocytic infiltration²⁶. Such change in echogenicity in the gland is also observed in adults positive for thyroid autoantibodies but with normal thyroid function²⁷. Likewise, a correlation between hypoechogenic pattern on ultrasound and higher TSH levels is noted in euthyroid individuals²⁸. Obviously, the size of the thyroid can be determined more precisely by ultrasound, a technique that has been reported to be superior to palpation in diagnosing mild to moderate goiter in children and adolescents²⁹. In addition to being of value for diagnosis of Hashimoto's thyroiditis, ultrasound provides essential information that can aid differential diagnosis of other diseases.

Thyroid scintigraphy and fine needle aspiration. Unless there is suspicion of malignancy, thyroid scintigraphy and biopsy are rarely used in children and adolescents with goiter. However, scintigraphy can be indicated in children presenting with hyperthyroidism and negative for autoantibodies against thyroid-stimulating hormone receptor (TSHrAb), because radioiodine uptake is decreased rather than increased in true hyperthyroidism.

Treatment

Overt hypothyroidism. Treatment of hypothyroidism involves remedying the deficiency in thyroid hormone to improve symptoms and avoid adverse consequences. Thyroxine is the recommended treatment in children and adolescents, as well as adults, and the goal is to maintain serum TSH within the reference interval. In infants, special care must be taken because thyroid hormone is essential for neurological development, and therefore full-dose replacement is recommended at diagnosis. In older children and adolescents with long-standing hypothyroidism, some recommend using a graded approach in order to avoid side effects, whereas others favor estimation of dose by body weight^{30,31}. Thyroxine has a long half-life of 5–7 days, which ensures a gradual equilibration over 5–6 weeks, the dosing can subsequently be individualized based on biochemical monitoring.

Subclinical hypothyroidism. Whether or not subclinical hypothyroidism should be treated is a matter of debate. A child's age, general condition, and presence of other diseases are clearly of importance in follow-up and treatment decisions. Children positive for thyroid autoantibodies in combination with subclinical hypothyroidism are more likely to develop overt disease compared to those who are negative for such antibodies³²⁻³⁵. The European Thyroid Association recommends annual follow-up of children negative for thyroid autoantibodies but more frequent

follow-up of children positive for thyroid autoimmunity³⁶. The recommendations for children presenting with goiter in combination with subclinical hypothyroidism are discussed below.

Euthyroid with goiter. Goiter is a common presentation of Hashimoto's thyroiditis, and some of these patients are euthyroid. Thyroxine treatment to reduce goiter is now generally accepted, since studies of children and adolescents have confirmed that this approach can significantly decrease the size of the thyroid^{12,37}

Transient hyperthyroidism. As mentioned above, some children and adolescents with Hashimoto's thyroiditis present with hyperthyroidism and related symptoms. Such patients are positive for autoantibodies against TPO and/or TG but negative for autoantibodies to thyroid-stimulating hormone receptor (TSHr). Antithyroid drugs are rarely required, because in most cases the toxic phase resolves spontaneously over 8–14 months³⁸. Symptomatic treatment with beta blockade can be indicated in severe symptoms.

Type 1 diabetes in children

Autoimmune destruction of the pancreatic islet beta cells (discussed below) results in type 1 diabetes, which is one of the most common chronic diseases in childhood and adolescents, making serious demands on the patients, their families, and healthcare. The goals of treatment are to establish good metabolic control and well-being for these young patients and help them avoid complications later in life.

Incidence and prevalence

There is evidence that the incidence of type 1 diabetes is increasing in many parts of the world and also shifting to a younger age³⁹. Reports indicate that the incidence in Sweden has remained constant at a rate of 44–45/100,000 annually over the last 5 years⁴⁰. Globally, it is estimated that more than half a million children under the age of 15 years are living with type 1 diabetes⁴¹.

Clinical presentation

The symptoms of high blood sugar levels caused by insulin loss include abnormal thirst and dry mouth, frequent urination, nocturia, fatigue, sudden weight loss, and blurred vision. Affected individuals often seek healthcare a number of weeks after the onset of the mentioned symptoms. Other patients present with diabetic ketoacidosis, a potentially fatal condition that can entail diffuse symptoms such as nausea, vomiting, abdominal pain, and altered consciousness. Diabetic ketoacidosis is more common in children presenting at < 2 years of age⁴². Furthermore, a limited number of patients are diagnosed in a pre-symptomatic phase in studies of type 1 diabetes birth cohorts.

Diagnosis of type 1 diabetes.

Diabetes is diagnosed according to the following criteria stipulated by the World Health Organization (WHO) and the American Diabetes Association ⁴³:

1. classic symptoms of diabetes with plasma glucose concentration ≥ 11.1 mmol/L
OR
2. fasting plasma glucose ≥ 7.0 mmol/L
OR
3. plasma glucose ≥ 11.1 mmol/L 2 hours after glucose load in oral glucose tolerance test.

In the absence of classical symptoms, repeated testing is recommended on a subsequent day. The diagnosis of type 1 diabetes is further confirmed by analyzing islet autoantibodies, as described below in the section concerning islet autoimmunity

Treatment

Patients with type 1 diabetes are dependent on lifelong injections of exogenous insulin. There is no cure for this disease, and studies aimed at interfering with the autoimmune process that leads to type 1 diabetes have not yet been successful.

To date, there is no insulin regimen that can completely mimic the normal physiology. Nonetheless, enormous progress is being made in the development of insulin pumps and strategies for continuous glucose measurements, which can aid optimization of glucose control and help affected children avoid many injections per day. Moreover, recent advances and improvement in both management and regular screening have minimized the late complications of type 1 diabetes, including nephropathy, retinopathy, neuropathy, and macrovascular disease ⁴⁴. Achieving metabolic control is a demanding process, and children and adolescents with type 1 diabetes require regular follow-up with review of treatment, education, and support, and such assistance is best provided by a diabetes healthcare team.

Etiology and pathogenesis

It is assumed that children at genetic risk of type 1 diabetes and autoimmune thyroid disease are affected by an environmental insult that leads to autoimmunity against the target cell. This autoimmunity is easily monitored with autoantibodies, which serve as biomarkers of the pathogenesis. The pathogenic process (i.e., the autoimmune response discussed in this chapter) may be ongoing for months to years before clinical onset. The literature offers no clear definition of what factors are etiological triggers and what factors belong to the pathogenic process in autoimmunity. Hence it is possible that the pathogenic factors reported thus far are etiological factors and vice versa, as the view of autoimmune diseases is changing, suggesting the etiology to be related to an environmental insult in individuals born with susceptible genes as discussed below.

Autoimmunity

The human immune system is designed to protect against foreign pathogens such as viruses and bacteria. The adaptive immune system mounts attacks on specific target antigens recognized on antigen-presenting receptors displayed on antigen-presenting cells. A potential negative consequence of this process is the generation of self-reactive receptors capable of responding to self-antigens. Interference with the regulatory mechanisms designed to counter the response to self-antigens results in immunity to self. The following section presents a short introduction of central players and genes in the immune response to self-antigen.

Auto reactivity

Environmental stimuli such as infections can cause cell damage and thereby liberate intracellular antigens. Antigen-presenting cells (APCs) are B lymphocytes, dendritic cells, or macrophages that phagocytose and process the antigens into peptides, which are subsequently presented together with a major histocompatibility complex (MHC) (Figure 1). The antigen complex is recognized by a T cell receptor on a CD4+ T helper cell, and thereafter the helper cell may differentiate into different subsets of effector cells, depending on co-stimulation by APCs at antigen presentation. Two subsets of T helper cells were originally described, which are designated T helper 1 (Th1) and T helper 2 (Th2) cells. Th1 cells mainly produce interferon gamma along with IL-2 and TNF-alfa⁴⁵, which stimulate phagocyte-mediated ingestion and killing of microbes by cytotoxic CD8+ cells, a process that is a key component of cell-mediated immunity. Th2 cells produce IL-4, IL-5, IL-10, IL-13⁴⁵ and other cytokines that stimulate B cells and the humoral mediated immunity with production of antibodies. A third subset of T helper 17 cells, denoted

Th17 cells were later identified, which produce the inflammatory cytokines IL-17, IL-22, and IL-23 that in turn synergize other pro-inflammatory cytokines ⁴⁶. Nevertheless, it is important to note that there is considerable plasticity in these subpopulations, so that one subset can differentiate into another, and likewise cytokines can be produced by cells other than T cells.

Tolerance

Immune tolerance is divided into central and peripheral tolerance. Central tolerance is established during lymphocyte development in the thymus for T cells and in bone marrow for B cells, whereas peripheral tolerance takes place in the lymph nodes and peripheral tissues. In central T cell tolerance, immature lymphocytes that react to self-antigens presented on MHC II molecules in the thymus are eliminated by apoptosis. The same applies to B lymphocytes that react to self-antigens in the bone marrow. Peripheral tolerance is necessary to prevent T cell response to self-antigen that is not presented in the thymus, or when the central tolerance is incomplete. The regulation of co-stimulators is thought to be a significant factor in peripheral tolerance. The differentiation and proliferation of T cells are normally activated by two signals: the first being the antigen presented on the APC, and the second provided by a variety of co-stimulators on the APC ⁴⁷ (Figure 1). Important co-stimulators are cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and CD28, which, respectively, downregulate and stimulate T cell activation. Both these co-stimulators play an important role in the presentation of self-antigens.

Additional participants in peripheral tolerance are the regulatory T cells that develop in both the thymus and the peripheral lymphoid organs and suppress the immune response with cytokines or expression of CTLA-4. The mature B cells that recognize a self-antigen and reach the peripheral are inactivated in a similar manner, that is, by not receiving stimulation from T cells, apoptosis, or expression of inhibitory receptors.

Defects in central and/or peripheral immune tolerance constitute the hallmark of autoimmunity.

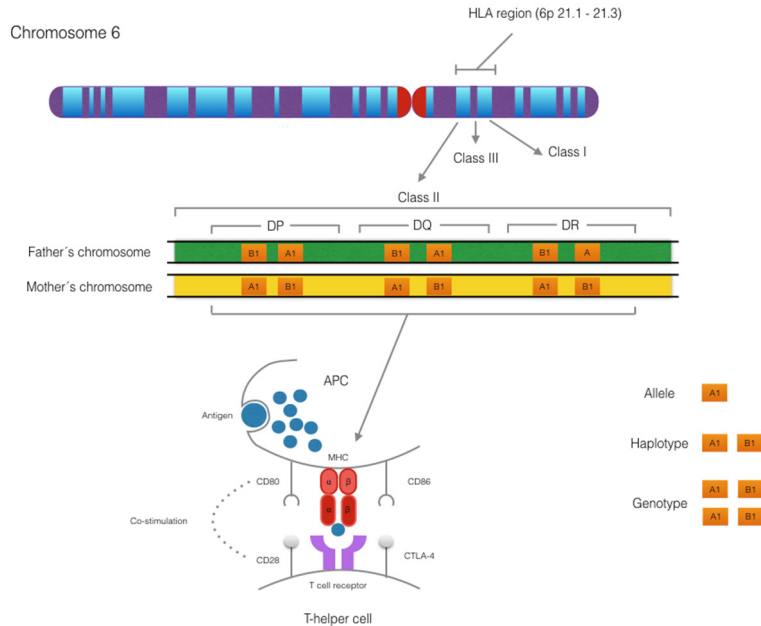


Figure 1. HLA genes and antigen presentation.

Schematic illustration of chromosome 6, the HLA class II heterodimer encoded by HLA DR-DQ and DP genes, and the antigen presentation. An antigen-presenting cell (APC) phagocytoses the self-antigen and presents it together with the HLA class II molecule to a T helper cell. The T helper cell recognition of and response to the antigen are dependent on co-stimulators found on the surface of both the APC and the T helper cell.

Genetic susceptibility

Inherited risk for most autoimmune diseases is attributable to multiple gene loci, with the largest contribution made by human leukocyte antigen (**HLA**) genes on chromosome 6. The above-mentioned MHC molecule is encoded by HLA genes. There are different classes of HLA heterodimers that encode different classes of MHC molecules: class I molecules are present on all cells, and class II molecules are expressed on dendritic cells, B cells, macrophages, and activated T cells. The HLA class II heterodimer is encoded by three different genes inherited from both parents: *HLA-DR*, *HLA-DQ*, and *HLA-DP*, each consisting of an alpha and a beta chain⁴⁸ (Figure 1). One HLA genotype has two haplotypes, one inherited from each parent, whereas one haplotype consists of two alleles. Relative risk of a disease can be reported for genotypes, haplotypes, or alleles. Discovery of the association between HLA alleles and autoimmune diseases was the first indication of the significance of T cells in autoimmune disease, because the only known function of the MHC molecule is the presentation of antigens to T cells⁴⁹. Although the incidence of an autoimmune disease or a preceding autoimmunity is often greater among individuals who inherit a particular allele, it is essential to point out that having a risk HLA allele for a certain disease does not automatically mean that an

individual will develop the disorder in question. HLA genes and the MHC molecule represent an example of factors for which there is disagreement in the literature; regarding the role of HLA as a predisposing factor only or as a factor of etiology or pathogenesis. The results obtained in the Environmental Determinants of Diabetes in the Young (TEDDY) study indicate that HLA association is involved in the etiology of type 1 diabetes since the HLA type determines the fate of the autoimmune reaction against a cell or organ^{50,51}.

Polymorphism in non-HLA genes is associated with various autoimmune diseases and may contribute to failure of self-tolerance or abnormal activation of lymphocytes. Central in this context are proteins encoded by numerous loci, such as protein tyrosine phosphatase non-receptor type 22 (PTPN22), CTLA-4, and CD28. **PTPN22** limits activation of naïve and effector T cells and the development of memory T cells in response to low affinity antigens (e.g., self-antigens)⁵². The mechanism of **CTLA-4** action is not fully understood, although it is known that this protein has a vital function on the surface of both T regulatory cells and other T cells, because it is essential in the recognition of antigens and, as mentioned, is central in peripheral tolerance. The CTLA-4 protein is thought to directly antagonize the co-stimulation of **CD28** and regulates the access of CD28⁵³. As stated, CD28 is a co-stimulator that is important in proliferation and influences the effector function of T regulatory cells⁵⁴. Manipulation of the genes encoding these proteins in animal models has led to discovery of their significance in the immune response and revealed that the polymorphism of these genes makes them risk alleles in the heritability of human autoimmunity. Various other genes and proteins are certainly involved in the autoimmune process, although they are not discussed here.

Autoimmunity does not necessarily lead to disease. Indeed, the factors regulating progression have not yet been identified, although various hypotheses have been proposed in attempts to explain different autoimmune diseases.

Endocrine autoimmunity

The endocrine system is often targeted by the immune system, and progressive destruction of the affected organ can lead to disease. The shared genetic risk in autoimmune endocrine disorders is apparent, when considering that the diseases tend to cluster in families or in the same patient, then referred to as autoimmune polyendocrine syndromes (APSs). The following classification of APSs was suggested nearly 40 years ago⁵⁵.

Type 1- chronic candidiasis, chronic hypoparathyroidism, Addison's disease (*at least two present*).

Type 2- Addison's disease (*obligate*), autoimmune thyroid disease and/or type 1 diabetes.

Type 3- autoimmune thyroid disease associated with other autoimmune diseases (*although not Addison's disease or hypoparathyroidism*).

Type 4- combination of organ-specific autoimmune disease not included in previous groups ⁵⁶.

Over the years, many modifications of this classification have been reported in which APS types 1 and 2 are most consistently referred to as syndromes. Studies of the immunological basis of these syndromes have provided fundamental information on immune regulation and autoimmunity. As an example, APS 1 is a rare monogenetic disorder in which the defective gene (autoimmune regulator [AIRE]) encodes a transcription factor required for the expression and presentation of self-antigens to lymphocytes in the thymus ⁵⁷. This results in failure to delete autoreactive T cells within the thymus, leading to widespread multi-organ autoimmunity.

Clearly the immune system can affect most endocrine glands and thus lead to autoimmune disease. Irrespective of the affected gland, this process can be divided into stages beginning with genetic susceptibility, followed by environmental factors triggering active autoimmunity that leads to metabolic derangements with evident symptoms of disease.

A variant of APS 3 comprises the two most common autoimmune endocrine disorders, autoimmune thyroid disease and type 1 diabetes, occurring both separately and in combination.

Thyroid autoimmunity

The thyroid gland consists of follicular and parafollicular cells. The follicular cells, or the thyroid cells, gather around a core of colloid that consists primarily of precursors of thyroid hormone, the main product of the follicles (Figure 2).

As in other autoimmune responses, a trigger leads to thyroid cell damage that results in the appearance of thyroid autoantigens, and this represents the onset of autoreactivity. Once established, the pathogenesis causes an immune-mediated killing of the thyroid cells, which may lead to disease involving alteration of thyroid function (i.e., either hypo- or hyperthyroidism). The appearance of thyroid antigen leads to the accumulation of APCs in the thyroid, which later migrate to the lymphoid organs where the fragmented autoantigen on the MHC molecule is presented to Th-cells. If, as described above, self-tolerance is lost, the T cells activate and infiltrate the thyroid along with B lymphocytes. Measurements of cytokines in patients with the two phenotypes of autoimmune thyroid disease, Hashimoto's thyroiditis and Graves' disease, have revealed the action of both cellular and humoral immune responses, and that different responses predominate in different diseases ⁵⁸. In Hashimoto's thyroiditis, it has been suggested that the

Th1 immune response dominates, causing activation of cytotoxic cells that results in progressive destruction of most follicles and fibrosis leading to glandular atrophy and hypothyroidism. On the other hand, the Th2 immune response prevails in Graves' disease, with production of autoantibodies that bind to and stimulate the receptors on follicular cell membranes and thereby alter the function of the follicular cells. Although it is tempting to dichotomize the response in this simple way, it does not explain the number of autoantibodies involved in Hashimoto's thyroiditis. Correspondingly, analysis of thyroid tissue in autoimmune thyroid disease has not demonstrated the presence of dominating Th1 or Th2 cells in different phenotypes of the disorder⁵⁹.

The main thyroid autoantigens are thyroid peroxidase (TPO), thyroglobulin (TG), and TSHr. Additional thyroid antigens include the sodium iodide symporter (NIS) and pendrin (Figure 2). There are currently no assays available in clinical practice that can measure the cell-mediated autoimmune response described above, although analysis of autoantibodies is widely used.

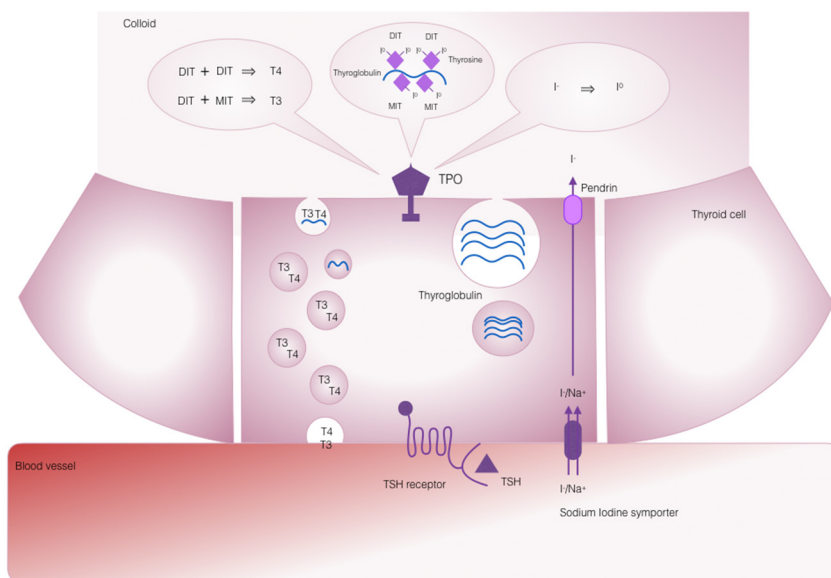


Figure 2. Thyroid cell antigens.

Schematic illustration of the thyroid antigens and their functions in thyroid hormone production.

TPO, thyroid peroxidase; TG, thyroglobulin; TSHr, thyroid hormone receptor; TSH, thyroid-stimulating hormone; T₃, triiodothyronine; T₄, thyroxine; MIT, mono-iodinated tyrosine; DIT, di-iodinated tyrosine.

TPO and TPOAb

In 1985, TPO was identified as the microsomal antigen involved in autoimmune thyroid disease⁶⁰. TPO plays an important role in thyroid hormone synthesis by catalyzing the oxidation of iodine, the iodination of tyrosines on TG, and the

coupling of iodotyrosines to form the hormones T4 and T3 (Figure 2). TPO is a 105–110-kDa membrane-bound protein that is localized to the apical surface of the follicular cell with a large extracellular domain and a short transmembrane and intracellular domain (Figure 2). Stimulation of TSH increases the amount of TPO transported to the apical membrane of a follicular cell ⁶¹. TPO autoantibodies (TPOAb) belong predominantly to the IgG class and are produced mainly by lymphocytic infiltrate in the thyroid gland ⁶², and they can interact with different epitopes in the restricted immunodominant region on TPO ⁶³, hence, the TPOAb are heterogeneous and may block the TPO enzyme activity in different ways. Studies also indicate that TPOAb are directly toxic to follicular cells through complement fixing ⁶⁴ or an antibody-dependent cytotoxic process ⁶⁵. Moreover, TPOAb titers are correlated with the production of cytokines that enhance cytotoxic immunity, which reflects a high level of disease activity ⁶⁶. The human TPO gene is located on chromosome 2.

TG and TGAb

TG is a 670-kD protein that is produced by follicular cells and constitutes the major component of the thyroid colloid (Figure 2). TG plays an important role in thyroid hormone synthesis. As described above, tyrosines attached to TG are iodized and coupled to yield T4 and T3, and then transported from the colloid into the follicular cell, where the hormones are released from TG and finally into the circulation upon TSH stimulation (Figure 2). The human gene that encodes TG is located on chromosome 8.

Autoantibodies against TG (TGAb) were first described in 1956 ⁶⁷. Like TPOAb, TGAb are generally IgG class autoantibodies that bind to various epitopes in numerous immunodominant regions on TG. The epitopes on TG are assumed to be heterogenic, because the TG molecule naturally changes configuration upon iodination. TGAb are not believed to be complement fixing or cytotoxic, and the role of TGAb in autoimmune thyroid disease have not yet been fully elucidated.

TSHr and TSHrAb

TSHr is a G-protein-coupled receptor with seven transmembrane units localized to the follicular basolateral cell membrane (Figure 2). TSH binds to TSHr and stimulates various thyroid functions, such as iodine uptake and production, and the release of iodothyronines from the gland.

There are two types of autoantibodies to TSHr (TSHrAb): the more common TSHr-stimulating autoantibody (TSAb) and the TSHr-blocking autoantibody (TBAb). TSAb is the hallmark of Graves' disease, and it binds to TSHr and stimulates thyroid function, which results in hyperthyroidism. In contrast, TBAb binds to and blocks the receptor for TSH, which leads to hypothyroidism. Both types of TSHrAb are IgG class autoantibodies that bind to epitopes across the TSH binding pocket, and in animal models it has been shown that some of these epitopes

are shared by stimulating and blocking antibodies ⁶⁸. Both the stimulating and blocking autoantibodies can be found in the same patient, resulting in a switch from hyperthyroidism to hypothyroidism and vice versa ⁶⁹.

Unlike TGAb and TPOAb, TSHrAb have a clearly pathological effect.

NIS and Pendrin

NIS is a glycoprotein that is located on the basolateral membrane of a follicular cell and transports iodine from the bloodstream into the cell (Figure 2). This protein is also found in the pancreas, breast, gastric/colonic mucosa, thymus, and salivary/lacrimal glands. Autoantibodies against NIS have been found in 22% of patients with Graves' disease and 24% of patients with Hashimoto's thyroiditis ⁷⁰.

The protein pendrin is located in the apical end of a follicular cell, where it mediates the efflux of iodine from the cell into the colloid. In one study ⁷¹, autoantibodies against pendrin were found in 81% of patients with autoimmune thyroid disease compared to 3% of controls, and these autoantibodies were more common in Hashimoto's thyroiditis than Graves' disease. Autoantibodies against pendrin and NIS are not currently used in clinical practice.

Islet autoimmunity

The endocrine part of the pancreas consists of the pancreatic islets, which contain clusters of endocrine cells that produce glucagon, somatostatin, pancreatic polypeptides, and insulin. The beta cells in the pancreatic islets express insulin, a critical hormone that regulates the metabolism of carbohydrates, fat, and proteins. Autoimmune destruction of the beta cells leads to a gradual loss of insulin production that results in type 1 diabetes. The detailed mechanism of the autoimmune response in type 1 diabetes, is not completely clear but is assumed to be mediated primarily by T cells. Studies of pancreas samples from patients with newly diagnosed type 1 diabetes and donors with this disease have demonstrated infiltration of predominantly CD8+ and CD4+ T cells, as well as B lymphocytes and macrophages, although not in all subjects ^{72,73}.

One hypothesis is that islet antigens available after beta cell damage are taken up by dendritic cells, which subsequently expose the antigens on MHC class II molecules on the cell surface. This complex is then recognized by CD4+ T cells that initiate the autoimmune response by activating CD8+ T cells, macrophages, and B lymphocytes, which in turn produce autoantibodies against pancreatic islet antigens, a detectable sign of the autoimmune process ⁷⁴.

Autoantibodies to pancreatic islet cells were first identified over 40 years ago in patients suffering from diabetes and other coexisting endocrine disorders, and were given the name islet cell autoantibodies (ICA) ⁷⁵. Four decades later, a heterogeneous group of islet autoantibodies was also recognized, which includes

those directed against glutamic acid decarboxylase 65 (GADA), insulinoma-associated protein 2 (IA-2A), insulin (IAA), zinc transporter 8 (ZnT8A), and presumably additional unknown autoantibodies. To date, 95% of children with type 1 diabetes are positive for at least one of the mentioned islet autoantibodies at the time of diagnosis ⁷⁶. Although extremely useful for predicting type 1 diabetes, as described below, it is believed that the islet autoantibodies do not contribute directly to beta cell damage.

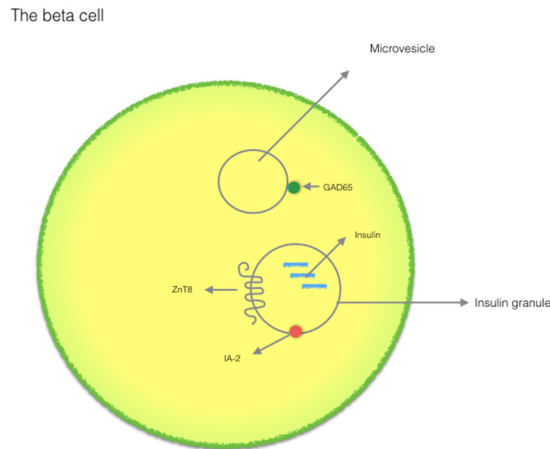


Figure 3. Beta cell antigens

Insulin is found inside the insulin secretory granule. ZnT8 is a transmembrane protein with six transmembrane regions. IA-2 is a single transmembrane protein in the secretory granule. GAD65 is a protein attached to membranes of microvesicles in beta cells.

GAD65 and GADA

Glutamic acid decarboxylase 65 (GAD65) is a 585-amino-acid enzyme that is encoded on chromosome 10p11^{77,78}, and is involved in the synthesis of gamma-aminobutyric acid (GABA), which is an inhibitory neurotransmitter found in neurons and the pancreas. GAD65 is attached to membranes of synaptic vesicles in neurons and microvesicles in beta cells, where the function of this enzyme is not entirely understood (Figure 3).

Autoantibodies against GAD (GADA) were identified 35 years ago ⁷⁹, and they are mainly IgG1 class autoantibodies that are directed toward middle and C-terminal epitopes on GAD65 ⁸⁰. GADA are found in 63–79% of type 1 diabetes patients at clinical onset of the disease ^{81,82}, and data indicate a positive correlation with age and an association with *HLA DQ2* ⁸³.

IA-2 and IA-2A

Insulinoma-associated protein 2 (IA-2) is a 979-amino-acid that is member of the protein tyrosine phosphatase (PTP) family and was first described in 1994⁸⁴. IA-2 is encoded on chromosome 2q35 and expressed in the membrane of the secretory granules (Figure 3). This protein is also found in the alpha cells of the pancreas and in other neuroendocrine cells, such as those in the pituitary and adrenal cortex. No enzymatic activity of IA-2 has been described, and the role of the protein is still unclear. Autoantibodies against IA-2 (designated IA-2A) belong mainly to IgG1 subclass⁸⁵, and have been shown to recognize primarily the juxtamembrane and PTP-like epitopes in the intracellular domain of the IA-2 molecule⁸⁶. IA-2A are detected in 85% of children and adolescents at diagnosis of type 1 diabetes⁸⁷. An IA-2A most often appears in combination with another islet autoantibody and seldom alone, which explains why it is often regarded as a marker of extensive beta cell destruction. High frequency and levels of IA-2A have been found to be associated with *HLA DQ8* genotype in patients with newly diagnosed type 1 diabetes⁸⁷.

Insulin and IAA

Insulin is a small 51-amino-acid protein that is encoded on chromosome 11q15, often considered to be the primary antigen in type 1 diabetes. The precursor of insulin is called proinsulin. The mature insulin molecule is formed after cleavage of the C-peptide fragment of proinsulin, and at that time consists of an alpha and a beta chain. C-peptide is a valuable marker of remaining insulin production.

Insulin autoantibodies (IAA) are IgG autoantibodies, and different subclasses of which have been reported to be involved in the progression to disease⁸⁸. IAA were first described in 1983 in patients with type 1 diabetes who had not yet begun exogenous insulin treatment⁸⁹. The epitopes recognized on the antigen have not been clearly defined, although the affinity of the autoantibodies is for regions 8–13 on the alpha chain and regions 1–3 on the beta chain⁹⁰. Autoantibodies to proinsulin have also been described in patients with newly diagnosed type 1 diabetes prior to start of insulin treatment, and found to recognize the C-peptide part of the proinsulin molecule⁹¹.

IAA are fairly age related, with a high frequency in children diagnosed with type 1 diabetes at a young age, and they are frequently identified as the first autoantibodies appearing in children in birth cohort studies, with a peak between age 6 months and 2 years⁹²⁻⁹⁴. The IAA have been observed to be associated with *HLA DQ8/DR4* genotypes⁹⁵.

ZnT8 and ZnT8A

Zinc transporter 8 (ZnT8) is the most recently discovered antigen in type 1 diabetes. This 369-amino-acid protein is located in the membrane of the secretory vesicles in

beta cells and is encoded by the SLC30A8 gene on chromosome 8q24.11 ⁹⁶. The SLC30A8 gene codes for three different amino acid variants of ZnT8: ZnT8R (arginine), ZnT8W (tryptophan), and ZnT8Q (glutamine). In contrast to GAD65 and IA-2, the function of ZnT8 and zinc is understood rather well. ZnT8 catalyzes transport of the zinc ion into the insulin granule, where zinc is essential for the processing, storage, secretion, and action of insulin ⁹⁷. ZnT8 is expressed not only in the beta cells, but also in the pancreatic alpha cells, peripheral blood lymphocytes, subcutaneous fat tissue, cells in the adrenal cortex, and the cubical follicular cells ⁹⁸. The function of ZnT8 in other endocrine cells is not as well elucidated as in the beta cells, although clarification of this aspect may facilitate understanding of the association between different autoimmune endocrine disorders.

ZnT8A recognizes epitopes on the N and C terminus of the antigen and is found in 60–80 % of patients at diagnosis of type 1 diabetes ⁹⁹. The addition of ZnT8A has significantly reduced the number of antibody negative individuals at type 1 diabetes diagnosis from 7.5–5.4 % ⁷⁶, although it has also been found that levels of these autoantibodies rapidly decline after the diagnosis ¹⁰⁰. In one investigation ¹⁰¹, it was noted that ZnT8A was unrelated to age in a pediatric population, with the exception of seldom appearing in children < 2 years of age. The same study also showed that ZnT8A was positively associated with *HLA DQ 6.4* and *DQ8*, but negatively associated with *HLA DQ2*.

Thyroid and islet autoantibodies as disease predictors: natural history

Autoimmune markers are highly valuable for clinical diagnosis of autoimmune diseases. Thyroid and islet autoantibodies are also useful for predicting such disease, particularly in risk populations. In clinical practice, screening for thyroid autoantibodies is often recommended in patients with an additional autoimmune disease, as well as in children with Down's and Turner's syndrome. It can be difficult to compare the results of investigations focused on prediction of thyroid versus islet autoantibodies. Elucidation of the natural history and thereby also the prediction of islet autoantibodies has made it possible to determine these proteins in a number of prospective birth cohort studies. Thus far, the natural history and the prediction of thyroid autoantibodies have been analyzed mainly in adults. Comparable investigations of these aspects in children and adolescents are often limited by both follow-up time and the number of patients.

The Wickham Survey is a large prospective study of thyroid autoimmunity in which thyroid autoantibodies, thyroid function, and goiter were first assessed in 2,779 randomly selected adult individuals and then re-evaluated in the same subjects

20 years later^{102,103}. At the 20-year follow-up, 55% of the women who had originally been found to be positive for thyroid autoantibodies and have elevated TSH, and 27% of those who had initially been positive for thyroid autoantibodies but shown normal TSH had developed Hashimoto's thyroiditis¹⁰³. Similarly in other studies of adults, TPOAb were found to be predictive of Hashimoto's thyroiditis in patients with type 1 diabetes, in relatives of patients with autoimmune thyroid disease, and of post-partum thyroid disorder in pregnant women¹⁰⁴⁻¹⁰⁶. The TPOAb titers have been reported not only to predict the risk of later disease, but also to be correlated with TSH in euthyroid subjects^{25,107}. However, the existence of TPOAb positivity in euthyroid individuals is a matter of debate, because it seems that all such individuals do not develop thyroid disease. Different epitopes on thyroid peroxidase (TPO) have been described in euthyroid subjects and those diagnosed with autoimmune thyroid disease, which supports the existence of a difference in the autoimmune response¹⁰⁸. Although autoantibodies to TG are found in most patients with hypothyroidism, the value of these proteins in predicting later autoimmune thyroid disease in euthyroid individuals has not been established¹⁰⁷.

Prospective studies assessing children and adolescents are primarily aimed at evaluating the spontaneous course of subclinical hypothyroidism (TSH above the normal range and normal FT4), and hence unfortunately do not always analyze thyroid autoantibodies. A recent investigation of subclinical hypothyroidism in girls compared the progression to overt disease in those positive and those negative for thyroid autoantibodies, and the results after 5 years showed that progression to disease occurred in 63.5% of the girls positive for thyroid autoantibodies but in 23.8% of those who were thyroid autoantibody negative³². In another study of children positive for thyroid autoantibodies and with normal or slightly elevated TSH¹⁰⁹, it was observed that 28% had higher TSH levels after 3 years, and 14% of those subjects required treatment. Also, a very similar assessment of children with subclinical hypothyroidism and thyroid autoantibodies found the risk of deterioration in thyroid status to be 53% over a period of 2 years³³. Some assessments have indicated that most children with subclinical hypothyroidism but no thyroid autoimmunity have normal TSH on follow-up^{34,35}. Comparisons of children and adults regarding prediction of thyroid autoimmunity suggest that such autoimmunity detected in childhood progresses more often to overt disease in children than in adults.

The serological markers of islet autoimmunity are of great value, not only as a diagnostic tool for distinguishing different types of diabetes, but also for screening of high-risk populations and to enable research in prevention studies. Prospective longitudinal investigations, mainly in children with genetic susceptibility, make it possible to evaluate the chronological appearance of islet autoantibodies and the eventual loss of beta cell function. Such analyses have revealed that maternal autoantibodies tend to fade slowly in a child during the first 6 months after birth, and that the first islet autoantibodies may appear as early as at age 6–9 months and

peak during the first 2 years ^{88,94,110}. As mentioned, IAA usually peaks as the first autoantibody in children younger than 2 years, whereas GADA appearing as the first autoantibody peaks a few years later, and thereafter the rate of seroconversion declines ^{94,111}. IA-2A seldom occurs as a first autoantibody, and the progression to disease is rapid in cases in which IA-2A appears early ¹¹¹. Likewise, ZnT8A is seldom the first autoantibody ¹¹². The significance of multiple islet autoantibodies for prediction of type 1 diabetes became quite clear when these factors were analyzed together in three birth cohorts in Finland, Germany, and the United States ¹¹³. Of the 13,377 children assessed in the cited study, 4.4% developed multiple islet autoantibodies, and, among those subjects, type 1 diabetes had developed in 44% over the next 5 years, in 70% within 10 years, and in 84% after 15 years of follow-up. The prodromal period from appearance of islet autoantibodies to clinical disease is clearly individual, although an average time of 30–36 months has been reported in children younger than 10 years ⁸⁸. Follow-up assessment of numerous children from birth to clinical disease has led to a new staging system for type 1 diabetes that includes two stages before the classical diagnosis of type 1 diabetes. Stage 1 comprises individuals that have developed two or more islet autoantibodies but are normoglycemic and without symptoms. Individuals in stage 2 have developed dysglycemia due to beta cell loss but are asymptomatic. The final stage is the classical diagnosis of type 1 diabetes including hyperglycemia and symptoms ¹¹⁴. This novel staging system is valuable for use in future intervention and prevention studies and to help clinicians develop standardized treatment for individuals that are now diagnosed with prediabetes.

Although the progression from autoimmunity to disease, considering both the risk and the time frame of developing the disease, is not as well established for Hashimoto's thyroiditis as for type 1 diabetes, there are similarities as suggested in the schematic diagram in Figure 4. The term prediabetes is now recognized, and similarly the designation prethyroiditis is suggested here, with the main difference between the two being that individuals positive for thyroid autoantibodies, TPOAb, and/or TGAb in stage 1 do not necessarily progress to stage 2. Correspondingly, all individuals in stage 2 do not progress to stage 3 (i.e., overt disease) as in type 1 diabetes. Moreover, it is proposed that subclinical hypothyroidism occurs between stages 2 and 3 in individuals with detectable autoantibodies, change in thyroid morphology and function, but no symptoms.

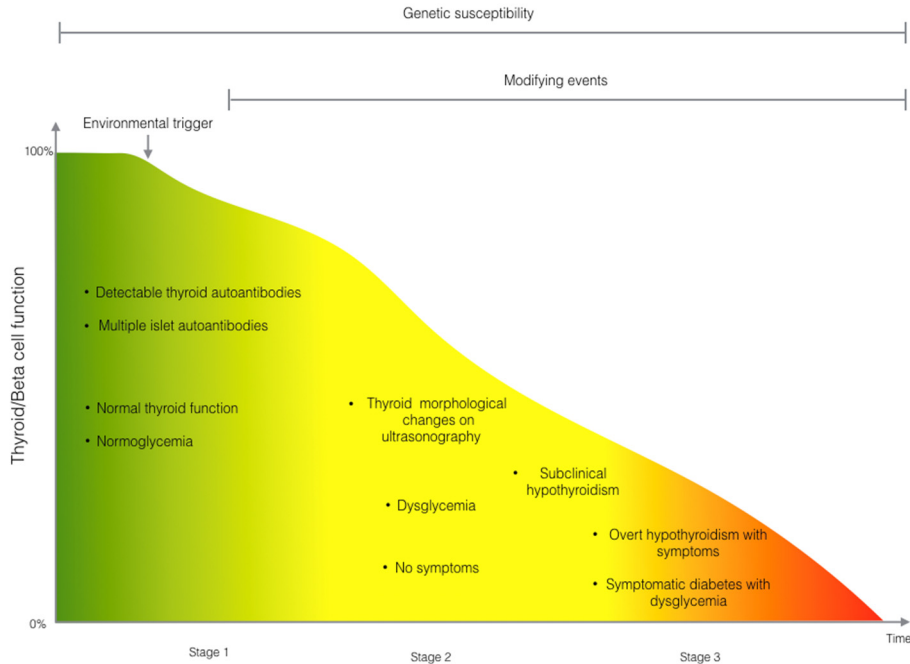


Figure 4.

Different stages of autoimmune Hashimoto's thyroiditis and type 1 diabetes

In this model of the disease progression in Hashimoto's thyroiditis and type 1 diabetes, subclinical hypothyroidism due to Hashimoto's thyroiditis is suggested between stages 2 and 3, with detectable thyroid autoimmunity and morphological changes on ultrasound but no symptoms. It is important to note that progression from autoimmunity to disease, considering both the risk of and the time to disease, is not as well established for autoimmune thyroid disease as for type 1 diabetes.

Predisposing factors: genes and environment

Genes

Hashimoto's thyroiditis

The hereditary component of autoimmune thyroid disease is apparent, because the disease and the preceding thyroid autoimmunity accumulate in families. The comparison of concordance rates between monozygotic and dizygotic twins provides a clear indication of a genetic component for which biometric twin modeling has revealed that 75% of the total phenotypic variance in autoimmune thyroid disease is caused by the effects of genes¹¹⁵. The identified susceptibility genes in autoimmune thyroid disease can be divided into immune-modulating genes

and thyroid-specific genes. Results are often presented separately for Hashimoto's thyroiditis and Graves' disease, because the genetic phenotypes appear to differ, being less defined for the former disorder, possibly due to difficulties in achieving a correct disease description.

Immune-modulating genes encode proteins involved in the immunological synapse, which is the interface between the MHC molecule on APC and T cell receptors on a T helper cell, as shown in Figure 1. **HLA** class II genes are among the first genes identified as entailing an increased risk of autoimmunity in general, including thyroid autoimmunity. Reports concerning high-risk HLA genotypes, haplotypes, or alleles in Hashimoto's thyroiditis vary and lack replication. The haplotype *HLA-DR3* has been identified as being positively related to Hashimoto's thyroiditis, whereas other investigations have shown the haplotypes *DR4* and *DRB1*04-DQB1*0301* to be associated with that disease¹¹⁶⁻¹¹⁸. Studies assessing HLA have to some extent shifted to pinpoint the amino acids in the pocket-binding domain on the MHC antigen-presenting molecule, and have shown that the *HLA-DR* amino acids Tyr-26, Tyr-30, Gln-70, and Lys-71 are strongly associated with Hashimoto's thyroiditis¹¹⁹. The proteins encoded by the **CTLA-4** gene and the **PTPN22** gene are negative T-cell regulators in the antigen presentation response and are related to several autoimmune diseases^{120,121}. Other studies have also revealed that polymorphisms of the CTLA-4 and PTPN22 genes are positively related to Hashimoto's thyroiditis¹²²⁻¹²⁴. Insofar as there are also **CD28** genes in the CTLA-4 gene region, it has been assumed that this region plays an important part in the genetic susceptibility to autoimmune thyroid disease. However, mapping of the genes in this region in patients with autoimmune thyroid disease and their families showed that only the CTLA-4 gene (i.e., not the CD28 genes) was related to autoimmune thyroid disease and thyroid autoimmunity in the patients' relatives¹²⁵. The X-chromosome-linked **FOXP3** gene is also vital in immune regulation in that it is involved in the development of T regulatory cells; studies have shown that it is associated with Hashimoto's thyroiditis in females, and that a polymorphism in this gene is related to the severity of the disease^{126,127}.

Autoimmune thyroid disease occurs chiefly in females and Turner syndrome with X isochromosome¹²⁸, and thus the importance of the **X chromosome** has been the subject of further research. Female tissue is a mosaic of two cell lines, one with the maternal X chromosome and the other with the paternal X chromosome, usually at a ratio of 50 to 50. Inactivation of the same X chromosome in over 80% of the cells is referred to as skewed X chromosome inactivation (XCI)¹²⁹, the consequence of which might be that self-antigens on one X chromosome are not expressed at sufficient levels in the thymus or on the peripheral sides involved in tolerance induction. It is plausible that loss of immunological tolerance to X-linked antigens can induce autoimmunity, because studies have suggested that XCI plays a role in autoimmune thyroid disease¹³⁰⁻¹³².

The thyroid-specific genes that have been identified include those encoding TSHR and TG, but not TPO. Multiple single-nucleotide polymorphisms (SNPs) in TG have been associated with both Hashimoto's thyroiditis and Graves' disease^{133,134}, and various SNPs in TSHR have been reported to be related to Graves' disease^{135,136}.

Type 1 diabetes

A hereditary component and clustering in families are not as noticeable in type 1 diabetes as in autoimmune thyroid disease, and indeed only about 15% of children diagnosed with type 1 diabetes have a first degree relative with the disease¹³⁷. In contrast, risk genes for type 1 diabetes have been described much more extensively in research showing that **HLA** class II genes account for approximately 50% of the genetic susceptibility¹³⁸. High-risk and negatively-associated genotypes, haplotypes, and alleles have been identified. Considering genotypes, the highest risk is conferred by *HLA-DQ*, with *DR* alleles modifying the risk^{139,140}, among the haplotypes, *DQA1*0301-B1*0302 (DQ8)* and *HLA-DQA1*0501-B1*0201 (DQ2)* confer the greatest risk. *HLA-DR3* is strongly linked to *DQ2* and *DR4* to *DQ8*. The probability of having specific genotypes has been found to be related to age¹⁴¹, in that young children diagnosed with type 1 diabetes more often have high-risk genotypes^{142,143}. The clinical course of those patients diagnosed early is not found to be related to the *HLA DR-DQ* genotype¹⁴⁴. In the Swedish population, alleles in the haplotypes *DR*03-DQA1*0501-DQB1*02* and *DRB1*04-DQA1*0301-DQB1*0302* are strongly associated with type 1 diabetes¹⁴⁵, and it is important to note that those haplotypes are not uncommon in this population. Negatively associated alleles and haplotypes may modify or influence the high-risk alleles and haplotypes. Given the large variability in the HLA class II genes, and considering differences between *DQ-DR* and *DQ-DP* alleles, there may be pronounced variation both within individuals and between family members. In addition, the HLA class I alleles *A*24* and *B*39*, which are known to be associated with type 1 diabetes, were recently found to affect the progression to clinical disease^{146 147}.

A number of other non-HLA genes are also a part of the complex genetic background of type 1 diabetes. For example, polymorphism in the **PTPN22** gene is associated with this disease in that it is assumed that a certain mutation allows T cells to remain functionally active for a longer time and thus renders them more prone to the destructive immune response that occurs after infections¹⁴⁸. This association is modified by GADA positivity and HLA genotype, resulting in a stronger relationship with GADA-positive type 1 diabetes patients and with low-risk as compared to high-risk HLA patients^{149,150}. A polymorphism in the **CTLA-4** gene, such as a susceptibility gene for type 1 diabetes, has been the topic of many studies, as illustrated by a meta-analysis revealing that different polymorphisms are associated with type 1 diabetes in different ethnic populations¹⁵¹. The **INS** gene encoding insulin is considered to be the second most common risk gene for type 1

diabetes¹⁵², and it may be associated with determining T cell tolerance to insulin in the thymus¹⁵³.

As mentioned, the haplotypes *DQ8* and *DQ2*, alone or in combination, show the strongest genetic relationship with type 1 diabetes, and almost 90% of Scandinavian children diagnosed with this disease have one or both of these haplotypes^{154,155}. This knowledge has been applied to select children at increased genetic risk to participate in prospective studies aimed at evaluating the immune progression and identifying possible modulating factors leading to type 1 diabetes. The DiPiS study is one of these projects.

Joint genetic susceptibility

Autoimmune thyroid disease and type 1 diabetes often cluster in families, as well as in individual patients, indicating joint genetic susceptibility. Not surprisingly, genes already identified as being associated with a risk of developing one or the other of these two diseases, and also genes involved in immune regulation, have been found to confer joint susceptibility; these include HLA class II, PTPN22, and CTLA-4 genes.

The **HLA class II** genes have been investigated to some extent in many cohorts of patients with both diseases and families with both diseases, often with quite inconsistent results.

One study of families with type 1 diabetes and autoimmune thyroid disease showed that the haplotype *DR3-DQB1*0201* was commonly transmitted to offspring affected with either type 1 diabetes or autoimmune thyroid disease¹⁵⁶, and another investigation found *DR3* to be most prevalent in patients with both these diseases¹⁵⁷. Moreover, in an evaluation of 105 patients with type 1 diabetes and autoimmune thyroid disease, *DR3* and *DR4* were found more often in the patients than in controls¹⁵⁸. In the search for genetic susceptibility in HLA class II genes, researchers have also evaluated relationship between thyroid autoimmunity and type 1 diabetes. Associations with the *DR3/DR4* genotype and with the haplotypes *HLA-DRB1*0404*, *DQB1*0301*, and *DPB1*0201* have been described^{159 160}. However, such observations have not been replicated in all subsequent studies, and thus the relationship is not quite clear. As mentioned, further investigation of the structure of the *HLA DR* peptide-binding pocket might help clarify this issue. One study has shown that the amino acid sequence comprising Tyr-26, Leu-67, Lys-71, and Arg 74 confers a joint risk of type 1 diabetes and autoimmune thyroid disease¹⁵⁸.

The **CTLA-4 gene** has also been reported to contribute to the susceptibility to both autoimmune thyroid disease and type 1 diabetes, and to be involved in positive thyroid autoimmunity in type 1 diabetes¹⁶¹⁻¹⁶³. Notably, the association with this gene was found to be stronger in patients with both diseases than in those with type 1 diabetes alone, indicating susceptibility to multiple autoimmunity¹⁶¹.

The role of the **PTPN22** gene in joint susceptibility has not been investigated as thoroughly, but a certain polymorphism has been found to be associated with having both type 1 diabetes and Hashimoto's thyroiditis^{162 164}.

The **FOXP3** gene has also been noted to contribute to the combined gene susceptibility, although further research is needed to confirm this finding¹⁶².

Environment

The genes listed above are highly critical in type 1 diabetes, autoimmune thyroid disease, and autoimmunity in general, although all relationships in this context have not yet been identified. Notwithstanding, the knowledge that the concordance rate for these diseases is not 100% in monozygotic twins, and that immigrants born and living in Sweden are more prone to develop type 1 diabetes compared to ethnic counterparts in their native country, emphasizes the significance of the environment¹⁶⁵. Identification of environmental factors is a challenging task, and although many exogenous factors have been suggested for both diseases, most of them are still controversial.

Pre- and perinatal factors

Factors present in the environment before and around birth are of special interest when studying autoimmunity in childhood, because these aspects may have an impact on the development of immunological tolerance. Pre- and perinatal factors have been studied quite extensively in relation to islet autoimmunity and type 1 diabetes, whereas such factors have been given somewhat less attention in studies of autoimmune thyroid disease in adults and hardly considered at all in childhood thyroid autoimmunity and autoimmune thyroid disease as prospective studies are sparse.

Prenatal studies begin by considering **maternal factors** during pregnancy, such as age, socioeconomic status, and health. Some investigations, albeit not all, have indicated that older maternal age is associated with increased risk of type 1 diabetes¹⁶⁶⁻¹⁶⁹. It has also been reported that poor socioeconomic status and low education of the mother increase the risk of type 1 diabetes in offspring^{168,169}, whereas the literature contains no studies concerning the mother's age and education in relation to autoimmune thyroid disease.

Autoimmune disease in the mother during pregnancy is of interest, because maternal IgG antibodies pass the placenta to reach the unborn child. Both thyroid and islet autoantibodies are of IgG type, and hence they are actively transported to the fetus in this manner. Autoantibodies against the TSH receptor can have clear pathological effects: TSAb can cause neonatal thyrotoxicosis, and TBAb can cause transient congenital hypothyroidism. One study showed that maternal TPOAb positivity was associated with TPOAb levels in 16-year-old adolescents, although

thyroid function at that age was found to be normal ¹⁷⁰. Furthermore, another investigation revealed that thyroid function in 6-year-old children did not differ between those born to TPOAb-positive mothers compared to those with TPOAb-negative mothers ¹⁷¹. In contrast, yet another assessment detected TPOAb in cord blood more frequently in children with Hashimoto's thyroiditis compared to controls ¹⁷². The availability of birth cohort studies of type 1 diabetes facilitates evaluation of the chronological appearance and the existence of islet autoantibodies in cord blood. As mentioned, maternally transmitted islet autoantibodies can be found in the offspring up to 6 months after birth, whereas subsequent development of islet autoimmunity in a child with positive antibodies at birth was not found ^{173,174}. However, the presence of IA-2A in cord blood was found to be linked to increased risk of later type 1 diabetes in the DiPiS study ¹⁷⁵. It is difficult to determine whether the maternally transmitted islet and thyroid autoantibodies actually have a pathological effect, or if they simply reflect inheritance.

Maternal infections and later risk of autoimmunity and disease in offspring has been studied more extensively in type 1 diabetes, and results indicate that various viruses are associated with development of this disease in children. Most of the research in this area has focused on maternal rubella and enteroviruses. The first suggestion of infection in utero and later risk of type 1 diabetes was described almost 50 years ago and concerned a relationship with congenital rubella infection ¹⁷⁶. The same research group subsequently reported that 20% of the children with congenital rubella developed type 1 diabetes before the age of 30 years ¹⁷⁷. Similarly, congenital rubella infection has been associated with both thyroid autoimmunity and Hashimoto's thyroiditis ^{178,179}. The mechanism behind the relationship with in utero rubella infection is not clear but has been suggested to be non-autoimmune in nature, because some of the patients in one investigation who were later diagnosed with type 1 diabetes required no insulin treatment and were not positive for islet autoantibodies ¹⁸⁰. Enterovirus, serologically diagnosed in the mother, and association with type 1 diabetes in the child has been observed in several studies ¹⁸¹⁻¹⁸³, this finding was although not confirmed in a more recent and larger investigation ¹⁸⁴. Only one study has assessed maternal enterovirus infection and Hashimoto's thyroiditis in offspring, and the results indicated a positive relationship ¹⁸⁵.

Compared to infections, **season of birth** represents a more indirect maternal factor that may influence development of autoimmunity in children. There is evidence that children born in the spring and summer months are at increased risk of type 1 diabetes ^{186,187}, but such an association was not confirmed in a large European study ¹⁸⁸. Moreover, results concerning the adult population in relation to season of birth and later autoimmune thyroid disease are inconsistent ^{189,190}, although one recent study did find an increased risk in adults born in June ¹⁹¹.

Research results concerning gestational age and type 1 diabetes risk are conflicting, with reports indicating an association with **gestational age** greater than 41 weeks as well as shorter than 38 weeks ^{169,192}. Regarding Hashimoto's thyroiditis

and gestational age, a large register study in Sweden demonstrated an association between being born prematurely and receiving a prescription for thyroxine in young adulthood¹⁹³.

Birth weight as a marker of poor fetal growth, which has been hypothesized to lead to permanent metabolic changes, has been found to be related to various disorders later in life, such as cardiovascular diseases, type 2 diabetes, and metabolic syndrome. Conversely, it has been suggested that increased birth weight due to overfeeding in utero can lead to overloading of the beta cells that renders them more prone to autoimmune attack and apoptosis¹⁹⁴. There are also results showing that high birth weight is associated with type 1 diabetes¹⁹⁵⁻¹⁹⁷. However, it is possible that the primary association exists between high-risk type 1 diabetes *HLA-DQ* genotypes and birth weight, which would exclude birth weight as a risk factor¹⁹⁸. The influence of birth weight has also been evaluated in relation to autoimmune thyroid disease, and some reports have indicated an association between low birth weight and thyroid autoimmunity in adulthood¹⁹⁹, whereas a large study of twins revealed no relationship with thyroid autoimmunity or autoimmune thyroid disease^{200,201}.

Infections

A great deal of research has concerned viruses as a trigger of autoimmunity, although the literature on this topic is confusing, because viruses may also affect the pathogenesis in individuals with thyroid or islet autoimmunity. If a causal role of viruses is identified, development of vaccines will constitute an option for preventing disease. The challenge in studies addressing this topic is the timing of sampling, considering that the viruses are present only temporarily in tissue of the host before they are cleared by the immune system. Consequently, it is important to identify viruses at an early stage in this process.

Currently, the focus in type 1 diabetes studies is on the group of enteroviruses that commonly cause infections in individuals of all ages, often with mild or moderate symptoms in the respiratory or the gastrointestinal tract, or no symptoms at all. Enteroviruses can cause pancreatitis and are present in pancreatic islets in patients with type 1 diabetes²⁰². In addition, various case-control studies have documented an association between enterovirus infection and type 1 diabetes²⁰³. Furthermore, GAD65 has been found to share an antigen epitope with a protein on the Coxsackie B virus, a type of enterovirus²⁰⁴. Other viruses that have been implicated in the risk of islet autoimmunity or type 1 diabetes include mumps virus, cytomegalovirus, adenovirus, Epstein-Barr virus, and rotavirus²⁰⁵⁻²⁰⁸.

Enteroviruses have also been detected in thyroid tissue in subjects with Hashimoto's thyroiditis, indicating a role in the development of this disease²⁰⁹. Likewise, it is possible that additional viruses such as parvovirus B19, Epstein-Barr virus, and herpes virus can participate in the pathogenesis of Hashimoto's thyroiditis²¹⁰⁻²¹². The Gram-negative bacterium *Yersinia enterocolitica* has long been

implicated in the pathogenesis of autoimmune thyroid disorders, particularly Graves' disease. Notably, a study of twins discordant for Graves' disease showed an association between Graves' disease and *Yersinia* infection ²¹³, whereas a prospective investigation found no association with either hypo- or hyperthyroidism ²¹⁴.

Although many studies have demonstrated an association between autoimmunity and infectious agents, firm evidence for causality is lacking. Therefore, it remains to be determined whether the viruses or their components present in the thyroid or other parts of a host are actually the cause of the disease or are simply "innocent bystanders".

Related to infections is the **hygiene hypothesis**, which was initially suggested in relation to atopy and asthma ²¹⁵. This hypothesis proposes that improvement in the living environment decreases children's exposure to infectious agents and other allergens, which in turn leads to insufficient maturation of the immune response and thereby also a lack of regulatory pathways, and thus renders the children prone to allergic and autoimmune diseases. The hygiene hypothesis has been offered as one explanation for the increasing incidence of type 1 diabetes, considering a reversed association with having an older sibling and the effects of the new environment on immigrants born in or moving to high-incidence countries ²¹⁶⁻²¹⁸. Supporting the hypothesis in the context of childhood thyroid autoimmunity is a study that compared the prevalence of thyroid autoimmunity in Russian Karelia and Finland, which are two adjacent regions with similar genetic background. The results of that analysis showed that the prevalence of thyroid autoimmunity was significantly lower in Russian schoolchildren, who lived in environments characterized by lower prosperity and standard of hygiene ⁶.

Stress

There is an essential difference in the approximation of psychological stress when considering type 1 diabetes and autoimmune thyroid disease. Stress in type 1 diabetes is studied primarily in children and their parents with a family perspective, whereas evaluation of stress in autoimmune thyroid disease is focused on the adult patient alone.

Psychological stress induces a hormonal response that includes a rise in cortisol and catecholamines, which increases the resistance to insulin and thus amplifies the demands on the beta cells. According to the beta cell stress hypothesis, the increased pressure on beta cells to produce insulin can initiate autoimmunity or accelerate autoimmunity that has already been induced. Stress might also disturb the immune-regulatory pathways and in that way, predispose to autoimmunity, a hypothesis that is more applicable to both autoimmune thyroid disease and type 1 diabetes. Evaluating stress is a complicated process due to the problem of recall bias in retrospective studies, and because it is difficult to define stress.

Serious life events very early in childhood were found to increase the risk of islet cell autoimmunity in children in a prospective study ^{219,220}, and negative life events during the first 2 years after birth were observed to increase the risk of type 1 diabetes in a retrospective analysis ²²¹. In contrast, a more recent retrospective assessment revealed no association between psychological stress early in life and later type 1 diabetes ²²². Also, stressful life events prior to Graves' disease have been noted in various studies, and one large population-based case-control investigation demonstrated such an association in subjects 12 months before diagnosis ²²³. Other researchers have found both more positive and negative life events prior to diagnosis of Graves' disease ²²⁴. By comparison, it is difficult to evaluate stress in Hashimoto's thyroiditis, because thyroid autoantibodies can exist years before symptoms of hypothyroidism and goiter develops. One study revealed no association between positive thyroid autoantibodies and stress in asymptomatic women ²²⁵.

Dietary factors

The dietary factors that are analyzed in type 1 diabetes and autoimmune thyroid disease differ greatly: supplements used by the mother and infant feeding are the main aspects of diet considered in evaluations of type 1 diabetes, whereas markedly different factors such as alcohol and smoking habits—obviously not relevant for infants—are in focus when studying diet in relation to autoimmune thyroid disease. No prospective studies of childhood thyroid autoimmunity have been conducted to evaluate maternal habits and infant feeding, and to date the only dietary factor that has been identified in this regard for both type 1 diabetes and autoimmune thyroid disease is vitamin D.

Vitamin D is a dietary factor and a hormone that exhibits seasonal variation, because sunlight is necessary for synthesis to reach adequate levels. Many of the immunocompetent cells mentioned above express a vitamin D receptor and the vitamin-D-activating enzyme CYP27B1. Vitamin D as a hormone is known to inhibit proliferation of T cells, production of cytokines, and production of antibodies by B cells ²²⁶. Furthermore, in the presence of active vitamin D, dendritic cells mature in the direction of tolerogenic cell that express less of the MHC class II molecules and adhesion molecules required for full T cell stimulation ²²⁷. The role of vitamin D in the immune system has been the subject of many studies, which have shown that low levels of vitamin D are related to numerous autoimmune diseases.

The seasonal clustering of type 1 diabetes in the winter months ²²⁸ and the higher incidence in the northern part of Europe draws attention to the possibility that vitamin D deficiency is an exogenous factor affecting this disease. A birth cohort study of the relationship between recommended intake of vitamin D supplements early in infancy and type 1 diabetes 30 years later showed a marked reduction in this disease in individuals who had received the recommended dose of vitamin D in

infancy²²⁹. Maternal intake of dietary vitamin D has also been inversely correlated to islet autoantibodies in children²³⁰. In contrast, assessments in the same project found no association between children's vitamin D intake or 25-hydroxyvitamin D levels and islet autoimmunity or type 1 diabetes²³¹. Vitamin D is currently under evaluation in type 1 diabetes prevention studies.

In recent years, there has been increased interest in vitamin D as a contributing factor in autoimmune thyroid disease. Results have shown that vitamin D deficiency occurs more frequently in patients with autoimmune thyroid disease, especially Hashimoto's thyroiditis, compared to controls, and that the degree of vitamin D deficiency is linked to levels of thyroid autoantibodies and thyroid function^{232 233}. A large prospective study following women for 5 years found no difference in serum vitamin D levels between those who did and those who did not develop thyroid autoimmunity²³⁴. No research has addressed the question of whether intake of D supplements prevents autoimmune thyroid disease or has any clinical effect in patients who have developed such disease.

Dietary factors in type 1 diabetes

Breastfeeding and its duration have been reported to protect against type 1 diabetes²³⁵⁻²³⁷. However, a recent analysis of data from 43 observational studies showed no clear relationship other than a possible reduction in type 1 diabetes in children who were exclusively breastfed during the first 2 weeks after birth²³⁸. In other studies, regardless of the duration of breastfeeding, early introduction of **cow's milk** was found to be related to increased risk of type 1 diabetes^{239,240}, and high consumption of cow's milk later in childhood was observed to raise the risk of type 1 diabetes in siblings of patients with that disease²⁴¹. Nonetheless, in a case-control study of children diagnosed with type 1 diabetes before the age of 5 years, current cow's milk consumption was noted to have a protective effect²⁴². Introduction of solid foods, such as gluten-containing, has been found to affect the development of islet autoimmunity in genetically susceptible individuals^{243,244}. In a randomized controlled trial, delaying the introduction of gluten had no effect on appearance of islet autoimmunity²⁴⁵. Gluten-free diet is one of the factors that has been studied as a means of prolonging the remission phase in type 1 diabetes, and the results indicate a potentially positive effect²⁴⁶. Larger trials are needed to evaluate the influence of gluten-free diet on autoimmunity and disease, although such assessment is difficult to accomplish due to problems with adherence.

Dietary factors and smoking in autoimmune thyroid disease

Iodine is an essential component of thyroid hormones. As described in previous section, iodine is coupled to tyrosines to form mono- and di-iodotyrosines that undergo an oxidative coupling to yield T4 and T3 (Figure 2). Iodine prophylaxis using iodized salt was implemented by the WHO to prevent iodine deficiency disorders. Interestingly, a rise in autoimmune thyroid disease has been noticed since

the start of iodine prophylaxis. This is illustrated by population-based studies in Denmark, which were performed before and after mandatory iodine prophylaxis, and revealed an increase in both thyroid autoimmunity and hypothyroidism^{247,248}. The explanation for the iodine-induced thyroid autoimmunity has been correlated to TG, because the configuration of this molecule is altered by iodination. Notably, it has been shown that posttranscriptional iodination of TG leads to unmarked cryptic B cell epitopes that are immunogenetic and induce the rise of TGAbs²⁴⁹.

Selenium is a trace mineral that is found in high concentrations in the thyroid gland and is required for antioxidant function and metabolism of the thyroid hormones. Selenium deficiency has been associated with poor immune function, possibly because T lymphocytes require significant amounts of selenium to function properly²⁵⁰. Low serum levels of selenium have been found to be related to increased thyroid volume and thyroid hypoechogenicity²⁵¹. Furthermore, in a double-blind randomized trial in patients with subclinical Hashimoto's thyroiditis, treatment with selenium caused a significant decrease in TPOAb titers²⁵². Similarly, adding selenium to treatment with thyroxine in patients with subclinical Hashimoto's thyroiditis has been shown to reduce TPOAb titers²⁵³. No trials have been conducted to determine the effects of selenium in children.

Smoking is a well-known risk factor for Graves' disease but has been shown to protect against Hashimoto's thyroiditis²⁵⁴. Moderate alcohol consumption has also been reported to decrease the risk of Hashimoto's thyroiditis²⁵⁵. No studies in the literature have assessed smoking and alcohol habits in mothers in relation to thyroid autoimmunity and disease in offspring.

Hashimoto's thyroiditis in children with type 1 diabetes

Several studies have examined the prevalence of thyroid autoimmunity, subclinical hypothyroidism, and overt disease in children and adolescents with type 1 diabetes (Table 1). The rates observed are generally quite similar in reports concerning thyroid autoimmunity with some correlation to duration of diabetes but vary more extensively in relation to disease, most likely due to use of different definitions.

Table 1.

Observations in various studies regarding thyroid autoantibodies, subclinical hypothyroidism, and overt disease in children and adolescents with type 1 diabetes

Age in years (SD)	Thyroid antibodies	SCH	Overt disease	n=	Mean T1D duration/years (SD or range)	Country
11.6±2.4	19% (TPOAb)		9.7%	330	2.2±1.2	Iran ²⁵⁶
1-20	21 % TPO or TG		7.7%	233	12.4 ±5.8	Brazil ²⁵⁷
12.4	21.6 % TPO or TG		10.6%**	7097	4.5	Germany ²⁵⁸
9.3±4.4	14.4 % TPO		2.1 %	382	Diabetes debut	Polen ²⁵⁹
12.3±4.6	17.4 % TPOAb and/or TGAb	10.4 %	none	144	4.7±3.9	Greece ²⁶⁰
10.9±3.6	34.5% TPOAb or TGAb	7.3%	4.3%	232	4 (0-16)	Kuwait ²⁶¹
12.8(2.3-13.3)	16.2% TPOAb and/or TGAb	2.9 %	1.9%**	105	4.8	Denmark ²⁶²
13.7±4.25	19.3 % TPOAb or TGAb	7.2 %		18431	5.7±3.9	Germany ²⁶³
14.2±3.7	26 % TPOAb			69	7.1± 3.5	Korea ²⁶⁴
1-20	16.5 % TPOAb			212	Diabetes debut	Italy ²⁶⁵

Abbreviations: T1D, type 1 diabetes; SCH, subclinical hypothyroidism; SD, standard deviation, TPOAb, thyroid peroxidase autoantibodies, TGAb, thyroglobulin autoantibodies, ** (Thyroxine treatment)

Thyroid hormones have a pronounced impact on glucose metabolism, and hypothyroidism has been reported to increase insulin resistance ²⁶⁶ and reduce hepatic gluconeogenesis ²⁶⁷. This means that having both Hashimoto's disease and type 1 diabetes complicates treatment of the latter. Measurable increases in HbA_{1C} and more frequent episodes of hypoglycemia, as well as higher lipid levels are reported, all of which can fortunately be resolved with thyroxine treatment ^{268,269}. There is evidence that subclinical hypothyroidism is associated with increased risk of coronary heart disease and mortality ²⁷⁰ and in addition reports indicate that dyslipidemia occurs in children with type 1 diabetes who are also diagnosed with subclinical hypothyroidism ²⁶³. These observations further stress the importance of early diagnosis and treatment of Hashimoto's thyroiditis in patients with type 1 diabetes to minimize long-term complications.

The benefit of screening children and adolescents for thyroid autoantibodies and disease is obvious. However, there is no consensus regarding what tests should be used for screening, the appropriate frequency of testing, or whether symptoms of hypothyroidism or goiter should guide testing. The latter is not a suitable choice, considering that in one study neither clinical and laboratory data nor the incidence of overt hypothyroidism differed between asymptomatic children with disease and antibodies discovered through screening and children with evident goiter and/or symptoms ¹¹. The International Society for Pediatric and Adolescent Diabetes (ISPAD) advocates screening with TSH and TPOAb at diabetes diagnosis and every other year in asymptomatic children without goiter, but offers no recommendations regarding follow-up in children positive for TPOAb ²⁷¹.

Aims

The overall objective of the research underlying this thesis was to investigate the prevalence of thyroid autoimmunity and the association with islet autoantibodies, *HLA-DQ* genotypes, and thyroid function in children with newly diagnosed type 1 diabetes, as well as in children and adolescents followed due to increased risk of type 1 diabetes. An additional objective was to assess predictive markers and factors that may have an impact on thyroid autoimmunity and thyroid disease.

The specific aims were as follows:

1. To determine the prevalence of thyroid autoimmunity in children and adolescents at diagnosis of type 1 diabetes and in 10-year-old children with increased type 1 diabetes risk.
2. To ascertain the age at which seroconversion of thyroid autoimmunity occurs and to elucidate the effect on thyroid function in children followed for increased risk of type 1 diabetes.
3. To investigate a possible association of thyroid autoimmunity and disease with *HLA-DQ* genotypes.
4. To investigate the influence of perinatal events and history of autoimmune disease in the family on thyroid autoimmunity at 10 years of age in children.
5. To study the relation of thyroid and islet autoantibodies and gender differences in children with type 1 diabetes and in those followed for their increased type 1 diabetes risk.
6. To optimize screening for autoimmune thyroid disease in children and adolescents with type 1 diabetes through analysis of the predictive value of thyroid and islet autoantibodies as well as thyroid function and *HLA-DQ* genotypes at type 1 diabetes diagnosis for later development of autoimmune hypothyroid disease.

Population and study design

Papers I and II

Population

Sweden has a population of 9.8 million, and there are 43 pediatric clinics in the country that are responsible for diagnosis and treatment of all children and adolescents with type 1 diabetes. Since May 2005, almost all children under the age of 18 years diagnosed with type 1 diabetes are included in the prospective Better Diabetes Diagnosis (BDD) study. At diagnosis, blood samples are collected and analyzed for autoantibodies against GAD65, IA-2, insulin, and ZnT8R/W/QA, and for *HLA-DQ* genotypes. The WHO criteria are used for diagnosis and classification of diabetes⁴³. The clinical diagnosis in the present cohort (see study group below) was reevaluated 6–12 months after inclusion at which time children with any of the following diagnoses were excluded: maturity-onset diabetes of the young (MODY; $n = 27$), type 2 diabetes ($n = 57$), neonatal diabetes ($n = 3$), secondary diabetes ($n = 32$), or diabetes of unknown type ($n = 46$).

Study group

Between May 2005 and October 2009, in the BDD, blood samples were also analyzed for thyroid autoantibodies and thyroid function in children and adolescents with newly diagnosed type 1 diabetes ($n = 2670$). After exclusion of subjects with missing data ($n = 237$), 2,433 remained for assessment, 44% girls ($n = 1,073$) and 56% boys ($n = 1,360$) with a median age of 10.3 years (range 0.7–17.9 years) (Figure 5).

In November 2014, data on thyroxine prescription for all 2,433 participants were obtained from the Prescribed Drug Register compiled by the Swedish National Board of Health and Welfare and were used to define the outcome of autoimmune hypothyroid disease.

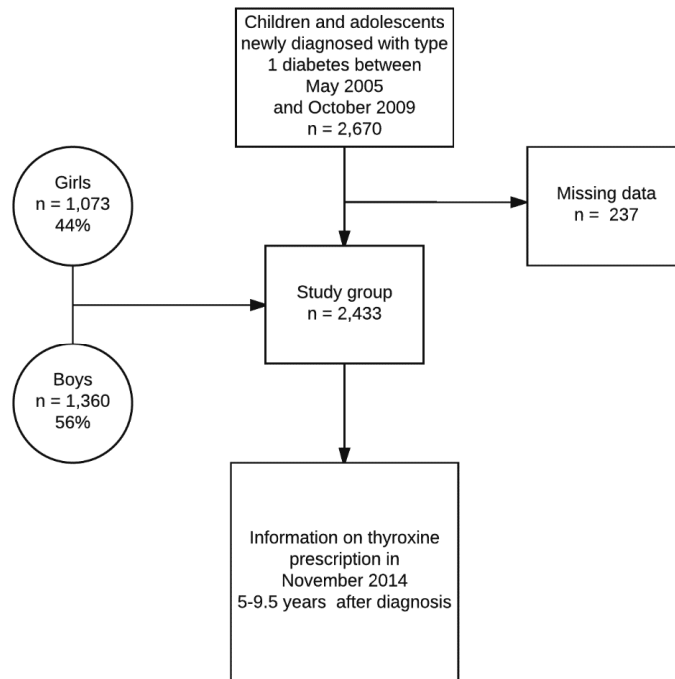


Figure 5.
Flow chart of the study population in Papers I and II.

Papers III and IV

Population

Skåne is the southernmost region in Sweden with a population of 1.3 million. All parents of children born between September 2000 and August 2004 in Skåne were invited to participate in the DiPiS study, which is a prospective population-based investigation of diabetes in children. The primary aim of DiPiS is to identify environmental factors that may trigger islet autoantibodies and to determine the value of those autoantibodies in combination with genetic factors for predicting type 1 diabetes. During the inclusion period, approximately 48,000 children were born in Skåne, and parents-to-be were given information about the DiPiS study during prenatal visits at the regional maternity health care clinics. After receiving oral consent from the mothers, 35,688 cord blood samples were obtained after deliveries

and analyzed for *HLA-DQ* genotypes. When a child was 2 months of age, an invitation to participate in the study and two questionnaires were sent to the parents. The first questionnaire (part 1) included items concerning the following: pregnancy, delivery, and the child's first two months after birth; the mother's infections and alcohol consumption and smoking habits during pregnancy; adverse life events occurring during pregnancy and the first 2 months postpartum (see supplementary material, Paper IV). The second questionnaire (part 2) included items requesting information on the child's birth weight and length, gestational age, and any family history of diabetes. The parents gave written informed consent for participation.

From the age of 2 years and primarily based on HLA genotypes, children at increased risk of type 1 diabetes were invited (via the parents) to undergo annual follow-up. At that time, additional information was gathered regarding other autoimmune diseases in the family. At annual follow-up visits, the parents completed additional questionnaires, and blood samples are collected from the children and analyzed for autoantibodies against GAD65, IA-2, insulin, and ZnT8R/W/Q. Children positive for multiple islet autoantibodies are also offered oral glucose intolerance testing every 6 months.

In 2015, 2,073 blood samples from 10-year-old children participating in DiPiS follow-up were available for thyroid autoantibody testing (Figure 6). The parents of all these children received a letter providing information on thyroid autoantibody testing and including contact information for those who did not want to participate, did not want the results, or had any questions. After exclusion of 28 children who chose not to participate and 150 whose samples could not be analyzed, 1,874 children remained to comprise the study group in Papers III and IV.

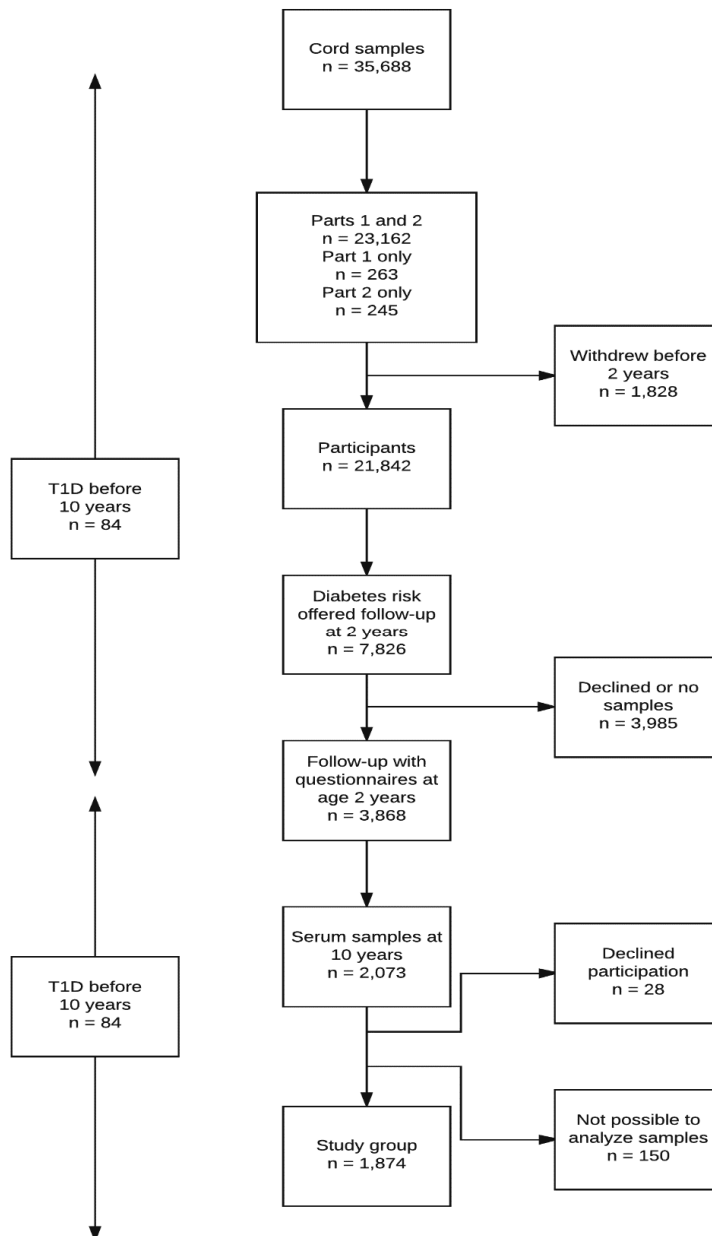


Figure 6.

Flow chart of the DiPiS study from collection of cord blood samples to analysis of thyroid autoimmunity at age 10 years. Part 1: first questionnaire, Part 2: second questionnaire, T1D: type 1 diabetes.

Study group

Paper III The parents of all children positive for thyroid autoantibodies ($n = 130$) at 10 years of age were contacted to request a confirmatory sample 1 year later, at which time the children were 11–16 years old. The confirmatory sample was also analyzed for TSH and FT4. Furthermore, samples collected during the prospective follow-up from 2 years of age were analyzed for TPOAb and TGAb.

Paper IV As shown in Figure 6, the parents of 23,670 children replied to the questionnaires, and 3,868 of those responding accepted an invitation for a child to continue follow-up at 2 years of age. Prospectively collected data from the questionnaires were correlated with the 1,874 children tested for thyroid autoimmunity at age 10.

Methods

Analysis of the thyroid autoantibodies TPOAb and TGAb

In the BDD study (Papers I and II), TPOAb (kit No L2KTO2) and TGAb (kit No L2KTG2) were analyzed in serum samples using the Immulite® 2000 immunoassay system according to instructions of the manufacturer (Simens Healthcare Diagnostics, Deerfield, IL, USA).

In the DiPiS study (Papers III and IV), TPOAb (kit No RS-TP/100) and TGAb (kit No RS-TG/100) were analyzed in plasma samples using RIA kits supplied by RSR Limited (Avenue Park Pentwyn Cardiff CF23 8HE, United Kingdom).

All analyses were performed according to the manufacturer's instructions and levels of both TPOAb and TGAb were derived from the standard curves included in the kits and were expressed as U/mL. Spline point-to-point curve fit was used for TPOAb, and cubic spline was applied for TGAb. The cutoff for TPOAb and TGAb was defined based on QQ plot analysis (Paper III).

Analysis of islet autoantibodies

Autoantibodies to GAD65 and IA-2

Recombinant GAD65 and IA-2 were produced by incorporation of ³⁵S-methionine (PerkinElmer, Waltham, MA, USA) by in vitro coupled transcription and translation using the TNT SP6 coupled reticulocyte lysate system (Promega, Madison, WI, USA), as described elsewhere²⁷². Full-length cDNA coding for human GAD65 in the pTNT vector (pThGAD65; Promega) or the intracellular domain (amino acids 606–979) of IA-2 in the pSP64 Poly(A) vector (IA-2ic; Promega) was used as template²⁷³. GADA and IA-2A were analyzed using a radioligand binding assay (RBA) reported in the literature²⁷². Duplicates of the samples were incubated with the radiolabeled antigen overnight at 4 °C and subsequently transferred to filtration plates (Millipore, Solna, Sweden), where IgG antibodies were bound to Protein A

Sephacrose (Zymed Laboratories Inc., San Francisco, CA, USA). Thereafter, all unbound antigen was washed away, and SuperMix scintillation cocktail (Perkin Elmer) was added to the plates. Radioactivity was counted in a Wallac Microbeta Trilux system (Perkin Elmer). GADA and IA2A levels were derived from the WHO standard 97/550 and expressed as U/mL.

Autoantibodies to insulin

Insulin auto-antibodies were analyzed by RBA using insulin labeled with ^{125}I (PerkinElmer) as described elsewhere²⁷⁴. Duplicates of the samples were assessed under conditions similar to those used to evaluate GADA and IA-2A, and the results were expressed in relative units related to positive controls. All positive samples were further analyzed in a competitive assay using non-radioactive human recombinant insulin (Actrapid® from NovoNordisk, Bagsværd, Denmark) to verify false-positive binding.

In 2010, the method was adjusted as previously reported²⁷⁵, to use a standard curve with seven different concentrations (in the range 3–358 U/mL) plotted against cpm values on a Log2scale in order to calculate U/mL instead of the relative units.

Autoantibodies to zinc transporter 8 variants

The three ZnT8A variants anti-ZnT8 arginine 325 (ZnT8RA), anti-ZnT8 tryptophan 325 (ZnT8WA) and anti-ZnT8 glutamine 325 (ZnT8QA) were analyzed in duplicate using a previously described RBA²⁷⁶. In short, the cDNA constructs with the rs13266634 C>T and rs16889462 G>A mutations were generated using a Phusion™ site-directed mutagenesis kit (Finnzymes Oy, Espoo, Finland), and recombinant antigens of all three variants were produced by incorporation of 35S-methionine (PerkinElmer) through in vitro coupled transcription and translation in a TNT SP6 coupled reticulocyte lysate system (Promega). The antibody titer was derived from in-house standards with ZnT8R, ZnT8W, or ZnT8Q reactivity and expressed as arbitrary U/mL.

Our laboratory is participating in the biannual Islet Autoantibody Standardization Program (IASP)²⁷⁷. Sensitivity and specificity for islet autoantibodies reported by the IASP in 2015 were as follows: GADA, 76% and 95.6%; IA-2A, 72% and 100%; IAA, 26% and 97.8%; ZnT8RA, 58%, and 100%; ZnT8WA, 50% and 100%; ZnT8QA, 38% and 100%.

HLA genotyping

Papers I and II

HLA DQB1 and *DQA1* alleles were determined on dried blood spots (DBSs) using a DELFIA hybridization assay (Perkin Elmer, Boston, MA, USA) as previously described²⁷⁸. The *DQA1* and *DQB1* alleles were amplified in a polymerase chain reaction (PCR), and sequence-specific oligonucleotide probes were used to identify the different alleles.

The first set of probes defines the presence of *HLA-DQB1**02, 03:02, 03:01, 06:02, 06:03, and 06:04, the second set of probes defines the presence of additional *DQB1* alleles. *HLA-DQA1* probes define the *DQA1**02:01, 03:01, and 05:01 alleles.

Papers III and IV

HLA-DQ genotyping in DiPiS was performed on cord blood samples as outlined above. Based on the presence of *DQ8* (*A1**0301-*B1**0302) and *DQ2* (*A1**0501-*B1**0201) haplotypes, the *HLA DQ* genotypes were categorized into four risk groups (X indicates neither *DQ8* nor *DQ2*): *DQ* 8/8 or 8/X, *DQ* 2/2 or *DQ2*/X, *DQ* 2/8 or *DQ* X/X.

All of the analyses mentioned above were performed at the laboratory of the Clinical Research Centre, Lund University, Malmö, Sweden.

TSH and FT4

Papers I and II

Thyroid-stimulating hormone (TSH) and free thyroxine (FT4) were assessed in serum samples using an Immulite 2000 analyzer according to the instructions of manufacturer (Siemens Healthcare Diagnostics, Deerfield, IL, USA). Since transient deterioration of TSH is fairly common at clinical diagnosis of type 1 diabetes, the cutoff values in Paper I were TSH > 8 mU/L and FT4 < 12 pmol/L for hypothyroidism, and TSH < 0.001 and FT4 > 22 pmol/L for hyperthyroidism. In Paper II, the reference value was 0.4–3.5 pmol/L for TSH and 12–22 pmol/L for FT4.

Paper III

TSH and FT4 in plasma were analyzed at the Clinical Biochemical Department of Malmö University Hospital. These assessments were carried out using electrochemiluminescence immunoassay according to the instructions of the manufacturer (Cobas, Roche diagnostics Ltd., Rotkreuz, Switzerland). The reference values were 0.4–3.7 pmol/L for TSH and 12–22 pmol/L for FT4.

Statistical analysis

Statistical analyses were performed using SPSS statistical software (versions 18.0 and 22.0; SPSS, Chicago, IL, USA), as well as R 3.1.1 and 3.4.1. Differences in proportions between groups were tested using the χ^2 test or Fisher's exact test when appropriate, and odds ratios (ORs) were calculated with 95% confidence intervals (CIs). The Mann-Whitney test was used to compare differences between two independent groups (Papers I, II, and III). All p values reported for the *HLA-DQ* gene associations in Paper I and for perinatal factors in Paper IV were corrected for multiple comparisons by the Bonferroni method (p_c). A p value of < 0.05 was considered statistically significant.

Association with future thyroxine prescription was analyzed by performing Cox proportional hazards modeling using the survival package in R. QQ plot analysis was performed using Microsoft Excel and GraphPad Prism 6.03 software (Paper III). Spearman's rank correlation was applied to analyze the relationship between islet autoantibody and thyroid autoantibody titers.

Ethical aspects

The BDD study was approved by the Regional Ethics Board in Stockholm, Sweden, and the DiPiS study was approved by the Regional Ethics Review Board of Lund University, Lund, Sweden. Additional approval for thyroid autoimmunity testing in the DiPiS study was obtained in 2015 from the Regional Ethics Review Board of Lund University, Lund.

Results and discussion

Prevalence of thyroid autoantibodies

In type 1 diabetes patients

In our large cohort comprising 2,433 children and adolescents up to 18 years of age consecutively diagnosed with type 1 diabetes between May 2005 and October 2009, we found the prevalence of thyroid autoimmunity (i.e., being positive for TPOAb or TGAb, or both) to be 12.3% (Figure 7). This observation agrees with the results of previous investigations of thyroid autoimmunity in children and adolescents at type 1 diabetes diagnosis, (Table 1). By comparison, even higher prevalence has been observed in adult populations with type 1 diabetes²⁷⁹, which concurs with the increasing prevalence of autoimmune thyroid disease with increasing age. In our cohort, children positive for both TPOAb and TGAb were older (median age 12.4 years) compared to children negative for thyroid autoantibody (median age 9.9) ($p < 0.001$) (Paper I).

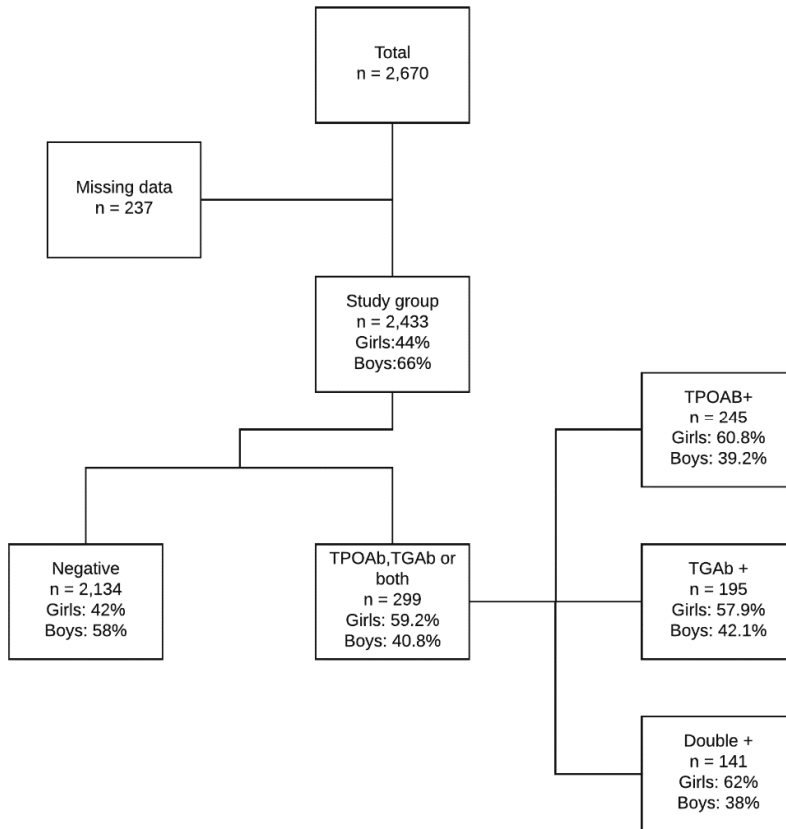


Figure 7.
The prevalence of thyroid autoantibodies in relation to gender in children at diagnosis of type 1 diabetes

In children followed for their increased risk of type 1 diabetes

In the DiPiS study, the prevalence of thyroid autoimmunity in 10-year-old children at increased risk of type 1 diabetes was 6.9% (130/1,874), and 4.4% of the subjects (83/1,874) were positive for TPOAb, 5.8% (109/1874) for TGAb, and 3.3% (62/1874) for both. This prevalence of TPOAb is somewhat higher than in the general population, considering that previous studies have shown TPOAb positivity in 2.8% of 12-year-olds in Sweden and 3.4% in children in Germany ^{7,9}. The difference in prevalence can be explained by the selection of our cohort, a selection primary based on risk genotypes for type 1 diabetes.

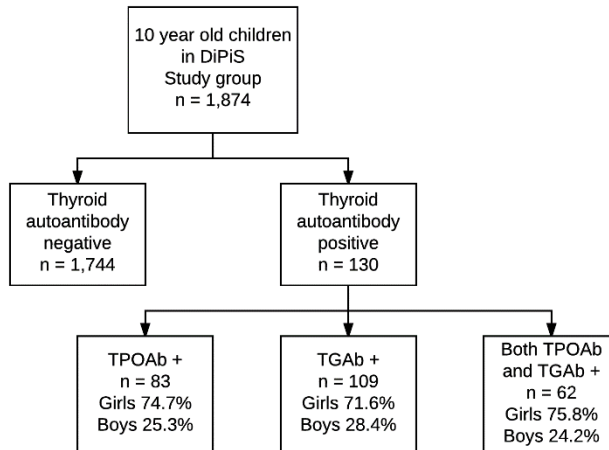


Figure 8.

The prevalence of thyroid autoantibodies in relation to gender in children in the DiPiS study at age 10 years.

Seroconversion and thyroid function

There are few prospective studies of thyroid autoimmunity in the literature, and therefore we further analyzed the seroconversion of thyroid autoimmunity in samples collected during prospective follow-up of children in the DiPiS study. As expected, our results showed that thyroid autoimmunity increased with increasing age, but, interestingly, children as young as 2 years old were positive for thyroid autoimmunity (Figure 9).

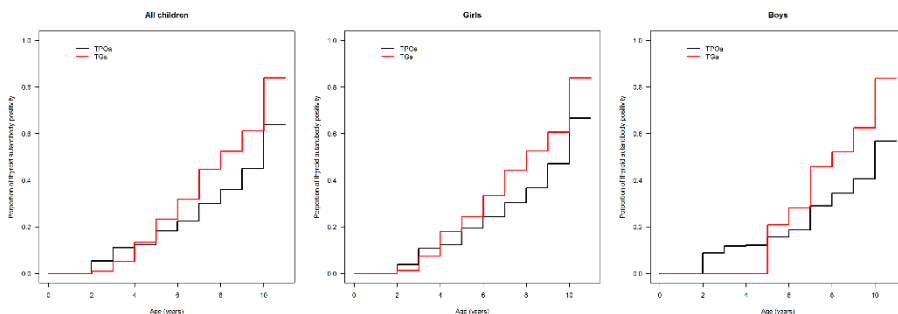


Figure 9.

Demonstration of the seroconversion of TPOAb and TGAb in children with thyroid autoimmunity at 10 years of age; all children, girls and boys.

Children found to be positive for thyroid autoantibodies at 10 years of age ($n = 130$) were asked to provide a confirmatory sample 1 year later, at which time the children were 11–16 years old. Nine of the 130 children in that group did not provide a confirmatory sample, because they were diagnosed with hypothyroidism (8/9) or hyperthyroidism (1/9) after the initial sampling. In this assessment, 96 of the 102 children with a confirmatory sample were still thyroid autoantibody positive. All six children who were negative for thyroid autoimmunity in the confirmatory sample had been positive only for TGAb (i.e., not for TPOAb) at 10 years of age. This finding suggests that TGAb is of inferior predictive value, that is, it may be less specific for later thyroid disease²⁸⁰.

Thyroid function was also analyzed in the 102 children, which showed that TSH was above the reference limit in 15 of these subjects (> 3.8 mIE/L) and below the limit in one child (< 0.01 mIE/L). Thus, thyroid dysfunction or established thyroid disease was found in 19% (25/130) of the children positive for thyroid autoantibodies at 10 years of age. The marked prevalence of thyroid dysfunction or disease is most likely explained by the high incidence of type 1 diabetes in the cohort (1.9%) compared to the Swedish population in general (0.8%)⁴⁰. These results demonstrate the occurrence of thyroid disease in a population at risk, even at a young age. The potential clinical implementation of these results might be in screening for thyroid dysfunction in children who are being followed in birth cohort studies due to increased risk of type 1 diabetes or children to parents with autoimmune disease.

Relation to HLA DQ genotypes

As discussed in a previous section, *HLA-DQ* genes play an important role in immunomodulation, because they encode the MHC molecule that an APC uses to present antigens to a T helper cell. The results of studies exploring *HLA-DQ* in relation to thyroid autoimmunity and disease are not conclusive, possibly due to the discrepancies in disease definition. An association between *HLA-DQ* genes and thyroid autoimmunity was investigated in the BDD cohort of patients with newly diagnosed type 1 diabetes and in the DiPiS cohort of 10-year-old children at risk of the disease. In the BDD study, it was also possible to analyze HLA genotypes in relation to later thyroxine treatment, a substitute endpoint for autoimmune hypothyroidism.

No associations were found between thyroid autoimmunity and *HLA DQ* genotypes in children followed due to increased risk of type 1 diabetes.

In patients with new-onset type 1 diabetes, genotypes containing *DQ5.1* were found to be negatively associated with TPOAb ($p = 0.004$, $pc = 0.012$), TGAb ($p = 0.01$, $pc = 0.07$), and both TPOAb and TGAb ($p = 0.002$, $pc = 0.014$). In addition, a

negative relationship was found between the genotype *DQ8/5.1* and TPOAb ($p = 0.019$, $pc = 0.057$) and, although somewhat weaker, also between that genotype and co-occurrence of TPOAb and TGAb ($p = 0.0085$, $pc = 0.06$) (Paper I). These results indicate a protective effect of the haplotype *DQ5.1* ($p = 0.004$) alone and in combination with *DQ8* in the genotype *DQ5.1/8* ($p = 0.03$), and we later extended this conclusion to include thyroxine prescription, a marker of autoimmune hypothyroid disease (Paper II).

The protective influence of *DQ5.1* on thyroid autoimmunity in children with type 1 diabetes was previously described in a small cohort of 285 children²⁸¹, and this observation was corroborated by the present results obtained in a cohort of 2,433 children and adolescents. Interestingly, we found the genotype *DQ8/5.1* to have a potentially protective effect. Earlier data are conflicting with regard to thyroid autoimmunity or disease and a relationship with *DQ8*, which is a high-risk haplotype in type 1 diabetes. Our findings also confirm the results of a previous study of families with both type 1 diabetes and Hashimoto's disease in which the haplotype *DQ8* was shown to protect against the latter disease²⁸². In contrast, a positive association was found with joint genetic susceptibility in families with both these diseases and the haplotype *DQ8*²⁸³. The disparities in these results may depend on different combinations of the *DQ8* haplotype; if *DQ8* is combined with *DQ5.1*, the binding affinity for the epitope may be altered in a protective manner, as has been described for *DQ8* joined with *DQ6* in type 1 diabetes²⁸⁴.

It seems that the only positive *HLA-DQ* correlation in our material was an association between TGAb and genotypes containing *DQ2* ($p = 0.014$, $pc = 0.098$), whereas no relationship was apparent for TPOAb (Paper I). This dissimilarity might be explained by there being no sequence homology between the autoantibodies and therefore dissimilarity in their association with *HLA-DQ* heterodimers. It was not possible to extend the relationship between TGAb and the haplotype *DQ2* to encompass thyroxine prescription (Paper II); *DQ2* has previously been found to be related to autoimmune thyroid disease²⁸³.

As described in the methods section, *HLA DQ* genotypes were not fully typed in the DiPiS study, a limitation that prevented further analysis and thereby precluded the possibility of replicating our earlier results regarding relationships involving *HLA-DQ*. This drawback might also explain why we found no correlations. Other plausible explanations are that the cohort in the DiPiS study was selected on the basis of high-risk HLA genotypes, and children diagnosed before the age of 10 years were not included in the investigation of thyroid autoimmunity.

Overall, similar to the findings of other studies, our results regarding the relationship between HLA genes and thyroid autoimmunity and clinical thyroid disease in children and adolescents with type 1 diabetes are weak. Consequently, HLA genotypes are still of no value for use in a screening regimen.

Family history of autoimmune diseases and relation to childhood thyroid autoimmunity

Autoimmune diseases are known to aggregate in families, and this aspect has been studied primarily in adult populations and led to reports suggesting various underlying genes. Accordingly, we addressed the question of whether a family history of autoimmune disease is related to thyroid autoimmunity by the age of 10 years in the children being followed in the DiPiS study due to an increased risk of type 1 diabetes. In addition, we performed separate evaluations of heredity in the following autoimmune disorders: thyroid disease, type 1 diabetes, rheumatic disease, and celiac disease.

Our results showed that having a father or a first-degree relative with thyroid disease was positively associated with TPOAb alone ($p = 0.05$ and $p = 0.03$, respectively) as well as with co-occurrence of TPOAb and TGAb ($p = 0.03$).

Reports of rheumatic disease were rare. Of the five children, reported having a father with rheumatic disease, two were positive for both thyroid autoantibodies; a positive relation was found to TPOAb ($p=0.017$), TGAb ($p=0.029$), any ($p=0.038$) and both ($p=0.001$).

It is notable that fathers with thyroid or rheumatic disease are more likely to have children with thyroid autoimmunity. Indeed, this picture suggests that the inheritance of thyroid autoimmunity is similar to the inheritance of type 1 diabetes, in which fathers are more likely than mothers to transfer the disease to their offspring²⁸⁵.

Our assessments showed that the children reported to have a sibling or a first-degree relative with insulin-treated diabetes were more likely to have any thyroid autoantibody ($p = 0.032$ and $p = 0.01$ respectively). Likewise, the risk for thyroid autoimmunity was increased in those reported to have a sibling ($p = 0.034$) or first-degree relative ($p = 0.033$) with celiac disease.

These findings indicating a positive relationship between childhood thyroid autoimmunity and having a sibling or first-degree relative with celiac disease agree with previous studies showing that children with celiac disease are more prone to thyroid autoimmunity compared to the general population⁷. Investigations have also demonstrated that patients with celiac disease and first-degree relatives of such patients are more likely to have autoimmune disease^{286,287}. Consequently, it is recommended that children diagnosed with celiac disease undergo screening for autoimmune thyroid disease²⁸⁶. Lastly, we also analyzed the relationship between occurrence of any autoimmune disease in the family and childhood thyroid autoimmunity, and the results showed that having a first-degree relative with any autoimmune disease increased the risk of being positive for TPOAb ($p = 0.012$), for either TPOAb or TGAb ($p = 0.04$), and for both thyroid autoantibodies ($p = 0.024$) (Paper IV).

It is very likely that the data collected regarding autoimmune diseases in the families of the children we investigated are underestimated, as this information has not been updated since the children were 2 years old. Furthermore, the statistical analysis was difficult due to the low number of cases, although the results do demonstrate an aggregation of childhood thyroid autoimmunity and other autoimmune disease in families.

The impact of perinatal events on childhood thyroid autoimmunity

It is conceivable that perinatal effects play a role in the development of childhood autoimmune diseases, because the triggers of autoimmune responses in genetically susceptible individuals may already be present before birth. Prospective studies of childhood thyroid autoimmunity are scarce, and hence knowledge concerning the effects of perinatal risk factors on later thyroid autoimmunity is limited.

We hypothesized that perinatal factors such as season of birth and maternal infections during pregnancy increase the risk of thyroid autoimmunity in offspring at 10 years of age. Investigating the effects of perinatal factors on thyroid autoimmunity in the DiPiS study revealed no relationship with maternal age, infections during pregnancy, use of alcohol or smoking during pregnancy, or season of birth or birth weight. We also analyzed possible influence of reported serious life events during and after pregnancy, and found no relationship with thyroid autoimmunity; the only plausible association was found between being born before 37 weeks of gestation and an increased risk that the child would develop TGAb at 10 years of age (OR 2.4, $p = 0.003$, $pc = 0.021$). Similarly, an increased risk was found to be related to developing either or both thyroid autoantibodies, although no specific relationship with TPOAb was observed (Paper IV).

The significance of this finding is uncertain. As previously mentioned, TGAb is not considered to be pathogenic²⁸⁸ and, compared to TPOAb, has been reported to be less predictive of later autoimmune thyroid disease. Notwithstanding, it is interesting that a large nationwide study in Sweden found prematurity to be related to thyroxine prescription in young adults¹⁹³. As mentioned, environmental factors impacting autoimmune thyroid disease have been examined quite extensively in adults, but we were unable to confirm the relationship that was recently described between such disease and season of birth or birth size in adults¹⁹¹. Stress, smoking, and alcohol consumption are factors that have been proposed to trigger autoimmune thyroid disease. Although well studied in adults, to our knowledge, no data have been published regarding possible effects of maternal stress or smoking and alcohol habits on a child's thyroid autoimmunity.

It may be that the children in our cohort are still too young to preclude an effect of prematurity, or any influence of the other investigated prenatal factors, on later autoimmune thyroid disease.

Relationship of thyroid autoantibodies with islet autoantibodies

Autoimmune hypothyroid disease is frequently associated with type 1 diabetes, as demonstrated in Table 1. It is not certain whether this relationship is due to joint genetic predisposition or the same environmental factors, or both. We investigated the relationship between thyroid and islet autoantibodies to further identify causation between the diseases.

In children at diagnosis of type 1 diabetes, we discovered a novel association between ZnT8A variants and both of the thyroid autoantibodies TPOAb ($p < 0.039$) and TGAb ($p < 0.015$). In part, this could be explained by a significant relationship between TPOAb and ZnT8RA ($p = 0.036$), one of the two most common variants of ZnT8Ab⁷⁶. Likewise, in 10-year-old children followed due to increased risk of type 1 diabetes risk, we observed that ZnT8A was associated with TPOAb ($p < 0.001$), with TGAb ($p = 0.021$), and with both TPOAb and TGAb ($p = 0.022$) (Papers I and III). Recently, a study of patients with latent autoimmune diabetes in adults showed higher ZnT8A titers to be correlated with TPOAb²⁸⁹, an association that is of interest considering that ZnT8 is expressed in the thyroid gland and in other endocrine tissues as well⁹⁸. The function of ZnT8 in beta cells is fairly well understood⁹⁷, and thus it can be speculated that this protein has an important secretory role in other endocrine cells such as in the thyroid.

In children at the time of diagnosis of type 1 diabetes and in 10-year-olds at risk of this disease, we found GADA to be positively associated with TPOAb ($p < 0.001$ and $p = 0.002$ respectively) (Papers I and III), which confirms a previously described relationship in children and adolescents with type 1 diabetes²⁹⁰. Analogously, two studies of islet autoantibodies in children and adolescents with Hashimoto's thyroiditis but not type 1 diabetes demonstrated a higher prevalence of GADA (4/41 [9.8%]²⁹¹ and 12/236 [5.1%]²⁹²) compared to controls, although the latter results were not statistically significant. At present, it is uncertain whether the existence of GADA in children with Hashimoto's thyroiditis is only indicative of a risk of later type 1 diabetes, or, in light of our results, if it also reflects thyroid autoimmunity.

We observed a previously undescribed negative association between IAA and TPOAb ($p = 0.030$) in children at the time of diagnosis of type 1 diabetes (Paper I), but this relationship was not detected in 10-year-old children in the DiPiS study. The most likely explanation for this disparity is the age difference in the appearance

of these autoantibodies: IAA is known to occur at an early age ⁹⁴, whereas TPOAb is more common in older children and adolescents. Hence children diagnosed with type 1 diabetes before the age of 10 years in the DiPiS study were not analyzed for thyroid autoimmunity. It is not known whether IAA occurred in children that later developed TPOAb, before diagnosis of type 1 diabetes diagnosis.

We also found that the presence of multiple islet autoantibodies at 10 years of age was associated with both TPOAb ($p < 0.001$) and TGAb ($p = 0.012$). Furthermore, a positive relationship was noted between TPOAb and IA-2A ($p = 0.001$) (Paper III).

We also analyzed the correlation between titers of thyroid autoantibodies and islet autoantibodies in children at age 10. This revealed that IA-2A titers were associated with TPOAb ($p = 0.021$, $rs\ 0.36$) and with TGAb ($p = 0.011$, $rs\ 0.40$) in IA-2A-positive children. Neither TPOAb nor TGAb was correlated with titers in children positive for GADA or ZnT8Ab (Paper III).

Gender differences

In thyroid autoimmunity and thyroxine prescription

In our cohort of children and adolescents diagnosed with type 1 diabetes, 44% were girls (1,073/2,433). Among those positive for thyroid autoantibodies, girls were overrepresented, constituting 60%, 57.9%, and 62% of those positive for TPOAb, for TGAb, and for both TPOAb and TGAb, respectively ($p < 0.001$; Figure 7). The female-to-male ratio (F:M) was 1.65:1 for TPOAb, 1.72:1 for TGAb, and 1.68 for co-occurrence of these autoantibodies (Paper I). Likewise, in the DiPiS study, girls constituted half of the study group but were overrepresented among the children positive for thyroid autoantibodies ($p < 0.001$) (Figure 8). In the children followed for an increased risk of type 1 diabetes, F:M was 2.95:1 for TPOAb, 2.5:1 for TGAb, and 3.13:1 for both TPOAb and TGAb (Paper III). TPOAb titers at 10 years of age were also higher in girls than in boys ($p < 0.0049$), and girls were younger when positive for both thyroid autoantibodies (Paper III). TPOAb titers have been observed to be correlated with cytokines produced by T cells in Hashimoto's thyroiditis, which reflects damage to thyroid cells ⁶⁶. Our data showing early detection of autoimmunity and higher titers of TPOAb in girls seem to suggest an increased susceptibility to autoimmune thyroid disease in girls that can appear even at a very young age.

Of the 147 children we studied who were prescribed thyroxine, 66% were girls, F:M 1.94:1, a ratio that is 2–3 times lower than that reported for children and adolescents with autoimmune thyroid disease in the general population ¹¹⁻¹³ (Paper II). Thus, boys with type 1 diabetes are at greater risk of developing thyroid disease.

The difference in F:M for thyroid disease found in children and adolescents with type 1 diabetes compared to the general population was surprising, we therefore wanted to determine whether a gender difference also exists in the association between thyroid and islet autoantibodies in the DiPiS study.

In the relationship between thyroid and islet autoantibodies

Among 10-year olds, 5.6% of the girls (52/931) were positive for islet autoantibodies. However, the only correlation found for girls was between TPOAb and multiple islet autoantibodies ($p = 0.022$), whereas there was no apparent association with islet autoantibody titers.

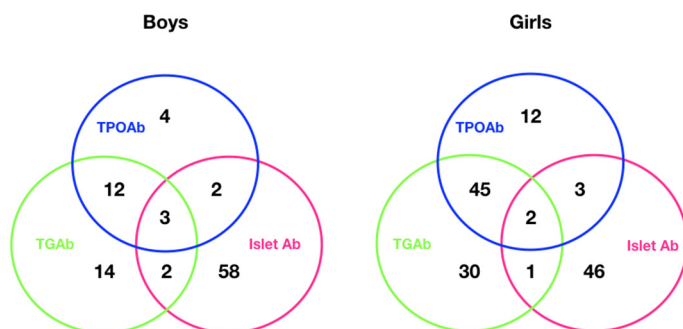


Figure 10. Venn diagram demonstrating the overlap between TPOAb, TGAAb, and islet autoantibodies in both genders at 10 years of age in the DiPiS study.

Considering 10-year-old boys, 6.9% (65/943) were positive for islet autoantibodies. TPOAb was positively associated with all the individual islet autoantibodies, including GADA ($p = 0.002$), IA-2A ($p = 0.001$), ZnT8Ab ($p = 0.001$), and IAA ($p = 0.009$), as well as with multiple islet autoantibodies ($p = 0.001$). TGAAb was positively related to GADA ($p = 0.013$), IA-2A ($p = 0.005$), ZnT8Ab ($p = 0.003$), and multiple islet autoantibodies ($p = 0.001$). Boys that were positive for both thyroid autoantibodies were also more likely to be positive for GADA ($p = 0.03$), IA-2A ($p = 0.005$), ZnT8Ab ($p = 0.003$), IAA ($p = 0.038$), and multiple islet autoantibodies ($p = 0.005$) (Paper III). Analysis of correlation between titers in boys showed a positive relationship between titers of GADA and TGAAb ($p = 0.009$, $r_s 0.38$) in those who were positive for GADA. A correlation was also found between titers of IA-2A and TPOAb ($p = 0.013$, $r_s 0.51$) in boys positive for IA-2A (Paper III).

The novel finding of a clear gender difference in the relationships between thyroid and islet autoantibodies in children at increased genetic risk of type 1 diabetes is fascinating. Moreover, when considering this observation together with the results indicating differences in F:M in children with type 1 diabetes and in those in the

DiPiS study compared with the general population, it seems evident that boys with autoimmune conditions, such as type 1 diabetes, are at increased risk of thyroid autoimmunity, and hence screening for autoimmune thyroid disease should be done in both genders.

Prediction of autoimmune hypothyroid disease in children with type 1 diabetes

Paper I addressed questions concerning the relationships between thyroid and islet autoimmunity in children at the time of diagnosis of type 1 diabetes and also discussed possible associations with HLA-DQ genotypes. The aim of our second investigation (Paper II) was to ascertain whether the findings reported in Paper I could be extended to autoimmune thyroid disease by using data on thyroxine prescription as a measure of clinical diagnosis. We hypothesized that such information obtained from a nationwide prescription register and covering our large cohort of children and adolescents with type 1 diabetes would allow us to identify factors that can predict autoimmune hypothyroid disease.

As stated, thyroid function is essential for growth and development in all children and adolescents, and it is of even greater significance in those diagnosed with type 1 diabetes²⁶³. Thus the importance of screening for autoimmune thyroid disease in type 1 diabetes is quite clear, but unfortunately the recommendations for screening, including advice on frequency and choice of test, vary between clinics and countries. Evaluation of TPOAb, TSH, and FT4 for screening is often recommended at diagnosis of type 1 diabetes²⁹³, although views regarding use of such testing varies during follow-up: some patients positive for thyroid autoimmunity are recommended screening with TSH^{294,295}, or both TSH and TPOAb annually^{296,297}, yet others are advised testing for TSH 6 weeks after diagnosis of type 1 diabetes and thereafter annual screening with TSH²⁹⁸, and in some cases it is recommended that all type 1 diabetes patients be screened for thyroid autoantibodies annually, and that those found to be positive for thyroid autoantibodies undergo testing for TSH²⁸⁰.

Moreover, islet autoantibodies are as described found in children with Hashimoto's why it was of interest to ascertain the prevalence of thyroxine treatment in our cohort before type 1 diabetes was diagnosed. Our analysis showed that thyroxine was prescribed for 6% of the subjects (147/2,433) 5.1–9.5 years after type 1 diabetes diagnosis, and for 10 children the prescription was issued from 4.3 years to 1 week before diagnosis (Figure 11), those 10 subjects were excluded from the prediction analysis.

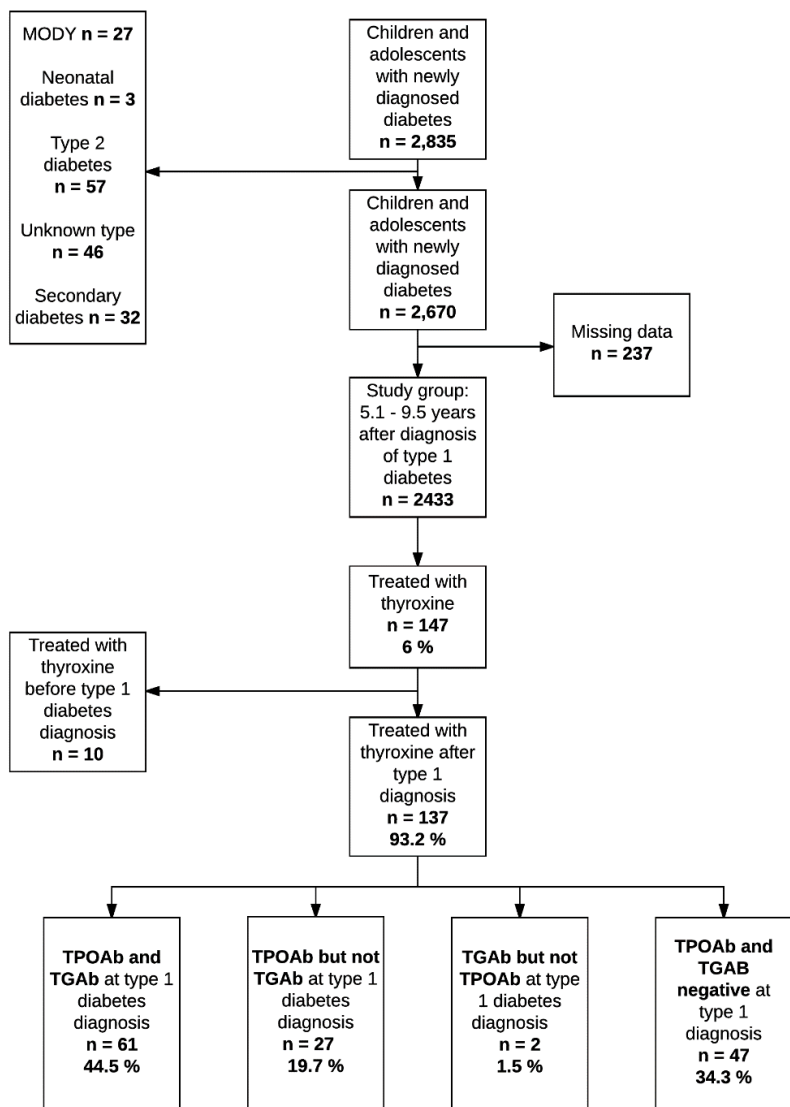


Figure 11.
Flow chart of the study population in relation to thyroxine prescription and thyroid autoantibodies.

The median age at thyroxine prescription was 12.9 years (range 3.6–25.7 years), and the children that later were treated with thyroxine were older at type 1 diabetes diagnosis compared to those not given thyroxine (median age 11.4 vs 10 years; $p = 0.002$). Nevertheless, age was not found to be an independent predictive factor.

Of the 421 children (270 boys and 151 girls) diagnosed with type 1 diabetes before the age of 5 years, 5% (19/421, 15 girls and 4 boys) were prescribed thyroxine after diagnosis, and for all but one of those children the prescription was issued before they reached the age of 10 years. As previously mentioned, the incidence of thyroid disease increases in puberty. Although there are no available records on puberty in our cohort, the age of our subjects suggests that children with type 1 diabetes more often develop thyroid disease that is not related to puberty.

In one fourth of our cohort, the time to thyroxine prescription was within 90 days of confirmation of type 1 diabetes, which definitely indicates simultaneous diagnosis of both the diabetes and autoimmune thyroid diseases. Furthermore, 25% of the patients (35/137) were prescribed thyroxine within 2 years of type 1 diabetes diagnosis. These findings stress the importance of screening for autoimmune thyroid disease at the time of type 1 diabetes diagnosis, regardless of the age of the patient.

Being positive for TPOAb or TGAb and having TSH levels outside the reference limits at the time of type 1 diabetes diagnosis were predictive of later thyroxine prescription, as shown by both univariate and multivariate Cox analysis, thus indicating the independent predictive value of these factors. Levels of TPOAb ($p < 0.001$), but not TGAb ($p = 0.10$), at type 1 diabetes diagnosis were also related to later thyroxine prescription. As mentioned, the value of thyroid autoimmunity for predicting autoimmune thyroid disease is well described. Only 1.5% of the patients in our cohort who were later prescribed thyroxine were positive for TGAb but not TPOAb at diagnosis of type 1 diabetes (Figure 11). Accordingly, to simplify routines, we have now recommended that only TPOAb be measured in screening for thyroid disease in patients with type 1 diabetes.

TSH outside the reference limit was found to predict later thyroid disease, even years after diagnosis of type 1 diabetes ($p < 0.001$). In a study of 110 children²⁹⁸, TSH levels were evaluated at diagnosis of type 1 diabetes and again 45 days later, which showed that aberrant TSH levels had normalized in most cases. TSH is a definite indicator of thyroid disease, and levels of this hormone can be markedly altered by critical illness such as the onset of type 1 diabetes. Clearly, prediction in the short term would not be a novel or remarkable finding, whereas it is quite interesting that abnormal TSH levels at diagnosis of diabetes, which presumably normalize after the diagnosis, can predict later thyroid disease, possibly indicating thyroid susceptibility.

When analyzing the predictive value of islet autoantibodies, we found that GADA positivity at detection of type 1 diabetes predicted future thyroxine prescription ($p < 0.001$), also significant as an independent variable ($p = 0.04$). As observed regarding TPOAb, higher levels of GADA was associated with thyroxine prescription ($p = 0.0013$). Notably, the risk of future thyroxine prescription in TPOAb-negative patients was increased in those who were positive for GADA compared to those who were negative for both TPOAb and GADA ($p = 0.003$).

We conducted further analyses of the independent predictive variables in relation to age, although age as mentioned was not identify as an independent predictive variable.

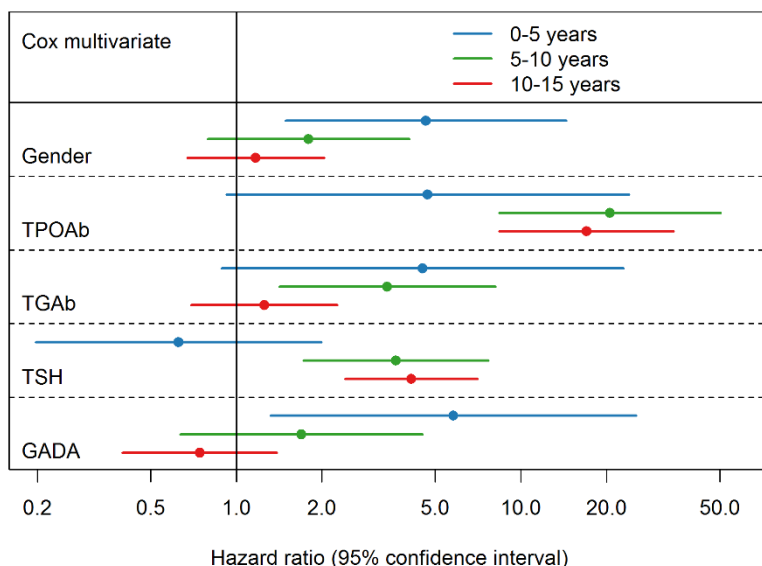


Figure 12. Combined predictive analysis of islet and thyroid autoantibodies and relationship with age at diagnosis of type 1 diabetes.

As shown in Figure 12, we found that the predictive value of islet and thyroid autoantibodies varied in different age groups. GADA positivity and gender predicted future thyroxine prescription in children younger than 5 years at diagnosis of type 1 diabetes, whereas TPOAb and TGAb positivity, as well as abnormal TSH levels, were of predictive value in patients diagnosed with this disease at age 5–10 years. In children and adolescents diagnosed between the age of 10 and 15 years, TPOAb and TSH outside the reference limits predicted later thyroxine prescription. The results indicating different risk factors in different age groups are interesting. The oldest group probably has similarities to adults where TPOAb has been found predictive¹⁰³. The gender disparity is also noteworthy in that it was not statistically significant in children older than 5 years, which may explain the lower F:M ratio compared to the general population. The dissimilar results in the youngest group draw attention to GADA positivity, because thyroid autoantibodies were not found to be significant predictors in that group. A critical limitation of the study is that no information was gathered regarding thyroid autoantibodies after type 1 diabetes diagnosis up to the time of thyroxine prescription. Hence the children may have seroconverted during that period, possibly indicating that GADA positivity precedes

thyroid autoimmunity in young children. Similarly, a previous study of 341 children and adolescents at diagnosis of type 1 diabetes demonstrated that GADA positivity predicted later thyroid disease, and the majority of the GADA-positive patients were diagnosed with autoimmune hypothyroidism within 4 years of the diabetes diagnosis ²⁹⁹. GADA positivity irrespective of thyroid autoimmunity has not been suggested in earlier screening protocols for type 1 diabetes patients. Our results based on a nationwide cohort of children and adolescents with type 1 diabetes further support the relationship between GADA and autoimmune thyroid disease, which is why we suggest that GADA be considered when designing a screening protocol.

Can screening for autoimmune thyroid disease in children with type 1 diabetes be improved?

The goal of the work reported in Paper II was to optimize screening after studying thyroid and islet autoantibodies, thyroid function, and *HLA DQ* genotypes in patients with type 1 diabetes to determine the value of these factors in predicting autoimmune thyroid disease. In light of our results, the protocol presented in Figure 13 is suggested for screening of autoimmune thyroid disease in children and adolescents with type 1 diabetes.

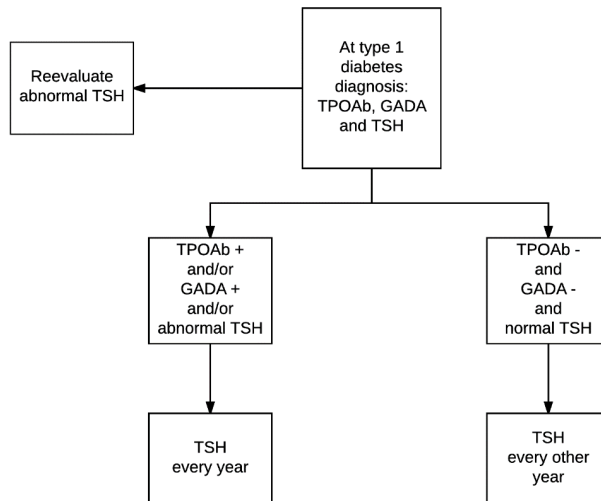


Figure 13. Suggested screening protocol for autoimmune thyroid disease in children and adolescents with type 1 diabetes.

Strengths and weaknesses

The strengths and weaknesses of the present studies are summarized below and are discussed in detail in the four papers included in this thesis.

A definite advantage of our study of children and adolescents at the time of diagnosis of type 1 diabetes is that we used a large nationwide cohort comprising nearly all children diagnosed with this disease over a period of 4.5 years to analyze all islet autoantibodies, including ZnT8A and thyroid autoantibodies, as well as *HLA-DQ* genotypes and thyroid function. This approach allowed us to investigate relationships between the thyroid and islet autoimmunity and associations with *HLA-DQ*.

One weakness of the study reported in Paper I is that information was missing regarding treatment for thyroid disease before and after diagnosis of type 1 diabetes. To compensate for this, in the evaluations presented in Paper II, data on thyroxine prescription in the cohort were obtained from the National Board of Health and Prescription to identify children prescribed thyroxine both before and after diagnosis of diabetes. When these analyses were conducted, the follow-up time after type 1 diabetes diagnosis in the study was 5.1–9.5 years, which enabled us to investigate factors predictive of autoimmune hypothyroid disease. A drawback of that approach is that it includes children treated with thyroxine in combination with thyrostatic drugs, or those treated after thyroidectomy and/or chemotherapy. Nonetheless, autoimmune hypothyroid disease is by far a more common diagnosis in childhood compared to the diagnoses mentioned above. Another limitation of this investigation is that information was missing with regard to thyroid autoimmunity present before diabetes diagnosis or between such diagnosis and treatment with thyroxine, which made it impossible to estimate the time from seroconversion of thyroid autoantibodies to prescription of thyroxine.

The strengths of our investigation of thyroid autoimmunity in children included in the DiPiS study due to an increased risk of type 1 diabetes were as follows: the large size of the cohort and the prospective design of the study using information on perinatal factors, autoimmune heredity, and the children's *HLA-DQ* genotypes, and annual sampling for analysis of islet autoimmunity. These aspects enabled us to investigate factors for development of thyroid autoimmunity. It was also possible to prospectively analyze samples collected from thyroid autoantibody-positive children at the age of 10 years to determine seroconversion and assess relationships with titers, because all autoantibodies are fully titrated. This approach also allowed

us to assess the overlap of thyroid and islet autoimmunity in non-diabetic children at risk of diabetes. Another advantage of this study is that it included follow-up of children positive for thyroid autoimmunity at 10 years of age, which made it possible to reexamine thyroid autoimmunity and investigate thyroid function to gain information on thyroid disease.

Weaknesses of our investigation of the DiPiS subjects are as follows: the use of a cohort selected on the basis of at-risk *HLA DQ* genotypes for type 1 diabetes; the exclusion of children diagnosed with type 1 diabetes before 10 years of age; and missing data on children who withdrew or did not participate in the annual assessments. These shortcomings may have led to underestimation of thyroid autoimmunity and its relationship with islet autoimmunity in the cohort.

A disadvantage in both studies is that TSHrAb were not analyzed, and consequently no information was available concerning the risk for hyperthyroidism and the preceding autoimmunity in children with type 1 diabetes as well as those followed due to an increased risk of that disease.

Conclusions

1. Thyroid autoimmunity was common in children and adolescents at diagnosis of type 1 diabetes, and in children followed due to increased risk of this disease.
2. Thyroid autoimmunity was found from 2 years of age in both genders, but was more common with increasing age. Asymptomatic thyroid dysfunction or established thyroid disease was detected at a later age in one fifth of the children in the DiPiS study who were positive for thyroid autoantibodies at age 10 years.
3. The haplotype *HLA-DQ 5.1* was protective against thyroid autoimmunity and hypothyroid disease in children and adolescents with type 1 diabetes. Overall, relationships with *HLA-DQ* were weak and hence cannot be used for screening children with type 1 diabetes for autoimmune hypothyroid disease.
4. Perinatal factors were not found to be related to thyroid autoimmunity, except regarding a possible association between prematurity and TGAb at 10 years of age. Children with thyroid autoimmunity were more likely to have a first-degree relative with autoimmune disease, fathers with thyroid or rheumatic disease, or siblings with celiac disease.
5. Thyroid autoimmunity was related to positive GADA as well as ZnT8A, and female gender. Girls developed thyroid autoimmunity independently of islet autoimmunity, whereas the risk in boys seemed to be related to presence of other autoimmunity, such as islet autoimmunity or diagnosis of type 1 diabetes.
6. Positive GADA, TPOAb and abnormal TSH at diagnosis of type 1 diabetes predicted autoimmune hypothyroidism in children and adolescents with type 1 diabetes, and this knowledge might be applied in screening for thyroid disease at the time of diagnosis of type 1 diabetes.

General discussion and future perspectives

In the research underlying this thesis, the association between childhood thyroid and islet autoimmunity was studied in children and adolescents at the time of diagnosis of type 1 diabetes, as well as in children followed due to an increased risk of this disease. Novel findings regarding the association between thyroid and islet autoimmunity are described and discussed in relation to gender, genes, risk factors, and prediction.

Notably, our work revealed a connection between thyroid autoantibodies and ZnT8A in children, both at diagnosis of type 1 diabetes and during follow-up for increased risk for this disease at 10 years of age. The secretory role of zinc in the beta cells and the existence of ZnT8 in other endocrine cells suggest that this protein is an endocrine antigen or a marker of multiple autoimmunity, as ZnT8A is rarely found in children under the age of 2 years and seldom appears alone. It would be interesting to determine whether autoantibodies against ZnT8 also occur in patients who have autoimmune thyroid disease but not type 1 diabetes.

In addition, we observed that GADA in children was related to thyroid autoimmunity at type 1 diabetes diagnosis and in 10-year-olds followed due to risk of this disease. This association has also been described in small studies of children diagnosed with autoimmune thyroid disease but not type 1 diabetes. There may be two plausible explanations for this relationship: first, GADA co-occurs with TPOAb or TGAb, or both those autoantibodies, because the immunogenetic risk is shared by beta cells and thyroid cells; second, the triggering factor (e.g., an infectious agent) is also shared by beta cells and thyroid cells. There is no evidence that GAD65 is expressed in thyrocytes, and therefore it is not known whether GADA positivity indicates thyroid autoimmunity. Further immunohistochemistry studies would reveal whether GAD65 occurs in the thyroid gland. Moreover, prospective assessments of children and adolescents, as exemplified by the Environmental Determinants of Diabetes in the Young (TEDDY) study that is presently also investigating thyroid autoimmunity, could disclose the chronological appearance of thyroid and islet autoantibodies representing the effects of possible environmental factors and relationship with later disease. It is also plausible that such investigations can help identify joint environmental and genetic factors involved in the co-occurrence of thyroid and islet autoimmunity and disease.

Defining a strong relationship with HLA in the joint genetic susceptibility has hitherto been difficult. We confirmed a significant protective effect of the haplotype *DQ5.1* on thyroid autoimmunity in children with type 1 diabetes and subsequently extended this finding to include thyroid disease. Divergent reports of susceptible and protective alleles in the joint genetic susceptibility compared to reports for the two diseases separately might be explained by change in epitopes and consequently the binding affinity. In the DiPiS study, we found that children can seroconvert at an early age and stay positive for thyroid autoantibodies for years without progressing to disease. We also observed that half of the thyroxine prescriptions given to children with newly diagnosed type 1 diabetes were issued within two years of diagnosis. Diabetes may be a risk factor for progression from thyroid autoimmunity to disease, perhaps because glycosylation can act as a promoter of the spread of specific thyroid epitopes and thereby modify the affinity for MHC molecules and induce progression of autoimmune response, and this may already occur during the preclinical phase of type 1 diabetes. Alternatively, the relative insulin deficiency influences and alters the function of the T cells, resulting in a defective immune defense. A third potential explanation is that children are diagnosed with autoimmune hypothyroid disease at the same time as they are diagnosed with diabetes simply due to awareness and screening.

Additional assessments of the relationships with HLA, such as modeling thyroid epitopes and analyzing the binding pockets in certain *DQ* haplotypes, might demonstrate differences in affinity, which would further identify susceptibility and risk alleles. Also, next generation sequencing might shed light on further associations with *HLA-DR-DQ-DP* in the co-occurrence of autoimmune thyroid disease and type 1 diabetes.

The predominance of the female gender in autoimmune diseases other than type 1 diabetes is well known. This thesis also shows that females are overrepresented among children positive for thyroid autoimmunity, both at the time of diagnosis of type 1 diabetes and in 10-year-olds followed for the risk of this disease, as well as in children who are later prescribed thyroxine. The novel finding here is a gender-related association between islet and thyroid autoantibodies, which might explain the difference in the female-to-male ratio in children with type 1 diabetes compared to those in the general population. Our results indicate that the risk of thyroid autoimmunity is increased in boys with concomitant autoimmune disease. However, gender was not predictive of later autoimmune thyroid disease in children with type 1 diabetes, except in those under the age of 5 years. Furthermore, it is known that fathers with type 1 diabetes are more likely to transfer the disease to their offspring. We found that fathers with thyroid and rheumatic disease are also more likely to have children positive for thyroid autoantibodies at 10 years of age, again highlighting the gender difference in an unexpected way. This might be explained by autoimmunity being less common in males.

Thyroid autoimmunity was observed in children as young as 2 years of age, and the prevalence was found to be higher both in children at diagnosis of type 1 diabetes and in children followed due to an increased risk of this disease. Interestingly, thyroid dysfunction was quite common in the children followed for increased risk, which underlines the importance of early awareness of asymptomatic hypothyroidism in such children being followed in birth cohort studies, as well as in children who have family members diagnosed with autoimmune disease.

The necessity of screening for autoimmune thyroid disease is unquestionable in children and adolescents with type 1 diabetes. Notwithstanding, current guidelines and data in the literature are not concordant regarding the need for and timing of thyroid testing or the tests of choice. In our large cohort of children, we found that autoimmune thyroid disease was diagnosed concurrently with type 1 diabetes in one fourth of the children, and, an additional one fourth were prescribed thyroxine during the first 2 years after diagnosis of type 1 diabetes. Predictive factors for later thyroxine prescription were positivity for TPOAb, TGAb, GADA, and abnormal TSH. The results presented here add strong practical evidence in favor of concurrent testing for TPOAb, GADA, and TSH at the time of diagnosis of type 1 diabetes, and continuous testing at follow-up.

Swedish summary

Kronisk inflammation i sköldkörteln och typ 1 diabetes, var för sig samt i kombination, är de vanligaste sjukdomarna i de hormonbildande organen hos barn och ungdomar. Sköldkörteln styr ämnesomsättningen i kroppen och är viktig både för barns mentala och fysiska utveckling. Kronisk inflammation i sköldkörteln medför störningar i körtelns hormonproduktion, där underproduktion av hormonet thyroxin (hypothyreos) är vanligast hos barn och ungdomar. Hypothyreos behandlas med Levaxin, thyroxin i tablettform. Barn med typ 1 diabetes har förlorat förmågan att tillverka det livsviktiga hormonet insulin, varför livslång behandling med insulin krävs för överlevnad - en mycket stor påfrestning för barnet och familjen. Båda sjukdomarna är autoimmuna, vilket innebär att kroppens immunförsvar angriper egna celler. Ett av immunförsvarets verktyg är antikroppar, som märker ut det som är främmande och skal avlägsnas. De två viktigaste autoantikropparna vid hypothyreos är riktade mot viktiga protein i produktionen av sköldkörtelhormon; thyroïdperoxidase (TPOA) och thyroglobulin (TGA). Vid typ 1 diabetes finns flera autoantikroppar, även dessa är riktade mot proteiner i betacellen - cellen som tillverkar insulin. Diabetesautoantikropparna är riktade mot glutaminsyra-decarboxylas (GADA), insulinoma-associerat-antigen-2 (IA-2A), insulin (IAA) och tre olika varianter av zinktransportör 8 (ZnT8A). Innan sjukdomsdebut kan det autoimmuna förloppet upptäckas och följas genom att mäta autoantikroppar i blodet. Autoantikropparna är prediktiva (kan förutsäga) för sjukdomens utveckling och används både vid screening och diagnos.

Det är fortfarande oklart vad som inleder den autoimmuna processen och leder till sjukdom hos vissa barn. Hitintills har forskning, framförallt på typ 1 diabetes, visat att en viss genetisk känslighet krävs men även att utlösande och påskyndande faktorer i omgivningen är viktiga för utveckling av sjukdom. Det samma gäller avseende grunden till att sjukdomarna samvarierar. Det är dock oklart om sjukdomarna samvarierar på grund av att de har samma genetiska bakgrund och påskyndande faktorer eller om den ena sjukdomen ökar risken eller har betydelse för att den andra utvecklas. De gener som är av största intresse är så kallade HLA gener. Dessa gener kodar för det försvarssystem som känner igen kroppsegna antigen.

Hypothyreos hos barn med typ 1 diabetes är viktigt att upptäcka i tid, då störningar i sköldkörtelfunktionen påverkar blodsockerkontrollen och möjligen även de allvarliga långtidskomplikationerna senare i livet. Internationellt finns

rekommendationer om screening av sköldkörtelfunktionen hos barn med typ 1 diabetes. Dock saknas konsensus om vilka prover som skall användas och hur ofta dom skall tas, då stora studier på prediktion av sköldkörtelsjukdom hos barn med typ 1 diabetes saknas.

Det övergripande syftet med avhandlingen är att studera frekvensen av sköldkörtelautoantikroppar hos barn och ungdomar med nydebuterad diabetes och hos barn som följs för ökad risk för typ 1 diabetes samt sambandet mellan diabetes autoantikroppar och sköldkörtelautoantikroppar, HLA-gener och kön. Vi ville även undersöka om faktorer vid diabetesdebut kunde förutse, prediktera, om barn och ungdomar med typ 1 diabetes skulle utveckla hypothyreos senare samt om faktorer under graviditet och runt förlossning samt hereditet för autoimmuna sjukdomar var relaterade till sköldkörtelautoantikroppar hos barn som följs för sin ökade risk för typ 1 diabetes.

Arbetena i avhandlingen är baserade på två studier: Better Diabetes Diagnosis (BDD) studien och Diabetes Prediktion i Skåne (DiPiS) studien. BDD är en nationell studie där nästan alla barn som insjuknar i diabetes i Sverige ingår. Vid diagnos analyseras alla diabetesautoantikroppar samt HLA-gener. Från maj 2005 till och med oktober 2009 analyserades även TPOA, TGA samt sköldkörtelfunktion - thyroidea stimulerande hormon (TSH) - hos 2433 barn. Diabetes Prediktion i Skåne (DiPiS) är en prospektiv longitudinell studie som följer barn med ökad risk för typ 1 diabetes. Studiens syfte är att studera samband mellan olika riskfaktorer och utveckling av typ 1 diabetes. I studien följs barn från födelsen fram till 15 års ålder med målet att identifiera faktorer i omgivningen som kan ha en betydelse för utvecklingen av typ 1 diabetes. Under åren 2000–2004 togs det prov från navelsträngen på drygt 35 000 nyfödda barn i Skåne för att identifiera de som hade riskgener för typ 1 diabetes. Totalt följs nu nästan 2000 barn. Barnen kommer årligen för kontroller och blodprov tas för analys av diabetesautoantikroppar. Föräldrar, och de äldre barnen, svarar på frågeformulär angående bland annat nutrition, hälsa, mediciner och välbefinnande.

I **arbete I** fann vi att prevalensen av sköldkörtelautoantikroppar hos barn och ungdomar med nydebuterad diabetes var 12%, vanligare hos flickor. TPOA och TGA var båda associerade med GADA och ZnT8A. Sambandet mellan sköldkörtelautoantikroppar och ZnT8A är inte tidigare rapporterat - ett ganska intressant samband då zinktransportör 8-proteinet även finns i sköldkörteln. Vi fann även att IAA var negativt associerat till TPOA, ett samband som inte finns beskrivit tidigare. Möjligen har dessa barn lägre risk för sköldkörtelautoantikroppar, men sannolikt kan det förklaras av att barn med IAA är unga vid diabetesdebut och att TPOA är vanligare hos äldre barn. Vi kunde också bekräfta att en variant av HLA genen är skyddande för sköldkörtelautoantikroppar hos barn med typ 1 diabetes. De beskrivna fynden är intressanta och ger en inblick i samvariationen av autoimmunitet mot sköldkörtel och betaceller.

Inga uppgifter fanns i vårt data som insamlades till arbete I om diagnos av sköldkörtelsjukdom. Vi ville **i arbete II** undersöka om resultaten från arbete I även gällde för klinisk sjukdom och därmed undersöka om det finns faktorer vid diabetesdebut som förutsäger att barnet senare utvecklar hypothyreos. I november 2014, 5.1–9.5 år efter diabetesdiagnos, inhämtade vi uppgifter från Läkemedelsverket, Socialstyrelsen, om vilka barn i studien som hade fått Levaxin utskrivet och när detta skrivits ut. Vi fann att 147 av 2433 barn hade fått utskrivet Levaxin varav 10 barn fick Levaxin före diabetesdiagnos. En fjärdedel av barnen som fick utskrivet Levaxin, fick det i anslutning till sin diabetesdiagnos och en fjärdedel inom 2 år från diagnos, ett intressant fynd då hypothyreos ofta ansetts vara en sjukdom associerad med lång diabetesduration. Flickor har en ökad risk för Levaxinbehandling jämfört med pojkar, men skillnaden mellan könen är inte lika stor som hos barn med hypothyreos utan samtidig typ 1 diabetes. Det samband som fanns mellan sköldkörtelautoantikroppar och ZnT8A i arbete I kunde inte bekräftas avseende senare Levaxinbehandling. Barn med GADA, TPOA, TGA och onormalt TSH vid diabetesdebut visade sig ha en ökad risk för senare Levaxinbehandling. Att TPOA och TGA förutsäger sköldkörtelsjukdom är redan känt men vi kunde i denna studie visa att enbart 2 barn som senare fick Levaxin var positiva för TGA men inte TPOA, varför TGA inte behövs i screeningsyfte. TSH er ett bra mått på hur sköldkörtelfunktionen ser ut, men TSH kan påverkas av annan sjukdom, som diabetesdebut. Av den anledningen har TSH inte rekommenderas av flera i screening vid diabetesdiagnos utan endast senare. Vi fann att onormalt TSH vid diabetesdiagnos var kopplad till Levaxinbehandling även flera år senare, vilket tyder på viss känslighet i sköldkörteln. Vi rekommenderar att TSH tas vid diabetesdiagnos och sedan regelbundet som led i screening för hypothyreos. GADA är tidigare beskrivit som riskfaktor för sköldkörtelsjukdom hos patienter med typ 1 diabetes och finns även beskrivet hos patienter med sköldkörtelsjukdom utan typ 1 diabetes. Det är oklart vad denna relation beror på. Möjligen beror det på att den genetiska känsligheten är densamma, eller att samma faktorer är utlösande. Ett annat alternativ är att GADA är ett tecken till mer utbredd autoimmunitet. Trots den kända relationen finns GADA inte med i rekommendationer när det gäller screening för sköldkörtelsjukdom hos barn och ungdomar med typ 1 diabetes. I vår studie fann vi att barn positiva för GADA, men negativa för TPOAb, TGAb och med ett normalt TSH vid diabetesdiagnos, hade en ökad risk för senare Levaxinbehandling. Därför föreslår vi att GADA tillsammans med TPOAb och TSH används vid screening av sköldkörtelsjukdom hos barn med typ 1 diabetes redan från diabetesdiagnos (Figur 13). Vi fann i arbete II att samma HLA-gener som i arbete I var skyddande för sköldkörtel-autoantikroppar, även var skyddande mot klinisk sjukdom.

Prospektiva studier på sköldkörtelautoantikroppar och sköldkörtelsjukdom finns inte på barn, varför man inte vet när barnen först blir positiva för dessa autoantikroppar. I DiPiS studien har vi en unik möjlighet att analysera sköldkörtelautoantikroppar hos en grupp barn med genetisk risk för typ 1 diabetes,

men som ännu ej insjuknat och där de flesta kommer att förbli friska. I denna prospektiva studie kan vi också undersöka när autoantikroppar mot sköldkörtel och betaceller utvecklas samt hur de samvarierar hos friska barn. I **arbete III** fann vi att 6,9% av 10 år gamla barn i DiPiS studien hade TPOA och/eller TGA. Vissa barn utvecklade sköldkörtelautoantikroppar redan från 2 års ålder, även om fler utvecklade autoantikroppar med åldern. Risken var betydligt högre för flickor. Vi fann liknande samband mellan autoimmunitet mot sköldkörtel och betaceller som i BDD-studien, fram för allt när det gäller TPOA, som var associerad med ZnT8A, GADA och IA-2A, medan TGA endast var associerad till ZnT8A. Det fanns en tydlig könsskillnad i sambandet mellan sköldkörtelautoantikroppar och diabetesautoantikroppar, då associationen endast fanns hos pojkar. Risken för sköldkörtelautoantikroppar och möjligen senare sjukdom är således sannolikt relaterad till annan autoimmun sjukdom som typ 1 diabetes hos pojkar, medan flickor har allmänt en större risk.

I **arbete IV** undersökte vi om händelser under graviditet och förlossning påverkade risken för att barnet senare utvecklade sköldkörtelautoantikroppar. Faktorer som födelselängd och vikt, graviditetslängd, infektioner, alkoholkonsumtion, rökning samt stress under graviditeten var undersökta. Vi fann ett möjligt samband mellan att vara tidig född och att ha TGA vid 10 års ålder, medan övriga faktorer inte påverkade risken. I samma arbete undersökte vi även sambandet mellan sköldkörtelautoantikroppar och rapporterade autoimmuna sjukdomar i familjen. Barn vars pappa rapporterade sköldkörtelsjukdom eller reumatisk sjukdom, samt om förälder eller syskon hade celiaki, hade högre risk för sköldkörtelautoimmunitet vid 10 års ålder. Samma sak gällde om en förälder eller ett syskon hade någon autoimmun sjukdom. Att autoimmuna sjukdomar samvarierar i familjer är välkänt sedan tidigare. Våra resultat visar nu också att risken att barn till föräldrar med autoimmuna sjukdomar eller barn vars syskon har autoimmun sjukdom, skulle kunna ha nytta av att screenas för sköldkörtelsjukdom.

Fler studier som DiPiS, där barn följs under längre tid, kan förhoppningsvis belysa varför barn utvecklar autoimmuna sjukdomar som sköldkörtelsjukdom och typ 1 diabetes samt ge möjlighet för intervention.

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