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Epidemiology and Risk Markers of
Autoimmune Diseases
in Russian Karelia and in Finland



ACADEMIC DISSERTATION

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1. List of original publications

This dissertation is based on the following original publications referred to in the text by their Roman numerals I–V:

- I Kondrashova A, Reunanen A, Romanov A, Karvonen A, Viskari H, Vesikari T, Ilonen J, Knip M, Hyöty H. A sixfold gradient in the incidence of type 1 diabetes at the eastern border of Finland. *Ann Med* 2005;37:67-72.
 - II Kondrashova A, Viskari H, Kulmala P, Romanov A, Ilonen J, Hyöty H, Knip M. Signs of beta-cell autoimmunity in nondiabetic schoolchildren: a comparison between Russian Karelia with a low incidence of type 1 diabetes and Finland with a high incidence rate. *Diabetes Care* 2007;30:95-100.
 - III Kondrashova A, Mustalahti K, Kaukinen K, Viskari H, Volodicheva V, Haapala AM, Ilonen J, Knip M, Mäki M, Hyöty H and the EPIVIR Study Group. Lower economic status and inferior hygienic environment may protect against celiac disease. *Ann Med* 2008;40:223-231.
 - IV Kondrashova A, Viskari H, Haapala A-M, Seiskari T, Kulmala P, Ilonen J, Knip M, Hyöty H. Serological evidence of thyroid autoimmunity among schoolchildren in two different socio-economic environments. *J Clin Endocrinol Metab* 2008;93:729-734.
 - V Viskari H, Kondrashova A, Koskela P, Knip M, Hyöty H. Circulating vitamin D concentrations in two neighboring populations with markedly different incidence of type 1 diabetes. *Diabetes Care* 2006;29:1458-1459.
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2. Abbreviations

AGA	antigliadin antibodies
AITD	autoimmune thyroid disease
APC	antigen-presenting cell
AT	autoimmune thyroiditis
CBV	coxsackie B virus
CI	confidence interval
CD	celiac disease
CTLA-4	cytotoxic T-lymphocyte antigen-4
DAISY	Diabetes Autoimmunity Study in the Young
DC	dendritic cells
DIPP	the Finnish Diabetes Prediction and Prevention Study
EAE	experimental allergic encephalomyelitis
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EMA	endomysial antibodies
GADA	glutamic acid decarboxylase antibody
GFD	gluten-free diet
GNP	gross national product
HLA	human leukocyte antigen
IAA	insulin autoantibody
IA-2A	islet antigen-2 antibody

ICA	islet cell antibody
IBD	inflammatory bowel disease
IDO	indoleamine 2,3 dioxygenase
IFN	interferon
Ig	immunoglobulin
IgA	immunoglobulin class A
IgE	immunoglobulin class E
IgG	immunoglobulin class G
IL	interleukin
JDF	Juvenile Diabetes Foundation
JDFU	Juvenile Diabetes Foundation units
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MS	multiple sclerosis
NOD mice	non-obese diabetic mice
NOD	nucleotide-binding oligomerization domain
NS	non-significant
PRR	pattern recognition receptor
PTP	protein tyrosine phosphatase
RIA	radioimmunoassay
RU	relative units
FT4	free thyroxin
SLE	systemic lupus erythemathosus

SNS	self-non-self discrimination
TCR	T-cell receptor
TG	thyroglobulin
TGAb	thyreoglobulin antibodies
TGF	transforming growth factor
TLR	toll-like receptor
TNF	tumor necrosis factor
TPO	thyroid peroxidase
TPOAb	thyroid peroxidase antibodies
TSH	thyroid stimulating hormone
TSHR	thyroid-stimulating hormone receptor
TSHRAb	thyroid-stimulating hormone receptor antibodies
tTG	tissue transglutaminase
tTGA	tissue transglutaminase antibody
T1D	type 1 diabetes
USD	United States Dollar
WHO	World Health Organization

3. Abstract

Autoimmune diseases are complex disorders in which susceptibility genes and environmental factors act together in the initiation of the autoimmune process. It is generally believed that genetic factors alone are not sufficient for the induction of these diseases but environmental factors play a role either increasing or decreasing the disease risk. The concordance rate of 20-50% in monozygotic twins suggests that environmental factors, such as virus infections and dietary factors, may have an influence on the appearance of the autoimmune response. The incidence rates of different autoimmune diseases vary a lot, and depend on age and sex as well as geographic and ethnic differences between populations.

Type 1 diabetes (T1D) is one of the most common organ-specific autoimmune diseases. It is more common in northern European countries than in southern Europe with the highest rate in Finland (62 per 100,000 in 2007). It has also rapidly increased worldwide over the past 50 years. The pathogenesis of T1D is mediated by an autoimmune process which selectively destroys the insulin-producing beta cells in the pancreas. This process may be subclinical for several months or even years and can be detected by the presence of autoantibodies against beta-cell antigens (islet cell autoantibodies, ICA; antibodies against insulin, IAA; glutamic acid decarboxylase, GADA; the protein tyrosine phosphatase (PTP) related IA-2 molecule, IA2A; and zinc-transporter 8, ZnT8A). In the past 30 years, a number of organ-specific autoantigens have been characterized in many other immune-mediated diseases such as autoimmune thyroid disease and celiac disease (CD), resulting in the improvement of strategies to detect subjects at risk in an early preclinical phase.

The aim of the present study was to address the role of genetic and environmental factors in the pathogenesis of autoimmune diseases such as T1D, CD and autoimmune thyroid disease. The interaction between genetic and environmental factors was analyzed in two neighbouring populations living in completely different socio-economic circumstances (Karelian Republic of Russia and Finland). The fact that these two populations share partly the same ancestry but differ in many lifestyle-associated factors creates an ideal setting to study the role of non-genetic (environmental and lifestyle-associated) factors in the pathogenesis of immune-mediated diseases. As a marker of these socioeconomic differences several microbial infections are known to be substantially more common in Russian Karelia than in Finland.

The incidence of T1D was studied among 0-14- year-old children in the Karelian Republic of Russia during the period 1990-1999. The study indicated a close to sixfold higher incidence of T1D in Finland compared to Russian Karelia. The incidence rate did not differ significantly between different ethnic groups in Russian Karelia (Finns/Karelians, Russians, others). Diabetes-associated autoantibodies (ICA, IAA, GADA and IA-2A) were screened in the background population including 3,652 nondiabetic schoolchildren in Finland and 1,988 schoolchildren in Russian Karelia. The frequencies of ICA, IAA, and GADA did not differ significantly between the Karelian (3.5%, 0.6%, and 0.9% respectively) and Finnish children (2.8%, 0.9%, and 0.5% respectively), while IA-2A were four times more common in Finland (0.6% vs. 0.15% in Russian Karelia; $P=0.03$). The frequency of multiple autoantibodies was similar in both countries (0.5% vs. 0.6%). The autoantibody prevalence did not differ significantly among the three ethnic groups in Russian Karelia. The genetic susceptibility to

T1D, as defined by major HLA risk genes (DQA1*05-DQB1*02/*0302, DQB1*0302/x; x ≠ DQA1*05-DQB1*02, DQB1*0301, DQB1*0602 or DQB1*0603), was about the same in both populations, suggesting that these risk genes cannot explain the conspicuous gradient in the incidence of T1D. These findings suggest that beta-cell autoimmunity is induced as frequently in the low-incidence Russian Karelia as in the high-incidence Finland, but progressive beta-cell autoimmunity is less common in Russian Karelia.

The prevalence of celiac-disease associated antibodies (transglutaminase and gliadin antibodies) and predisposing HLA-DR3-DQ2 (DQA1*05-DQB1*02) and HLA-DR5-DQ7/DR7-DQ2 haplotypes were screened in the same unselected cohorts of schoolchildren in Russian Karelia and Finland. A conspicuous gradient was observed in the prevalence of transglutaminase antibodies (0.6% in Russian Karelia vs. 1.4% in Finland, P=0.005). Immunoglobulin class G antigliadin antibodies were also less frequent in children from Russian Karelia (10.2% vs. 28.3%, P< 0.0001). Children positive for transglutaminase antibodies were invited to small-bowel biopsy to confirm the diagnosis of CD. The results indicated a fivefold difference in the prevalence of biopsy-proven CD between the two populations (a prevalence of 1 in 496 in Karelia compared to 1 in 107 children in Finland). The HLA-DQ risk alleles for CD showed minor differences between the two populations suggesting that genetic susceptibility cannot explain the huge gradient in the prevalence of the disease. In addition, the consumption of grain products did not differ between the two populations.

The prevalence of thyroid autoantibodies [thyroid peroxidase antibodies (TPOAb), thyroglobulin antibodies (TGAb)] was studied in a subgroup of 532 schoolchildren from Russian Karelia and 532 schoolchildren from Finland. The frequency of TPOAb was significantly lower in Russian Karelian children compared to Finnish children (0.4% vs. 2.6%, P=0.006). A similar difference was observed for TGAb (0.6% vs. 3.4%, P=0.002). Gender had a clear effect on thyroid autoimmunity in both populations, and the predominance of girls (88.5%) was seen for both TPOAb and TGAb (P<0.001). The frequency of the susceptible HLA-genotypes (DR3-DQ2/x, DR4-DQ8/y and other genotypes) was similar in Finland and Russian Karelia and thyroid antibodies showed no clear association with HLA alleles.

Vitamin D deficiency has been implicated in increasing the risk of autoimmune diseases. In the present study the 25-OH-vitamin D status was analyzed in schoolchildren and pregnant women as one possible environmental determinant of the observed difference in disease susceptibility in these two populations. The series of schoolchildren comprised 100 children from the Karelian Republic of Russia and 100 subjects from Finland matched for age, gender and month of sampling. The series of pregnant women included 103 samples obtained from Karelian pregnant women and 172 samples from Finland representing similar age and calendar time of sampling. Circulating concentrations of 25-hydroxy (25-OH) vitamin D did not differ between Finland and Russian Karelia among the schoolchildren (median 39.3 vs. 35.0 nmol/l, P=NS) or pregnant women (median 28.9 vs. 28.4 nmol/l; P=NS).

In conclusion, this study indicates a conspicuous difference in the prevalence of autoimmune diseases between the two adjacent countries. The difference observed was not associated

with HLA risk alleles suggesting that non-genetic (environmental) factors must play an important role in this phenomenon. There may be some driving environmental factors in Finland or a lack of protective environmental factors that are present in Russian Karelia. However, vitamin D status, which has been implicated as a modulator of the risk for autoimmune diseases, seems not to be among these factors as it did not differ between the two populations. The protective environment in Russian Karelia is characterized by inferior prosperity and standard of hygiene as well as high frequency of microbial infections. Accordingly, the present findings are in line with the so-called 'hygiene hypothesis' according to which the reduced exposure to microbes in Western countries leads to an imbalance in the immune system predisposing to the development of autoimmune and allergic diseases.

4. Finnish summary

Autoimmuunitautien epidemiologia ja riskimerkkiaineet Venäjän Karjalassa ja Suomessa.

Autoimmuunitaudit syntyvät kun elimistön oma puolustusjärjestelmä hyökkää omia kudoksia kohtaan. Aikaisempien tutkimusten perusteella tiedetään, että useimpien autoimmuunitautien syntyä säätelevät sekä geneettiset että elinympäristöön liittyvät tekijät. Autoimmuunitaudeille altistavia geenejä on tunnistettu jo lukuisia, mutta ympäristötekijöistä ja niiden vuorovaikutuksista riskigeenien kanssa tiedetään paljon vähemmän. Ympäristön vaikutuksen puolesta puhuvat mm. se, että vain 20-50 %:ssa sellaisista identtisistä kaksospareista, joista vähintään toisella on autoimmuunitauti, molemmat sairastuvat samaan tautiin. Lisäksi autoimmuunitaudit ovat yleistyneet niin nopeasti, että nousua ei voi selittää geneettisillä tekijöillä, vaan ympäristötekijöillä on todennäköisesti tärkeä osuus tässä ilmiössä. Näihin epäiltyihin ympäristöperäisiin tekijöihin kuuluvat erityisesti tietyt virusinfektiot (esim. enterovirukset) ja ravintotekijät (esim. D-vitamiini), jotka voivat vaikuttaa autoimmuunitautien riskiin.

Autoimmuunitautien patogeneesiä välittää immunologinen prosessi, joka etenee yleensä hitaasti ja joka voidaan todeta osoittamalla kohdekudokseen suuntautuvia autovasta-aineita verenkierrosta. Keliakiassa transglutaminaasi-entsyymiä vastaan kohdistuvien autovasta-aineiden (tTGA) osoittamisesta on tullut tärkeä diagnostinen testi ja lisäksi keliakiassa esiintyy muita autovasta-aineita sekä vasta-aineita ravinnon gliadiinia vastaan. Tyypin 1 diabeteksessä esiintyy puolestaan haiman saarekesoluihin kohdistuvia autovasta-aineita, joita voidaan käyttää riskiänsiöiden tunnistamiseen (saarekesoluvasta-aineet, ICA; insuliiniautovasta-aineet, IAA; glutamiinihappodekarboksylaasia vastaan kohdistuvat autovasta-aineet, GADA; proteiiniityrosiinifosfataasiperheeseen kuuluvaa insulinooma-antigeeni 2:ta kohtaan suuntautuvat autovasta-aineet, IA-2A; sekä sinkin kuljettajaproteiinia (sinkki-transporter 8, ZnT8A) kohtaan suuntautuvat autovasta-aineet. Kilpirauhasen autoimmuunisairauksissa todetaan puolestaan autovasta-aineita kilpirauhasen peroksidaasi-entsyymiä (TPO) ja thyreoglobuliinia (TG) vastaan.

Tämän tutkimuksen tavoitteena on selvittää geenien ja ympäristötekijöiden osuutta autoimmuunitautien synnyssä käyttäen hyväksi ainutlaatuista epidemiologista asetelmaa. Tutkimus perustuu autoimmuunitautien ja niille altistavien riskitekijöiden vertailuun kahdessa väestössä, jotka asuvat maantieteellisesti lähekkäin ja joilla on osittain samantyyppinen geneettinen tausta, mutta jotka kuitenkin elävät hyvin erilaisessa sosioekonomisessa ympäristössä. Nämä kaksi kohorttia koostuvat kouluikäisistä lapsista, jotka asuivat toisaalta Karjalan tasavallassa Venäjällä ja toisaalta vastaavanikäisistä lapsista Suomessa. Tutkimus kohdistuu kolmen yleisen autoimmuunitaudin (tyypin 1 diabeteksen, keliakian ja kilpirauhasen autoimmuuni-ilmiöiden) ja niiden riskitekijöiden esiintyvyyden selvittämiseen taustaväestössä. Näiden sairauksien yleisyyttä tutkittiin analysoimalla kliinisen sairauden ilmaantuvuutta (tyypin 1 diabetes) ja toisaalta seulomalla terveiltä koululaisilta ym. sairauksiin liittyviä autovasta-aineita ja HLA-geenejä. Lisäksi tutkittiin väestön D-vitamiinitasoa, jolla on epäilty olevan merkitystä ko. sairauksien synnyssä.

Tavoitteena on näin saada uutta tietoa geeni-ympäristö-vuorovaikutuksista, jotka säätelevät autoimmuunitautien riskiä.

Tutkitut autoimmuunisairaudet ja niihin liittyvät autovasta-aineet osoittautuivat olevan Suomessa monikertaisesti yleisempiä kuin Karjalan tasavallassa. Tämä ero ei selittynyt HLA-riskigeenien eroilla, sillä HLA-geenien jakaumassa havaitut erot olivat pienempiä kuin sairauksien esiintymisessä havaitut erot. Lisäksi autovasta-aineita esiintyi suomalaisilla korkean geneettisen riskin omaavilla lapsilla enemmän kuin vastaavilla korkean riskin lapsilla Karjalan tasavallassa viitaten siihen, että erot sairauksien esiintyvyydessä johtuvat pääosin muista tekijöistä kuin HLA-riskigeeneistä. Vaikka autovasta-aineissa oli tämänkaltaisen yleinen ero väestöjen välillä, diabetekseen liittyvät autovasta-aineet olivat siinä suhteessa poikkeus, että neljästä tutkitusta diabetes-autovasta-aineesta vain yksi (IA-2A) oli merkittävästi yleisempi suomalaisilla kuin Karjalan tasavallan lapsilla. Koska tämä vasta-aine liittyy pitkälle edenneeseen beeta-soluvaurioon ja koska tyyppin 1 diabeteksen ilmaantuvuus oli Suomessa kuusi kertaa suurempi kuin Karjalan tasavallassa, on mahdollista että Karjalan tasavallan lapsilla diabetekseen liittyvä autoimmuuniprosessi ei etene aggressiiviseen vaiheeseen yhtä usein kuin Suomessa.

Kaiken kaikkiaan tulokset viittaavat siihen, että ympäristötekijöillä on tärkeä merkitys autoimmuunitautien patogeneesissä. On mahdollista, että Suomessa vallitseva elinympäristö edistää autoimmuuniprosessin käynnistymistä ja etenemistä tai että Karjalan tasavallan ympäristössä on suojaavia tekijöitä. Tässä tutkimuksessa analysoitiin yhden mahdollisen ympäristötekijän merkitystä (D-vitamiini), sillä aiemmat tutkimukset ovat viitanneet siihen, että D-vitamiinin puutos voi altistaa autoimmuunitaudeille. Tulokset osoittavat, että elimistön D-vitamiinitasot eivät merkittävästi poikenneet väestöjen välillä, ja D-vitamiinin puutos oli jopa hieman yleisempää Karjalan tasavallassa. Näin ollen D-vitamiini ei näyttäisi selittävän näitä autoimmuunitautien esiintyvyydessä todettuja eroja väestöjen välillä.

Tutkimus on ensimmäinen kattava selvitys autoimmuunitautien esiintyvyydestä Karjalan tasavallassa. Autoimmuunitautien vertailu Suomen ja Karjalan tasavallan välillä osoittautui hedelmälliseksi tutkimusasetelmaksi, joka mahdollistaa uuden tiedon saamisen autoimmuunitautien syntymekanismeista, erityisesti ympäristötekijöiden ja geenien vuorovaikutuksista. On mahdollista, että tätä tutkimusasetelmaa käyttäen voidaan tunnistaa tekijöitä, jotka suojaavat Karjalan tasavallan lapset näiltä sairauksilta ja kehittää tältä pohjalta uusia hoitoja ja ennaltaehkäisykeinoja.

5. Introduction

Over the last few decades we have witnessed a dramatic increase in the prevalence and incidence of major immune-mediated diseases particularly in developed and industrialized countries around the world, including autoimmune diseases such as type 1 diabetes (T1D), celiac disease (CD) and autoimmune thyroid diseases (AITD). This rising trend in the rate of autoimmune diseases seems to continue. In contrast, there is a considerably lower incidence rate of autoimmune diseases in middle- and low-income countries.

Autoimmune disease is characterized by an immune-mediated attack on the target organ that is no longer recognized by the immune system as self. Currently, there are at least 60 known or suspected autoimmune disorders, affecting approximately 5 percent of the population in Western countries and it is evident that autoimmune diseases create a substantial and increasing public health concern. For some autoimmune diseases the population prevalence is conspicuously high, e.g. AITD, rheumatoid arthritis and the population morbidity is substantial for some others, e.g. for T1D, systemic lupus erythematosus (SLE), and multiple sclerosis (MS). In developed and increasingly so even in developing countries, autoimmune diseases rank well up with the major global health concerns of cancer, cardiovascular diseases and chronic pulmonary diseases (1).

Autoimmune T1D is among the most common of all chronic diseases in children. Since diabetes represents a major medical problem in terms of the increasing incidence and the high rate of diabetes-specific complications, there is an obvious need to develop measures for the prevention of the disease. Predisposition to beta-cell autoimmunity is under polygenic control, but studies on monozygotic twins demonstrate that environmental factors are equally important. In addition, the dramatic increase in the incidence of T1D in children under 15 years of age in developed countries cannot be explained by genetic factors alone. The countries that have had the most dramatic rise in the rate of T1D have over the same period experienced tremendous improvements in socio-economic status and sanitation. The same increasing trend has also been described for other autoimmune diseases such as CD and multiple sclerosis (MS). It has been proposed that continuous improvement in sanitation and living standards in developed countries may somehow predispose to autoimmune diseases. Rapid transformation of the environment and lifestyle has not allowed time for the human immune system to adjust to these changes, and the reduced exposure to childhood infections has been implicated in the increase in the incidence rate and prevalence of autoimmune and allergic diseases (hygiene hypothesis) (2).

Over the last decades considerable progress has been made in the identification of autoantigens and in the development of strategies to predict autoimmune diseases. There are consistent findings indicating that the autoimmune process precedes the clinical manifestation of autoimmune disorders by many months or even years. Thus, the appearance of organ-specific autoantibodies in the early preclinical state reflects an increased risk for future development of the disease. This has made it possible to diagnose subclinical

autoimmune diseases and predict the development of clinical symptoms on an individual level.

This study was carried out to evaluate the effect of environment on the development of common autoimmune diseases (T1D, CD and AITD) by analyzing immunological and genetic risk markers of autoimmunity in two adjacent populations, which live in completely different socio-economic environments.

6. Review of the literature

6.1. Regulation of immune tolerance

Immunologic tolerance is a state of unresponsiveness that is specific for a particular antigen. One of its most important biological implications is the regulation of self-tolerance, which prevents the immune system from mounting an attack against the host's own tissues. Self-tolerance is maintained by various mechanisms that prevent the maturation and activation of potentially self-reactive lymphocytes (1).

The primary mechanism of immunologic tolerance is central deletion when T cells that are strongly reactive to self-peptides are eliminated in a process termed negative selection. T cells that mature in the thymus and enter peripheral lymphoid organs must display TCRs with some affinity for self-peptide-self-MHC complexes in order to receive the necessary signals for survival, termed positive selection. T cells that express TCRs lacking any affinity for the self-peptide-self-MHC complexes fail to undergo positive selection and die. As positive selected thymocytes move from the cortex to the medulla of the thymus, they continue the maturation process and further test their TCRs for self-reactivity. These medullary cells express T cell costimulatory molecules, such as CD80 and CD86, ligands for CD28, which play a crucial role in ensuring self-tolerance. TCRs that bind strongly to self-peptide-MHC complexes trigger the death (negative selection) of thymocytes in the medulla of thymus (3).

Recent studies on intrathymic expression of peripheral autoantigens, termed promiscuous gene expression, showed a causal relationship between the transcriptional regulator autoimmune regulator (AIRE) and the promiscuous expression of antigens in medullary thymic epithelial cells. Mutations of the AIRE gene are associated with a multiorgan autoimmune syndrome known as autoimmune polyglandular syndrome type 1.

B cells similarly undergo a process of negative selection in the bone marrow and additionally in the spleen where B cells migrate after exiting the bone marrow. The repertoire of naïve B cells vary within individual with higher-affinity autoreactive B cells present during times of infection or inflammation.

A distinct subset of T cells, named regulatory T cells (Tregs), is the result of relatively high-affinity interactions in the thymus. The majority of these Treg cells express CD25 and constitute 1-2% of CD4⁺ T cells in humans. Development of these CD4⁺ CD25⁺ Tregs in the

thymus plays a key role in maintaining tolerance in the periphery. Recently, the expression of the forkhead transcription factor Foxp3 has been found in CD4⁺ CD25⁺ Tregs both in the thymus and periphery. Some of the naïve CD4⁺ CD25⁻ T cells may also differentiate into Tregs that express Foxp3 in the periphery. Extensive studies from many laboratories have shown that Foxp3 is specifically expressed by Tregs and programs their development and function. The Scurfy mouse strain, which develops an X-linked lymphoproliferative disease and dies by 3 weeks of age, has mutation in Foxp3 gene. Transfer of CD4⁺ CD25⁺ Tregs into neonatal Scurfy mice prevents severe disease. Mutations in gene Foxp3 in humans results in X-linked autoimmune syndrome known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) or XLAAD (X-linked-autoimmunity-allergic dysregulation syndrome), including type 1 diabetes, thyroiditis, immune dysregulation, severe atopy, eczema, food allergy (3; 4).

Peripheral tolerance is maintained by four main mechanisms (5), including functional anergy, deletion (death) by apoptosis, ignorance and suppression by regulatory T lymphocytes (Treg). *Anergy* describes a state of metabolic arrest that can lead to apoptosis. It occurs if lymphocyte receives an antigenic stimulus without costimulatory signal. *Deletion* is the mechanism mediating the tolerance of mature T cells by clonal elimination. This mechanism was demonstrated in mice deficient for genes involved in apoptosis, such as TNF-family receptors Fas and FasL. *Ignorance* occurs if tissue-specific self-antigens are not detectable by the immune system, when potential self-reactive T cells remain ignorant of the antigen expressed in the tissues. *Suppression by regulatory T lymphocytes* (CD4⁺ CD25⁺ Tregs) can be mediated by several molecular mechanisms. The role for the cytokines IL-10, transforming growth factor (TGF)- β and Foxp3 in the function of natural CD4⁺ CD25⁺ Tregs has been suggested by both human studies and animal models (1; 4).

Germline mutations or targeted deletions of several genes can lead to autoimmunity by disrupting one or the other pathway of tolerance. For instance, deletion of the transcription factor Foxp3 or the growth factor IL-2 interferes with the generation or function of CD4⁺ CD25⁺ T regs, and the absence of IL-2 may also reduce Fas-mediated apoptotic cell death. Each pathway may maintain tolerance to a subset of self-antigens, when a loss of any pathway will result in a limited set of autoimmune reactions. An alternative possibility is that multiple mechanisms must work together to maintain self-tolerance, when their dysfunctions alter the finely tuned balance between tolerance and autoimmunity.

Transgenic mouse models indicate that tolerance of CD4 T cells to a tissue antigen is maintained by at least two mechanisms. Eggena et al. examined the consequence of deleting CTLA-4 and eliminating Tregs on the development of diabetes in DO.11 TCR transgenic mouse which express TCR capable of specifically recognizing the protein ovalbumin (OVA) (6). Adoptive transfer of OVA-specific T cells from these mice into mice expressing OVA in islet cells induces acute insulinitis and diabetes only if their lymphocytes, including Tregs are removed. If not removed, then transfer of naive wild-type OVA-specific(CD25⁻) T cells into mice expressing islet antigen induces diabetes only following peripheral immunization with OVA together with an adjuvant. In contrast, naïve CTLA-4^{-/-} OVA-specific T cells (CD25⁻) induce diabetes after recognizing the self-antigen alone indicating that CTLA-4 controls the activation of autoreactive T cells. CTLA-4 and Tregs thus act cooperatively to maintain

tolerance and CTLA-4 functions independently of Tregs. Deficiency of both CTLA-4 and Tregs is needed to induce autoimmune response in this model. This indicates that these two mechanisms must work in distinct ways, and CTLA-4 cannot be solely a mediator of Treg functions. Cooperation between two distinct pathways of tolerance may account for the observation that many autoimmune diseases are associated with multiple gene polymorphisms.

It seems that tolerance to systemic (secreted) antigens does not require CTLA-4 or Treg (5). CTLA-4 does not appear to play an essential role in anergy to secreted self-antigens since CTLA-4^{-/-}DO.11 T cells also become anergic. Depletion of Tregs neither seems to prevent or reverse tolerance. The available data indicate that systemic antigens shut off lymphocyte responses by inducing a form of receptor “desensitization”, such that the lymphocytes reset their activation threshold and can no longer respond to the self-antigen. Anergy, in this case, is not a permanent genetic or biochemical alteration in the T cells, but a transient loss of responsiveness that lasts as long as the cells are exposed to the systemic antigen. Distinct mechanisms may be responsible for tolerance to tissue and secreted proteins, although there might be considerable overlap between different types of self-antigens. Genetic mutations that disrupt tolerance and promote autoimmunity provide valuable information about the normal pathways of tolerance.

6.2. Mechanisms of autoimmunity

6.2.1. Activation of the immune system

Autoimmune pathology can be caused by both antibody and cell-mediated components. Specific immune and autoimmune responses involve the same elements: an antigen (or autoantigen) and a response by subsets of immune cells and key molecules including antigen presenting cells (APCs), T lymphocytes, B lymphocytes, cytokines, chemokines and their receptors, signaling and costimulatory molecules on cell surfaces (7). B- and T-cell differentiation takes place in the central lymphoid tissues, which are principally the bone marrow for B cells and the thymus for T cells. Since only 3-8% of human population develops autoimmune disease, it is remarkable that the enormous burden of self-reactive receptors is so well regulated in most individuals (8). Each lymphocyte usually produces only a single receptor out of the billions possible and several strategies are employed to deal with autoreactive receptor specificities (9).

The invasion of a foreign agent induces a cascade of concerted events which usually begin with the activation of the innate immune system. The first action is up to macrophages or dendritic cells (DC), cells able to phagocytize and process foreign particles. When the foreign particle is a protein, the macrophage (or dendritic or any other cell with the same characteristics) will process it enzymatically into smaller pieces, i.e. peptides. Certain peptides, derived from the ‘processed’ foreign protein particle (the antigen), are then picked up by major histocompatibility complex (MHC) molecules that will expose them on the surface of the cell. Once properly exposed by activated macrophages on the cell surface, the peptide can be ‘presented’ to T cells. A particular T-cell clone with an appropriate T-cell

receptor (TCR) will eventually recognize the MHC molecule/peptide 'complex' and in so doing becomes 'activated'. The activated T-cell clone then starts to express certain receptors and to secrete various immunomodulators (9). This phase of the T-cell activation involves the expression of the interleukin-2 (IL-2) receptor by CD4 positive (CD4⁺) T lymphocytes and their secretion of the growth promoter IL-2 itself. IL-2 binds to its own receptor exposed on the surface of the same cell, generating a self-maintained system. Activated T cells do not only divide, but also differentiate to become able to secrete additional factors such as interleukin-4 (IL-4) and γ -interferon (IFN- γ).

This function of the activated CD4⁺ T cells is directed to "help" other cells of the immune system, such as B lymphocytes and CD8⁺ T lymphocytes, to proliferate. B lymphocytes activated against the foreign antigens differentiate into large granular antibody-secreting cells (i.e., plasma cells), while specifically activated CD8⁺ T cells (i.e., cytotoxic) reach the critical number necessary for a successful attack and the consequent removal of other cells of the self which have been "contaminated" (i.e., infected) by the foreign agent. Complex selection mechanisms allow the maturation of only those B and T cells which are able to spare self targets but efficiently attack nonself structures. When these selection mechanisms do not work properly, cells able to react against antigens expressed on an individual's own tissues are not completely eliminated and the possibility of antiself, "auto", aggression is much greater.

6.2.2. Role of different effector Th-cell subsets

The CD4⁺ T helper (Th)-cell population comprises functionally distinct subsets that are characterized by the patterns of lymphokines they produce following activation (10). Although these subsets were first identified by *in vitro* analysis of murine T-cell clones, strong evidence has been generated for similar subsets *in vivo* in mice, rats and, also in humans. Recently it has been demonstrated that in mice several CD4⁺ subsets do exist: Th1, Th2, Th0, Th17 and regulatory (Treg). The skewing of murine Th cells towards Th17 and Treg is mutually exclusive. The presence of transforming growth factor -beta (TGF- β) skews towards Treg and IL-6 and TGF- β towards Th17. These cell subsets are characterized by expression of specific transcription factors (11). Th1 cells secrete IL-2, INF- γ , and tumor necrosis factor (TNF), and support macrophage activation, delayed-type hypersensitivity responses and immunoglobulin (Ig) isotype switching to IgG2a. Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13, and provide efficient help for B-cell activation, for switching to the IgG1 and IgE isotypes, and for antibody production. Th0 cells are characterized by production of cytokines of both the Th1 and Th2 types, and are thought to be obligatory precursors of Th1 and Th2 cells (12). Tregs express forkhead box P3 (Foxp3) and Th17 cells express the orphan nuclear receptor ROR γ (11). Several factors, including the dose of antigen, the type of antigen-presenting cell (APC) and the major histocompatibility complex (MHC) class II haplotype, influence the differentiation of naïve CD4-T cells into specific Th subsets (10). Reciprocal regulation occurs between the Th1- and Th2-cell subsets. For example, IFN- γ inhibits the differentiation and effector functions of Th2 cells, and can lead to a dominant Th1 response. Conversely, IL-4 strongly directs the development of Th2 cells, both *in vitro* and *in vivo*, and mice in which the IL-4 gene has been disrupted have an

impaired ability to generate Th2 responses. Furthermore, IL-4, IL-10 and IL-13 inhibit Th1-cell proliferation, and oppose the effects of IFN- γ on macrophages.

It has been proposed that Th1-type responses drive the autoimmune process in organ-specific autoimmune diseases such as T1D. All non-obese diabetic (NOD) mice, an animal model of autoimmune diabetes, exhibit lymphocytic proliferation of the islets of Langerhans and 60-80% of female NOD mice become hyperglycemic by 30 weeks of age. These infiltrates comprise CD4⁺ and CD8⁺-T cells, B cells and macrophages, but in adoptive transfer experiments it has been shown that the T cells play the most prominent role in the induction of diabetes (12; 13). T-cell clones that are able to accelerate the manifestation of diabetes in young NOD mice produce Th1-type cytokines when challenged with islets and APCs *in vitro* (14). Further evidence for the role of Th1 cells in autoimmune diabetes derives from studies that have identified glutamic acid decarboxylase (GAD) as a key β -cell antigen recognized by T cells and B cells. NOD T cells produce large amounts of IFN- γ in response to this protein (15). Indeed, anti-IFN- γ antibodies can prevent the development of diabetes in NOD mice (16). Accordingly, Th1-type cells appear to be involved both in the early and late phases of diabetes development in the NOD mouse. Since NOD mice spontaneously develop diabetes, they may be useful for the identification of factors that can prevent disease. For example, systemic administration of IL-4 prevents diabetes in female NOD mice (17).

The protective role of Th2 cells in T1D has also gained support from studies on the I-A – transgenic NOD mice. Transgenic NOD mice carrying an I-A^{g7} allele that had been mutated at positions 56 and 57 (His-Ser→Pro-Asp) were observed to be protected against both diabetes and insulinitis (18). T cells from these mice can inhibit the adoptive transfer of diabetes. In addition, such T cells fail to proliferate or make IFN- γ in response to beta-cell antigen *in vitro*, despite the fact that these mice do contain T cells specific for beta-cell antigens. Furthermore, although the autoantibodies made by nontransgenic NOD mice to beta-cell antigens (such as GAD) are predominantly of the IgG2a subclass, as would be predicted from the IFN- γ (Th1) response of T cells specific for these same antigens, transgenic mice make autoantibodies comprising more IgG1 and IgE, consistent with the presence of IL-4-producing Th2 cells (19). Finally, using an adoptive transfer system, the prevention of diabetes was shown to be, at least partially, due to the production of IL-4 and/or IL-10 by T cells. Thus, the T cells appear to have been diverted from a pathogenic Th1 phenotype to a protective Th2 phenotype. This suggests that the genetic make-up of the individual can dictate whether autoreactive CD4⁺ T cells differentiate into disease-inducing Th1 cells or into non-pathogenic Th2 cells. This mechanism is termed ‘clonal diversion’ (12).

One of the major theories addressing why autoreactivity persists and causes disease is based on an imbalance in cytokines. There are two sets of opposing cytokine environments, pro-inflammatory and anti-inflammatory. Pro-inflammatory cytokines, such as IL-1, IL-2, IL-6, IL-8, IL-12, IL-17, TNF- β , IFN- γ , etc., are elevated in an active early immune response. Biological activities of this family of cytokines include fever, immune stimulation, constitutional symptoms such as malaise, aching, activation of cytokine networks, induction of nitric oxide and oxygen metabolites, and induction of proteolytic enzymes to help the inflammatory response, and fibroblast proliferation. All these activities also enhance the

ability of the antigen-specific arm of the immune response, T and B cells to work more effectively and faster. On the flip side, anti-inflammatory cytokines such as IL-4, IL-10, TGF- β , and IL-1 receptor antagonist, are decreased in autoimmune disease. These cytokines act by inhibiting the production or activity of the pro-inflammatory and growth promoting cytokines. In addition, there are many soluble receptors for pro-inflammatory cytokines that inactivate excess cytokines.

In animal models and human autoimmune diseases such as MS, rheumatoid arthritis, juvenile rheumatoid arthritis, T1D, inflammatory bowel disease (IBD) and others, the pro-inflammatory cytokines are unusually high, and the anti-inflammatory cytokines are unusually low leading to an imbalance that favours excessive inflammation. Another variation on this pro- and anti-inflammatory theme is the T-helper balance of cytokines. The Th1 subset produces IL-2 and interferon-gamma, which are pro-inflammatory cytokines. The Th2 subset produces IL-4, IL-5, and IL-10 which are primarily anti-inflammatory cytokines. The overall balance in a normal immune response probably requires the right combination of pro-inflammatory cytokines and anti-inflammatory cytokines. The balance of pro- and anti-inflammatory cytokines is related to gender bias in immune responses. Studies have shown that pregnancy is a state characterized by a predominance of anti-inflammatory Th2 cytokines, such as IL-4 and IL-10. The maternal immune system needs to be in a state of relative immune suppression to be able to tolerate the fetus. Relatively new data support the concept that cells expressing indoleamine 2,3 dioxygenase (IDO) can suppress T-cell responses and promote tolerance in pregnancy. IDO is an enzyme that degrades the amino acid tryptophan and is expressed on dendritic cells that use IDO mechanism at the fetomaternal interphase (placenta) (20). If the pro-inflammatory response is too strong with increased levels of IL-1, IL-6, TNF there is a higher likelihood of pregnancy loss. Nonpregnant women tend to have a predominance of pro-inflammatory or Th1 mediated responses (21), compared to pregnant women and men. This may contribute to the higher rate of most autoimmune diseases among women.

Based on cytokine phenotypes, initially the existence of two distinct effector Th subsets was proposed: Th1 and Th2 (22). Recently this paradigm has been updated following the discovery of Th17 cells, the third independent subset of effector Th cells (23) (see Figure 1). Th17 cells play an important role in host defence against specific extracellular pathogens and in the induction of tissue inflammation. Furthermore, recent reports have proposed that there is a reciprocal relationship between Foxp3⁺Tregs and Th17 cells. It was shown that IL-6 has a pivotal role in this differentiation pathway resulting either in the generation of pro-inflammatory Th17 cells and tissue inflammation or protective Tregs and therefore inhibition of autoimmunity and induction of tolerance (24).

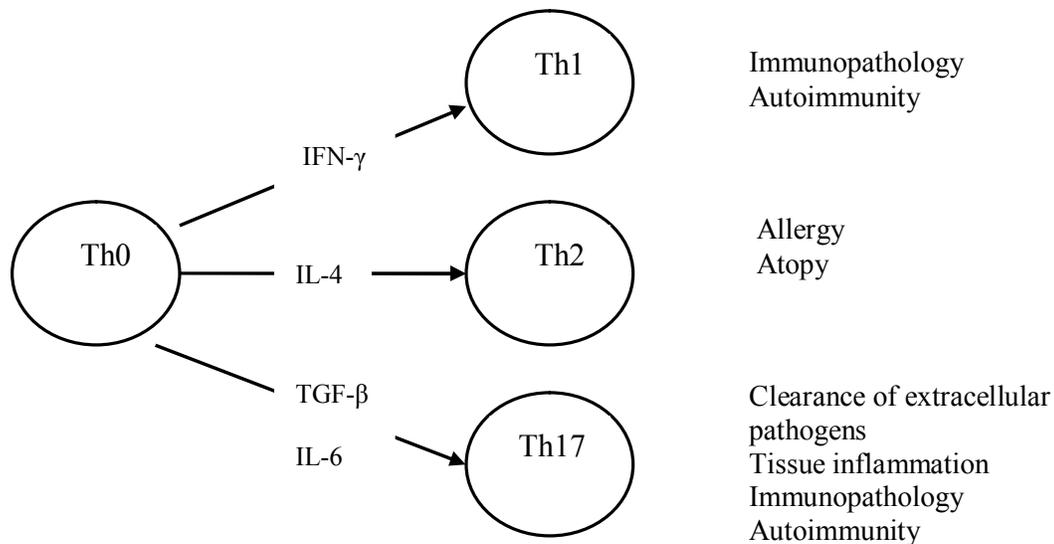


Figure 1. Distinct subsets of effector Th cells. Modified from Bettelli et al. (24)

6.2.3. Nature of the autoimmune process

One of the important functions of the immune system is the discrimination between “self” and “nonself”. Such discrimination is a complex process of multi-step interactions between various cells and components of the immune system which synergize to maintain tolerance and avoid the development of autoimmunity. If such immune reactions are vigorously self-directed, they may cause pathological damage to tissues and result in clinical autoimmune disease (25). Accordingly, autoimmune disease is characterized by an immune-mediated attack on a target organ that is no longer recognized by the immune system as self (26). This leads to the clinical signs of inflammation and infiltration of lymphocytes and macrophages into the affected tissues as well as the appearance of autoantibodies and/or autoreactive T lymphocytes into the peripheral circulation. As long as autoantigens and autoreactive lymphocytes persist, established autoimmune disease will be self-sustaining.

Autoimmune diseases can be divided into two main categories including “organ-specific” and “systemic” autoimmune diseases. In systemic autoimmune diseases such as vasculitis, rheumatoid arthritis and SLE, the immune attack is widespread throughout the blood vessels or connective tissues. In organ-specific autoimmune diseases the damage is directed to one specific organ or organ system. In the majority of the organ-specific autoimmune diseases, target organs are of endocrine character, and these diseases are also referred to as “endocrine autoimmune diseases”. The organ-specific autoimmune diseases include T1D and Graves’ disease, diseases of the central nervous system such as MS and myasthenia gravis, inflammatory bowel diseases such as Crohn’s disease, and skin diseases such as psoriasis and pemphigus. However, it is important to draw a distinction between “autoimmunity” and “autoimmune disease”. The presence of autoreactive T or B lymphocytes or autoantibodies is not necessarily associated with pathology and clinical disease (27). Environmental factors

and biochemical milieu are thought to be important determinants of whether autoimmunity progresses to clinical disease or not (26).

Several factors are involved in the initiation of the autoimmunity, including exposure to environmental factors, genetic predisposition and alterations in mechanisms of central and peripheral tolerance (27-30). Autoimmune diseases tend to coexist within individuals and within families. For example, there is an increased prevalence of autoimmune (Hashimoto) thyroiditis in patients with rheumatoid arthritis and those with T1D (31).

Both autoreactive T cells and autoantibodies can damage tissues. T-cell can mediate target cell damage through perforin-induced cellular necrosis or through granzyme B-induced apoptosis (32). It is now clear that cytokines produced by T cells can directly cause tissue injury. Autoantibodies can also induce damage through mechanisms that include the formation of immune complexes, cytolysis or phagocytosis of target cells, and interference with the function of target cells. Increasingly, the distinction made between T-cell-mediated and antibody-mediated autoimmune disease appears inappropriate for most autoimmune phenomena.

Different models have been proposed to explain the pathogenesis of autoimmune diseases. Burnet's model (33) suggested that each lymphocyte expresses multiple copies of a single surface receptor specific for a foreign entity, signaling through this receptor initiates the immune response, and the self-reactive lymphocytes are deleted early in life. This self-non-self (SNS) discrimination model has dominated the field, and it has been modified by later findings. It was first modified in 1969 after the discovery that B lymphocytes hypermutate, creating new, potentially self-reactive cells. Bretscher and Cohn added a new cell (the helper) and a new signal (help), proposing that the B cell would die if it recognized an antigen in the absence of help (34). In 1975, Lafferty and Cunningham proposed that T cells also need a second signal (costimulation), which they receive from "stimulator" cells (now called antigen-presenting cells [APCs]) and suggested that this signal is species specific (35). In 1989, Janeway (36) proposed that APCs have their own form of SNS discrimination and can recognize evolutionarily distant pathogens. He suggested that APCs are quiescent until they are activated via a set of germ line-encoded pattern recognition receptors (PRRs) that recognize conserved pathogen-associated molecular patterns on microbes. On activation, APCs up-regulate costimulatory signals and present antigens to T cells. The PRRs allow APCs to discriminate between "infectious-nonself" and "non-infectious-self". Matzinger (37) introduced the "danger model", which implies that the immune system is more concerned with damage than with foreignness, and is called into action by alarm signals from injured tissues, rather than by the recognition of non-self. The danger model proposes that APCs are activated by danger signals from injured cells, such as those exposed to pathogens, toxins, mechanical damage. The danger model has been supported by the discovery of endogenous, non foreign alarm signals, including mammalian DNA, RNA, heat shock proteins, interferon- α , interleukin (IL)-1 β , CD40-L, and breakdown products of hyaluron (38). Many organs harbor special populations of lymphocytes that appear to be evolutionary old and have been called 'innate lymphocytes' because they respond to these stress-induced self molecules rather than to foreign entities.

Toll-like receptors (TLRs) are a class of single membrane-spanning non-catalytic receptors that recognize structurally conserved molecules derived from microbes once they have breached physical barriers such as the skin or intestinal mucosa, and activate immune cell responses. TLRs play a key role in the innate immune system and recognize both endogenous and exogenous molecules. The binding characteristics of a newly discovered family of intracellular proteins, called nucleotide-binding oligomerization domain (NOD) receptors, can respond to both pathogen-related signals and normal physiological signals involved with apoptosis. Perhaps, TLRs and NODs originally evolved as receptors for injury-related signals, and the microbes subsequently evolved mechanisms to use these receptors to enhance their own survival.

6.3. Genetic factors

It is clear from epidemiologic studies and studies of animal models that there is a genetic component to essentially every autoimmune disease (31; 39-41). In most human autoimmune diseases, the concordance rates in monozygotic twins are less than 50% (40). It is not surprising that the genetic risk factors for autoimmunity have low penetrance (42) as during immune development the mature genes encoding immunoglobulins and T cell receptors for antigen both assemble from separate gene segments in an unpredictable manner. In addition, immunoglobulin genes somatically mutate throughout life. Genetically identical individuals have dissimilar immune systems, and thus should have different propensities toward autoimmunity. Most autoimmune diseases are multigenic, with multiple susceptibility genes working in concert to produce the abnormal phenotype. Established genetic risk factors include genes encoding MHC, complement proteins, immunoglobulins, peptide transporter proteins, and genes controlling the production of sex hormones (43-45). Each factor may independently enhance the immunogenicity of autoantigens, either by increasing their processing and presentation by B lymphocytes and macrophages or by increasing the chance for recognition by autoreactive T and B lymphocytes. Genetic factors may also influence immune responses to infectious agents that can trigger autoimmunity.

Animal models suggest that whether a particular gene or mutation causes a disease depends on the overall genetic background of the host. Some genetic defects can predispose patients to more than one autoimmune disease, so that several diseases may share common pathogenic pathways. Genetic studies in humans are consistent with these ideas. There are allelic variants of the gene encoding cytotoxic-T-lymphocyte-associated protein 4 (CTLA-4), a T-cell surface molecule that down-regulates activated T cells. Polymorphism in this gene causes a small decrease in the inhibitory signal mediated by CTLA-4 and is associated with T1D, thyroid disease, and primary biliary cirrhosis.

6.3.1. Major histocompatibility complex (MHC) genes

The genes of the major histocompatibility complex [human leukocyte antigen (HLA) in man] are extremely polymorphic. The class I MHC molecules primarily bind short peptides derived from abundant cytoplasmic proteins or from organisms that replicate in the

cytoplasm. The class II molecules bind a longer and more diverse group of peptides, which are generated from extracellular proteins. Peptides bound to class I MHC molecules are targets for cytotoxic T cells while the peptides on class II MHC molecules trigger the activation of T cells that regulate delayed hypersensitivity and antibody production (44). The strength of interaction between a T cell and an antigen-presenting cell is usually low, but it may be enhanced by increasing the density of the receptors on the cell surface and by strengthening the effects of costimulatory adhesive molecules (46). The endogenous peptides that bind to histio MHC molecules in the thymus play an important role in T-cell development (47). Peptides eluted from MHC molecules have shown that MHC molecules themselves are an important source of small peptides that bind to other MHC molecules (48). It is possible that MHC genes influence the T-cell repertoire by generating peptides in the thymus.

Most autoimmune diseases are linked to a particular class I or class II HLA molecule, but this association may require linkage with another gene such as that encoding TNF- α or complement. In the case of T1D, rheumatoid arthritis, and ankylosing spondylitis, however, the class I or class II molecule itself confers susceptibility to disease. Some HLA alleles protect against disease even when a susceptibility allele is present. For example, the HLA-DQB1*0602 allele protects against T1D even if the HLA-DQB1 0302 susceptibility gene is present. When this haplotype was examined in MS, the presence of the same haplotype was instead shown to predispose to disease. This difference could help to explain why it is rare to see the clustering of MS in patients with T1D, and vice versa. The association of HLA alleles with a particular disease may vary among different populations. The class II HLA-DRB1*0401 and DRB1*0404 alleles are strongly associated with rheumatoid arthritis in persons of Northern European ancestry, but not in black or Hispanic populations.

The association of the HLA-DR3 extended haplotype is not unique to T1D but appears to be a general autoimmunity haplotype. For example, it has been observed to be associated with SLE (along with the DR15 and DR8 haplotypes), Graves' disease and CD (49). Simmonds and Gough (39) have shown that the predisposing HLA-DR3 haplotype can be differentiated from the protective DR7 haplotypes (DRB1*07-DQB1*0302-DQA1*0201 and DRB1*07-DQB1*02-DQA1*0201) by the presence of different amino acids at position β 74 of the DRB1 binding pocket. Most HLA-DR3 subtypes have arginine at position β 74, whereas DR7 alleles have glutamine at this position. However, the role of HLA-DQA1 in disease susceptibility remains to be elucidated. An association with position β 74 is also seen in T1D, in which variation at that position has been shown to differentiate between the lower-risk HLA-DRB1*0403 and DRB1*0406 alleles, containing a negatively charged glutamine, and the high-risk DRB1*0401 allele containing a non-charged polar alanine. The shared epitope associated with rheumatoid arthritis also encompasses position β 74. The mechanism for the involvement of position β 74 in so many autoimmune diseases has not yet been resolved. It is probably due to the fact that position β 74 encompasses several binding pockets that play crucial roles in both TCR docking and antigen presentation to Th cells, suggesting that position β 74 mediates its effects on autoimmunity by altering antigen recognition. The association of the HLA region with other autoimmune diseases is less clear. However, it is worth noting that autoimmune hypothyroidism (Hashimoto's thyroiditis) has been linked to DR3 and DR4 and autoimmune Addison's disease has been linked to DR3. Taken together,

these data suggest that the HLA class II region contributes to most autoimmune diseases. The mechanisms by which variations lead to autoimmunity remain unknown, but are likely to be different for each disease. The clinical outcome of disease seems more likely to be the result of changes in the amino acids that compose the binding pockets of the DR and DQ molecules which enable antigen presentation and TCR interaction and the subsequent effect that this has on autoreactive T-cell deletion during central tolerance and generation of Tregs.

CD, like many other autoimmune disorders shares a common genetic predisposition, i.e. HLA-DQ2 or -DQ8 (50). CD is a polygenic disorder and HLA is the single most important genetic factor. The primary HLA association in the vast majority of CD patients is with HLA-DQ2 (DQA*05/DQB1*02) and in a minority of patients with HLA-DQ8 (DQA1*03/DQB1*0302) (51). The HLA association in CD can be explained by a superior ability of DQ2 to bind the biased repertoire of proline-rich gluten peptides that have survived gastrointestinal digestion and that have been deamidated by tissue transglutaminase. Gluten-reactive T cells recognize peptides from gluten in the context of HLA-DQ2 or HLA-DQ8, but not in the context of any other HLA molecules expressed by patients (52).

Most T cells generated in the thymus never survive. Autoimmunity is normally prevented because T cells that react avidly with the MHC molecule-autoantigen complex are deleted. Two consequences of T-cell selection are relevant to the pathogenesis of autoimmunity. First, the regions of foreign antigens that preferentially elicit T-cell responses may be similar (but not identical) to the self-peptides that were important for positive selection (47). Second, all T cells may be autoreactive to some degree, but their avidity of binding may be too low to trigger an immune response under normal conditions. Amino acid sequence comparisons have shown that many autoantigens have regions of homology with common environmental antigens (53). Such molecular mimicry between self- and foreign peptides should not be viewed as a peculiarity of autoimmunity, but rather as one basis for the construction of the functional T-cell repertoire. Triggering of autoreactivity by T cells exposed to exogenous antigens that share sequences with the self-peptides may be favoured if the exogenous antigen were part of a microorganism that replicated intracellularly to produce high peptide concentrations on HLA class I molecules, or one that made a superantigen or other adhesive proteins that could bridge T lymphocytes with antigen-presenting cells. For example, the peptide sequence in the HLA complex that confers susceptibility to rheumatoid arthritis is duplicated exactly in major protein antigens of *Escherichia coli* and Epstein-Barr virus (54).

6.3.2. Immunoglobulin genes

Antigen-binding site of antibodies is encoded by hundreds of separate light- and heavy-chain variable region genes that can somatically mutate throughout life. A mounting body of evidence indicates that immunoglobulin genes can influence autoimmunity. In addition to their role as secretory proteins, antibodies function as specific cell-surface receptors for antigen on antigen-presenting B lymphocytes and by antibody-secreting plasma cells. The immunoglobulin variable region genes from many different autoantibody secreting cell lines have been sequenced and a relatively small group of genes is used repeatedly in individuals of diverse ethnic backgrounds (55). During development, both preferential recombination and antigenic selection lead to the expansion of B cells expressing autoantibody genes and

the heavy and light variable region genes that most frequently encode autoantibodies are highly expressed in human fetal liver (56). It is likely that the initial B lymphocyte repertoire is also skewed towards autoreactivity by a process of positive selection. (57). The reasons for the selection of autoreactive B cells during development are a subject of ongoing debate. A low level of autoreactivity may provide a survival stimulus for B lymphocytes during long periods between antigen exposures. Antibodies released by autoreactive B cells may assist in removing senescent cells, cellular debris, and immune complexes from circulation. As antigen-presenting cells, autoreactive B lymphocytes could be very important in the maintenance of immunologic nonresponsiveness to self, because they do not normally express costimulatory molecules. Experiments with mice transgenic for autoantigens and autoantibodies indicate that the fate of an autoreactive B cell depends on its affinity for antigen, the concentration and physical form of the antigen, and the milieu in which antigen-antibody reactions take place. In general, cells expressing antibodies with high affinity for abundant antigens are efficiently deleted. Thus, the early B-cell repertoire is biased towards low-affinity autoreactivity (58). If some immunoglobulin genes encoding autoantibodies are important, then deletions or polymorphisms in these genes could increase susceptibility to autoimmunity. In support of this notion, a homozygous deletion of an antibody heavy-chain variable region gene encoding both anti-DNA and anti- IgG autoantibodies has been associated with SLE and rheumatoid arthritis (45; 59).

6.3.3. *Cytotoxic T-lymphocyte-associated 4 gene (CTLA-4)*

T-cell activation occurs via a two-stage process. The first step involves the interaction of a presented antigen with the TCR-CD3 complex, leading to the generation of an initial signal. The second step involves a co-stimulatory signal by the interaction of the CD28 molecule with B7 molecules (CD80 or CD86) expressed on activated antigen-presenting cells, such as dendritic cells and macrophages, producing a positive co-stimulatory signal to the T cells. This stage of T-cell activation is downregulated by the CTLA-4 molecule. Owing to the negative control function of CTLA-4, functional mutations within this gene could increase susceptibility to autoimmune disease. The CTLA-4-CD28 interaction controls the rate of T-cell activation and is likely to play a major role in the development of autoimmune disease. The genes encoding CD28 and CTLA-4 have been mapped to human chromosome 2q33 and there are only four known polymorphisms of the CTLA-4 gene. These polymorphisms are linked with T1D, Graves' disease, Hashimoto's thyroiditis, Addison's disease and CD. Accordingly, this gene seems to have important implications for the mechanisms of the autoimmune disease. Other genes in this chromosomal region, such as CD28 and Inducible Costimulator (ICOS) could also be responsible for the observed genetic effect. Several potential mechanisms whereby polymorphism of the CTLA-4 gene could lead to autoimmune disease have been postulated. Soluble CTLA-4 appears to be present in human serum, and binding to CD80/CD86 may inhibit T-cell proliferation via increased activation of CD28 (39). It has also been suggested that CTLA-4 is expressed by Tregs. CTLA-4 has been shown to be associated with both B-cell antibody-mediated autoimmune diseases such as Graves' disease (60) and T-cell mediated autoimmune diseases such as T1D (61). Additionally, CTLA-4 was shown to be associated with organ-specific autoimmune diseases, e.g. T1D, Graves' disease and MS (MS) (60), and systemic autoimmune diseases (62). Thus, it seems that CTLA-4 is a general autoimmunity gene. However, the relative risk conferred

by CTLA-4 is low (1.1-1.5) (63) , demonstrating that other genes must play a role in the development of autoimmunity, possibly by interaction with CTLA-4.

6.3.4. Lymphoid- specific phosphatase (LYP) gene

The PTPN22 gene encoding the LYP molecule is located on chromosome 1p13. LYP is a 110 kDA PTP expressed in lymphocytes where it physically associates with the SH3 domain of the Csk kinase, an important suppressor of the Src family of kinases which mediate downstream T-cell activation. LYP is one of the most powerful inhibitors of T-cell activation and it has a clear effect on the risk of T1D. The proposed mechanism of action for LYP is believed to be related to the change from arginine in codon 620 (Arg620) to a tryptophan (Trp620). Several recent studies have shown that PTPN22 is associated with rheumatoid arthritis (64), SLE (65), T1D (66; 67), and Graves' disease (68). Accordingly, like CTLA-4, PTPN22 seems to be a general autoimmunity gene that predisposes to both B and T-cell-mediated autoimmune diseases. PTPN22 is an important regulator of TCR signaling in memory and effector T cells (69). Similar to CTLA-4 the relative risk conferred by PTPN22 is relatively low (approximately 2). Therefore, it seems that other autoimmunity genes must play a role in the development of autoimmunity, and it is likely that many genes with small effects (like CTLA-4 and PTPN22) cause susceptibility to autoimmunity, and that predisposition is not due to a single or a few major genes.

6.3.5. The autoimmune regulatory gene (AIRE1)

The AIRE gene, located on chromosome 21q22.3, was first identified in 1997 in an attempt to find the gene responsible for autoimmune polyendocrinopathy-candidiasis ectodermal dystrophy (APECED) or Autoimmune polyendocrine syndrome type1. The AIRE1 gene appears to play a vital role in presentation of organ-specific antigens which are not normally present in the thymus for naïve T cells during negative selection, thus enabling self-reactive T cells to be deleted before entering the circulatory system. AIRE1 is critical in controlling autoimmunity but polymorphism in the AIRE1 gene leads specifically to the development of APECED and not other autoimmune diseases. APECED or Autoimmune polyendocrine syndrome type 1 is also known as candidiasis-hypoparathyroidism-Addison's disease-syndrome, Autoimmune Polyglandular Syndrome I. Its main features are mucosal and cutaneous infections with candida yeasts, autoimmune dysfunction of the parathyroid glands and the adrenal glands, other symptoms include vitiligo, alopecia, hypogonadism, hypothyroidism (70). There are also several other autoimmune diseases known to be due to mutations in a single gene such as immunodysregulation polyendocrinopathy X-linked syndrome (IPEX) caused by a defect in FoxP3 gene, autoimmune lymphoproliferative syndrome (ALPS) results from mutations in FAS and FASL genes and familial hemophagocytic lymphohistiocytosis (FHLH) caused by mutations in PRF1 (FHL2), UNC13D (FHL3), STX11 (FHL4) genes.

6.3.6. Insulin gene

The insulin gene (INS) on chromosome 11p15 was first associated with T1D in 1984. This region was narrowed down to a 4.1 kb region and has been attributed to the variable number of tandem repeats (VNTR). The INS VNTR clusters into 30-60 repeats (class I), 60-120 repeats (class II) and 120-170 repeats (class III). Homozygosity for class I alleles was observed to be associated with T1D and the INS VNTR-associated trait is inherited in a dominantly protective fashion with one dose of a class III allele providing 60-70% protection from disease. The INS VNTR is believed to contribute to T1D through regulation of the transcription of INS within the thymus. Transcriptional activity in the thymus has been shown to be ~200-300% higher in INS transcripts encoded by the resistant class III alleles compared with levels of INS transcripts produced by the class I predisposing alleles. Higher insulin levels produced by class III alleles in the thymus may induce negative selection of insulin-specific T lymphocytes more efficiently.

6.3.7. Complement genes

Homozygous deficiency of complement component C1q encoded by chromosome 1p23 has been shown to carry a 98% risk of developing SLE. Complement genes C2 and C4, located within the MHC class III region, have also been shown to be associated with SLE (75% of C4 homozygous subjects and 33% of C2 homozygous subjects). As the major autoantigens in SLE have been observed to be generated by apoptotic cells, incomplete removal of apoptotic cell debris because of deficiencies within the complement system, could enable the apoptotic debris to be presented to the immune system, triggering an autoimmune response. Several studies associate C4 polymorphism/deletion with other autoimmune diseases such as T1D, rheumatoid arthritis, systemic sclerosis, postpartum thyroiditis. A 8-year prospective study by Deschamps et al. (1992) demonstrated a significant risk for siblings to develop T1D if they had DR3,4 genotype, autoantibodies to islet cells and C4B deficiency (71).

6.3.8. Genetic polymorphisms predisposing to more than one autoimmune disease

Epidemiologic studies have suggested that many autoimmune diseases share some causative genetic factors. For example, prevalence of CD is higher in patients with T1D than that in the general population: at most, 10% of children and 2% of adults with T1D test positive for tissue-transglutaminase antibodies (tTGA) (72; 73). Several genomewide association studies have recently been performed in order to determine the localization of shared non-HLA genetic risk factors in these two disorders. Smyth et al. performed a genomewide search to assess an association between eight alleles and the risk of CD and between 15 alleles and the risk of T1D (74). According to that study about half of the non-HLA genetic risk alleles were common to both diseases. Additionally, a new risk allele for both diseases was identified: a 32-bp insertion-deletion variant in the CCR5 gene. These results confirm that genetic polymorphisms may predispose to more than one autoimmune disease.

6.3.9. Gender

The relationship between sex steroids and autoimmunity has been demonstrated in animal models of many autoimmune diseases such as SLE, T1D and autoimmune thyroiditis (AT) (75; 76). In all these studies estradiol accelerated the progression of humoral immune response-dependent autoimmune diseases via enhancing the Th2 pathway, while androgen had a protective effect. It has been proposed that specific responses of different immune cell subsets to androgens and oestrogens could be one of the contributing factors to the sexual dimorphism characteristic of the immune system (77). It has been shown that sexual dimorphism in the thymocyte subsets persist after gonadectomy in adult rats, suggesting that gonadal steroids are more important for the induction than for the maintenance of the gender difference in immune responsiveness (78).

Androgens exert considerable effects on the size and composition of the thymus. Removal of androgens by castration results in thymic enlargement (79) and testosterone specifically targets double positive ($CD8^+CD4^+$) thymocytes for apoptosis via upregulating TNF- α production (80). The net effect of androgen action (direct or indirect) seems to be an enhanced suppressor effect (81). Estrogen has been shown to cause significant thymic atrophy and to decrease the number of thymocytes in mice (82). Estrogen also stimulates $CD4^+CD8^+$ -cells and can activate an extra-thymic pathway of autoreactive T-cell differentiation (82-84). Estrogen treatment increases the number of antibody-producing cells and the levels of circulating autoantibodies in normal mice (85) as well as immunoglobulin production (86). In summary, androgens and estrogens are potent immune modulators. Sex steroids act as negative regulators in both the thymus and bone marrow, but androgens and estrogens tend to affect different subsets of immune cells. In general, androgens seem to inhibit immune activity, while estrogens appear to have a more powerful effect on immune cells and to stimulate immune activity.

6.4. Microbes

The role of environmental factors in the etiology of autoimmune diseases is obvious given the disease concordance rate between monozygotic twins. More than 50% and sometimes 70 or 80% of monozygotic twins are discordant for major autoimmune diseases (87). Several other observations also support the idea that environmental factors influence the occurrence and/or course of certain autoimmune diseases: i) there is a North/South gradient with a higher rate of autoimmune disease in northern countries, (ii) the incidence of certain autoimmune diseases is increasing rapidly, and (iii) migrant populations acquire the incidence and prevalence of autoimmune diseases seen in the areas to which they move (88-92). Many different environmental factors can have such an effect including dietary factors, toxins, microbiota and acute or chronic microbial infections. Infections may have two kinds of effects on the risk of autoimmune diseases: They can either trigger the process or they may be protective.

6.4.1. Microbes in the breakdown of immune tolerance

In animal models several viruses can induce autoimmune diseases (93; 94). It is conceivable that several viruses showing a tropism for the same organ could trigger an autoimmune response specific to the organ. Human studies have been complicated by the difficulties in getting samples from the target organs (e.g. pancreas in T1D) and the fact that these infections may take place many years before the clinical presentation of the autoimmune disease. It is possible that the triggering infection heals rapidly and that its virological and serological traces have disappeared by the time of clinical diagnosis. There may still be antibodies to the pathogen, but their specificity to the autoimmune disease is doubtful, the more so since the infection in question may be relatively common in general population (87). Immunological studies performed on animal models of autoimmune diseases strongly suggest that infections are among the best candidates for the environmental factors triggering human autoimmune diseases. The following three main mechanisms (a-c) have been proposed for the triggering role of infections:

a. Polyclonal lymphocyte activation

Certain microbes are able to induce strong polyclonal activation of the immune system and activate autoreactive lymphocytes (e.g. Epstein-Barr virus). Polyclonal activation may be caused by a cytokine storm induced by microbes or by microbial superantigens and may explain some autoimmune states. Superantigens are powerful microbial toxins that target the immune system causing massive T-cell activation, cytokine release and systemic shock. Superantigens share the common ability to bind simultaneously to the class II MHC expressed on antigen presenting cells and the variable region of the T-cell receptor β -chain (TCR V β). Superantigens stimulate any T-cells that have the correct TCR V β on its surface. They cause a number of diseases characterized by fever and shock. Among bacterial superantigens the best studied ones are the family of staphylococcal enterotoxins (SE) and streptococcal pyrogenic exotoxins secreted by Gram-positive bacteria *Staphylococcus aureus* and *Streptococcus pyogenes* are commonly found on the skin, in the nose and upper respiratory tract of humans. The entero- and exotoxins are potential virulence factors in diseases caused by these bacteria. The characteristic hallmark of all superantigens is their V β -restricted expansion (i. e. activation and proliferation of T cells that express restricted number of TCR V β domains). For example, staphylococcal enterotoxin B stimulates T cells bearing V β 1.1, 3.2, 6.4, 15.1. The number of streptococcal and staphylococcal superantigens has increased considerably over recent years and is likely to grow further. Similarity in amino acid sequences between the staphylococcal and streptococcal superantigen family members varies from 20 to 90%, as they have all evolved from a common ancestral gene. Other bacterial superantigens have also identified from *Yersinia pseudotuberculosis* and *Mycoplasma arthritidis*. Viral superantigens include the mouse mammary tumour virus (MMTV) products and superantigens from cytomegalovirus and Epstein-Barr virus. Viral superantigens are produced by endogenous murine retrovirus-mouse mammary tumor virus and are type II proteins with no sequence similarity to bacterial superantigens. In conclusion, superantigens display remarkable variations in structure and function to bring TCR and MHC class II molecules together (95; 96).

b. Antigen mimicry

It has been noted that the protein sequence of a number of bacterial or viral proteins present structural homology with autoantigen sequences (97). There is a significant homology between the Coxsackie B4 virus protein and the glutamic acid decarboxylase sequence which may have relevance in T1D and between the hepatitis B virus polymerase sequence and a segment of myelin-basic protein which has been incriminated in the pathogenesis of MS (98; 99). Another example is rheumatic fever where common antigenic determinants have been identified between streptococcal proteins and heart autoantigens (100). In Guillain-Barre syndrome (an acute polyradiculoneuritis) antibodies cross-reacting with *Campylobacter jejuni* and peripheral nerve gangliosides have been detected (101).

However, no evidence of functional homology has been consistently confirmed so far.

c. Increased immunogenicity of organ autoantigens secondary to infection-mediated inflammation.

Two experimental models have illustrated this mechanism. In Theiler's murine encephalomyelitis (virus-induced demyelination disease) the infection initially provokes a virus-specific encephalomyelitis associated with T-cell reactivity to viral proteins. However, within a few weeks, the virus-specific response is replaced by an autoimmune response, including myelin-basic protein and proteolipid protein-specific T-cell reactivity, which mediates the chronicity of the disease (102). Similarly, infection of mice with the Coxsackie B3 virus induces a long-term cardiomyositis which develops in two phases, the first viral phase followed by immune-mediated tissue pathology. In these models, it is assumed that the initial virus-induced inflammation triggers overexpression of molecules participating in autoantigen recognition by T cells. These molecules include MHC molecules (class I and class II) and costimulatory and adhesion molecules.

Mechanisms that could result in increased immunogenicity of autoantigens include also breakdown of humoral tolerance and autoantibody production via "betrayal" of helper T-cells. Autoantigen-specific B-cells that bind both microbial antigen and self-antigen, ingest, process, and present microbial antigens to its MHC molecules on the cell surface. The activated T cells provide T-cell help or costimulatory signals to these B cells without knowing that the B cells are in fact autoreactive which contribute to a cascade of autoreactivity by secreting autoantibodies.

In addition, there is evidence indicating the existence of mechanisms of intramolecular help by which foreign structures (also a microbe) could trigger antibody-mediated autoimmunity. Such a mechanism is very important, for example, in patients with CD, when complexes of gluten and tTG2 permit the gluten-reactive T cells to provide help to the tTG2-specific B cells (see page 49).

6.4.2. Microbes in the protection against autoimmunity

It has become increasingly apparent that infections could also protect against autoimmune diseases. This assumption is in line with the hygiene hypothesis which was initially formulated to explain the increase in allergic diseases (103; 104). As we live in a 'cleaner environment' the decreasing pressure of natural infections in general population may contribute to these immune abnormalities because the developing immune system is not

exposed to stimulation that might be necessary to generate regulatory cells involved in the modulation and prevention of autoimmunity. The incidence of most autoimmune diseases has been steadily increasing over the last three decades in Europe and North America. This trend has been particularly spectacular for T1D, inflammatory bowel disease, CD and MS. In the case of T1D, the increasing incidence rate is associated with a decrease in age of clinical diagnosis with more frequent involvement of very young children (less than 2-3 years old) (105). This “diabetes epidemic” is observed in most developed countries being more pronounced in Northern than Southern European countries. At the same time, the incidence of major infectious diseases has decreased in these countries. In this context particular attention should be given to gastrointestinal infections, which are very frequent in developing countries and relatively rare in western countries. This trend is clearly explained by the dramatic improvement in the quality of drinking water and food (cold chain). The findings in human populations are paralleled with the fact that NOD mouse colonies around the world have a wide range of diabetes incidence reflecting the environmental effects with a greater incidence of diabetes in those colonies tested to be pathogen free (2). The disease incidence appears to be vary from 20 to 90% in female mice by 300 days, while males have an incidence of 1-65%. There appears to be a reciprocal relationship between the incidence of diabetes and the level of microbial exposure within the NOD mouse colonies. Conversely, deliberate infection of NOD mice with various bacteria, viruses, or parasites totally prevents the manifestation of clinical diabetes if the infection occurs at an early age.

The following three orders of mechanisms of the protective effect conferred by infections can be discussed which are neither mutually exclusive nor independent (a-c):

a. Competition

It is now apparent that lymphocyte proliferation and survival depend on a number of homeostatic signals, including cytokines such as IL-7 and self-peptide MHC recognition. One may postulate that the strong immune responses that are elicited by infectious agents compete for homeostatic signals with immune responses against weaker antigens, such as autoantigens and allergens (106).

b. Regulation

Regulatory CD4 T cells (Tregs), lymphocytes with suppressor capability are naturally present in a normal organism. Like any other CD4 T cells, they mature in the thymus. However, contrary to naive CD4 T cells, they exit the thymus already functionally committed and seem to undergo TCR selection for high self-avidities, possibly because they are committed on thymic epithelial presenting cells. Tregs acquire, already in the thymus, an activated phenotype and express a set of cell surface markers also found on conventional, activated CD4 T cells (e.g., CD25, GITR, CTLA4), indicating their intra-thymic activation, concomitantly or after commitment (107). The expression of the transcription factor Foxp3 seems to be sufficient to confer regulatory function on conventional CD4 T cells, and Foxp3 expression is currently considered the best specific marker for Tregs (108). Conventional CD4 T cells can differentiate in the periphery when exposed to particular stimuli and/or cytokine cocktails to postthymically induced regulatory T cells, e.g. those named Tr1 or Th3, which do not express Foxp3 and differentiate from conventional naive cells much in the same way as Th1 and Th2 cells do. Foxp3 Treg cells can also be induced *de novo* from naive

precursors in the periphery. Recently, Coombes et al. have identified a population of CD103⁺ mesenteric lymph node dendritic cells that induce the development of Foxp3⁺ T reg cells after antigen activation in the intestine. They also demonstrated that promotion of T regs is dependent on TGF- β and the dietary metabolite, retinoic acid (109). It has been demonstrated that the beneficial effects of Tregs in microbial infections are accompanied by suppression of protective immune response. Tregs were shown to increase microbial loads in mice infected with fungi (*Pneumocystis carinii*), yeast (*Candida albicans*) and parasites (*Leishmania major*) and limit the associated immunopathology (lethal pneumonia, gastric inflammation and skin lesions) (110; 111). Infections increase the number and activity of Tregs which accumulate and proliferate at the site of infection/inflammation, dampen the driving immune response and limit the tissue damage. There are several mechanisms of suppression by Tregs, ranging from the cytokines IL-10 and TGF- β to cell-cell contact via the inhibitory molecule CTLA-4. The importance of IL-10 is demonstrated in IL-10^{-/-} mice spontaneously developing colitis. It was possible to abrogate colitis by blocking IL-10 *in vivo*. TGF- β generally resulted in a picture similar to that of IL-10. The identity of Treg-expressed CTLA-4 inhibitory molecule has shown that CTLA-4, aside from its high affinity to B7.1 and B7.2, triggers the induction of the IDO enzyme when it interacts with the ligand on DC. IDO catalyzes the conversion of tryptophan to kynurenine and other metabolites, having immunosuppressive effects. Another mode of suppression by Tregs is through their action on APCs. Purified CD25⁺CD4⁺ Tregs are able to downregulate the expression of CD80 and CD 86 on DC, converting them into inefficient APCs (112).

c. Innate immunity

Innate immune responses are important regulators of adaptive immunity, and it has been proposed that stimulation of a certain type of TLR leads to activation of tolerogenic dendritic cells. In line with this, various TLR agonists (TLR2, 3, 4 and 9) prevent diabetes manifestation in young NOD mice in association with TLR-dependent production of IL-10 and TGF- β (113).

6.4.3. Effect of microbes on regulatory T cells

The microbial specificity of Tregs has been suggested to result from peripheral conversion of conventional CD4⁺ Foxp3⁻T cells into Foxp3⁺ cells. The crucial question regarding the repertoire of Tregs limiting infection-associated immunopathology and protective immune responses to microbes is also central to the mechanisms of immunological memory. In addition, this alternative is fully compatible with the so-called 'hygiene hypothesis' (114). An increased frequency of Tregs during various microbial and parasitic chronic infections in humans has been reported. It is likely that individuals best capable to clear acute infections have limited numbers of Tregs and are thus more prone to the immunopathology associated with infection (115; 116). In experimental systems, Tregs have clearly proven to be extremely efficient in preventing the establishment of chronic or sub-acute pathological conditions caused by infection-associated tissue inflammation.

6.4.4. Effect of microbes on antigen presentation

To become pathological, the self-reactive T cells have to be activated to expand and to become effector cells. Effector T lymphocytes can induce tissue damage by helping in the generation of pathogenic autoantibodies or helping in the development of cellular immune responses mediated by macrophages and cytotoxic T lymphocytes. Dendritic cells (DC) play a crucial role in the activation of autoreactive lymphocytes (117). DC are highly specialized antigen-presenting cells which can induce tolerance or specific immunity and are necessary and sufficient to induce tolerance to peripheral antigens (118). In the presence of IL-10, monocyte-derived human DC do not produce IL-12 and can anergize T cells. Recent observations suggest that immature DC might also induce regulatory T cells. Moreover, some DC might induce the death of autoreactive T lymphocytes. In humans, monocyte-derived DC activated during viral infection can kill sensitive lymphocyte targets through the TNF-related apoptosis-inducing ligand (TRAIL). Microbes and pro-inflammatory cytokines induce the maturation of DC, a process characterized by an up-regulation of co-stimulatory molecules necessary to optimally activate naïve T cells, expression of the chemokine receptors allowing DC to migrate to the lymph nodes and stimulation of cytokine production. In particular, some microbes or microbial products such as a lipopolysaccharide (LPS) are potent stimulators of IL-12 production by DC. It is interesting to note that DC can respond to LPS only in the presence of the soluble form of CD14, a factor present in serum. Thus, increased vascular permeability associated with inflammation is likely to enhance DC activation by microbial products (118). In experimental allergic encephalomyelitis (EAE; animal model for MS) pre-existing autoreactive T cells remain in a quiescent state unless microbial products such as LPS or CpG oligonucleotides are present. Such microbial factors are necessary to allow the development of effector Th1 cells and induction of EAE through the stimulation of IL-12 production. (119).

6.4.5. Effect of microbes on the exposure of self antigens

Infection could also favour the development of autoimmune disease by increasing the availability of self-antigens and by allowing their presentation to the immune system. The generally accepted hypothesis is that infection results in the migration of DC loaded with self-antigens to draining lymph nodes. This, in turn, leads to the activation of autoreactive T cells. Infection can cause this scenario by inducing tissue damage and liberation of autoantigens which are picked up by DC.

The availability of self-antigens also depends on the control of the clearance of apoptotic cells (117; 120). The high predisposition for lupus-like syndromes among patients or mice deficient in some complement factors (particularly C1q and C4) may be explained by their impaired capability to clear apoptotic cells. By opsonizing apoptotic cells, complement factors promote their clearance. Additional evidence supporting the importance of the clearance of apoptotic cells and their byproducts, such as chromatin, is inferred from the observation of mice deficient in serum amyloid protein. Such mice develop a lupus-like syndrome, which can be explained by a defect in chromatin clearance in the absence of serum amyloid protein. Thus, when apoptotic cells accumulate, autoantigens that are normally cleared persist and could be presented by DC. DC can phagocytose apoptotic cells

and cross-present autopeptides. Normally apoptotic cells do not induce DC maturation and thus apoptotic cell-derived peptides would be present in a neutral or tolerogenic fashion. Additional factors such as pro-inflammatory cytokines or microbial products should induce DC maturation for the activation of autoreactive T cells.

Apoptotic cells were initially described as anti-inflammatory since they inhibit the liberation of pro-inflammatory molecules by macrophages. However, some forms of apoptosis may be inflammatory and induce DC maturation, especially when apoptotic cells are coated with antibodies. There is also evidence that post-apoptotic cells can be as pro-inflammatory as necrotic cells (121). When apoptotic cell death takes place in the context of inflammation, in the presence of autoantibodies or in association with secondary necrosis, then presentation of self-peptides can lead to autoimmunity.

6.4.6. The concept of the hygiene hypothesis

The hygiene hypothesis was first proposed in the late 1980s to explain the rise of allergic conditions (122). The incidence of these disorders in the USA and Europe increased from the late 19th century, and appears to have doubled in some decades, particularly during the 1960s and 1970s. Epidemiological correlations with the modern way of life prompted the assumption that modern hygiene was reducing contact with pathogens that prime T helper 1 (Th1) responses. At that time it was believed that this would result in a compensatory increase in T helper 2 (Th2) activity that characterizes allergic disorders (123). The hygiene hypothesis as an explanation for increasing atopy was first postulated by an epidemiologist, Strachan, in 1989, who reported an inverse relationship between family size and development of atopic disorders. He proposed that the rise in allergic diseases could be explained if these diseases were prevented by infection in early childhood transmitted by unhygienic contact with older siblings or acquired prenatally (122; 124).

At birth, the infant's immune system is biased towards Th2 responses (125-127) and is therefore particularly susceptible to developing allergic conditions. This is a consequence of the Th2 immune environment during pregnancy, which is necessary to minimize the possibility of rejection of the growing fetus *in utero* (128). Maternal exposure to pathogens can alter the development: pathogen exposure may result in maturation of the fetus by IL-12 in the amniotic fluid, stimulating development of Th1 cells. The overall bias towards Th2 response continues after birth until about the age of 2 years. Normal development involves activation of the Th1 pathway to provide the appropriate balance: Th1 responses develop gradually in response to the immunological challenges of life, such as infections.

The unifying hypothesis that can explain the simultaneous increase in autoimmunity and inflammatory bowel disease (IBD) (Th1 mediated) and allergies (Th2 mediated) is that modern living conditions can lead to defective maturation of regulatory T cells (Tregs) and regulatory antigen presenting cells (APC_{reg}). Therefore, rather than Th1/Th2 balance, the crucial factor is likely to be the effector T cells (T_{effector})/Treg balance. In the absence of optimal immunoregulation, the individual may develop a Th1 or Th2-mediated inflammatory disorder, depending on his/her own particular Th1/Th2 bias, immunological history, and genetic background. The argument that a similar lack of Treg activity could

underlie the increases in such diverse disorders as allergies, IBD, and autoimmunity is given added weight by the observation that mice and humans who have genetic defects of the transcription factor Foxp3, which is required for the natural Treg development and function, have a syndrome that induces components of all of these disease types. Thus diminished immunoregulation can lead to inappropriate immune responses to allergens, gut contents, or self (129; 130). In addition to this hypothesis, many studies have shown that IBD has its own pathogenetic background. The identification of NOD-like receptors demonstrated that mutations in NOD2 are associated with the development of IBD. NOD 2 mutations result in impaired clearance of invasive bacteria due to defective recognition by intestinal phagocytes (131).

According to the hygiene hypothesis the exposure to relatively harmless microorganisms (including helminths, saprophytic mycobacteria, and lactobacilli) that have been present throughout mammalian evolution (the so-called 'old friends' hypothesis) is crucial for the maintenance of immune regulation (129). Contact with 'old friends' is greatly diminished in wealthy countries but increased on farms, in cowsheds, and through contact with pets. Accordingly, allergic disorders are less frequent in individuals with helminth infections, and atopic sensitisation increases after treatment of intestinal helminths. Similarly, there are less lactobacilli in the gut of children with allergies, and high doses of lactobacilli may inhibit the development of atopic eczema. In addition, the saprophytic mycobacterium *M vaccae*, originally isolated from a cow, drives maturation of Tregs that cure pre-existing allergy in a mouse model (124). It is possible that because of our long evolutionary association with these organisms, they are recognized by the innate immune system as harmless or, in the case of some helminths, treated as 'friends' because a response would merely lead to immunopathology. Therefore, rather than priming aggressive immune responses, these organisms prime immunoregulation by inducing the maturation of DC which possess the ability to drive Tregs. The increased DCreg and Tregs lead to two immunoregulatory mechanisms mediated in part by release of IL-10 and TGF- β . Firstly, continuing exposure to 'old friends' will cause continuous background activation of Tregs specific for the 'old friends' themselves, resulting in a constant background of bystander suppression. Secondly, DCreg inevitably sample self, gut contents, and allergens and so induce Tregs specific for the target antigens of the three groups of chronic inflammatory disorder. These mechanisms may be aborted when there are legitimate 'danger' signals. For example, Treg function can be turned off by appropriate 'danger signals' *in vitro* (124). TLR and other PRR may play an important role in these phenomena and polymorphism in NOD2 (an intracellular receptor for bacterial peptidoglycan) are linked to increased susceptibility to both Crohn's disease and asthma. Thus an extension of the 'old friends' mechanism suggests that in an environment that less actively primes Treg activity, immunoregulatory disorders will occur first in those individuals whose innate immune systems are least efficient at driving Tregs (103; 124).

6.5. Human autoimmune diseases

6.5.1. General epidemiology

Autoimmune diseases are the third most common category of disease in the United States after cancer and heart disease. Autoimmune diseases are among the leading causes of death among young and middle-aged women (ages < 65 years) in the USA (116). The incidence of autoimmune diseases ranges from less than 1 per 100,000 person-years (e.g. myasthenia gravis, scleroderma) to more than 20 per 100,000 person-years (thyroiditis, adult-onset rheumatoid arthritis). The prevalence range from less than 5 per 100,000 (e.g. chronic active hepatitis, uveitis) to more than 500 per 100,000 (Graves' disease, rheumatoid arthritis, thyroiditis). The estimated total (summed across diseases) incidence is about 90 per 100,000 person-years and prevalence about 3-8% (8; 132).

Almost all autoimmune diseases disproportionately affect women and it has been estimated that about 78% of those with autoimmune diseases are female. In some diseases (e.g. thyroiditis, scleroderma, SLE), 85% or more of the patients are female. The gender disparity is smaller in other diseases (e.g. 60-75% female patients in MS, rheumatoid arthritis). A relatively equal risk between males and females is seen in some childhood onset autoimmune diseases (e.g. T1D). Studies on adult-onset T1D indicate a higher risk among men than among women (132).

There are notable differences between autoimmune diseases in the age distribution at diagnosis. Although most diseases can present at any age, there are clear peaks in clinical manifestation. The mean age at diagnosis of two childhood-onset diseases, juvenile rheumatoid arthritis and T1D is approximately 8-10 years (133; 134). Other diseases that generally become manifest between the age of 30 and 50 years include MS, Graves' disease and myasthenia gravis. An older age at diagnosis (40-70 years) is seen in myositis, thyroiditis, Sjögren's disease, rheumatoid arthritis, Wegener's granulomatosis and other types of systemic vasculitis (135; 136). Gender differences in age at diagnosis of specific autoimmune diseases may provide evidence of different disease pathways. SLE becomes manifest 5-10 years earlier among women than among men (137). In contrast, hyperthyroidism occurs earlier among men (respective mean age 35 and 48 years for men and women).

Differences in risk of specific autoimmune disease between countries or between ethnic groups living in the same area have been reported. The pattern is not consistent across autoimmune diseases, however, as specific ethnic groups may be at higher risk for some diseases but at lower risk for others (138). The most country-specific data are available for T1D. T1D is more common in Northern European countries than in Southern European countries. Incidence rates are very low in Asian countries. A similar pattern is suggested for MS. Incidence rates for T1D among blacks and Hispanics appear to be lower than among Caucasians, and blacks and Asians living in the USA also have a lower risk than Caucasians of MS (139). In addition, several studies have reported increasing rates of T1D over periods ranging from last 10 to 40 years (139; 140). MS and CD appears to be increasing at least in

some areas (141; 142). Rheumatoid arthritis (juvenile and adult-onset) appears to have declined over the same period of time (143).

Basic descriptive information (incidence, prevalence, age, gender, ethnicity or racial distributions, temporal changes in incidence) is lacking for many diseases. Autoimmune diseases as a group do not follow consistent demographic patterns. Disease-specific research, as well as studies that focus on potentially related diseases, needs to be conducted.

6.5.2. Type 1 diabetes

Epidemiology

Numerous epidemiological studies have reported that the incidence of T1D has increased worldwide over the past few decades (90; 139; 144). Diabetes itself was an uncommon diagnosis in the 19th century. Until 1851, the diagnosis was based on the taste of urine. Blood glucose remained difficult to measure until the introduction of the Folin-Wu method in 1920, which enabled blood glucose to be measured on fingerstick samples. Joslin reported in 1923 that 86% of children presenting under 16 years of age had previously died in ketoacidosis, providing useful internal evidence that T1D was truly rare. Accordingly, childhood diabetes resulting in ketoacidosis was uncommon but well recognized in the decades before the introduction of insulin, and mortality statistics from the USA, Denmark and Norway suggest an incidence in the range of 2-7/100,000/ year under the age of 15 years for the time period 1900-1920 (105).

Insulin changed childhood diabetes from a rare and fatal disease to a condition in which prolonged survival was possible. The main source of incidence and prevalence data for the period 1920-1950 comes from Scandinavia. A Norwegian government survey in 1934 gave a minimum prevalence of 0.28/1,000. More detailed information about the incidence of diabetes in Norway is available from retrospective surveys in Oslo and Bergen. Westlund examined all cases of diabetes admitted to hospital in Oslo over the period 1925-1954. The average incidence under the age of 15 years can be estimated as 4.1/100,000/ year. Per Hansen made an effort to identify all cases of the disease over the period 1925-1941. The incidence was estimated at 7.9/100,000/year for individuals under the age of 20 years (105). Finland had a wartime registry of patients receiving insulin or diet supplements. A national population of 3.64 million was reported to comprise 250 individuals under 20 years of age with diabetes, equivalent to a prevalence of 0.2/1,000 for this age-group. A more complete subanalysis based on case records from the Children's Hospital in Helsinki showed that a total of 223 children born after 1939 had attended. A nationwide study estimated the annual incidence to be 12.5/100,000/year in 1953 among children under the age of 15 years. However, it should be kept in mind that studies on the incidence and prevalence of childhood diabetes before 1950 may underestimate the true frequency of diabetes as the access to medical care was variable (105; 145).

A steep rise in the incidence of T1D occurred in many populations over the latter part of the 20th century. In Europe, the best evidence comes from Norway. The 1925-1954 Oslo survey was extended to 1964, the incidence increased from a stable baseline of 4.1

cases/100,000/year to a new level of 8.4/100,000/year over the period 1955-1964. Data from Denmark showed that the incidence of diabetes doubled over a 30-year period from the 1950s, apparently reaching a plateau in the late 1970s. In Finland retrospective analysis of the period 1965-1984 showed a predominantly linear trend, equivalent to a 2.4% annual increase. The best US data for this period come from the Erie County Study. The incidence rose from 6.6/100,000/year in 1950-1952 to 7.4 in 1953-1955, 10.6 in 1956-1958, and 11.3 in 1959-1961. These data indicate that the incidence of T1D was relatively low and stable until mid-century (105; 146).

More rigorous epidemiological studies came into use in the second half of the 20th century. During the period 1960-1996 a significant rise in incidence was recorded for 24 out of 37 longitudinal studies from 27 countries (147; 148). The average annual increase was 3.0% (95% CI 2.6-3.3%) with a greater relative increase in low incidence countries. Extrapolation of these trends indicated that the global incidence would increase by 40% over the time period 1998-2010 (147; 148). The increase was similar in the age ranges 0-4, 5-9, and 10-14 years, but the most rapid increase was seen in the youngest age group. There is data indicating that a rising incidence first became apparent in those countries with the highest initial rates of diabetes (Finland, Norway) and reached lower incidence populations at a later stage. High incidence rates are now also reported from non-European populations. Kuwait has the seventh highest rate in the world (149). This suggests that genetic susceptibility may not vary as widely among ethnic groups as was previously anticipated. A rising incidence in a stable population implies an etiological role for environmental factors. The relevant environmental exposures are likely to be encountered very early in development, since clinical diabetes frequently appears within the first years of life. Recent comparisons of incidence trends in the 0-14 and 15-39 year age-groups in Belgium (1989-2000) and in Sweden (1983-1998) have shown that in both cases the increase in the youngest age group has been balanced by a fall in the older age group (150).

The DIAMOND Study (151) examined the incidence of T1D in children under 15 years of age among 114 populations from 112 centres in 57 countries. The age-adjusted incidence varied from 0.1 per 100,000/year in China and Venezuela to 40.9 per 100,000 /year in Finland, representing a variation more than 350-fold (2006) (151). The rapid increase in the incidence of T1D appears to be a global phenomenon in recent decades (152). The incidence of the disease has increased worldwide by 2.8% per year during the years 1990-1999, and there are no signs that this trend is abating. The increase in incidence has been very rapid, particularly in the youngest age groups in European populations.

In the recent cohort study by Harjutsalo et al. from Finland, 10,737 children were diagnosed with T1D before 15 years of age during the period 1980-2005 (140). The average incidence increased from 31.4 per 100,000 per year in 1980 to 64.2 per 100,000 per year in 2005. The increase was most conspicuous among children under the age of 5 years, on average 4.7% per year. The authors concluded that the increase in the incidence of T1D in childhood might be due to differences in age at disease presentation rather than to differences in the cumulative lifetime risk. The authors also reported a sex bias in incidence in puberty and thereafter. The overall boy-to-girl ratio at the age of 13 years was 1.7. The prognosis for the incidence of T1D in Finland is that it will double in the next 15 years and age at diagnosis

will become younger. The general conclusion of this study is that the steep increase in incidence noted in the latter half of the 1990s might represent changes in our everyday environment that affect the penetrance of T1D susceptibility genes. The increasing incidence trend also shows some variation between the continents, being 4% per year in Asia, 3.2% in Europe and 5.3% in North America. However, a decreasing trend at 3.6% was observed in Central America and the West Indies. The increasing trend was strongest in centers with very high and intermediate incidence. During the second study period from 1995 to 1999, the annual increase was higher (3.4%) than during the first 5-year study (2.4%) but this difference was not statistically significant (140). In the pooled data from this study the increase was less pronounced in older age groups: 4% in the youngest age group, 3.0% in the 5-9-year age group and 2.1% in the 10-14-year age group.

The constantly increasing incidence of the disease over such a short period of time cannot be explained by shifts in genetic susceptibility alone. Thus, causative agents should be sought from the environment and gene-environment interactions. Recent studies have indicated that environmental factors may have a greater role in progression to clinical diabetes among genetically non-susceptible individuals than among those genetically susceptible (153). Experts recommend that studies on environmental agents should be extended to cover completely new areas, including lifestyle, social circumstances, stressful life events, and health behaviour (154-156). Many attempts have been made to identify the reasons for the rapid increase in the rate of T1D over the past 30 years. The leading hypotheses are related to early exposure to cow's milk or to enterovirus infections (92). The alternative possibility is that protective factors have been lost from the childhood environment. The hygiene hypothesis argues that exposure to a range of infections in early childhood is necessary for successful maturation of the neonatal immune system. Therefore, a number of recent reviewers have attempted to link the increasing rate of asthma and atopy to that of autoimmune diseases (103; 124; 157; 158).

Autoimmunity

At disease presentation, 85-98% of patients with T1D have the presence of one or more autoantibodies, which are known to be detectable up to 10 years before diagnosis (159). Earlier studies on diabetes-associated autoimmunity (160-162) have already demonstrated that the expression of two or more autoantibodies is associated with progression to clinical diabetes (163) over the next decade in a majority of first-degree relatives of affected patients. Data from the Finnish Diabetes Prediction and Prevention (DIPP) Study, which is a prospective birth cohort study in subjects carrying HLA-conferred susceptibility to T1D has shown that 3.3% of the children with the high-risk genotype became autoantibody positive within the first 2 years of life compared to 1.6% in the moderate-risk group (164). Children with one antibody remained diabetes-free at least up to the age of 5 years (164), whereas children with two or more antibodies have a cumulative risk of approximately 50% to develop T1D by the age of 5 years and a risk of about 77 % by the age of 10 years (165). The analysis of a sib cohort from the DIPP Study (166), has shown that positivity for multiple autoantibodies is associated with the HLA genotypes predisposing to T1D. Recent data from the Diabetes Autoimmunity Study in the Young (DAISY) Study also revealed that the persistent production of autoantibodies (ICA, GADA, IAA and IA-2A) is associated with

T1D, and the individuals who have a family history of T1D have an increased risk over the general population (6% compared with 0.4%)(167-169). In this study the presence of a family history of T1D was found to be associated with confirmation of autoantibody positivity. Levels of autoantibodies were associated with the subsequent risk of diabetes, in particular, higher peak levels of IAA were related to a higher progression rate to overt disease. In addition, in both studies (DIPP and DAISY) a group of transiently autoantibody-positive children was identified (45% in DAISY Study and almost 50% in the DIPP Study). Transient expression may represent “transient” islet cell autoimmunity and may be associated with the reappearance of autoantibodies in later childhood and adulthood and/or development of diabetes (167) .

The study by Marciulionyte et al. (170) in children from the UK and Lithuania with a two-to threefold gradient in the incidence of T1D showed no huge differences in the prevalence of islet autoantibodies in the background population of these two countries except for a significantly reduced frequency of IA-2As in Lithuanian children (0.2% vs. 2.4% among British children; $P < 0.001$). Several studies in young children and in siblings of children with diabetes from Finland have indicated that IA-2As appear as the last autoantibody reactivity during subclinical progression of the autoimmune process to overt diabetes, and that these autoantibodies have the highest predictive value for clinical T1D during the pre-diabetic disease process (164; 171; 172).

The role of autoantibodies to a newly identified autoantigen, zinc-transporter 8 (ZnT8), is currently being assessed in T1D (173; 174).

Genetic factors

There is an inverse association between age at diagnosis and HLA genotypes conferring susceptibility to T1D (175-177). About 30-60% of the genetic risk for T1D is considered to be due to the effect of HLA genes. The rest of the genetic risk is conferred by other genes including the insulin gene, the CTLA-4 gene, the PTPN22 gene, the MDA-5 gene and some other genes, each of them contributing by 5-10 % to the genetic disease susceptibility (178). Previous studies (179) have shown that the distribution of T1D – related HLA-DQ allele combinations in four populations in the Eastern Baltic region demonstrated a lower frequency of risk-associated genotypes in the Russian population. In another study genetic susceptibility defined by HLA-DQ alleles was found to correlate with the geographical differences in T1D incidence (180). However, it is still unclear to what extent genetic factors can contribute to the international variation in the incidence of T1D. The traditional view that genes directly determined the phenotype has also been challenged and growth and early adaptation to the environment are now viewed as an interactive process between genes and environment. The biological signature of each individual thus derives from a dynamic process of adaptation (181).

Environmental factors

Since only a small fraction of genetically susceptible individuals develop T1D, a role for the environment has been implicated in the initiation and/or acceleration phase of islet

autoimmunity. Studies on monozygotic twins have shown that the concordance rate for the development of T1D is not higher than 25 to 53% (182-184). A Danish study has indicated that there is no difference between the prevalence of islet cell autoantibodies in di-and monozygotic twins of T1D patients who themselves are non-diabetic, implying that islet cell autoimmunity is strongly environmentally determined (185). Migration studies represent another line of evidence that suggests a role for environmental factors in the development of T1D (186-188). It has been shown that children living in South Asia have a low incidence of T1D, but second-generation migrants from there to the United Kingdom have overall rates similar those seen in the indigenous population (188). Further support for the impact of environmental factors on the disease process is derived from the marked geographical variations in disease incidence and the rapid increase in incidence rates. Taken together, these epidemiological and genetic studies are consistent with a model in which a common environmental factor, or set of related factors, operates on a genetically susceptible pool of individuals to give rise to the disease.

Virus infections

Enteroviruses have been considered the prime suspect even though other viruses, such as rubella virus (congenital rubella), mumps, cytomegalovirus, rotavirus have also been cited as potential etiological agents (177; 189; 190). Some viruses cause persistent or latent infections while others are efficiently cleared. Notably, a virus does not necessarily have to infect the host but may, in fact, already reside within the genome. Even different strains of the same virus may use distinct pathways to induce autoimmune disease. Secondly, several factors may influence the outcome of an infection, and a virus may cause disease only under certain conditions (191). Studies in animals have shown that factors such as the timing of the infection, the host's previous history of viral infections and the type of immune response raised by the host, including that of the target cells, can dramatically alter the risk for disease development. Some studies instead pointed to situations where a viral infection can induce protection against self-reactivity and autoimmunity (192).

Coxsackie B4 virus (CBV4) can cause diabetes in animals. There are also a few reports of isolates of CBV4 obtained from patients at diagnosis of T1D. Coxsackie B4 was isolated from the pancreas of a child who died at the presentation of T1D and the isolate was diabetogenic in mice (193; 194). Successful viral isolation from infected pancreas would require the presence of infective viral particles in sufficient amounts to induce a cytopathic effect. As has been shown in animal models, this is only achieved in a very narrow window after primary infection (usually 8 days), depending on the enterovirus strain used (195). New lines of evidence have emerged that point to a consistent association between enterovirus infection and T1D (196). Enterovirus genome and proteins have repeatedly been detected in the pancreatic islets of patients with T1D and patients who have died of severe enterovirus infection. Several studies have found CBV-specific IgM antibodies to be more common in patients with newly diagnosed T1D than in healthy individuals (197; 198). During follow-up of at-risk subjects, it has been observed that children who later developed T1D had almost twice as many enterovirus infections as the control individuals, and those infections were associated with the emergence of islet cell autoimmunity, reflected by the appearance of several islet cell autoantibodies (199; 200). In addition to childhood infections, enterovirus

may infect women in the prenatal period and increase the risk of diabetes in the offspring (200). However, not all studies have reported such an association and therefore this remains a controversial area of research. These discrepancies could be due to the fact that in many of these studies the determination of enterovirus infection was carried out indirectly through the determination of virus antibodies, and studies that use multiple approaches to identify infection (serology, RT-PCR, feces analysis) appear more likely to report an association with T1D or islet autoimmunity, suggesting that the sensitivity of the viral detection is an important factor (201). Molecular approaches have been used to identify virus with higher sensitivity and possibly to identify the virus involved. These studies have shown a higher frequency of enterovirus RNA in the serum of patients with diabetes compared to healthy control subjects (201; 202). In some of the cases, the detection of enterovirus RNA preceded the synthesis of islet cell autoantibodies. A more refined approach has been to examine T-cell responses to individual viral proteins. These studies have reported that T-cell proliferation to viral proteins in patients with T1D is either similar to that in control subjects or reduced (203; 204). One explanation is that decreased responses are due to a re-distribution of virus-specific T cells, as virus-responder cells are presumed to have homed to the pancreas and therefore being unavailable for detection in peripheral blood. An alternative explanation for normal or reduced proliferation of T cells to enterovirus antigens is that a significant proportion of responder cells do so not by proliferation but by other effector functions. In fact, it was found that the quality of the response of anti-enterovirus T cells differed between patients with T1D and control subjects. Patients with newly diagnosed disease produce more IFN- γ , a pro-inflammatory cytokine generated by effector memory CD4 T cells, but show less T-cell proliferation (204). T-cell proliferation is dependent upon IL-2 secretion, a response typically associated with central memory T cells. This implies that the evolution of diabetes is characterized by a state in which anti-CBV4 effector cells are mobilized from the central memory pool (192). There are several potential mechanisms for virus-induced immunopathology. These include molecular mimicry, bystander activation, and a persistent virus infection (205). A mechanism for CBV involvement by induction of islet-cell autoimmunity has been implicated, mediated via molecular mimicry between the human GAD65 protein and the CBV P2-C protein (181; 206). This has, however, not been confirmed (207).

Vitamin D

The function of vitamin D was largely considered to be in the area of calcium, phosphorus, and bone metabolism until 1980. In 1968, the idea appeared that vitamin D itself is biologically inactive and must be metabolically activated before it can function. This led to the chemical identification of the active forms of vitamin D in 1968-1971. The attempt to understand how the active form of vitamin D [1 α , 25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃)] carries out its functions led to the discovery of the vitamin D receptor (VDR) in the 1970's. It became clear that this hormone is localized almost entirely in the cellular nucleus in a specific fashion in target tissues. This localization was also detected in other tissues not previously considered targets of vitamin D (208; 209). Thus, the specific nuclear localization of 1,25-(OH)₂D₃ and the presence of the VDR was observed in the keratinocytes in the skin, in pancreatic islet cells, lymphocytes and promyelocytes. Abe et al. (1981) and Tanaka et al. (1982) demonstrated that the vitamin D hormone can suppress the proliferation of

promyelocytes and cause their differentiation into monocytes. The work of Manolagas and coworkers (1985,1986) provided the first strong evidence that activated lymphocytes contain significant quantities of the VDR (210; 211). Numerous *in vitro* studies showed that peripheral lymphocytes, macrophages, and thymus tissue contain the VDR (212; 213). The work of DeLuca and his group (1992) demonstrated that the CD8⁺ lymphocytes have the highest concentrations of the VDR. The CD4 lymphocytes and macrophages contain relatively small but significant amounts of the VDR. The structure of the VDR was identified relatively recently (214).

Several epidemiological studies have described an inverse correlation between sunlight and the incidence of T1D and MS. Vitamin D is an obvious candidate as a mediator of this sunshine effect. Cod liver oil taken during the first year of life reportedly reduced the risk of childhood-onset T1D (215-217). The EURODIAB Substudy 2 Study Group also showed in 1999 an association between vitamin D supplementation in infancy and a decreased risk of T1D. The 1966 birth cohort study from Northern Finland reported that both regular and irregular supplementation with vitamin D during infancy was associated with a conspicuously reduced risk of later T1D, and that suspicion of rickets by the age of 2 years was related to an increased risk of T1D (218). The DAISY Study reported that the presence of islet auto-antibodies in offspring was inversely correlated with vitamin D intake during pregnancy (219). A protective effect of vitamin D in MS was implied by recent studies which related the intake of vitamin D to reduction in the risk of MS (220; 221). However, the results of genetic studies investigating the possible relationship between VDR polymorphism and T1D are inconsistent: no correlation can be observed in some populations (222), whereas a clear correlation has been reported in some others (223-225). The identification of VDR on almost all cells of the immune system, especially macrophages and DC and activated T lymphocytes prompted the investigation of 1,25 (OH)₂ D₃ as a potential immunomodulator (226-228). Cytokines secreted by antigen-presenting cells for the recruitment and activation of T cells are directly influenced by 1,25 (OH)₂ D₃ and some authors have described the inhibition of IL-12 (229; 230).

6.5.3. Celiac disease

Epidemiology

Celiac disease (CD) represents a wide spectrum of clinical features. It may present in childhood soon after the introduction of gluten-containing food. Previously, a dramatic and even fatal clinical picture with diarrhea, anorexia, failure to thrive, abdominal distension, and growth retardation was regarded as typical. Today, most children present with a milder, pauci- or mono-symptomatic disease that resembles the picture in adults (231). Many of the symptoms associated with the milder disease form, like chronic fatigue, joint pain, and neuropsychological problems do not directly point to a small intestinal disorder (232-234), and neither do complications such as osteoporosis, reduced fertility, peripheral neuropathy, epilepsy with cerebral calcifications or the blistering skin disorder dermatitis herpetiformis (235-238). It appears that most patients have atypical CD (fully developed gluten-induced villous atrophy discovered in an asymptomatic patient by serologic screening or perhaps during an endoscopy performed for another reason), although classic CD (i.e. fully developed

gluten-induced villous atrophy and classic features of intestinal malabsorption) is described most commonly (237). The true prevalence of CD is difficult to estimate because of this variable presentation, particularly when many patients may have little or no symptoms. In North America the prevalence has been estimated to be 1:3000, but a recent American study found it to be 1:105 among the general population, suggesting that the disease is underrecognized (237).

More than half of the cases are now diagnosed in adult life, even after the age of 60 years. Many of these cases have had undetected CD in childhood, whereas in others the disease appears to have started later. Recent serologic studies have shown a uniform prevalence throughout Europe of around 1 in 130 to 1 in 300 in the USA (237). Even higher prevalence (1 in 100) have been observed in Finnish and UK schoolchildren (73; 239), and among 45-76-year-old subjects in the UK (240). Between 1985 and 1995 there was an epidemic of CD in Sweden among children younger than 2 years of age with a threefold increase in incidence (241). The sharp rise in incidence was likely related to changes in infant feeding habits resulting in increased amount of gluten given in infancy. Notably, the risk for CD was reduced in those children who were breastfed at the time of gluten introduction (242).

The prevalence of CD in the general unselected populations of North America and Western Europe as assessed by serology ranged widely from 152 per 100,000 (0.152% or 1:658) to 2,670 per 100,000 (2.67% or 1:37). The prevalence as assessed by biopsy examination ranged from 152 per 100,000 (0.152% or 1:658) to 1,870 per 100,000 (1.87% or 1:53) (237; 243; 244). Among the studies conducted in the United States, the prevalence ranged from 0.00312 (0.312% or 1:320-only child population in this group) to 0.00949 (0.949% or 1:105) (180; 237). The largest of these, by Fasano et al., observed a prevalence of CD in not-at-risk populations to be 0.95% in adults, 0.31% in children, and 0.75% overall (0.0075 or 1: 133). The prevalence of CD reported by seven Italian studies was similar to that seen in the American studies, ranging from 0.2% to 0.94% (245; 246). One of these, a recent large study by Tommasini et al. in 3,188 school-aged children, found the prevalence of CD to be 1:106, which is similar to that reported for not-at-risk American adults by Fasano et al. but higher than the reported rate for children. Only four studies showed a prevalence of CD greater than 1.5% (from the United Kingdom, Sweden, Germany), and an additional six studies showed a prevalence of between 1% and 1.5% (from the United Kingdom, Sweden, The Netherlands, Ireland, and Finland) (247-249). Overall, the prevalence by serology ranged from 0.17% to 2.67% for 13 small studies, whereas 12 of the 18 large studies reported a prevalence in the range of 0.5% to 1.26% (1:200-79). Among the studies from the United States, the prevalence ranged from 0.4% to 0.95% in adults, and was 0.31% in children. In Italy, the prevalence varied from 0.2% to 0.94%, and a recent large study showed the prevalence in school-age children to be 1:106 (0.94%). The Scandinavian countries, Ireland, and the United Kingdom tended to show a higher prevalence of CD in the range from 1.0% to 1.5%. Overall, the prevalence of CD is likely close to 1% in Western populations but may be higher in Northern European countries (250). The observed prevalence of CD in patients with symptoms or conditions associated with the disease ranged between 1% and 4%. The prevalence of CD among first-degree relatives undergoing intestinal biopsy examination varied from 5.5% to 22.5%, the pooled prevalence was 16% (251). One American study and one Hungarian study provided data on the prevalence of CD in second-degree relatives. The

EMA-based prevalence of CD in those groups was 2.6% and 5.5%, respectively (237; 252). Patients with T1D are also known to have increased risk of CD. The majority of studies have showed a prevalence in the range of 4%-6% among patients with T1D (253; 254).

Immunopathology

The diagnosis of CD is defined by typical gluten-induced alterations of the small intestinal mucosa. These changes can be classified into three stages (introduced by Marsh in 1992): the infiltrative, the hyperplastic, and the destructive lesion. In the infiltrative lesion the mucosal architecture is normal, but there is an increased infiltration of intraepithelial lymphocytes in the villous epithelium. The hyperplastic lesion is similar to the infiltrative lesion, but in addition has hypertrophic crypts. The last stage is now often subgrouped into partial, subtotal or total villous atrophy (Marsh 3A-C); the latter corresponding to the classic flat lesion (255). The classic flat lesion, in addition to the increased intraepithelial lymphocytes, is characterized by swelling of the lamina propria and infiltration of CD4⁺ αβ T-cells, plasma cells, macrophages-dendritic cells, mast cells, and neutrophils in the same compartment. Several molecules with immune function have altered expression in the celiac lesions. The epithelial expression of HLA class II molecules with strong expression of DR and DP molecules but little or no expression of DQ molecules is very noticeable (256; 257). In previous studies it has been shown that a clear subepithelial IGA deposits can be found below the basement membrane along the villous and crypt epithelium and around mucosal vessels in CD (258).

The coexistence of autoimmune diseases in CD is striking, particularly that of T1D (259; 260), Sjögren's syndrome (261), AITD (261), connective tissue diseases (261), and IgA deficiency (262). Like many other autoimmune diseases, pediatric and adult CD show a gender bias with a female-to-male ratio of approximately 2:1 (263; 264).

Untreated CD patients usually have increased levels of antibodies against wheat gluten and autoantigens present in the mucosa. The autoantibodies in CD are primarily directed against the Ca²⁺-activated form of the enzyme tissue transglutaminase 2 (tTG2) (265), but antibodies to calreticulin and actin have also been reported (266). The antibodies to tTG2 are both of the IgA and IgG isotypes, but the IgA antibodies, which are primarily produced in the intestinal mucosa demonstrate the highest disease specificity (267). The role of the tTG2 enzyme in the pathogenesis of CD is not completely understood. There are high levels of active tTG2 in the inflamed lesions of patients with untreated CD, and the gluten proteins are excellent substrates for tTG2 (Larre et al., 1993). Most importantly, the T cells within the celiac lesions predominantly recognize gluten peptides modified by tTG2 (268; 269). tTG2 catalyzes a post-translational transamidation or deamidation of specific glutamine residues within its substrate proteins (Lorand and Graham, 2003). In the transamidation reaction, the glutamine becomes cross-linked to a protein-bound lysine or a polyamine, whereas the deamidation reaction results in conversion of the glutamine to glutamic acid. The enzyme is involved in the selection of HLA-DQ2-restricted T-cell epitopes. The fact that the glutamic acid residues created by TG2-mediated deamidation play a critical role in DQ2/DQ8 binding and T-cell recognition provides indirect evidence that tTG2 is involved in the mucosal deamidation of gluten peptides. Besides its enzymatic activity, tTG2 is involved in the

attachment and motility of fibroblasts and monocytes via interactions with integrins and fibronectin (270; 271). Hence, the celiac villous atrophy could be caused by TG2 autoantibodies disturbing the migration of fibroblasts and epithelial cells from the crypts to the tips of the villi (272). Autoantibodies to tTG2 may also be involved in the extra-intestinal manifestations of CD, although the mechanisms are not understood. It appears that celiac patients with dermatitis herpetiformis in addition to their anti-tTG2 antibodies, have antibodies that target tTG3, a transglutaminase that is uniquely found in the dermal papillae of dermatitis herpetiformis patients (273). The mechanism underlying the formation of the autoantibodies in CD has remained open. An explanation could be that the complexes of gluten and TG2 permit the gluten-reactive T cells to provide help to the TG2-specific B cells by a mechanism of intramolecular help. This model can explain why the serum tTG2 antibodies in CD disappear when the patients are put on a gluten-free diet. When the gluten goes, so does the T-cell help needed for the B cells to switch isotype and differentiate into plasma cells (274).

CD is also characterized by an increased density of proliferating T-cell receptor (TCR) $\alpha\beta^+CD8^+CD4^-$ and TCR $\gamma\delta^+CD8^-CD4^-$ cells in the villous epithelium. All these IELs express the epithelial homing marker CD103 (integrin $\alpha E \beta 7$) (275). Many IELs co-express innate (natural killer NK cells) receptors recognizing non-classical HLA molecules. The expansion and activation of IELs in CD seems to be driven by interleukin IL-15 (275) but the mechanisms leading to this increased expression are poorly understood. Gluten proteins comprise multiple T-cell epitopes, and T cells with several distinct specificities can be isolated from small intestinal biopsies of a single patient. Among the α -gliadins there are epitopes that are immunodominant and are recognized by T-cells from almost all patients (268), whereas reactivity to other epitopes can only be found in a minority of patients with CD.

Despite the absence of a single pathogenic motif, the gluten epitopes recognized by gut T cells are generally very rich in proline and glutamine residues. In several of the epitopes, proline residues are present in four of the nine residues in the core region (276). Studies of biopsies taken from patients following oral challenge with gluten proteins (277) have shown that morphologic changes are induced and the mucosal response was directly related to the amount of gluten given. There seems to be two waves of immune activation: a rapid wave that occurs within hours, including overexpression of HLA-DR molecules on enterocytes, and a later wave dominated by activation of resident T cells (278).

Different approaches have been taken to establish an animal model of CD, but none have demonstrated mucosal immunopathology and genetic features mimicking the human disorder. It has not been possible to induce gluten-sensitive enteropathy in mice transgenic for the HLA-DQ2 gene (279; 280) or HLA-DQ8 gene (281). Of interest for CD, transgenic mice overexpressing human IL-15 in intestinal epithelial cells develop intestinal inflammation confined to the proximal small intestine with reduced villous length, marked infiltration of lymphocytes, and vacuolar degeneration of villous epithelium (282).

Genetic factors

The high prevalence of CD among first-degree relatives indicates that the susceptibility to this disease is strongly influenced by inherited factors. The strong genetic influence in CD is further supported by a very high concordance rate (around 75%) in monozygotic twins (283). The overall susceptibility to develop CD is associated with two conventional HLA-DQ molecules: DQ(α 1*05, β 1*02)=DQ2 and less pronounced to DQ(α 1*03, β 1*0302)=DQ8. These HLA-DQ molecules bind peptides and present them to CD4⁺ helper T cells carrying the $\alpha\beta$ TCR. This genetic evidence points towards a central role for CD4⁺ T cells in controlling disease development. About 85% of patients with CD carry the DR3-DQ2 haplotype (the DRB1*0301-DQA1*0501-DQB1*0201 haplotype). A proportion of the remaining ones are DR5-DQ7/DR7-DQ2 heterozygotes (i.e., they carry the DRB1*11/12-DQA1*0505-DQB1*0301/DRB1*07-DQA1*0201-DQB1*0202 haplotypes) (284; 285). CD patients with these DQ-DR haplotype combinations thus share the same functional DQ molecule on the cell surface, encoded by genes carried in the cis (e.g., DQA1*05 and DQB1*02 carried on the same haplotype) or trans position (e.g., DQA1*05 carried on a different haplotype from DQB1*02) (286). CD patients who are DQA1*05 and DQB1*02 negative frequently carry the DRB1*04-DQA1*03-DQB1*0302 haplotype (i.e., DR4-DQ8 haplotype). Genetic data accordingly favor DQ8 as the primary disease-susceptibility determinant in those patients. The very few remaining CD patients who are neither DQ2 nor DQ8 carry either the α or the β chain of the DQ2 heterodimer (i.e., DQA1*05 or DQB1*02) (287).

Much less is known about the non-HLA genes in this disease. The region that has most consistently been linked to CD is on the long arm of chromosome 5 (5q31-33) (288; 289). There is also evidence for a susceptibility factor on chromosome 11q (290) and on chromosome 19p13 (291). Recent genome-wide association study by Hunt et al. has identified eight chromosomal regions (1q31, 2q11-2q12, 3p21, 3q25-3q26, 3q28, 4q27, 6q25, 12q24) outside the HLA region as being associated with CD (292). A subsequent study by Smyth et al. identified a new risk allele for both CD and T1D: 32-bp insertion-deletion variant in the CCR5 gene. That study also reported that about half of the genetic risk alleles for CD (at least four of the eight validated) had been shown to have a definite association with both CD and T1D. Of the 15 validated alleles conferring an increased risk of T1D, at least two contributed to a risk of CD, with five alleles showing highly suggestive associations (74).

Environmental factors

The main environmental determinant of the pathogenesis of CD is the amount of dietary gluten. Due to their high content of proline and glutamine residues, the proteins of wheat gluten are collectively referred to as prolamines. They are usually classified into α -, γ -, ω -gliadins and high and low molecular weight (HMW and LMW) glutenins (293). A single wheat cultivar may express more than 100 different gliadin proteins (294). This high content of proline residues makes the prolamines particularly resistant to gastrointestinal digestion. Clinical observations suggest that the prolamines of barley and rye are also toxic for celiac disease patients (234). With the oats prolamines the situation is more complex. Several

feeding studies have suggested that oats are safe for CD patients. (295; 296), but it is now clear that some celiac patients are oat intolerant as well (297).

A possible role of infections in the development of CD was implicated by a report from Sweden. In children under 2 years of age, a positive correlation was observed between CD risk and being born during the summer, which may be related to the fact that children born in summer are first exposed to dietary gluten during the winter when infections are more common (264). In addition, case-control data indicate that celiac patients experience an excess of three or more episodes of infections when compared to control children. Adenovirus 12 has been proposed as a candidate factor because one of its proteins displays partial linear homology over 12 amino acids with an γ -gliadin (298). Subsequent epidemiologic studies, however, have not confirmed this hypothesis. Recent prospective studies carried out in USA have suggested that rotavirus infections may increase the risk of CD (299).

6.5.4. Thyroid autoimmunity

There are several type of AITD. The prevalence of autoimmune hypothyroidism in general Caucasian population is 1-2 % among women, whereas thyroid autoantibodies can be detected in up to 20 %, reflecting the presence of focal subclinical thyroiditis. Pathologic changes in autoimmune thyroiditis (AT) range from mild focal thyroiditis to extensive lymphocytic infiltration and scarring (300). In Hashimoto's thyroiditis there is a dense infiltration by lymphocytes, plasma cells, and macrophages, and germinal center formation. Thyroid follicles are progressively destroyed and cells undergo hyperplasia and oxyphil metaplasia. For the diagnosis of autoimmune hypothyroidism biochemical evidence and thyreoglobulin (TG) and /or thyroid peroxidase (TPO) antibodies are needed and thyroid ultrasound pattern as well as needle aspiration biopsy are used.

Immunopathogenesis

Circulating autoantibodies against thyreoglobulin (TGAb) and thyroid peroxidase (TPOAb) are found, often at very high levels. TG is the major protein product synthesized in the thyroid gland (301). At four-eight hormonogenic sites, iodinated tyrosine residues couple to form T4 or tri-iodothyronine (T3). Oxidative stress during TG synthesis may be particularly critical in the generation of immunoreactive C-terminal fragments of the molecule (302). TGAb are detected in most patients with AITD, both Graves' disease and Hashimoto's thyroiditis (303), and there is evidence that TGAb in AITD patients are restricted in their epitope specificity, in contrast to the polyclonal nature of TGAb present in healthy individuals (304). There are two major and one minor antibody epitopes on each 330 kDa subunit, and wide spacing of these prevents IgG cross-linking and therefore complement fixation. Antibody reactivity is predominantly of the IgG1 and IgG4 subclasses but is not light chain restricted. TPO is a 100-150 kDa apical protein responsible for tyrosine iodination and coupling in the formation of thyroid hormones. TPOAb have a similar IgG subclass distribution to TGAb, but are k-light chain restricted (305). 80% of TPOAb recognize an immunodominant region involving overlapping, conformational epitopes in two extracellular domains and specific patterns of TPOAb recognition are genetically transmitted in families

with AT (305). Thyroid stimulating hormone (TSH) receptor (TSH-R)-blocking antibodies are found in around 20% of patients with AT (306). Autoantibodies against T4 and T3 occur in 15-35% of AT patients.

Immune complexes are deposited in the basement membrane around the thyroid follicles in Hashimoto's thyroiditis (307), and the presence of terminal complement complexes indicate the formation of membrane attack complexes (308). Thyroid follicle cells are relatively resistant to lysis, through enhanced expression of multiple regulators of complement activation, especially CD59, in response to cytokines derived from the infiltrating lymphocytes and macrophages (309). After a sublethal complement attack, thyroid follicle cells are less able to respond to TSH stimulation and also release cytokines (IL-1, IL-6), prostaglandin E2 and reactive oxygen metabolites, which may have proinflammatory effects (310). TPOAb are assumed to be major mediators of complement fixation and activation within the thyroid gland (311). Cytokines such as IL-1 may be critical in dissociating the junctional complex and allowing access of autoantibodies to apically expressed TPO and other antigens thus implying a secondary role for these antibodies in the disease pathogenesis (312). Autoantibodies may also cause damage by antibody-dependent cell-mediated cytotoxicity and placental transfer of TSH-R blocking antibodies causes transient neonatal hypothyroidism.

T cells may provoke thyroid dysfunction by release of cytokines. IFN- γ inhibits the response to TSH stimulation and has in combination with TNF a marked inhibitory effect on TPO and TG expression (313). CD8⁺ T-cell clones have also been derived from patients with Hashimoto's thyroiditis (314). The importance of cytotoxic T cells in the pathogenesis is emphasized by the presence of frequent perforin-containing T cells in the intrathyroidal CD8⁺ T-cell population in Hashimoto's thyroiditis (315). The major pathway for the destruction of thyroid follicular cells is based on Fas expression and is upregulated by IL-1 β . Accordingly cytokines might induce cytotoxicity by suicide as Fas interacts with FasL (316). Another proposal is that the infiltrating lymphocytes cause apoptosis of thyroid follicular cells through their expression of TNF-related apoptosis-inducing ligand (TRAIL) engaging with various death receptors, induced by cytokines (317). Treg cells are important in experimental autoimmune thyroiditis, but mechanisms which control thyroid-specific T cells that have escaped central tolerance in human autoimmune thyroiditis are elusive. In conclusion, further studies are needed to identify the earliest T-cell responses and to determine the exact genetic basis for thyroid autoimmune diseases.

AT can be induced experimentally in animals by immunizing them with TG and can also occur spontaneously with features most closely resembling Hashimoto's thyroiditis (318). Both these models are characterized by the development of TGAb (319; 320). The thyroiditis after immunization consists of CD4⁺ and CD8⁺ T cells and macrophages, with only a small percentage of B cells. The disease can be transferred by T cells but not by TGAb and the critical effector cells are CD8⁺ cytotoxic T cells which require specific CD4⁺ T cells for their induction (321). An extreme example of T-cell modulation has been the generation of mice transgenic for the T-cell receptor of a T-cell clone specific for a cryptic epitope of TPO, derived from a patient with AT (322). These mice developed thyroiditis and hypothyroidism despite this being in the context of murine TPO and antigen presentation of the relevant

epitope by H-2 rather than HLA. Thus, the development of disease in these animals indicates that a cryptic TPO epitope can have a significant pathogenic role in autoimmunity.

Genetic factors

The role of genetic factors in AT is suggested by the frequent presence of thyroid autoantibodies in other family members and the association of thyroiditis with other endocrinopathies such as the type 2 autoimmune polyglandular syndrome. Twin studies show a 55% concordance rate in monozygotic but not dizygotic twins, and similar findings have been reported for the aggregation of thyroid autoantibodies in euthyroid twins of individuals with AT (323; 324). Associations with HLA alleles have been extensively investigated. Hashimoto's thyroiditis is associated with HLA-DR3 and to a lesser extent HLA-DR4. Another established susceptibility locus besides HLA is CTLA-4 with polymorphisms in this gene conferring a relative risk of around 2. Since the first description of an association between CTLA-4 and Graves' disease in 1995 (325) CTLA-4 has been shown to be associated with a series of autoimmune conditions, which is not unexpected in view of the critical role of CTLA-4 in the immunologic synapse. Later studies demonstrated the association of CTLA-4 with Hashimoto thyroiditis also in populations of diverse ethnic origins, such as Caucasian populations (326-328), and Japanese (329; 330). The protein tyrosine phosphatase (PTPN22) gene is the latest autoimmunity gene to be identified and it has been reported to be associated with Graves' disease (68). Genome-scanning methods have implicated a number of susceptibility loci on chromosomes 6p, 8q, 10q and 12 q (331). Two whole genome screens have shown strong evidence for linkage of AITD with the chromosome 8q24 region which comprises the TG gene (332; 333). Association studies also showed an association of TG microsatellite markers with AITD (332; 334; 335). It can be hypothesized that TG sequence variants are involved in the susceptibility to AITD; such variants may act by modifying the interaction of TG peptide profiles, produced by digestion of TG by cathepsins in endosomes, with HLA class II molecules.

The female preponderance of AT is in part due to the influence of sex steroids. Pregnancy ameliorates AT, in some women the enhanced autoimmune response is sufficient to cause biochemical or clinical thyroid dysfunction and this is termed postpartum thyroiditis (336). Fetal microchimerism may play a role via intrathyroidal chimeric cells breaking immunologic tolerance (337).

Environmental factors

There is no convincing evidence for a direct role of infections in the etiology of AT (338). The prevalence of AT is rising in developed countries, possibly as a result of increasing iodine intake. Iodine may be involved at an early stage in causing thyroid injury through the generation of reactive oxygen metabolites (339). Indirect evidence for iodine-induced injury is provided by studies of iodization programs in iodine-deficient regions showing an increase in thyroid autoantibodies and lymphocytic thyroiditis shortly after supplementation. Radioactive fallout from the Chernobyl nuclear disaster led to an almost 10-fold increase in the prevalence of thyroid autoantibodies in girls aged 9-10 years at the time of the accident, presumably through the release of thyroid autoantigens from the damaged thyroid (340; 341).

Certain viruses have also been studied as potential triggers of AITD, but none has shown constant association with the disease (342).

7. Aims

The main aim of the present study was to assess the contribution of genetic and environmental factors to the incidence of autoimmune diseases. The study included two populations living close to each other but differing conspicuously in their socio-economic circumstances (Finland and the adjacent Russian Karelia). This comparison is particularly interesting, since due to population mixing during earlier centuries, these populations share partly the same kind of ancestry. Accordingly, this epidemiological setting is optimal for the detection of possible gene-environment interactions underlying the pathogenesis of autoimmune diseases.

The present work focuses on the following main aims:

- 1) to evaluate the contribution of environmental and genetic factors to T1D by comparing the incidence of the disease in children younger than 15 years of age living in Russian Karelia and in Finland (Substudy I);
- 2) to define the immunologic and genetic risk markers for T1D, i.e. the prevalence of autoantibodies to various islet cell antigens and the frequency of HLA-risk genotypes in the background population of these two countries (Substudy II);
- 3) to compare the prevalence of CD and immunological and genetic risk markers for the disease in two neighbouring populations, i.e. in Russian Karelia and Finland (Substudy III);
- 4) to assess the frequency of thyroid autoimmunity in these two adjacent regions, i.e. the prevalence of thyroid autoantibodies and the frequency of HLA-DQ alleles (Substudy IV);
- 5) to assess the effect of the ethnic background on the risk of these autoimmune diseases by analyzing humoral signs of autoimmunity and HLA-defined genetic disease susceptibility in children with either Finnish-Karelian, Russian, or other ancestry in Russian Karelia (Substudies I-IV);
- 6) to analyze the vitamin D status in a series of schoolchildren and pregnant women from the Russian Karelia and Finland and to determine whether vitamin D could contribute to the difference observed in the risk of autoimmune diseases between these two countries (Substudy V);

8. Subjects and methods

8.1. General characteristics of the two study populations

The Karelian Republic of the Russian Federation is located in the northwestern part of Russia (area 172,400 km²) and shares a territorial border with Finland (see Figure 1 in Substudy IV). Thus, climatic conditions are very similar in Russian Karelia and Finland (e.g. sunshine hours, ultraviolet radiation, rain, temperature, etc). The population of the Karelian Republic of Russia amounted to 794,921 in 1990 and to 765,098 in 1999, and the number of children younger than 15 years of age was 189,754 and 137,713 respectively. Petrozavodsk is the capital city with a total population of about 280,000. In the census the ethnic background was recorded according to that of the mother. Russians comprise the largest ethnic group with 73.6%, Karelians and Finns 12.3%, others including Ukrainians, Belorussians and some other ethnic groups altogether 14.1%.

8.2. Incidence of type 1 diabetes (Substudy I)

There was no national diabetes registry in Russian Karelia but all children with newly diagnosed T1D were referred for initial care to the Republic Hospital in Petrozavodsk. After initial treatment, children with diabetes are also observed and treated in outpatient clinics in other parts of Russian Karelia. In the present study cases with newly diagnosed T1D were primarily identified from the records of the Republic Hospital in Petrozavodsk. Children diagnosed with T1D between 1990 and 1996 were identified retrospectively, while cases diagnosed from 1997 to 1999 were identified prospectively.

To assess the completeness of ascertainment of the incidence cases we used a capture-recapture method by comparing the results obtained from the hospital records with the annual reports made by two outpatient clinics to the Ministry of Health in Russia during the years 1990-1999 (as a secondary source). No additional or missing cases were observed when these two sources were compared. Accordingly, the overall ascertainment rate was estimated to be 98-100%. Case definition was based on the criteria used by EURODIAB ACE (89). In all cases insulin treatment was initiated before the 15th birthday. Information on the ethnic background of the parents and the grandparents was collected by a personal interview with the family. Maternal ethnicity was used in the estimation of the incidence rate of T1D in relation to ethnic group, as it was used for the determination of ethnicity in the census. Average annual incidence rates were calculated in three age groups (0-4, 5-9 and 10-14 years) and for both genders using the ascertainment-corrected number of incidence cases and the average mid-year population data from the National Statistical Committee of Russian Karelia. The incidence of T1D in Finnish children aged 0-14 years was based on data derived from the National Drug Reimbursement Register described earlier (88; 343).. The degree of ascertainment of these data has been shown to be close to 100 % (88). All population data were derived from the National Registry of the Statistical Committee of the Karelian Republic and were based on the census carried out in 1989.

8.3. Subjects

Study participants were recruited among schoolchildren in both countries according to the following design.

In the Karelian cohort serum samples were obtained from altogether 1988 randomly selected schoolchildren living in different regions of Russian Karelia during the period 1997-2001 as part of the T1D-related EPIVIR Project (EU INCO-Copernicus Programme, contact number IC15-CT98-0316). The series included 1004 girls (50.5%) and 984 boys (49.5%), mean age 11.6 (2.4; SD) years (range 6.2-18.3 years). In addition, an EDTA (ethylenediamine tetraacetic acid) whole blood sample was obtained from each child for genetic analyses. Serum and blood samples were stored at -20°C until analyzed. In Substudy II the ethnic background of the children was recorded according to the mother's ethnicity and was categorized into three ethnic groups: those of Finnish-Karelian ancestry ($n=906$), those of Russian ancestry ($n=815$), and others ($n=267$) based on questionnaire data. In Substudy III the ethnic background of the children was recorded according to the ancestry of both the mother and father and classified as follows: 500 children with both parents having a Finnish or Karelian background, 382 with a Russian background, and 1,106 children with a mixed or other ethnic background. All children had informed parental consent to take part in the study, and the study was approved by the Ministry of Health in the Karelian Republic of Russia.

The Finnish cohort comprised 3,652 non-diabetic schoolchildren living in five communities (Haapajarvi, Ii, Oulainen, Yli-Ii, and Yli-Kiiminki) in the province of Northern Ostrobothnia [mean 11.7 (2.6; SD) years; range 7.0-18.0 years] and included 1,867 girls (51.1%) and 1,785 boys (48.9%) recruited during 1994. Serum and EDTA blood samples were collected from all children in the same way as in Russian Karelia and samples were stored at -20°C until used. The data on diabetes autoantibody frequencies in the Finnish children has been reported previously (344) likewise the results of their tTGA and HLA screening (73). All children had written parental consent to take part in the study, and the study was approved by the ethical committee of the Faculty of Medicine, University of Oulu, Oulu, Finland.

In Substudies II and III the whole cohort of Finnish and Karelian children was screened for diabetes or CD-associated autoantibodies while Substudies IV and V were based on a subcohort of children described above. A total of 1,064 schoolchildren (532 schoolchildren from Russian Karelia and 532 from Finland) were included in Substudy IV. Children from both countries were matched pair-wise for age, gender and season of the blood sampling. The mean age was 11.4 (2.0; SD) years (range 7.1-15.0) and 57% ($n=304$) were girls. In Substudy IV the children from Russian Karelia included 302 (56.8%) children with both parents having Finnish or Karelian background, 57 (10.7%) children with Russian background and 173 (32.5%) with a mixed or other ethnic background (the ethnic background recorded according to the ancestry of both parents). In Finland all children were of Finnish ancestry. Serum

samples and EDTA whole blood samples were taken from all children and stored at -20°C as described above.

The series of Substudy V included cohorts representing the background population (schoolchildren and pregnant women). The series of schoolchildren comprised 100 children from the Karelian Republic of Russia (the region of Petrozavodsk) and 100 subjects from Finland (Oulu and Tampere regions) matched for age, gender and month of sampling [mean age 10.9 (1.7; SD) years; 52% male subjects]. The series of pregnant women included 103 samples obtained from Karelian pregnant women (the region of Petrozavodsk) and 172 samples from Finland (the Joensuu region) matched for age and date of sampling [mean age 26.7 (5.3; SD) years]. These serum samples were collected at the end of the first trimester of pregnancy as part of the routine prenatal follow-up during 2000, and 81% of the samples were drawn during October-December. The study was approved by the local ethical committees and the Finnish Maternity Cohort Steering Group.

8.4. Autoantibody analyses

Diabetes-associated autoantibodies.

All autoantibody measurements were carried out in the Research Laboratory, Department of Pediatrics, University of Oulu, and identical methods were used to analyze the samples of both the Finnish and Karelian cohorts. Islet cell antibodies (ICA) were measured with a standard immunofluorescence method using sections of frozen human group O pancreas. End-point titers of each sample were converted into Juvenile Diabetes Foundation units (JDFU) relative to an international reference standard. The detection limit was 2.5 JDFU. The assay achieved 100% sensitivity and 98 % specificity in the most relevant workshop for the standardization of the ICA assay (345). All samples initially positive for ICA were retested for confirmation.

Insulin autoantibodies (IAA) were analyzed using a microassay modified from that described by Williams et al. (346). The limit for IAA positivity [1.56 relative units (RU)] was set at the 99th percentile in a separate series of 374 non-diabetic Finnish children and adolescents. This assay had a disease sensitivity of 58% and a specificity of 98% in the 2005 Diabetes Autoantibody Standardization Program (DASP) Workshop. Specific radioligand assays were used to quantify GADA and IA-2A levels as described (171), (347). The cut-off limit for antibody positivity, which was set at the 99th percentile in more than 370 non-diabetic children and adolescents, was 5.36 RU for GADA (n=373) and 0.43 RU for IA-2A (n=374). The disease sensitivity of the GADA assay was 82% and the specificity 96%, and the corresponding characteristics of the IA-2A assay were 72% and 100% respectively in the 2005 DASP Workshop. All samples with IAA, GADA and IA-2A levels between the 97th and 99.5th percentiles were reanalyzed to confirm their status. Multiple autoantibody positivity was defined as positivity for at least two antibodies out of ICA, IAA, GADA and IA-2A.

Both the Russian-Karelian and Finnish samples were analyzed for IAA and IA-2A at the same time. Finnish samples were assayed for ICA and GADA 2 years earlier than the

Russian-Karelian samples. The same pancreas was used, however, as the substrate in the ICA assay for both the Finnish and Russian Karelian samples, and the standard curve for the transformation of the ICA titers into JDF units remained unchanged when retested before starting the analysis of the Russian Karelian samples. The performance characteristics, in particular the specificity, of our GADA assay has remained very stable over the last 3 years according to the DASP workshops: The disease sensitivity of the GADA assay was 80% and the disease specificity 96% in 2003, with corresponding values of 82% and 96% respectively in 2005. The possible drift over time of the assays for molecular autoantibodies is continuously monitored based on both the standard curves and internal quality control samples run on each plate.

Celiac disease- associated antibodies

All schoolchildren from both Russian Karelia and Finland were screened for serum IgA (immunoglobulin class A)-class antibodies to tTGA using an enzyme-linked immunosorbent assay (ELISA)-based Celikey assay (Pharmacia Diagnostics, Freiburg, Germany) according to the manufacturer's instructions, and the cut-off limit for tTGA positivity was set at 5 U/ml (73). Serum IgA-class endomysial antibodies (EMA) were additionally analyzed from all tTGA positive subjects by indirect immunofluorescence assay as previously described (73; 348). A characteristic staining pattern at a serum dilution $\geq 1:5$ was considered positive. IgA- and IgG-class antigliadin antibodies were analyzed using an in-house ELISA method (349) in a subgroup of 265 Finnish and 265 Karelian children [median age 11.4 (2.0; SD) years in both groups], the latter being selected based on Finnish-Karelian ethnicity in both parents. Values equal to or above 10 EU/ml for IgG-class antigliadin antibodies and 0.2 EU/ml for IgA-class antigliadin antibodies were considered positive. All antibody analyses of CD-associated antibodies were carried out at Medical School, University of Tampere, Finland, using identical methods for both series.

Thyroid disease-associated autoantibodies

IgG class serum TPOAb and TGAb were measured using the UniCAP assay (Thyroglobulin ImmunoCAP and Thyroid Peroxidase ImmunoCAP, Phadia, Freiburg, Germany) according to the manufacturer's instructions in the Center of Laboratory Medicine, Tampere University Hospital, Tampere, Finland. The cut-off limit was 60 IU/ml for TPOAb positivity and 220 IU/ml for TGAb positivity. Thyrotropin (TSH) and free thyroxin (F T4) levels were analyzed using a chemiluminescence assay (Abbott, Architect TSH and free T4, Abbot Park, IL). The reference range for TSH in healthy children was 0.35-5.0 mU/l and for free T4 9.0-19.0 pmol/l.

Vitamin D status

Circulating concentrations of 25-hydroxy (25-OH) vitamin D were analyzed using a commercial enzyme-immunoassay kit (Immunodiagnostic Systems Limited, Boldon, UK) according to the manufacture's instructions, and the cut-off limit for vitamin D deficiency (serum 25-OH vitamin D) was set at <25 nmol/l as previously proposed (216; 350).

8.5. Genetic analyses

Diabetes-associated HLA class II alleles were typed by polymerase-chain reaction and microtiter-well plate based hybridization with lanthanide-labeled oligonucleotide probes as described (179). For Substudies II and III samples from 1,977 schoolchildren (99%) from Russian Karelia and from 3,649 Finnish schoolchildren (99.9%) were available for genotyping. The HLA genotypes were categorized into four risk groups for T1D: (i) high risk (DQA1*05-DQB1*02/*0302); (ii) moderate risk (DQB1*0302/x; x \neq DQA1*05-DQB1*02, DQB1*0301, DQB1*0602 or DQB1*0603); (iii) low risk (DQA1*05-DQB1*02/y, DQA1*03-DQB1*02/y, DQB1*0301/*0302, DQB1*0302/*0603; y \neq DQA1*0201-DQB1*02, DQB1*0302, DQB1*0301, DQB1*0602 or DQB1*0603); and (iv) decreased risk (all other genotypes). For Substudies III and IV samples that were positive for the HLA-DQB1*02 allele were further analyzed for the presence of the associated HLA alleles DQA1*0201 and DQA1*05 to define the HLA DR3-DQ2 haplotype. For Substudy IV EDTA samples were available for genotyping from all Finnish schoolchildren and from 99.6% (530) of the schoolchildren from Russian Karelia. All HLA-analyses were performed in the Immunogenetics Laboratory, University of Turku, Turku, Finland.

8.6. Endoscopy and small intestinal biopsies.

In Substudy III all subjects who tested positive for serum tTGA were offered an option for upper gastrointestinal endoscopy and small intestinal mucosal biopsy to confirm the diagnosis of CD. In Russian Karelia the endoscopy was performed at the Municipal Children's Hospital in Petrozavodsk. In Finland the endoscopy was carried out as a part of an earlier study (73) at the Department of Pediatrics, Oulu University Hospital, Finland. For histological analysis three duodenal mucosal biopsy samples were fixed in formalin and one snap-frozen in liquid nitrogen for cryostat sections. Light microscopy and morphometric techniques were used to study the formalin-fixed biopsy samples stained with hematoxylin and eosin. A ratio of villous height to crypt depth less than two was considered an indicator of CD (i.e. villous atrophy with crypt hyperplasia). The frozen biopsy specimens were stained for intraepithelial lymphocytes bearing CD3⁺, α/β ⁺ and γ/δ ⁺ T-cell receptors, and corresponding cell densities were determined as previously described (351). Small bowel mucosal tissue transglutaminase-specific IgA deposits were investigated from frozen sections by direct immuno-fluorescence and the intensity of the deposits was graded 0-3 as previously described (258; 352). In CD a clear subepithelial IgA deposit can be detected below the basement membrane along the villous and crypt epithelium and around mucosal vessels. This is in contrast to normal small bowel samples, where IgA is detected only inside plasma and epithelial cells. All histological analyses were centralized to the CD Research Unit, University of Tampere, Finland, and were performed without prior knowledge of disease history or laboratory findings.

8.7. Statistical analyses

In Substudy I the crude annual incidence rates per 100,000 were assessed by sex and 5-year (0-4, 5-9, 10-14) age groups using mid-year population of the country as the denominator. The age-adjusted rates were calculated by the direct method using proportions of 5-year age

groups of Finnish children aged 0-14 years in 1990 as weights. Testing of statistical differences of age-adjusted incidence rates were based on 95 percent confidence intervals (CI). The frequency of susceptible and protective *HLA DQ* genotypes was compared with the Chi-square test. In Substudy II the Chi-Square test and Fisher's exact test were used to compare the frequency of autoantibodies and Mann-Whitney U-test in the analysis of nonparametric data (SPSS, version 10.1, SPSS Inc., Chicago, IL, USA). The 95% CI were calculated with the exact method. The power of this study to detect a difference in the frequency of positivity for at least one autoantibody of the same magnitude as the sixfold difference in disease incidence was 100%, while the power to detect a sixfold difference in the frequency of multiple autoantibodies was 80%. In Substudy III Student's t test was used for the analyses of continuous variables and the Chi-square test for the analyses of distributions (SPSS, version 12.1, SPSS Inc., Chicago, IL, USA). In Substudy IV the chi-square test and Fisher's exact test were used to compare the frequency of autoantibodies. For the comparison of paired data, McNemar's test was used. Autoantibody levels were compared between the groups with Mann-Whitney U-test. The SPSS statistical package (version 12.1, SPSS Inc.) was used for the data analyses. In Substudy V Wilcoxon's test and the conditional logistic regression test were applied to compare circulating concentrations of 25-hydroxy (25-OH) vitamin D between the groups. A two-tailed P-value of less than 0.05 was considered statistically significant in all the studies.

8.8. Ethical aspects

The study protocols (I-IV) were approved by the Ministry of Health in the Karelian Republic of Russia and by the ethical committee of the Faculty of Medicine, University of Oulu, Oulu, Finland. Informed parental consent was obtained from all participants. Substudy V was additionally approved by the local ethical committees and the Finnish Maternity Cohort Steering Group. Families with children who were observed to be positive for diabetes – associated autoantibodies were informed, and the family was invited to visit a pediatric endocrinologist. If the child tested positive for tTGA the family was informed about the result and the child was invited for an intestinal biopsy to confirm or exclude the diagnosis of CD.

9. Results

9.1. Epidemiology of type 1 diabetes in Russian Karelia and in Finland (Substudy I)

The incidence of T1D in children under 15 years of age in Russian Karelia was analyzed during the period from January 1990 to the end of December 1999. A total number of 133 children with newly diagnosed T1D were registered. The age-adjusted mean annual incidence of T1D was 7.4 per 100,000 (95% CI 3.5-11.3) in Russian Karelia compared to 41.4 per 100,000 (95% CI 37.3-45.5) in the corresponding age group in Finland (see Figure 1 in Substudy I). The incidence of T1D in Russian Karelia varied from 4.9/100,000 in 1990 to 10.2/100,000 in 1999 with two peaks observed in 1992-93 and in 1998-99. No clear increase was observed. The mean incidence was slightly lower in the later period (1995-99) than the incidence rate in the first 5-year period (1990-94) (6.1 /100,000, 95% CI 2.0-10.2; vs. 7.1/100,000, 95% CI 3.4-10.8). In Finland the incidence was constantly at a higher level also showing a clear increase from 34.4 in 1990 to 49.1 in 1999. When the incidence was related to age clear peaks were observed at the ages of 2 years and 5 years, followed by a third peak in the pubertal period (Figure 2). In Finland, the incidence rose rapidly at the age of 2 years and kept increasing gradually along with increasing age.

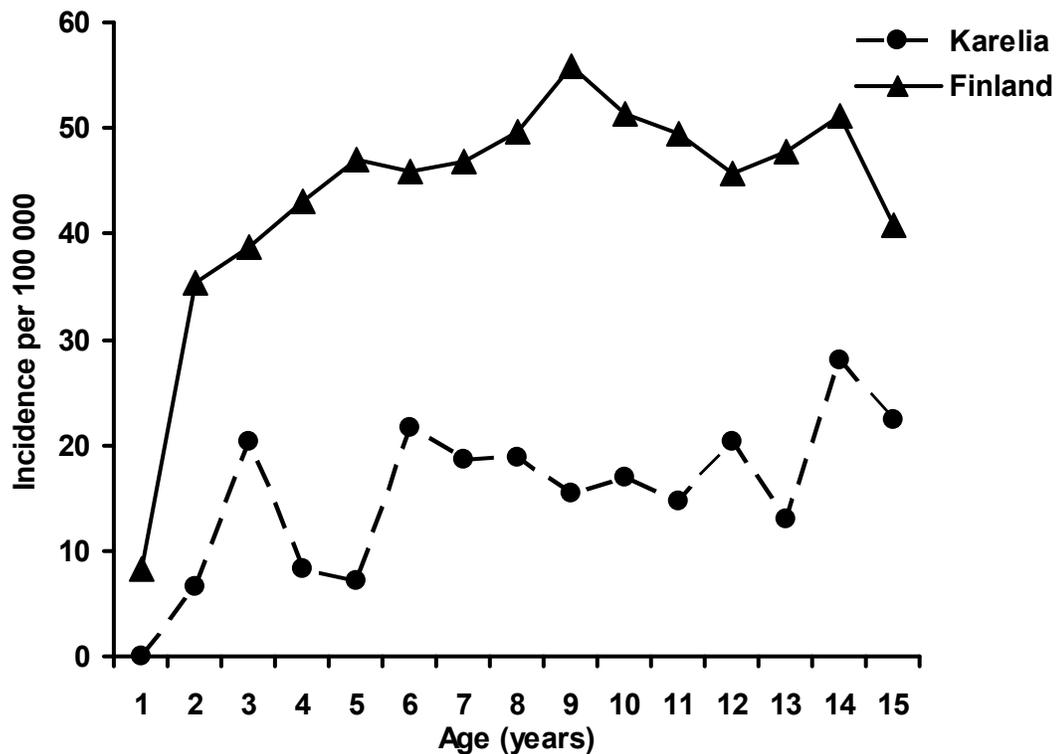


Figure 2. Mean annual incidence of T1D by age in 0-14 year-old children in Russian Karelia (circles) and Finland (triangles) during the period 1990-99.

The incidence of T1D among girls in Russian Karelia was 9.4/100,000 (95% CI 2.7-16.1), while it was 5.9/100,000 (95% CI 0.8-11.0) among boys, the difference remaining non-significant. Interestingly, we found that the difference between boys and girls in Russian Karelia was more conspicuous in the young age group, especially in 2-3 year-old and in 6-7 year-old children (Figure 3). In Finland the same gender difference was also observed in 4 year-old and 6 year-old children (Figure 4). We found the reverse gender difference between Finland and Russian Karelia in the incidence of T1D among children in the pubertal period with clear predominance of boys in Finland at the age of 13 and 14 years old (Figure 3 and 4). Girls from Russian Karelia showed a higher incidence of T1D over almost the total study period, except for the beginning of the study (1990-1991 and 1993; Figure 5). By contrast, data from Finland showed that the incidence of T1D was higher in boys during almost the whole study period (Figure 6). There was a clear seasonal variation in the disease incidence characterized by a nadir in summer (May-July) and a peak in the fall (see Figure 2 in Substudy I).

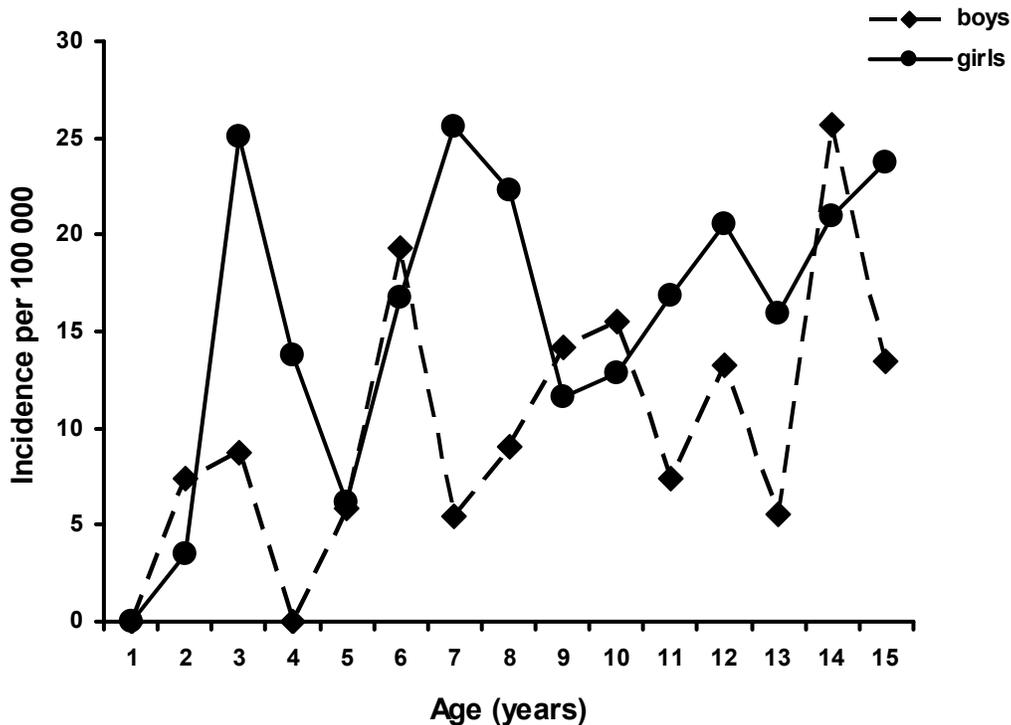


Figure 3. Age-adjusted mean annual incidence of T1D (per 100,000) by age at diagnosis in boys (diamonds) and girls (circles) in 0-14 year-old children in Russian Karelia during the period 1990-1999.

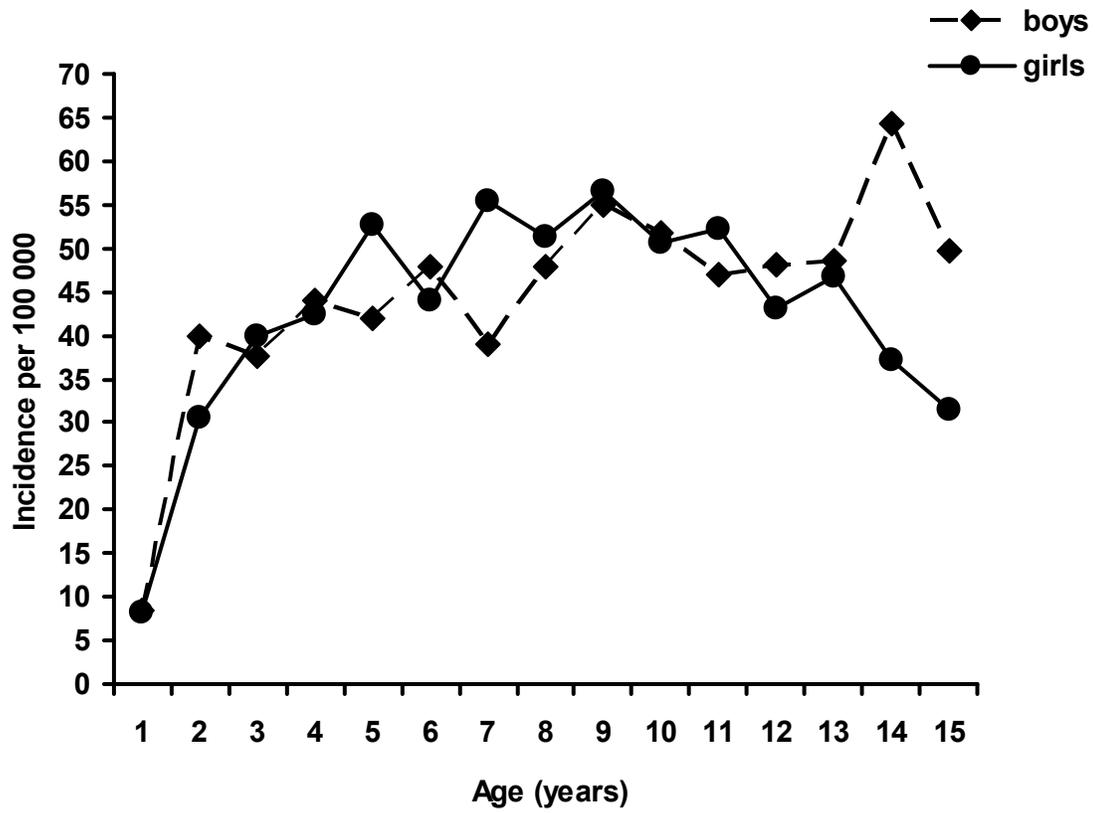


Figure 4. Mean annual incidence of T1D (per 100,000) by age at diagnosis in boys (diamonds) and girls (circles) in 0-14 year-old children in Finland during the period 1990-1999.

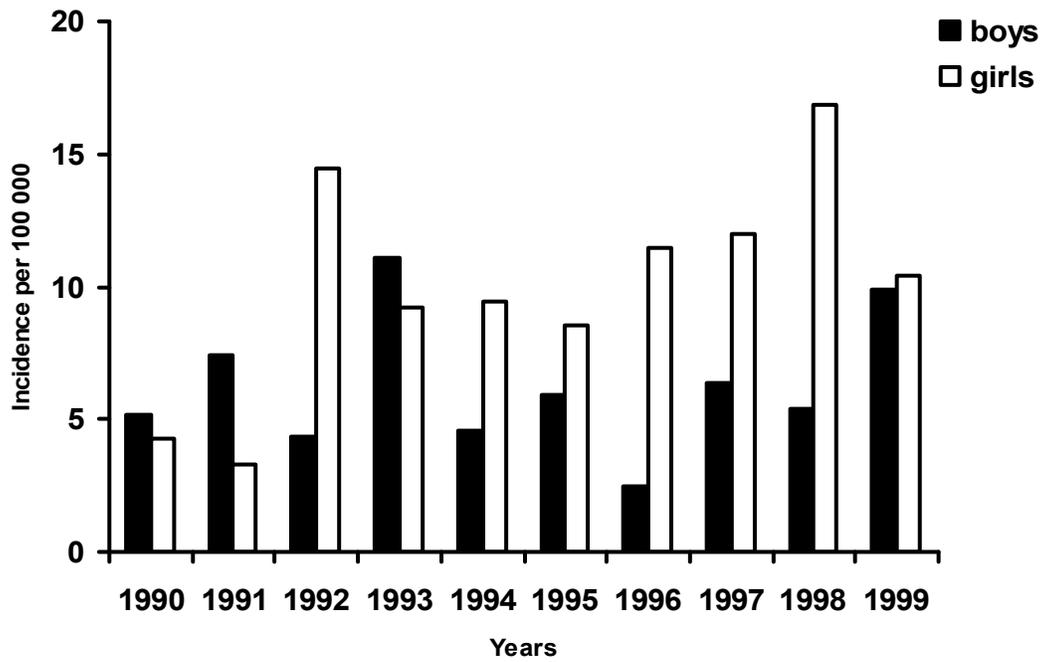


Figure 5. Mean annual incidence of T1D in boys (black bars) and girls (white bars) in 0-14 year-old children in Russian Karelia during the period 1990-1999.

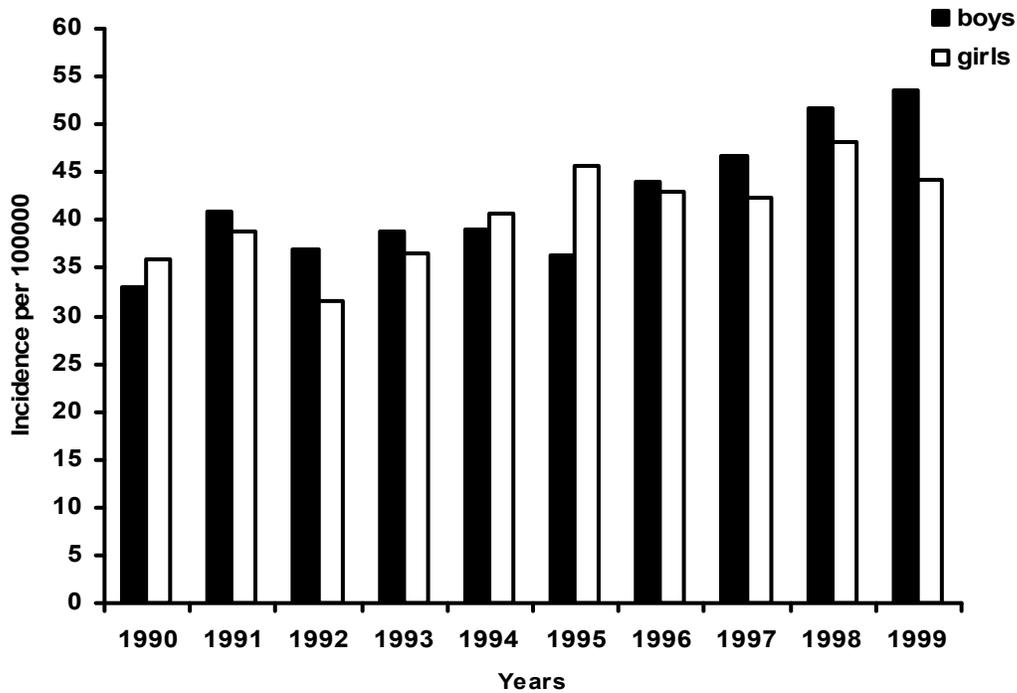


Figure 6. Mean age-adjusted annual incidence rates (per 100,000) of T1D in Finnish children in boys (black bars) and girls (white bars) aged 0-14 years 1990-1999.

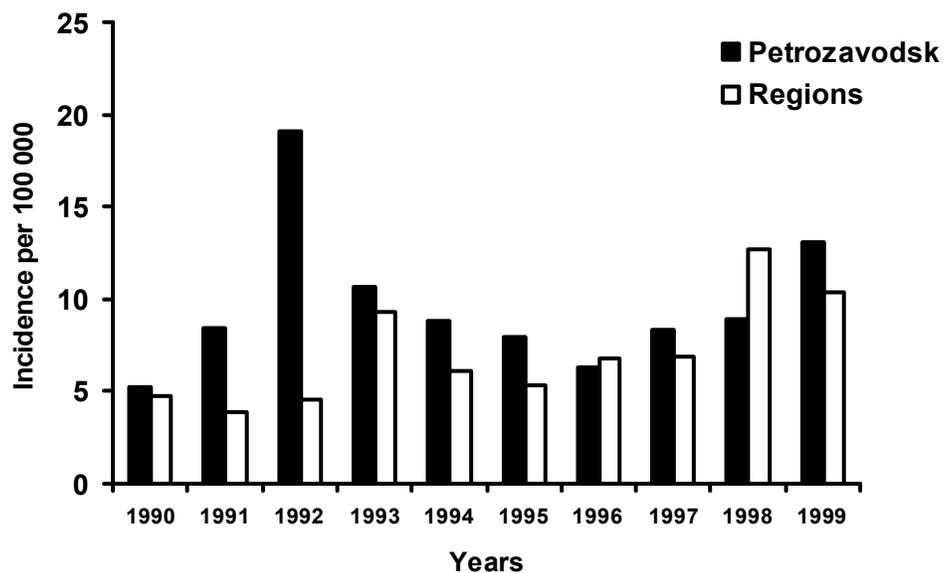


Figure 7. Mean annual incidence of T1D according to urban/rural population in Russian Karelia 1990-1999. Petrozavodsk represents urban population (black bars) living in the city of Petrozavodsk, while people living in the regions around the city of Petrozavodsk represent the rural population (white bars).

We also observed a difference in the incidence of the disease, when analyzing the incidence separately in urban and rural populations in Russian Karelia with a somewhat higher rate among children from urban area (Figure 7). Ethnic background had no significant effect on the incidence of the disease in children in Russian Karelia. Thus the mean age-adjusted annual incidence in the Finns/Karelian ethnic group was 11.1 (95% CI 0-24.8) compared to 6.7 (95% CI 2.4-11.0) among Russians (see Table 1 in Substudy I). The frequency of HLA-DQ genotypes divided into four risk groups did not differ significantly between children in Russian Karelia and in Finland. HLA frequencies did not differ either when compared between different ethnic groups among children in Russian Karelia (see Table 2 in Substudy I).

9.2. Immunological and genetic risk markers of type 1 diabetes in Russian Karelia and Finland (Substudy II)

The frequency of autoantibody-positive children did not differ significantly between Russian Karelia and Finland (4.5%, 95% CI 3.7-5.5 vs. 3.9%, 95% CI 3.3-4.5 had at least one autoantibody). In Russian Karelia, 4.1% of the children (N=81) were positive for a single autoantibody and 0.5% (N=9) for multiple autoantibodies. In Finland, 3.3% of the children (N=120) tested positive for a single autoantibody and 0.6% (N=21) for multiple autoantibodies (Figure 1 in Substudy II). The frequency of individual antibodies including ICA, IAA, and GADA did not differ significantly between the Karelian (3.5%, 0.6% and 0.9% respectively) and Finnish children (2.8%, 0.9% and 0.5% respectively). However, the frequency of IA-2A was four times higher in Finland (0.57% vs. 0.15%; P= 0.03).

The comparison of the frequency of single autoantibodies in three ethnic groups in Russian Karelia (Finnish-Karelian, Russian, or other ancestry) indicated that there were no significant differences (Table 1 in Substudy II) whereas multiple autoantibodies were slightly more frequent among children of Finnish-Karelian ancestry (0.8%, range 0.3-1.6 in children of Finnish-Karelian ethnicity vs. 0.1%, range 0.03-0.7 among those of Russian ethnicity; P= 0.07).

ICA titers were higher among Finnish ICA-positive schoolchildren than in those from Russian Karelia (median 6 JDFU, range 4-514 in Finland vs. 5 JDFU, range 4-1,027 in Russian Karelia; P=0.002). GADA-positive schoolchildren in Finland also had higher GADA titers (median 41.7 RU, range 6.64-113.6 RU) when compared with corresponding figures in Russian Karelia (median 10.3 RU, range 5.4-215.7 RU; P= 0.038). IAA and IA-2A levels did not differ significantly between the countries. However, IA-2A levels in the Karelian schoolchildren (N=9) with multiple autoantibodies were significantly lower (P= 0.006) than those in Finnish children (N=21) (Figure 2 in Substudy II)

HLA-conferred susceptibility to T1D was compared between the two populations based on categorization into four risk groups. The frequency of the high-risk genotype (DQA1*05-DQB1*02/*0302) did not differ significantly between Finnish and Russian Karelian children [2.2% vs. 1.7%, difference 0.5% (0.3-1.3); P=0.271]. The frequency of the moderate-risk genotypes (DQB1*0302/x; x ≠ DQA1*05-DQB1*02, DQB1*0301, DQB1*0602 or DQB1*0603) and low-risk genotypes (DQA1*05-DQB1*02/y, DQA1*03-DQB1*02/y, DQB1*0301/*0302, DQB1*0302/*0603; y ≠ DQA1*0201-DQB1*02, DQB1*0302, DQB1*0301, DQB1*0602 or DQB1*0603) was higher among Finnish children than in Russian Karelian children: 11.5 vs. 9.4%, difference 2.1% (0.5-3.8); P= 0.016 and 15.1 vs. 13.2%, difference 2.0% (0.1-3.9); P= 0.048 respectively. The Karelian children more often carried genotypes conferring decreased risk [75.7 vs. 71.1%, difference 4.6% (2.2-7.0); P= 0.003].

The high-risk children tested positive for autoantibodies more often in both countries except for IAA (Figure 3 in Substudy II). High risk and moderate-risk children in Finland also tested positive more frequently for GADA than those carrying low or decreased risk

genotypes. Finnish children with the high-risk genotype tested positive for multiple autoantibodies more frequently than children with low or decreased risk genotypes. Karelian children demonstrated the same trend in terms of GADA positivity and positivity for multiple autoantibodies but the differences between the genotypes remained non-significant due to the relatively small number of autoantibody-positive children.

9.3. Prevalence and genetic risk markers of celiac disease in Russian Karelia and Finland (Substudy III)

Serological screening

Twelve (0.6%, 0.3-1.1%) of the 1,988 children in Russian Karelia tested positive for tTGA compared to 52 (1.4%, CI 1.1-1.9%) of the 3,654 Finnish schoolchildren ($P=0.005$). Ethnic background had no significant effect on the frequency of tTGA in children from Russian Karelia: 0.4% (CI 0.05-1.5%; antibody range 5-9.7 U/ml) of Finnish and/or Karelian children tested positive for tTGA compared to 0.7% (CI 0.3-1.1%; antibody range 5.6-56 U/ml) of children in other ethnic groups ($P=0.735$). The median tTGA titer was significantly lower among tTGA-positive children in Russian Karelia compared to corresponding figures in Finland (10.0 U/ml; range 5 to 56 U/ml vs. 70.3 U/ml; range 5.7 to 1048 U/ml; $P<0.0001$).

In Russian Karelia nine (75%) of the 12 tTGA-positive children also tested positive for EMA, and the three remaining EMA-negative children had very low tTGA levels (Table 1 in Substudy III). Fifty (96%) of the 52 tTGA-positive Finnish children were positive for EMA. The two tTGA-positive but EMA-negative children had very low tTGA levels (5.7 and 7.0 U/ml).

Gliadin antibodies (IgA and IgG) were compared in two subgroups of children from the two countries: in 265 children of Finnish and/or-Karelian ancestry and in 265 Finnish children. The frequency of IgA-class gliadin antibodies did not differ between Russian Karelia and Finland (12.1%, CI 8.4-16.6% vs. 16.2%, CI 12.0-21.2%; $P=0.212$). However, there was a 2.7-fold difference in the frequency of IgG-class gliadin antibodies between Russian Karelia and Finland (10.2%, CI 6.8-14.5% vs. 28.3%, CI 23.0-34.1% respectively; $P<0.0001$). The levels of IgA-and IgG-class gliadin antibodies did not differ significantly between two cohorts.

Biopsy- proven celiac disease

Eight out of 12 Karelian children (10 girls and two boys) agreed to undergo small bowel biopsy. The biopsy samples indicated clear villous atrophy (three total and one partial atrophy) and crypt hyperplasia in 50% ($N=4$) of those eight subjects who underwent biopsy (Table 1 in Substudy III). Thus, the estimated prevalence of biopsy-proven CD is 1:496 in Russian Karelia. CD had been diagnosed previously in one boy at the age of 2 years, but this boy had not followed a gluten-free diet (case 3, Table 1). Mucosal tissue transglutaminase IgA deposits and marked intraepithelial infiltrations of CD3⁺-expressing lymphocytes (range 46 – 89 per millimetre), and of α/β ⁺ and γ/δ ⁺ TCR-bearing lymphocytes (range 21-38.8 per

millimeter of epithelial tissue) were detected in three of the biopsy-proven celiac subjects. The biopsy-proven celiac patients did have clinical symptoms. Three children suffered from recurrent abdominal pain and intermitted diarrhea or constipation, two had nausea and vomiting, one had tiredness. One child had anemia, one had vitiligo and one suffered from fainting fits. There were three children of mixed ethnic background and one child of Finnish/Karelian ancestry among the four biopsy-proven patients. Altogether, when the prevalence of biopsy-proven CD in Karelia was compared to that in Finland using the same screening and diagnostic criteria there was a close to five-fold gradient in the prevalence of the disease between Finland (1:107) and Russian Karelia (Figure 1 in Substudy III).

Children with normal small bowel mucosal morphology in spite of positive tTGA result

The diagnosis of CD was not confirmed in half of the Karelian children (n=4) who underwent biopsy since their biopsies showed no signs of villous atrophy and crypt hyperplasia (cases 5,6,7,8; Table 1 in Substudy III). Three of these subjects (cases 6,7,8; Table 1 in Substudy III) had actually turned tTGA negative after the initial screening. Three of four children (cases 5,6,7; Table 1 in Substudy III) had increased density of γ/δ^+ intraepithelial lymphocytes. One subject (case 5; Table 1) had high density of CD3⁺ cells and tissue transglutaminase-specific IgA-deposits in the mucosa. Thus, these children had signs of ongoing mucosal inflammation.

HLA risk alleles for celiac disease

Finnish children carried the CD-associated HLA-DR3-DQ2 (DQA1*05-DQB1*02) risk genotypes more frequently than the Karelian schoolchildren (15.2%, CI 14.1-16.5% vs. 10.6%, CI 9.3-12.0%; P<0.001) (Figure 2 in Substudy III). In Finland, 7.4% (CI 5.4-9.9%) of the children who carried this haplotype tested positive for tTGA compared to 2.9% (CI 1.1-6.1%) in the Russian Karelian cohort (P=0.03) (Fig. 8). The other risk associated HLA-DR5-DQ7/DR7-DQ2 genotype was three-times more frequent among the Karelian children (1.9%, CI 1.4-2.6% vs. 0.6%, CI 0.4-0.9%; P<0.001). This genotype was associated with tTGA positivity in three schoolchildren in Russian Karelia. In contrast to tTGA, gliadin antibodies did not show any association with the HLA-DR3-DQ2 haplotype in either population. All patients with biopsy-proven CD in Russian Karelia carried the major susceptibility haplotype, i.e. DR3-DQ2. One of the four Karelian children with normal small bowel mucosal villous morphology carried the HLA-DQB1*02 allele being present together with DQA1*0201 in the DR7-DQ2 haplotype. Data on the HLA genotyping in four Russian children who did not undergo biopsy are presented in Table 1 in Substudy III.

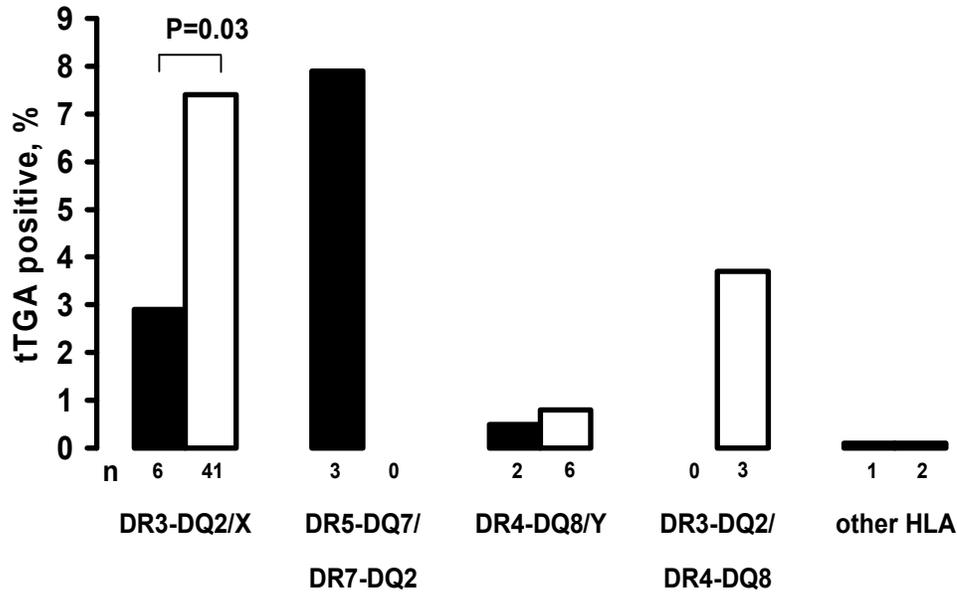


Figure 8. Proportion of tTGA positive subjects (%) in Russian Karelia (black bars) and in Finland (white bars) among subjects with different HLA risk genotypes. x other than DQ7 or DQ8, y other than DQ2. n = absolute number of tTGA positive children carrying different HLA-genotypes.

9.4. Prevalence of thyroid autoimmunity in Russian Karelia and in Finland (Substudy IV)

Clear differences were observed in the frequency of thyroid autoantibodies (TPOAb and TGAb) between the countries when analyzed in the series of 532 Karelian children and the same number of Finnish children. Fourteen Finnish children (2.6%; 95% CI 1.5-4.4%) and two Karelian children (0.4%; 95% CI 0.05-1.4%; $P=0.006$, Figure 1 in Substudy IV) tested positive for TPOAb. TGAb were detected in 18 Finnish children and three Karelian children (3.4%, 95% CI 2.0-5.3% vs. 0.6%, 95% CI 0.1-1.6%; $P=0.002$, Figure 1 in Substudy IV).

Autoantibody positive Finnish and Karelian children had no significant differences in the autoantibody levels even though antibody levels tended to be higher in Finnish children: median TPOAb levels were 225.5 IU/ml and 148 IU/ml ($P=0.63$) accordingly, and TGAb levels 545.5 IU/ml in the Finnish children vs. 359 IU/ml in Karelian children ($P=0.55$) (Table 1 in Substudy IV). Seven (29.2%) out of 24 (92.3%) autoantibody- positive children analyzed for serum TSH had increased levels as a marker of subclinical hypothyroidism. Serum FT4 concentrations were normal in all children. None of the autoantibody-positive Karelian children had elevated TSH levels.

Gender had a clear effect on thyroid autoimmunity in children from these two populations. The predominance of girls (88.5%) was seen in children who tested positive either for TPOAb or TGAb ($P<0.001$). In Finland thirteen out of all girls (4.3%, CI 2.5-7.6%) and one out of all boys (0.4%, CI 0.01-2.4%; $P=0.01$) tested positive for TPOAb. Sixteen Finnish

girls and only two boys were TGAb positive (5.3%, CI 3.0-8.4% vs. 0.9%, CI 0.1-3.1%; $P=0.01$). Two of the Karelian girls (0.6%, CI 0.08-2.4%) tested positive for TPOAb and three for TGAb (1.0%, CI 0.2-2.9%). Two Finnish girls had been diagnosed with hypothyroidism (cases 11 and 13, Table 1 in Substudy IV). One girl (case 13) developed hypothyroidism at the age of 5.2 years, while the other girl had been diagnosed at the age of 12.2 years.

The frequency of previously described susceptible HLA genotypes (DR3-DQ2/x, DR4-DQ8/y and other genotypes) was similar in Finland and Russian Karelia (see Table 2 in Substudy IV). In this study thyroid antibodies showed no clear association with HLA alleles. Thyroid autoantibodies were more frequent in Finland than in Karelia in all HLA groups.

9.5. Vitamin D status in Russian Karelia and Finland (Substudy V).

Vitamin D status was analyzed in cohorts representing the background population of schoolchildren and pregnant women in Russian Karelia and Finland. Serum samples of schoolchildren (86%) were collected in March- April during the years 1994-2000 and 81 % of serum samples of pregnant women were collected during the first trimester of pregnancy in October-December in 2000 in both countries. Circulating concentrations of 25-hydroxy (25-OH) vitamin D did not differ markedly between Finland and Russian Karelia (39.3 nmol/l in Finland vs. 35.0 nmol/l in Karelia, $P=NS$; Wilcoxon test) among the schoolchildren. There were no sex differences in circulating 25-hydroxy (25-OH) vitamin D concentrations among schoolchildren. Nor was any difference observed in the serum concentration of 25-OH vitamin D between Finland (median 28.9 nmol/l) and Russian Karelia (median 28.4 nmol/l; $P=NS$, conditional logistic regression test) among pregnant women.

10. Discussion

10.1. Genetic differences between children living in Russian Karelia and in Finland

The occurrence of autoimmune diseases is associated with a confluence of genetic predisposition and environmental influence. The large international variation between countries as well as the rapid change in the incidence of autoimmune diseases reflect dynamic interactions between these two factors. Mechanisms that could result in such a scenario include environmental influences, somatic mutations, and random receptor mutations (1).

The importance of heredity in autoimmune disease is evident from human studies. Familial aggregation of autoimmune diseases has long been recognized. In addition, comparisons between genetically identical and non-identical twins show higher frequency of autoimmune diseases in the former group. In animal models the genetic predisposition is also significant: A series of inbred animal strains have been produced which spontaneously develop autoimmune disease at high frequency. Many of these underlying susceptibility genes are already identified and their functions defined. The susceptibility alleles in the HLA-DR and/or-DQ loci in the MHC class II region (see Table 1) are the dominant contributors to autoimmune diseases. The association between autoimmune diseases and class I genes has not been firmly confirmed with the exception of HLA-B27, which is associated with predisposition to a group of diseases named ‘spondyloarthropathies’. In addition, numerous other non-HLA genes have more recently been implicated in affecting the susceptibility to autoimmune disease, including CTLA-4, PTPN22, and apoptosis genes, such as Fas and FasL.

Table 1. Association of various autoimmune diseases with HLA class II alleles and haplotypes

Disease	Alleles	Haplotypes
Type 1 diabetes	DRB1*03, DRB1*0401, *0402, *0404, *0405, DQB1*0302	DRB1*0401/2/4/5-DQB1*0302, DRB1*0301-DQB1*02
Celiac disease	DRB1*0301, DRB1*0701, DQB1*0302	DRB1*0301-DQB1*02, DRB1*0701-DQB1*02, DRB1*04-DQB1*0302
Autoimmune thyroiditis	DRB1*0301	DRB1*0301-DQB1*02
Graves’ disease	DRB1*0301, DQB1*02	DRB1*0301-DQB1*02

* Modified from Noel R. Rose and Ian R. Mackay (1).

Despite the established role of genetic susceptibility in autoimmune diseases, its total effect seems to be relatively minor, since not more than about one third of the risk for human autoimmune disease is attributable to genetic factors (1). Both genetic susceptibility factors (such as predisposing HLA genotypes) and environmental risk factors may differ between populations and contribute to the international variations in the prevalence and incidence of autoimmune diseases, e.g. T1D (1; 353). Part of this variation may be explained by diabetes-associated HLA-DQ genotypes (179; 354). By contrast, the rapid increase in the incidence of immune-mediated diseases in most Western countries cannot be explained by an enrichment of specific genetic factors in the population indicating that environmental factors must play a decisive role in the observed increase (89).

The important strength of the present study is that it provides a unique opportunity to evaluate the reasons underlying the marked differences in autoimmune diseases between two adjacent populations. The geographical setup is particularly interesting, since the populations in Finland and in the neighboring Russian Karelia live close to each other. In addition, due to population mixing during earlier centuries these populations share partly the same kind of ancestry (Karelians and Finns comprise approximately 12 %, while ethnic Russians comprise the majority group (about 74%) in Russian Karelia).

The present study shows that the frequency of HLA genes conferring susceptibility to or protection against autoimmune diseases is quite the same in the background populations in Finland and Russian Karelia even though all autoimmune diseases studied were significantly more frequent in Finland (Table 2). This data together with the fact that these two populations share partly the same ancestry suggests that non-genetic (environmental and lifestyle associated) factors must play a major role in the development of autoimmunity. Populations in Russian Karelia and Finland are living in completely different socioeconomic circumstances. This is clearly reflected by the difference in gross national product which was USD 1,660 in Russian Karelia in 2001 compared to USD 25,130 in Finland (in 2004 the corresponding figures were USD 3,410 and USD 32,790). This has a marked influence on the socioeconomic circumstances of these populations, which is reflected in a substantially higher frequency of microbial infections (such as *Helicobacter pylori*, hepatitis A virus, *Toxoplasma gondii* and enterovirus infection) in Russian Karelia than in Finland (355-357).

Table 2. Differences in the distribution of disease-susceptibility HLA-genotypes between Finland and Russian Karelia. The table represents a summary of HLA data analyzed in the Substudies I-IV.

Autoimmune disease	Risk classification of different HLA combinations	Number (%) of carries in Finland	Number (%) of carries in Russian Karelia	Difference in the HLA distribution between two countries (%), 95% confidence interval and statistical significance
Type 1 Diabetes	High risk: DQA1*05-DQB*02/*0302	2.2%	1.7%	0.5%; N.S.
	Moderate risk: DQB1*0302/x; x ≠ DQA1*05-DQB1*02, DQB1*0301, DQB1*0602 or DQB1*0603	11.5%	9.4%	2.1%; P=0.016
	Low risk: DQA1*05-DQB1*02/y, DQA1*03-DQB1*02/y, DQB1*0301/*0302, DQB1*0302/*0603; y ≠ DQA1*0201-DQB1*02, DQB1*0302	15.1%	13.2%	2.0%; P=0.048
	Decreased risk: DQB1*0301, DQB1*0602 or DQB1*0603	71.1%	75.7%	4.6%; P=0.003
Celiac disease	High risk: DR3-DQ2 (DQ*05-DQB1*02)	15.2%	10.6%	4.6%; P<0.001
	Risk: DR5-DQ7/DR7-DQ2	0.6%	1.9%	1.3%; P<0.001
	DR4-DQ8/y	20.9%	18.5%	2.4%; P=0.03
	DR3-DQ2/DR4-DQ8	2.2%	1.7%	0.5%; N.S.
	Other HLA	61.1%	67.2%	6.1%; P<0.001
Thyroid autoimmunity	Risk: DR3-DQ2/X (DQA1*05-DQB1*02)	16.4%	15.1%	1.3%; N.S.
	Risk: DR4-DQ8/Y (DQA1*03-DQB1*0302)	24.8%	21.1%	3.7%; N.S.
	Other HLA	61.3%	64.7%	3.4%; N.S.

10.2. Risk of autoimmune diseases in children living in Russian Karelia and in Finland

10.2.1. Type 1 diabetes

Finland has the highest incidence rate of T1D in the world, the figure was 40.8 per 100,000 over the period 1991-1998 (358) and has continued to increase to 63 per 100,000 in 2006 (359). By contrast, Russia and the Baltic countries have a low incidence of the disease (360). Our results (Substudy I) indicate that the incidence of T1D is markedly lower in Russian Karelia than in Finland, and close to that reported previously from other parts of Russia (91; 360). The sixfold gradient in the incidence of T1D between these countries suggests that either the genetic risk or diabetogenic environmental factors, or both, differ between the two populations. It is also clear that the geographical variation in diabetes incidence within Finland is much lower than what was observed between the two countries in the present study (361). Altogether, our findings suggest that environmental and lifestyle-related factors contribute to the sharp gradient in the incidence of T1D between Finland and Russian Karelia.

About 30-60% of the genetic risk for T1D is considered to be due to the effect of HLA genes. The rest of the genetic effect is mediated by other genes including the insulin gene, the CTLA4 gene, the PTPN22 gene, the MDA-5 gene and some other genes, each of them contributing to the genetic risk by 5-10 %.

There are not many studies available on the frequency of HLA alleles in the Russian population and none has been published about HLA genes in Russian Karelia. A previous analysis (179) of the distribution of T1D – related HLA-DQ allele combinations in four populations in the Eastern Baltic region demonstrated a lower frequency of risk-associated genotypes in the Russian population. In Substudy I the frequency of different diabetes-associated HLA-DQ combinations did not differ significantly between the background population in Russian Karelia and Finland based on the relative small population cohorts analyzed. In that study we also evaluated the contribution of genetic factors by comparing the incidence of T1D in subjects of either Russian or Karelian/Finnish ancestry. The incidence did not differ significantly between these ethnic groups. Accordingly, we concluded that differences in HLA risk genes might contribute to the six-fold gradient in the incidence rate of T1D between Russian Karelia and Finland, but their effect must be minor. In Substudy II, where a considerably larger cohort of children were analyzed for HLA genotypes, there was a small but statistically significant difference in HLA distribution between the two populations: HLA allele combinations which are associated with increased risk of T1D were more frequent in Finland, while protective HLA combinations were more common in Karelia. Accordingly, it is possible that these differences in HLA genes may contribute to the higher incidence of T1D in Finland. However, it is evident that they have only a modest effect because the difference in the incidence of T1D is of much greater magnitude.

In the second study (Substudy II) we evaluated data on genotype-specific autoantibody frequencies based on an extensive unselected cohort of schoolchildren representing the general population. We analyzed the frequency of subclinical beta-cell autoimmunity, i.e., the prevalence of diabetes-associated autoantibodies, and HLA-risk genotypes in the background population in Russian Karelia and Finland. Earlier studies on diabetes-associated autoimmunity (160-162) have already demonstrated that the expression of two

or more autoantibodies is associated with a high risk of progression to clinical diabetes over the next decade in a majority of first-degree relatives of affected patients. The DIPP Study, which is a prospective birth cohort study in subjects carrying HLA-conferred susceptibility to T1D, has shown that 3.3% of the children with the high-risk genotype became autoantibody positive within the first 2 years of life compared to 1.6% in the moderate-risk group (164). Children with one antibody remained diabetes-free at least up to the age of 5 years (164), whereas children with two or more antibodies have a cumulative risk of approximately 50% to develop T1D by the age of 5 years and a risk of about 77 % by the age of 10 years (165). In addition, some studies (362; 363) have indicated that the prevalence of diabetes-associated autoantibodies differs between countries, probably to some extent due to different assay methods used, but this is not the case in the present study.

Similar data on the frequency of subclinical beta-cell autoimmunity have been reported in the study by Marciulionyte et al. (170). A comparison between children from the UK and Lithuania with a two- to threefold gradient in the incidence of T1D has shown no huge differences in the prevalence of beta-cell autoantibodies in the background population of these two countries except for a significantly reduced frequency of IA-2A among Lithuanian children (0.2% vs. 2.4% among British children; $P < 0.001$). These data are consistent with the observation on the reduced frequency of IA-2A among schoolchildren in Russian Karelia in the present study (0.15% vs. 0.6%; $P = 0.01$).

In our study risk markers of T1D were also analyzed in three ethnic groups of children in Russian Karelia. We observed that multiple autoantibodies were slightly more frequent, although not significantly so, among children of Finnish-Karelian ancestry whereas the prevalence of single autoantibodies was similar in all three groups. HLA alleles did not significantly differ between the ethnic groups.

Several studies in young children and in siblings of children with diabetes have indicated that IA-2A appear as the last autoantibody reactivity during subclinical progression of the autoimmune process to overt diabetes, and these autoantibodies have the highest predictive value during the pre-diabetic disease process. Thus, the decrease in the prevalence of IA-2A in Russian schoolchildren suggests that progressive beta-cell autoimmunity is less common in Russian Karelia than in neighboring Finland. One can speculate that such a difference may be due to decreased exposure to driving exogenous factor(s) or the presence of protective factors in the Karelian population. On the other hand, it is also possible that in the Finnish population autoantibody-positive children progress to T1D substantially faster than Karelian children and therefore such children have dropped out from the current series which did not include affected patients. The finding that autoantibody-positive children had higher autoantibody levels in Finland than in Karelia suggests that the autoimmune process may indeed be more aggressive in Finnish children.

The present study (Substudy II) is the first one to analyze genotype-specific autoantibody frequencies in a large cohort of schoolchildren representing the general population. Our results showed that there is a strong association between autoantibody positivity with the high-risk HLA genotype in both Russian Karelia and Finland. IAA was the only exception in this pattern, which may be partly due to the low overall frequency of IAA-positive subjects. Similarly, the low-risk HLA genotype was associated with the lowest frequency of various autoantibodies with the exception of ICA. It is of note that the

difference was statistically significant for GADA and multiple autoantibodies in relation to HLA-conferred susceptibility to T1D among Finnish schoolchildren. The difference remained non-significant among Russian children due to the smaller cohort size. As in a study of a sib cohort from the Finnish Diabetes Prediction and Prevention Study (166), positivity for multiple autoantibodies was associated with the HLA genotypes predisposing to T1D. A more prominent gradient was observed between high- and moderate-risk genotypes in the present study when compared to the sib cohort in the DIPP Study. Thus, this comparison suggests that the gradient increases with age since the sib cohort from the Finnish Diabetes Prediction Prevention Study was almost two times younger than the children in the present study (mean age 6.2 vs. 11.7 years).

In conclusion, the present study (Substudies I and II) showed that the incidence of clinical T1D is six times higher in Finland than in Russian Karelia, but this difference is not reflected in the frequency of subclinical beta-cell autoimmunity, which did not differ conspicuously between the countries except for IA-2A, the prevalence of which was lower in Russian Karelia. This may indicate that progressive beta-cell autoimmunity is rarer among children in Russian Karelia. Altogether, these findings favor the assumption that high-incidence Finland is characterized by a more rapid disease progression, while disease initiation is as frequent in schoolchildren from low-incidence Russian Karelia as in peers from high-incidence Finland.

10.2.2. Celiac disease

The present study (Substudy III) is the first extensive epidemiological analysis to evaluate the prevalence of gluten intolerance among children in Russian Karelia. To the best of our knowledge there are no earlier publications on this issue in Russia. The frequency of biopsy-proven CD was 1:496 in Karelian children compared to 1:107 in Finnish peers using identical criteria. The prevalence of CD in Russian Karelia was found to be close to that reported from Croatia (1:500). However, the prevalence is higher in many European countries, South America, USA, North Africa, Iran, and India, while gluten intolerance is very rare in Japan and China (73; 364-366). Altogether, CD seems to be prevalent among Caucasians affecting on an average approximately 1 in 100 individuals (250; 367-369).

The present study was based on tTGA screening in an unselected background population of schoolchildren followed by confirmation of the diagnosis by small-bowel biopsy in antibody-positive cases. The advantage of this study design is that it excludes the possibility of any selection bias caused by different diagnostic routines in different countries. The analyses were based on identical study design and methodology in Russian Karelia and Finland. It is also noteworthy that the steep gradient persisted when the prevalence was estimated based on extrapolated data. The uptake of biopsy can be estimated to be 83% in the Finnish population: 43 of the 52 tTGA- positive Finnish children were biopsied compared to eight (67%) out of 12 tTGA-positive Karelian children. Extrapolation from the biopsied antibody-positive children in whom the diagnosis was confirmed in 50% in Karelia would give an estimated prevalence of 1 in 331 (6 out of 1,988 Karelian children). Extrapolation from the biopsied antibody-positive children in whom the diagnosis was confirmed in 79% (34 out of 43 biopsied subjects) in Finland would give an estimated prevalence of 1 in 89 (41 out of 3,654 Finnish schoolchildren). This results in a 3.7-fold difference in the prevalence of biopsy-proven

CD between these populations, which is in line with the 4.6-fold difference obtained from actual data.

In our study the major susceptibility haplotype HLA-DR3-DQ2 was associated with biopsy-proven disease and transglutaminase antibody positivity both in Russian Karelia and Finland. Finnish children carried the CD-associated HLA-DR3-DQ2 haplotype somewhat more frequently than the Russian-Karelian children (15.2% vs. 10.6%; $P < 0.001$). In contrast, the other risk-associated genotype HLA-DR5-DQ7/DR7-DQ2 was more common in Russian Karelia than in Finland (1.9% versus 0.6%; $P < 0.001$). This genotype is rare among patients in Scandinavian countries but is common in southern Europe (370). It is obvious that the relatively small difference in the frequency of HLA genotypes predisposing to CD between Finland and Russian Karelia cannot totally explain the conspicuous gradient seen in the prevalence of CD between these two adjacent countries. This is also supported by the fact that tTGAs were significantly more frequent in Finland when analyzed in the high risk HLA group separately in each country. Accordingly, the present study suggests that nongenetic factors must contribute to the observed difference in the prevalence of CD.

10.2.3. Thyroid autoimmunity

Thyroid autoantibodies (TGA_b, TPOA_b, TSHRA_b) are widely used as markers of thyroid autoimmunity being an important diagnostic tool in AITD (371-373). The autoimmune process detected by the presence of these autoantibodies usually progresses slowly (374; 375). The appearance of thyroid autoantibodies in children and adolescents indicates a moderately increased risk (5-6%) for progression to overt disease over the following 4-5 years (376). This is in contrast to diabetes-associated autoantibodies which are more strongly associated with disease progression. In previous population studies TPOA_b were detected in about 10% of middle-aged individuals, out of whom 2-3% had overt thyroid disease and 5-7% had increased TSH levels (377; 378). Our study is the first report on the prevalence of thyroid autoantibodies in a large cohort of children representing the background population. The present study showed that these autoantibodies are less frequent in a younger age group compared to those previously reported among older subjects. In addition, only 29 % of the 24 thyroid autoantibody-positive children had increased TSH levels and two had been diagnosed with hypothyroidism, suggesting that in most cases these autoantibodies were present in the early subclinical period before the clinical manifestation of AITD. Like other organ-specific autoimmune diseases AITD has a multifactorial etiology and multiple genes, environmental and endogenous factors may be involved in the disease pathogenesis (306; 379-381).

The frequency of thyroid autoantibodies was more than five times lower in Russian Karelia than in Finnish children, suggesting that the predisposition to thyroid autoimmunity differs in these adjacent populations. We analyzed genetic factors as one of the possible explanations for such a marked difference in the frequency of thyroid autoimmunity. Previous studies have indicated that there is an association between AITD and the HLA-DR3 and HLA-DR4 genotypes (179; 382-385), and we compared the frequency of these alleles in the two populations. However, there was no significant difference in the distribution of HLA genotypes either between the two countries or between autoantibody-positive and negative subjects. Accordingly, we conclude that the risk HLA alleles have little or no effect on the early stage of thyroid autoimmunity. It is

also notable that the prevalence of autoantibodies was low among children of Finnish/Karelian ancestry in Russian Karelia supporting the role of non-genetic factors.

Female sex has been shown to increase the risk of AITD (386). Thyroid antibodies have been observed also to be predictive for postpartum thyroiditis, which occurs in 35-85% of TPO-positive pregnant women compared to an overall frequency of 4-8% among pregnant women (336; 387). The present study showed that thyroid autoantibodies were almost eight times more frequent in girls than in boys. This implies that gender has a strong influence on thyroid autoimmunity already in the early subclinical phase. It is of note that in the present study the gender difference was already present in young children (mean age 11 years) suggesting that factors other than the hormonal changes during puberty may contribute to the thyroid autoimmune process. However, we cannot exclude the effect of pubertal hormonal changes since most of the antibody-positive children were older than 11 years of age.

10.3. Gene-environmental interactions in the pathogenesis of autoimmune diseases - what can be learned from comparisons between different countries

Although the mechanisms for the development of autoimmune diseases remain poorly understood, mounting evidence suggests that these diseases are the result of environmental exposures in genetically susceptible individuals. This suggests that certain environmental exposures must play a role in the breakdown of tolerance in genetically susceptible individuals. This environmental influence may either target the early initiation phases of the autoimmune process or later phases when it can regulate the progression of the subclinical autoimmune process to clinical disease. It is also important to realize that environmental factors may either promote the process or have a protective effect. It has been difficult to identify the exact nature of such environmental determinants but it is generally believed that they may include both infectious and non-infectious agents.

A variety of epidemiologic and experimental data have linked a number of exposures to autoimmune diseases, such as virus infections and several dietary components (190). These factors may have a major influence on the appearance and quality of the autoimmune response (388) (Table 3). Taken together, the following arguments have been considered as indicators of the role of environmental factors in the development of autoimmune disease (Modified from Noel R. Rose and Ian R. Mackay, *The Autoimmune Diseases*, Fourth Edition, 2006):

- Disease concordance in monozygotic twins is less than 50%
- Geographic clustering in disease incidence or prevalence
- Temporal changes in the incidence and prevalence of disease and migrant studies
- Seasonality in disease onset and in birth dates
- Strong temporal associations with some environmental exposures and disease onset
- Disease improvement after removal of the suspect agent
- Disease recurrence after re-exposure to the suspect agent
- Epidemiologic associations between particular exposures and certain diseases
- Animal studies

Table 3. Examples of exposures proposed as possible risk factors for autoimmune diseases

Exposure	Disease	Interpretations and references
Virus infection	Type 1 diabetes	Homology between Coxsackie B4 virus protein and GAD (98); Temporal relationship between virus infections and diagnosis of T1D (389); Enterovirus RNA correlation with islet-specific autoantibodies in subjects at risk for T1D (201); Animal studies: diabetes develops after LCMV infection in the RIP-LCMV transgenic mice (390)
Microchimerism	Autoimmune thyroid disease	Fetal cells or target tissue specimens detected years after pregnancy (391)
Foods (gluten, cow's milk)	Celiac disease, type 1 diabetes	Immune response to ingested wheat gluten and related proteins of rye and barley leads to villous atrophy and inflammation in the intestine in CD (392) Bovine albumin was shown to cross-react with beta-cell autoantigen P69 as a possible form of antigen mimicry in T1D (393)
Tobacco smoke	Autoimmune thyroid disease	Meta-analyses suggest 2-3 fold increased risks of Graves' and Hashimoto's diseases (394)

Stress	Graves' disease	Stressful life events in the 12 months preceding overt disease were significantly higher compared to controls (395); other diseases poorly studied
Toxic agents	Experimental type 1 diabetes	Streptozotocin induces diabetes in susceptible strains of mice and rats (396; 397)
Vaccines	Multiple syndromes	Arthritis after rubella virus vaccines; thrombocytopenia after measles vaccines; controversy remains over others (398)

The repeated activation of the immune system by environmental factors may play an important role in the loss of tolerance, which culminates in the progression to overt disease. However, it is still unclear to what extent each factor - the environment, genetic susceptibility and regulation of the immune response itself - contributes to the loss of tolerance and the subsequent progressive autoimmune destruction (399).

In Substudy V we compared vitamin D status in young children and pregnant women in Russian Karelia and Finland based on the findings of the recent studies indicating that vitamin D deficiency may increase the risk of autoimmune diseases (227). Vitamin D supplementation is an important factor in the prevention and treatment of rickets in young children, and regular supplementation in infancy has long been recommended in both countries (400-402). There is also data suggesting that a considerable proportion of pregnant women suffer from vitamin D deficiency, and supplementation during pregnancy is recommended. In fact, we did not find any marked difference in the circulating vitamin D concentrations between Finland and Russian Karelia, suggesting that vitamin D status may not contribute to the conspicuous difference in the incidence of autoimmune diseases between these two countries. Accordingly there must be other driving environmental factors in Finland or protective factors which are present in Russian Karelia.

Substudy III demonstrated that CD is considerably less common in Russian Karelia than in Finland. The pathogenesis of CD is multifactorial. Predisposing HLA alleles and non-HLA genes together with environmental factors contribute to the process (50; 403; 404). Possible explanations for the observed difference in the prevalence of CD include dietary

gluten exposure, genetic disease susceptibility, and/or other factors modifying disease risk (duration of breastfeeding, the use of antibiotics in children) (405; 406). The population in Russian Karelia and Finland are equally exposed to grain products. According to Food and Agriculture Organization Statistics the annual consumption of grain and grain products per person is 106 kg in Finland compared to 155 kg in Russia (407). There is no evidence that exposure to gluten differs markedly between the two countries, although there is no detailed data available on the age at introduction of cereals and on the amount of gluten introduced in young children in Russian Karelia. The only available information is on the recommended age for introducing cereals in infants in both populations. The recommended age at first exposure to cereals is 4-5 months in children in the Russian Federation (408; 409). The data from the DIPP Nutrition study in Finland showed that the median age at introduction of oats is 5 months, whereas it is 6 months for wheat, barley, and rye (410). Thus it seems unlikely that different age at first exposure to gluten is the reason for the considerable difference in the disease prevalence.

Previous studies on the role of breastfeeding have shown that the lack of breastfeeding is associated with several chronic childhood diseases such as CD, T1D and others (242; 411; 412). In fact, the duration of breastfeeding in Finland is twice as long as in Russia. Thus, infants in Finland are breastfed for 6-10 months, while infants in the Russian Federation are breastfed for 3-5 months according to the data from the WHO Global Data Bank on Breastfeeding and Complementary Feeding (years of survey 1983-1997, in Finland; 1992- 1996, in the Russian Federation). Accordingly these data imply that long duration of breastfeeding is not associated with low risk of autoimmune diseases at the population level.

The fact that a similar gradient existed in all autoimmune diseases suggests that these diseases may have some common pathogenetic mechanisms. In fact, our recent study has shown that a similar gradient also exists for allergic sensitization (355). The results suggest that factors other than vitamin D status, consumption of grain products or breastfeeding are probably responsible for the disease gradient observed between Russian Karelia and Finland. The conspicuous difference in socioeconomic status does have a profound effect on lifestyle and living conditions in the two countries, such as the frequency of enteral infections in young children and the use of antibiotics. However, there are no comparable data available on the consumption of antibiotics in children in these two countries.

In fact the results fit well with the concepts of the hygiene hypothesis. The inferior hygienic conditions and lower economic status in Russian Karelia is reflected by a conspicuously higher frequency of microbial infections. In our previous studies we have observed that 73% of schoolchildren were seropositive for *Helicobacter pylori* in Russian Karelia compared to 5% in Finland, and a similar difference was observed in hepatitis A virus, *Toxoplasma gondii* and enteroviruses (357; 413). Altogether, these findings could well be explained by the hygiene hypothesis, suggesting that the lack of microbial exposures may play a role in the pathogenesis of a wide range of immune-mediated diseases (87; 114). Frequent microbial exposures in Russian Karelia could protect the children from autoimmune diseases and allergies by stimulating the Tregs and other regulatory elements of the immune system. In a recent study we observed that IgE-mediated allergic sensitization is also considerably less common in Russian Karelia (413). However, Karelian schoolchildren had significantly higher concentrations of total IgE than Finnish children, which may reflect an increased exposure to parasite infections

in Russian Karelia. The prevalence of parasite infections is very high in Russian Federation. For example, approximately 3,086 cases of Enterobiosis per 100,000 children were registered in the Russian Federation in 2000 (414). More than 8 % of children under 6 years of age and 11% of schoolchildren suffered from Enterobiosis. In 2006 more than 340,618 cases of Enterobiosis were registered in the Russian Federation, and the total prevalence of Ascariidosis was 39.6 per 100,000 (415). Parasite infections may protect against autoimmune diseases as suggested for pinworms in T1D (416). Autoimmune animal models also point to mechanisms related to the hygiene hypothesis, because unclean mice are less prone to the development of spontaneous autoimmunity (417; 418).

However, there are also opposing relationships between microbial exposures and subsequent autoimmunity, allergy and asthma. One of these opposing paradigms is that the reciprocal relationship between Th1 and Th2 immune development and autoimmunity and allergy seems too simplistic because commonly classified Th1 and Th2 diseases coexist in the same individuals. Thus, according to several epidemiologic observations asthma seems to be more common in children with autoimmune diseases. The study by Kero et al. (2001) indicated that the cumulative incidence of asthma in Finnish children with T1D was 5% and 24.6% among children with CD compared to 3.4% among children in whom these autoimmune conditions were absent (419).

Secondly, biologically the influence of microbe-driven immune development on autoimmune disease is not well understood. For the hygiene hypothesis to be relevant to autoimmunity, there is a fundamental immune paradox, because bacteria and viruses typically induce Th1-type immune responses, however, many autoimmune diseases appear also to be mediated by auto-specific Th1 cells (420). Accordingly, the mechanisms by which microbes can reduce the development of autoimmune diseases are not well understood. Additionally, the original paradigm of a reciprocal relationship between Th1 and Th2 might only be relevant before disease development: early microbial exposure might prevent subsequent disease development, and microbial exposure exacerbates already established disease.

In conclusion, the fact that a similar gradient exists in several immune-mediated diseases implies that this effect is mediated by general immunoregulatory pathways. Microbial infections are thought to be important in the maturation of the immune system early in life and a certain degree of exposure to microbes may be needed for the development of a proper balance and regulation of the proinflammatory and allergic immune responses. The increased risk for autoimmunity in Finland might indicate that there are some driving environmental factors or a lack of protective elements which are present in Russian Karelia. These factors are probably related to differences in socioeconomic environment and microbial exposure between these two countries.

11. Limitations of the present study

The present study led us to generate the hypothesis that the environment in Russian Karelia includes factors which may protect against a series of immune-mediated diseases, and that this protection might be linked to a strong microbial exposure. On the other hand, it remains to be ascertained whether the high prevalence of these diseases in Finland is a consequence of an increased exposure to driving exogenous factors. In the

present study it was not possible to identify such environmental factors and to analyze their effects on the age at disease manifestation or the maturation of the immune system in young children. The study cohorts mostly comprised schoolchildren, and accordingly this limited the ability to detect early events in the development of immune-mediated diseases, which can occur soon after birth and which may have an important effect on immune regulation. In addition, only serum and genetic samples were taken in this study and therefore gene expression analyses were not possible.

12. Future prospects

The present study offers an impetus to focus future research on the most interesting aspects of the gene-environment interactions which may play a role in the pathogenesis of immune-mediated diseases. Our results support the hygiene hypothesis and further studies are needed to determine microbe-host interactions which can modulate immunoregulatory pathways and play a role in a wide range of immune-mediated diseases. The study setting provides an opportunity to define the impact of contrasting standards of hygiene and living on the appearance of signs of autoimmunity and allergy in young children. Finns and Karelians are genetically related, providing an opportunity to assess the direct impact of environmental factors. Future research should focus on the comparison of the ontogeny of the immune system in infants and young children living in these contrasting environments including the impact of gut microbial colonisation and effect of various infections and dietary factors on immune responses.

New insights into the pathogenesis and natural course of immune-mediated diseases might result in improved strategies to detect subjects at risk in an early preclinical stage and to develop novel immunotherapies to prevent disease manifestation. Most important is the identification of environmental factors affecting the incidence and prevalence of immune-mediated diseases in different populations. For example, the identification of protective factors should be much more likely in Russian Karelia than in Finland, as their effect on the disease process should be more prominent in such protective environment. The prevention of autoimmune diseases will be more effective if intervention therapies are given early, while most target tissues and cells are still intact.

13. Conclusions

The present investigation is the first international comparison of the epidemiology, immunology and genetic risk factors of organ-specific autoimmune diseases in Finland and in the neighbouring Karelian Republic of Russia. The study was carried out in collaboration between the University of Petrozavodsk (Department of Paediatrics, Medical School) in Karelian Republic of Russian Federation and the University of Tampere (Department of Virology, Medical School) in Finland as part of the international EPIVIR Project. The main aim was to evaluate the role of genetic and environmental factors in the pathogenesis of T1D, CD and thyroid autoimmunity. The study focuses on humoral signs of autoimmunity as well as the effect of ethnic background and HLA-defined genetic disease susceptibility. An additional aim was to

assess the contribution of environmental and genetic risk factors to the exceptionally high and continuously increasing incidence of T1D in Finland.

The special advantage of the study is the unique epidemiological setting based on two populations which share the same geographical and climatic factors and have partly the same ancestry, but due to the sharp welfare gradient across the border, are exposed to completely different socioeconomic environments, reflected e.g. by the marked differences in the frequency of gastrointestinal infections, early feeding in childhood, childhood vaccination practices and the consumption of antibiotics by children. In addition, the study was carried out in unselected background populations (schoolchildren), which made it possible to find true differences in genetic and environmental risk factors for these diseases.

The results indicate that the incidence of T1D is six times higher in Finland compared to Russian Karelia. The incidence was also low among such children in Karelia who had a Finnish/Karelian ancestry, suggesting that differences in ethnic background do not explain this phenomenon. In spite of the clear difference in clinical T1D, there were no such differences in the frequency of diabetes-associated autoantibodies (ICA, IAA, GADA and IA-2A) or HLA-DQ risk alleles for T1D between schoolchildren in Finland and Russian Karelia. The only difference observed was in the frequency of IA-2A, which were significantly more frequent in Finland, suggesting that the higher risk of clinical diabetes in Finland may be related to a more aggressive progression of the autoimmune process rather than more frequent initiation of the disease process. Altogether, the results suggest that environmental factors contribute to the variable incidence of T1D in different populations. Thus, the environment in Finland may have some features which increase the disease susceptibility of the Finnish population, or alternatively, the environment in Russian Karelia may include some protective elements which reduce the disease risk.

The analyses of other immune-mediated diseases such as CD and autoimmune thyroiditis indicated a similar gradient between the two countries. This study is the first extensive epidemiological survey of gluten intolerance among children in Russian Karelia. The result indicated a fivefold gradient in the prevalence of CD between Russian Karelia and Finland. The frequency of biopsy-proven CD was 1:496 in Russian Karelia compared to 1:107 in Finland when using identical criteria. The fact that this gradient was observed in all ethnic groups and that the HLA-DQ risk alleles for CD showed only minor differences between the two populations suggests that genetic factors can hardly explain this conspicuous gradient in CD. Accordingly, some non-genetic environmental factors probably contribute to the low incidence of CD in Russian Karelia. The consumption of grain products did not significantly differ suggesting a role for other lifestyle-related factors. The study on thyroid autoimmunity demonstrated a similar gradient between the two populations. Again, this difference was not related to HLA-associated genetic susceptibility.

Vitamin D status was analyzed in infants and pregnant women from both countries as one possible environmental determinant of the risk of autoimmune diseases. Vitamin D deficiency was not more common in Finland, which thus argues against its contribution to the higher incidence of autoimmune diseases in Finland. Vitamin D levels were actually quite similar in Russian Karelia and in Finland.

The parallel gradient in several autoimmune diseases suggests that general immunoregulatory pathways are involved in this phenomenon. The results of this study support the idea that the likelihood for developing autoimmunity depends on complex interactions of the individual exposures to environmental factors and the presence or absence of genetic risk factors. Combined with our recent studies showing a several-fold higher frequency of microbial infections in Russian Karelia, the results of the present study are also in line with the concept of the hygiene hypothesis. It is possible that certain environmental changes and subsequently complex gene-environment interactions can be responsible for the conspicuous differences seen in the frequency of autoimmune diseases between the two populations. Environmental factors to which children are exposed in early life (such as microbial infections) can interact with the specific genotype of a child and influence the developing immune system in a way that either predisposes to or protects from the development of autoimmune disease. However, in the present study it was not possible to identify potential protective and /or risk factors for these autoimmune diseases and further studies are needed to clarify the underlying mechanisms of this interaction.

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