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To my family

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ABSTRACT

Type 1 diabetes is an autoimmune disease, and it is one of the most common chronic diseases of childhood. An asymptomatic pre-diabetes period preceding the clinical onset is characterized by autoantibodies against pancreatic islet antigens in the peripheral blood. The major genetic contribution comes from the highly polymorphic Human Leukocyte Antigen (HLA) genes, of which the HLA class II (DR-DQ) genes confer the strongest effect, while others, such as the HLA class I, comprise a minor effect. This thesis focused on the association of specific HLA class I alleles with type 1 diabetes risk and with diabetes-associated autoimmunity. This was established by utilizing two large datasets: nuclear families from The Finnish Pediatric Diabetes Register (FPDR) and a follow-up cohort collected in the framework of The Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study.

The results from the FPDR showed that the HLA-A*24, B*39:01 and B*39:06 alleles were associated with type 1 diabetes, but the risk was restricted to specific HLA-DR-DQ haplotypes. The results from the DIPP cohort elaborated that the HLA class I effect was pinpointed to the pre-diabetes phase characterized by multiple autoantibodies. The presence of HLA-A*24 or B*39:01 allele was associated with faster progression to disease onset. No effect was detected with early stages of autoimmunity specified by the first appearance of autoantibodies indicating that class I variants are not involved in the triggering events initiating the autoimmune reaction but affect the later stages of the ongoing autoimmune process. Supporting this, the A*24 allele was associated with steeper decline of first-phase insulin response in children with multiple autoantibodies whereas no effect was observed in children with one or zero autoantibodies.

This thesis highlights the heterogeneous etiology of type 1 diabetes with multiple pathways to the same clinical disease. The results also emphasized population differences in type 1 diabetes risk with the HLA-B*39:01 effect being characteristic to the Finnish population whilst the effect seem to be absent in many other parts of the world.

KEYWORDS: Type 1 diabetes, genetics, HLA, risk, autoimmunity, autoantibodies

TURUN YLIOPISTO
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TIIVISTELMÄ

Tyypin 1 diabetes on autoimmuunisairaus, joka on yksi yleisimpiä lapsuusiän kroonisia sairauksia. Ennen kliinistä diabetesta tautia edeltää oireeton esidiabetes, jonka aikana perifeeriseen verenkiertoon ilmaantuu autovasta-aineita haiman saarekesoluja vastaan. Tärkein genettinen vaikutus on hyvin polymorfisella Human Leukocyte Antigen (HLA) -geenialueella, jonka luokan II (DR-DQ) geeneillä on selkeästi voimakkain vaikutus, kun taas toisilla, kuten luokan I varianteilla, vaikutus on vähäisempi. Tässä väitöskirjassa keskityttiin tiettyjen HLA luokan I varianttien vaikutukseen tyypin 1 diabeteksen sairastumisriskiin sekä siihen liittyvän autoimmuunireaktion eri vaiheisiin. Työssä hyödynnettiin kahta suurta data-aineistoa: Suomen pediatrisen diabetesrekisterin ydinperheaineistoa sekä Suomen tyypin 1 diabeteksen ennustamista ja ehkäisyä (DIPP) tutkivan projektin puitteissa kerättyä seurantakohorttia.

Diabetesrekisterin tulokset osoittivat, että HLA-A*24, B*39:01 ja B*39:06 alleelit assosioituivat tyypin 1 diabetekseen, mutta vaikutus oli rajoittunut tiettyihin HLA-DR-DQ haplotyyppeihin. DIPP-kohortin tulokset osoittivat luokan I varianttien vaikutuksen kohdistuvan esidiabeteksen vaiheeseen, jolle on tunnusomaista useiden autovasta-aineiden läsnäolo. HLA-A*24 tai B*39:01 alleelin läsnäolo liittyi nopeampaan tautiprogressioon henkilöillä, joilla oli useita autovasta-aineita. Vaikutusta ei havaittu autoimmuunireaktion varhaisissa vaiheissa kun autovasta-aineiden ilmentyminen alkaa. Tämä viittaa siihen, että HLA luokan I variantit eivät osallistu autoimmuunireaktion laukaisevaan tapahtumaan, vaan vaikuttavat sen sijaan käynnissa olevan autoimmuuniprosessin myöhäisempiin vaiheisiin. HLA-A*24 alleelilla havaittiin myös yhteys alenevaan ensivaiheen insuliinivasteeseen henkilöillä, joilla oli useita autovasta-aineita.

Nämä tulokset tukevat ajatusta tyypin 1 diabeteksen heterogeenisesta etiologiasta. Tulokset viittaavat myös populaatioeroihin tyypin 1 dabeteksen perinnöllisen riskin suhteen. Esimerkiksi HLA-B*39:01 alleelilla näyttää olevan huomattavasti suurempi riskivaikutus suomalaisessa väestössä kuin useissa muissa populaatioissa.

AVAINSANAT: Tyypin 1 diabetes, genetiikka, HLA, riski, autoimmuniteetti, autovasta-aineet

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Abbreviations

AFBAC	affected family-based association controls
B2M	beta-2 microglobulin
BCE	before Common Era
CTLA4	cytotoxic T-lymphocyte associated protein 4
CTSH	cathepsin protease
DELFIA®	dissociation-enhanced lanthanide fluorescence immunoassay
DiMe	Childhood Diabetes in Finland
DIPP	Finnish Type 1 Diabetes Prediction and Prevention study
DNA	deoxyribonucleic acid
FPDR	Finnish Pediatric Diabetes Register
FPIR	first-phase insulin response
FUT2	fucosyltransferase 2
GADA	glutamic acid decarboxylase antibodies
GWAS	genome-wide association study
HBDI	Human Biological Data Interchange
HLA	human leukocyte antigen
IA-2A	islet antigen 2 autoantibodies
IAA	Insulin autoantibodies
ICA	islet cell autoantibodies
IFH1	Interferon-induced helicase
IKZF4	IKAROS family zinc finger 4
IL10	Interleukin 10
IL2	Interleukin 2
IL2RA	Interleukin-2 receptor subunit alpha
IMGT	ImMunoGeneTics
INS	insulin
IVGTT	intravenous glucose tolerance test
JDFU	juvenile diabetes foundation units
LADA	latent autoimmune diabetes in adults
LD	linkage disequilibrium
MHC	major histocompatibility complex

MODY	maturity onset diabetes of the young
NOD	non-obese diabetic
OR	odds ratio
PCR	polymerase chain reaction
PEDIA	Pediatric Diabetes Research Group
PTPN2	protein tyrosine phosphatase non-receptor 2
PTPN22	protein tyrosine phosphatase non-receptor 22
SNP	single nucleotide polymorphism
T1D	type 1 diabetes
T1DGC	Type 1 Diabetes Genetic Consortium
T2D	type 2 diabetes
TCR	T cell receptor
WHO	World Health Organization
ZnT8A	zinc transporter 8 autoantibodies

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I M.-L. Mikk, M. Kiviniemi, A.-P. Laine, T. Härkönen, R. Veijola, O. Simell, M. Knip, J. Ilonen and the Finnish Paediatric Diabetes Register. The HLA-B∗39 allele increases type 1 diabetes risk conferred by HLA-DRB1∗04:04-DQB1∗03:02 and HLA-DRB1∗08-DQB1∗04 class II haplotypes. *Human Immunology*, 2014; 79: 65–70.

II M.-L. Mikk, T. Heikkinen, M.M.I. El-Amir, M. Kiviniemi, A.-P. Laine, T. Härkönen, R. Veijola, J. Toppari, M. Knip, J. Ilonen and the Finnish Paediatric Diabetes Register. The association of the HLA-A*24:02, B*39:01 and B*39:06 alleles with type 1 diabetes is restricted to specific HLA-DR/DQ haplotypes in Finns. *HLA*, 2017; 89: 215–224.

III M. Koskinen, M.-L. Mikk, A.-P. Laine, J. Lempainen, E. Löyttyniemi, P. Vähäsalo, A. Hekkala, T. Härkönen, M. Kiviniemi, O. Simell, M. Knip, R. Veijola, J. Ilonen, J. Toppari. Longitudinal Pattern of First-Phase Insulin Response Is Associated with Genetic Variants Outside the Class II HLA Region in Children with Multiple Autoantibodies. Brief report. *Diabetes*, 2020; 69: 12–19.

1 Introduction

Type 1 diabetes (T1D) is an autoimmune disease often manifesting at a young age. In contrast, type 2 diabetes (T2D) is not immune mediated but is associated with overweight and obesity and is often symptomatic in adulthood. The incidence rate of T1D started to increase in western countries after World War II, and to date, it is one of the most common chronic diseases of childhood. Finland has one of the highest incidence rates in the world, which is why great efforts have been made for T1D research in Finland. For example, Finland has an extensive national diabetes register (The Finnish Pediatric Diabetes Register, FPDR) and a large follow-up cohort of children at genetic risk collected in the framework of the Finnish Type 1 Diabetes Prediction and Prevention study (DIPP) providing immense data for T1D research.

T1D is a multifactorial disease meaning that genetic predisposition, as well as specific external factors are needed for disease development, and the events triggering the disease process seem to start very early in life. However, the symptoms that manifest after the insulin producing β-cells in the pancreas have been reduced to critical level due to autoimmune reaction, can appear several years after the cascade towards the disease has been set to motion. Despite decades of extensive research, the actual causative factors have remained ambiguous. One reason for that may be heterogeneity of the disease. This means that there are probably several different disease mechanisms (triggered by different internal and external factors) leading to the same outcome of clinical T1D. This thesis focuses on the genetic side of this heterogeneity.

It is well known that polymorphisms in genes within HLA (Human Leukocyte Antigen) class II region contribute about half of the genetic risk for T1D. Genetic factors in other parts of the genome as well as in the HLA region outside the class II area have been associated with the disease as well. However, the specific properties of the HLA gene area and the overpowering risk effect of the HLA class II genes has hindered the exact localization (and characterization of the effect) of other risk variants in this region. This study examines the association of specific HLA class I alleles with T1D disease risk as well as the association with different stages of the autoimmune process leading to clinical disease. The strong HLA class

II effect and the heterogeneity hypothesis are taken into account by data adjustment with class II genotype. Large sample collections from the Finnish Pediatric Diabetes Register and the DIPP study has made it possible for this study to aim to characterize associations of variants with low frequency.

On a larger scale this study aims to increase the knowledge of the predictive value of the genetic risk for T1D. This could have a significant effect on the life of a person at risk by early identification of symptoms and avoiding sometimes fatal ketoacidosis at the early stages of the disease. On the other hand, the success of future prevention trials would benefit from very accurate genetic screening for participants by taking into account disease heterogeneity which could possibly impede with study results.

2 Review of the Literature

2.1 Epidemiology and diagnosis

The earliest known mention of diabetes was in an ancient Egyptian papyrus from the 1550 BCE with the symptoms of excessive urination and weight loss. The distinction between type 1 and type 2 diabetes was stated already in the fifth century by Indian and Chinese physicians with the notation that the latter one inflicted mainly wealthy and heavy adults. Before the discovery of insulin treatment by Frederick Banting and Charles Best in Toronto in 1922 (Banting et al., 1922) various kinds of care tactics (exercise, diet, drugs) were used throughout history but usually the disease resulted in fatality within months from onset. Today, with modern insulin treatment and blood glucose monitoring the life expectancy of a patient with T1D can be no different from the non-diabetic population (Orchard et al., 2010) but the risk of severe complications is still substantial.

After World War II the incidence rate of T1D started to rise in Europe and in the U.S. and later on in other parts of the world as well (Gale, 2002; Tuomilehto, 2013). Today it is one of the most common chronic diseases of childhood (Gale, 2002). The International Diabetes Federation estimated that in 2019 over one million children and adolescents in the world had T1D and the global incidence rate is still growing by 3 % annually (Patterson, Harjutsalo, et al., 2019; Patterson, Karuranga, et al., 2019). However, the incidence of T1D has drastic demographical differences. For example, Finland has the highest incidence rate in the world with over 60 cases per 100,000 people per year but countries such as China and Venezuela have only 0.1 cases per 100,000 people annually (Tuomilehto, 2013). Despite the overall global increase in incidence, some high incidence areas such as Finland seem to have reached a plateau in the incidence rate in recent years (Patterson, et al., 2019; Tuomilehto, 2013). In addition to geographical variation, T1D shows a distinct seasonal and periodical variation in the incidence. Children born in the spring are more likely to be affected with T1D than children born in other seasons, and most cases are diagnosed in autumn and winter (Kahn et al., 2009; Moltchanova et al., 2009; Patterson et al., 2015; Weets et al., 2004). Also, evidence of incidence rate fluctuation in cycles of 4-6 years has been shown in literature (Chobot et al., 2017; Haynes et al., 2012; McNally et al., 2010; Patterson, et al., 2019; Staines et al., 1993).

Type 1 diabetes has traditionally been characterized as a disease of children and the young. Indeed, globally the incidence rate has increased especially in the very young age group (0-4 years) but the peak in incidence seems to be in ages 5-14 (Dabelea et al., 2007; DIAMOND Project Group, 2006; EURODIAB ACE Study Group, 2000; Harjutsalo et al., 2008). However, T1D can manifest at any age with substantial amount of diagnoses given in adulthood (Thunander et al., 2008; Vandewalle et al., 1997). In fact, it has been estimated that approximately 10% of adults diagnosed with type 2 diabetes show characteristics of T1D (Palmer et al., 2005; Tuomi, 2005) meaning that the number of T1D cases could be highly underestimated. Another interesting epidemiological feature is that, unlike most autoimmune diseases, T1D afflicts more males than females, especially in the age group of 15 and older whereas in younger children the male to female ratio is essentially 1 (Harjutsalo, et al., 2008; Kyvik et al., 2004; Weets et al., 2002).

According to the American Diabetes Association the criteria for diagnosis of T1D (and T2D) include fasting blood glucose higher than 7 mmol/l, 2-hour glucose tolerance test 11.1 mmol/l or higher, hemoglobin-A1c 6.5% or higher and any blood glucose level of 11.1 mmol/l or higher with symptoms of hyperglycemia (American Diabetes Association, 2012). However, as aforementioned, particularly among adults distinguishing T1D from T2D can be challenging, which has resulted in a proposal of new disease classification such as the latent autoimmune disease of adults (LADA) and ketosis-prone diabetes (Leslie et al., 2008; Naik et al., 2009). T1D can also be classified to type 1A which is the classical autoimmune T1D and to type 1B or idiopathic for those patients with no apparent characteristics of autoimmunity (Eisenbarth, 1986, 2007; Gianani et al., 2010). The T1BD includes also monogenic forms of diabetes like maturity onset diabetes of the young or MODY (Hattersley et al., 2009). In 2019 the World Health Organization published new guidelines for diabetes classification where subtypes of T1D were removed and a new category was established (World Health Organization, 2019). In this frame diabetes is classified into five categories; *Type 1 diabetes*, *Type 2 diabetes*, *Hybrid forms of diabetes* (including slowly evolving immune mediated diabetes of adults which was formally referred as LADA and ketosis-prone type 2 diabetes), *Other specific types of diabetes* (including monogenic forms of diabetes among others) and *Hyperglycemia first detected during pregnancy*.

2.2 Etiology and pathophysiology

Type 1 diabetes is (in contrast to type 2 diabetes) an autoimmune disease with genetic and environmental factors needed for onset. The disease manifests when insulin-producing β-cells in the islets of Langerhans in the pancreas are selectively destroyed and therefore the secretion of insulin becomes critically low. This disrupts the proper

regulation of blood glucose levels leading to hyperglycemia and, if untreated, ketoacidosis and eventually death. The classical symptoms of hyperglycemia are polydipsia, polyphagia and polyuria which are typically present at diagnosis. The disease is chronic, and patients are dependent on lifelong insulin treatment.

George Eisenbarth proposed a pathogenic model of T1D in 1986 (Eisenbarth, 1986) which has been updated later (Atkinson, 2012; Atkinson & Eisenbarth, 2001) as studies revealed new information about the disease. Essentially the model (Figure 1) describes how putative external factors trigger the autoimmune process that can eventually lead to clinical T1D in genetically susceptible individuals. The initial events towards autoimmunity may happen as early as in perinatal life or in early childhood and is followed by an asymptomatic phase of highly variable duration (from months to years), ending in disease onset with clinical symptoms (Knip, 2002; Knip et al., 2017). In this pre-diabetes phase autoreactive T cells are targeting β-cells. When β-cell death reaches critical point insulin production is essentially lost. Characteristically pre-diabetes starts with the appearance of antibodies specific to islet autoantigens in peripheral blood. The best-known diabetes-associated autoantibodies are against insulin (IAA), glutamic acid decarboxylase 65 (GADA), protein phosphatase like protein IA-2 (IA-2A) and zinc transporter 8 (ZnT8A).

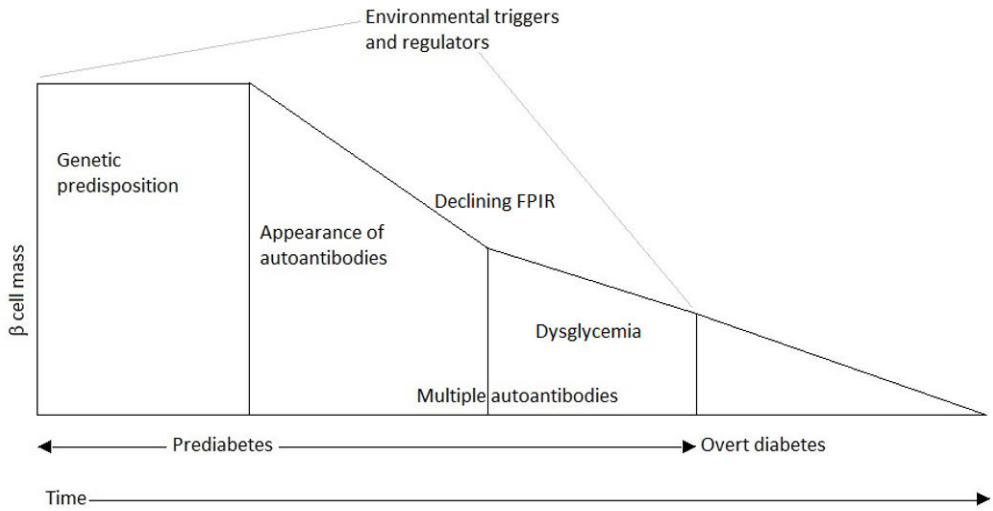

Figure 1. Model of the pathogenesis and natural history of type 1 diabetes (modified from Atkinson 2012). Environmental factors (unknown) can trigger autoimmune reaction in genetically susceptible individuals. First signs of diabetes associated autoimmunity is the emergence of diabetes-associated autoantibodies in blood. If the right external and internal factors (also largely unknown) are met, the autoimmunity persists and autoantibodies against several autoantigens emerge and persist. First-phase insulin response declines and dysglycemia appears. When β-cell mass reaches critical point and glucose metabolism becomes disrupted overt diabetes occurs.

Usually, autoantibodies specific to one islet antigen appear first, and as the autoimmune reaction advances, autoantibodies against other antigens emerge and become persistent.

The pathogenesis of T1D is a combination of genetic and external factors. Both aspects have been studied extensively over the last decades and the genetic research has yielded in results with considerable predictive value for T1D. Although the genetic model of T1D cannot be classified as dominant, recessive or intermediate inheritance of a specific set of genes (Todd, 1999), the genetic component of the disease is evident. Despite the fact that most cases of T1D are sporadic, the risk for developing the disease is 8-15 times higher for first-degree relatives (Harjutsalo et al., 2006; Hemminki et al., 2009; Sipetic et al., 2002; Tuomilehto et al., 1995; Weires et al., 2007) and two-fold for second-degree relatives of T1D patients (Allen et al., 1991; Weires, et al., 2007). The long-term concordance rate of monozygotic twins can be as high as 65 % for T1D incidence and 78 % for developing persistent autoantibody positivity or T1D or both (Redondo et al., 2008). Dizygotic twins however, have the concordance for progression to T1D or developing islet autoantibodies similar to non-twin siblings of T1D patients (Hyttinen et al., 2003; Redondo et al., 1999).

The several-fold increase of incidence in 50 years, the demographical and seasonal variance and the fact that less than 10% of individuals with high susceptibility associated gene variants are afflicted by T1D (Ilonen et al., 2002; Van der Auwera et al., 2002) strongly suggest that environmental factors play an important role in the pathogenesis of T1D. This is supported by the fact that people migrating from low-incidence country to high-incidence country tend to acquire the T1D risk of their destination country (Hussen et al., 2013; Oilinki et al., 2012; Peled et al., 2017; Soderstrom et al., 2012). This area has been under extensive research, with infections and dietary factors presented for T1D triggers, but has yet failed to reach exact consensus. Most compelling evidence for viral infections in disease etiology has been presented for enteroviruses (Coppieters, Wiberg, et al., 2012; Stene & Rewers, 2012). Dietary factors, such as maternal vitamin D intake, breast feeding or early exposure to cereals or cow's milk have all been suggested to be involved in either diabetes risk or protection but the results have been contradicting (Rewers & Ludvigsson, 2016). The fact that no definite environmental factor causing the disease has been proven yet is another indication for disease heterogeneity.

2.2.1 Autoimmunity and islet specific autoantibodies

The clinical T1D manifests when the amount of insulin produced by remaining β-cells is insufficient to maintain correct blood-glucose level. Typically, at diagnosis

patients have detectable levels of one or more type of antibodies against diabetes-associated autoantigens in the sera indicating autoimmunity in disease pathogenesis (Bingley, 2010; Knip et al., 2016; Sorensen et al., 2012). The process starts when a triggering (unknown) factor initiates the autoimmune reaction leading to gradual loss of insulin producing β-cells and finally to overt T1D. The first sign of an ongoing autoimmune process is the appearance of islet specific autoantibodies in an event called seroconversion. The islet autoantibodies were first discovered by indirect immunofluorescence assay detecting cytoplasmic islet cell autoantibodies (ICA) (Bottazzo et al., 1974) which is a heterogeneous group of autoantibodies against islet cell proteins. Specific antigens for autoantibodies were identified later with radio-binding assays, and the best known diabetes-associated biochemically defined autoantibodies recognize insulin (IAA) (Palmer et al., 1983), glutamic acid decarboxylase 65 (GADA) (Baekkeskov et al., 1990), protein phosphatase like protein IA-2 (IA-2A) (Lan et al., 1996) and zinc transporter 8 (ZnT8A) (Wenzlau et al., 2007).

Typically, autoantibodies specific to only one autoantigen emerge first, and as the autoimmunity progresses to asymptomatic pre-diabetes, autoantibodies against other autoantigens appear and persist. Islet autoantibodies can appear as early as 6 months of age with the peak incidence being 1 to 2 years of age in genetically susceptible children (Parikka et al., 2012; Ziegler & Bonifacio, 2012). It is generally acknowledged that autoantibodies are not directly involved in β-cell death but function as biomarkers of an ongoing autoimmune process. The presence of only one type of autoantibody has a low predictive value for developing T1D but the presence of autoantibodies against two or more autoantigens leads to disease almost inevitably (Bingley et al., 1994; Siljander et al., 2007; Ziegler et al., 2013). Other markers for impending diabetes are specific lipid and metabolite profiles (Oresic et al., 2008; Pflueger et al., 2011) and declining first-phase insulin response (FPIR) (Koskinen et al., 2016; Siljander et al., 2013; Sosenko, 2016).

A great part of knowledge about the natural course of T1D comes from longitudinal follow-up studies where genetically susceptible individuals are recruited from birth and are periodically monitored for autoantibodies, or from studies concerning first-degree relatives from T1D patients. These studies have shown that IAA is usually the first autoantibody to appear in young children whereas GADA is often the first autoantibody in older children and adults (Giannopoulou et al., 2015; Ilonen et al., 2013; Krischer et al., 2015). This indicates that there are at least two different pathways to islet autoimmunity. Autoantibodies against IA-2 and ZnT8 appear closer to clinical onset and are rarely first autoantibodies to appear (Ilonen et al., 2018; Wenzlau, et al., 2007; Vermeulen et al., 2012). The positivity for IA-2A and/or ZnT8A in IAA and GADA positive individuals is acknowledged as a high-risk autoantibody profile with tendency to

progress more rapidly to T1D compared to IAA and GADA positive subjects without IA-2A or ZnT8A (De Grijse et al., 2010; Gorus et al., 2017; Gorus et al., 2013; Regnell & Lernmark, 2017; Vermeulen et al., 2011).

The autoimmune mechanism(s) leading to T1D remains mostly unclear, but it is thought to be orchestrated by autoreactive T cells. It is evident that, at first, autoantigenic T cells escape the thymic selection and enter periphery. Several mechanisms by which this thymic escape takes place have been considered. Low or restricted expression of diabetes associated autoantigens in the thymus, as well as, relatively weak affinity of the T cell receptor (TCR) to MHC-autoantigen-complex, or weak affinity of the autoantigen to the MHC molecule are all possible ways for autoreactive T cells to escape the thymic selection (Ilonen et al., 2019; Roep & Peakman, 2012). In fact, it seems that naïve autoantigen-specific T cells are commonly found in the circulation of healthy individuals indicating that this is not an atypical pathogenic phenomenon (Danke et al., 2005; Skowera et al., 2015).

For initiation of the autoimmune process, however, other (unknown) internal or external factors are needed. The β-cells are metabolically active cells, and it is thought that when insulin demand is increased (during infection, for example) the β-cells become stressed and damaged which can attract immune cells to the islets (Sims et al., 2018). The infiltration of immune cells into pancreatic islets is called insulitis and is a hallmark of the immune destruction of β-cells (Gepts, 1965). What causes insulitis and what are the mechanisms within the insulitis lesions that lead to β-cell destruction are unfortunately obscure, to which there are several reasons (Morgan & Richardson, 2018). Firstly, the examination of the human pancreas in living individuals is not easily achieved, and research has relied largely on post-mortem samples which have been rare at best. Secondly, although there are useful rodent models for T1D, there are fundamental differences between the species in T1D pathogenesis. And thirdly, heterogeneity is evident in human insulitis, since the proportion of islets with inflammation varies according to age at disease onset.

Evidence, that will be reviewed in following sections, support the hypothesis that the CD8+ cytotoxic T cells are likely to be directly involved in β-cell killing, and that CD4+ helper T cells are important in the initiation of the autoimmune process and activation of the humoral autoimmunity. In short, a probable model suggests that in pancreatic lymph nodes autoreactive naïve CD4+ T cells recognize islet antigens transported there and presented by antigen-presenting cells (APCs) such as macrophages (Ilonen, et al., 2019). The activated CD4+ T cells can then activate B cells to become autoantibody-producing plasma cells. The autoreactive CD4+ T cells can possibly activate CD8+ T cells which can probably directly kill β-cells. Finally, when the autoimmune attack against β-cells has led to prolonged disrupted glucose metabolism, the clinical symptoms of T1D manifest.

2.3 Genetic susceptibility to type 1 diabetes

The pattern of inheritance of T1D is complex. Majority of T1D cases are sporadic but 12% of patients have an affected first-degree relative and the same amount have a second-degree affected relative (Parkkola et al., 2013). Among first-degree relatives (monozygotic twins excluded), siblings are at highest risk with up to 10% by the age of 20 (Steck et al., 2005) but the lifetime risk of offspring varies whether the affected parent is father (7.8%) or mother (5.3%) (Harjutsalo, et al., 2006). Interestingly, female offspring of afflicted mother seem to be relatively protected against T1D (Harjutsalo et al., 2010).

As early as the 1970s the most predominant genetic risk for T1D was localized to the HLA gene area (Nerup et al., 1974; Singal & Blajchman, 1973) and was later pinpointed to specific HLA class II DR and DQ alleles (Noble et al., 1996; Thomson et al., 1988). Genes in the HLA class I and III regions have been associated with the disease as well, and more than 50 genes outside the HLA have been localized as predisposing variants, some with stronger effects than others.

2.3.1 HLA genes and HLA molecule function

The strongest genetic association with T1D comes from the HLA-DR-DQ genes with variants conferring susceptibility as well as protection. The HLA genes are located on the Major Histocompatibility Complex (MHC) which is a large, almost 4 Mb gene area on chromosome 6p21 (Figure 2), containing genes mainly involved in the immune system. This gene area holds characteristics unlike elsewhere in the genome. It contains the most polymorphic loci and has strong linkage disequilibrium (LD) which creates notable challenges for disease association studies.

The HLA region is composed of three parts: the HLA class I, II and III. The so-called classical HLA genes are located in the HLA class I and II regions (Figure 2). These genes encode HLA class I (called A, B and C) and class II (called DR, DQ and DP) molecules that are involved in antigen recognition in the immune system. These cell surface proteins present antigen derived peptides to T cells that recognize the peptide/HLA molecule combination via TCR to activate the immune response (Figure 3). Both types of HLA molecules are quite similar in structure and function but the most notable difference is that class I molecules are expressed in all nucleated cells and they present antigens to cytotoxic CD8+ T cells whereas class II molecules are expressed (almost) solely in antigen presenting cells such as macrophages, dendritic cells, B cells and activated T cells (and thymic epithelial cells), interacting with CD4+ helper T cells (Klein & Sato, 2000).

The HLA molecules are heterodimers with highly variable peptide binding groove to which the peptide binds and is presented to the TCR. The shape of the

peptide binding groove determines the repertoire of the antigen peptides that can bind to the HLA molecule. The HLA-A, B and C genes encode the α-chain that forms the HLA class I molecule together with an invariant β-2 microglobulin encoded by the B2M gene on chromosome 15q21.1. The class II genes encode both chains of the heterodimer as the A genes (DRA, which is relatively invariant, DQA1 and DPA1) encode the α-chain and the B genes (DRB1, DQB1 and DPB1) the β-chain. The peptide binding groove in class I molecules is located only in the α-chain, but the class II molecule groove is formed in the combination of α- and β-chain. All classical HLA genes have a similar structure with exon 1 encoding the signal peptide and exons 2 and 3 (and 4 for class I) encoding immunoglobulin-like domains of the extracellular portion of the molecule. The HLA gene variance (that translates to amino acid sequence) comes to a large extent from exons encoding the peptide binding groove (exon 2 and 3 for class I and exon 2 for class II). These exons are the basis of DNA based genotyping of the HLA alleles.

Since the number of foreign antigens in contact with the human body can be huge, it is no surprise that the HLA genes are the most polymorphic genes in the genome. For example, according to the ImMunoGeneTics (IMGT/HLA) database in January 2019, the number of HLA-B alleles with nucleotide changes affecting the amino acid sequence of the gene product was 4,572 and the total number of B alleles was 7,053. Therefore, a specific nomenclature system has been established. Each gene is marked after the prefix HLA separated with a hyphen (e.g. HLA-B) following an asterisk and the allele group (e.g. HLA-B*39). The allele subtype is then marked after colon (e.g. HLA-B*39:06). Each four-digit allele translates to changes in the amino acid sequence. All other changes in the nucleotide sequence not translating to different gene product are then separated with colons. (e.g. HLA-B*39:06:02). When this polymorphic nature is combined with tight LD characteristic to the HLA the outcome is a vast number of conserved haplotypes.

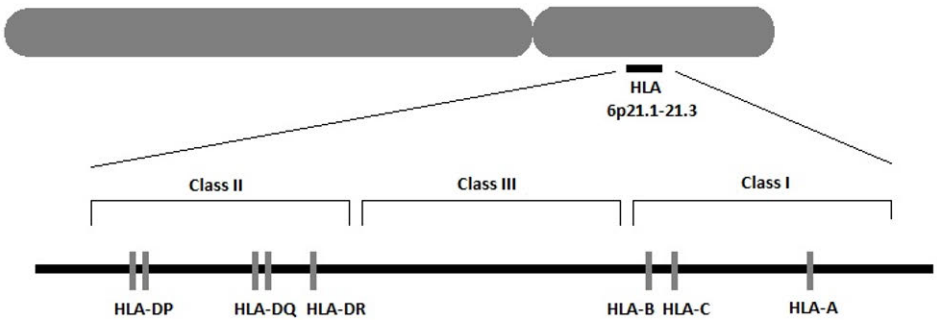

Figure 2. Schematic picture of the HLA (MHC) region on chromosome 6. The HLA-DP consists of two genes: DPA1 and DPB1. The HLA-DQ consist of two genes: DQA1 and DQB1. The HLA-DR gene is DRB1.

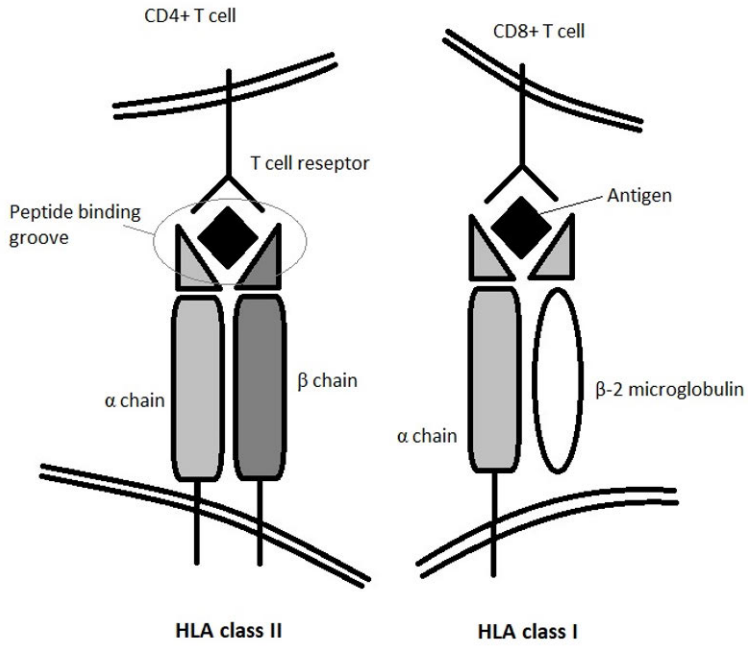

Figure 3. Schematic picture of the HLA (MHC) molecules

Conserved haplotype means that one long haplotype with multiple loci can be deduced by genotyping only one or two genes. This can result to convenient and cost-effective detection system. However, this poses problems to association studies trying to localize the exact causative variants in disease etiology because it can be difficult to distinguish whether a variant is causative or merely a marker.

2.3.2 HLA class II association with type 1 diabetes

The strongest genetic association with T1D is attributable to HLA-DRB1, DQA1 and DQB1 loci. Due to LD the alleles of these genes are often found in fixed compositions and two specific haplotypes confer the highest risk. The DRB1*03:01 allele is found almost exclusively coupled with DQA1*05:01 and DQB1*02:01 alleles forming one of these high risk haplotypes, the DRB1*03:01-DQA1*05:01-DQB1*02:01 (abbreviated as DR3-DQ2) haplotype (Erlich et al., 2008; Hermann et al., 2003; Ilonen et al., 2016; Noble & Valdes, 2011). The other high risk haplotype holds DRB1*04, DQA1*03:01 and DQB1*03:02 and is abbreviated as DR4-DQ8 (Erlich, et al., 2008; Hermann, et al., 2003; Ilonen, et al., 2016; Noble & Valdes, 2011). The abbreviations are serotypes from era before modern genetic methods for detecting HLA variants. The nomenclature between

serotypes and haplotypes can be confusing but the serotypes are well established terms for HLA haplotypes in the literature and therefore utilized here as well.

The DR4-DQ8 haplotype can have different DRB1*04 subtypes with distinct risk effects each. The highest risk is conferred by 04:01 and 04:05 alleles whereas 04:02 and 04:04 propose a lesser risk but are considered as risk alleles, nonetheless (Erlich, et al., 2008; Hermann, et al., 2003; Ilonen, et al., 2016; Noble & Valdes, 2011). However, the presence of DRB1*04:03 allele turns this haplotype to a protective one. This might lead to the assumption that the DRB1 gene is the main risk factor for T1D and the DQA1 and DQB1 to a lesser extent, but that is not the case. Generally, variation in each locus is important for T1D risk since, for example, the DRB1*04:01 (with DQA1*03:01) can also be found on a haplotype together with DQB1*03:01 but this haplotype is actually neutral or even protective from T1D (Erlich, et al., 2008; Hermann, et al., 2003; Ilonen, et al., 2016).

The presence of a protective haplotype is usually dominant in effect meaning that even if it is coupled with high-risk haplotype the effect of the whole genotype is mostly protective against T1D. Most notable protection is conferred by DRB1*15:01-DQB1*06:02 haplotype which is almost never detected in T1D cases but is one of the most common haplotypes in Caucasian populations (Noble & Valdes, 2011). Other haplotypes conferring strong protection are DRB1*14:01-DQB1*05:03 and DRB1*07:01-DQB1-03:03, whereas DRB1*11/12/13-DQB1*03:01 and DRB1*13:01-DQB1*06:03 provide moderate protection (Erlich, et al., 2008; Hermann, et al., 2003; Ilonen, et al., 2016).

The combination of the DR3-DQ2 and DR4-DQ8 haplotypes, the DR3-DQ2/DR4-DQ8 genotype, confers the highest risk for developing T1D (Erlich, et al., 2008; Hermann, et al., 2003; Ilonen, et al., 2016; Noble & Valdes, 2011). The DR3-DQ2/DR3-DQ2 and DR4-DQ8/DR4-DQ8 homozygous genotypes are predisposing as well but the risk is considerably lower than the risk of the heterozygous genotype. This has likely something to do with the fact that the heterozygous genotype can produce DQ heterodimer *in trans.* These heterodimers can be formed by combination of either DQA1*03:01 and DQB1*02:01 or DQA1*05:01 and DQB1*03:02 encoded molecules. The former combination is present *in cis* in some African populations but the latter one has not been found *in cis* on any haplotype. It seems that the DQA1*03:01/DQB1*02:01 combination is highly diabetogenic and the DQA1*05:01/DQB1*03:02 to a lesser extent (Erlich, et al., 2008).

Vast majority of class II association studies considering T1D have concentrated on the risk of developing the disease. However, follow-up studies have made it possible to examine the role of HLA class II in the autoimmune process leading to T1D. In 2016, Ilonen et al. showed that the HLA-DR-DQ genotypes correlated strongly with the appearance of first autoantibody. The high DR-DQ risk groups

were associated with younger age at seroconversion, and the association weakened as the risk of the genotype group declined. No correlation was found between the risk groups and progression time from seroconversion of multiple autoantibodies to clinical disease indicating that the class II molecules are important actors at the early stages of autoimmunity. It is likely that autoantigen peptide presentation by HLA class II molecules to the CD4+ T cells is a key step in the loss of tolerance and initiation of the autoimmune process. Studies have demonstrated the presence of at least two different pathways to islet autoimmunity associated with high risk class II haplotypes (Giannopoulou, et al., 2015; Ilonen, et al., 2013; Krischer, et al., 2015). The DR4-DQ8 is associated with IAA (and younger age of seroconversion) whereas the DR3-DQ2 is associated with GADA (and older age of seroconversion). Recently, the DR4-DQ8 haplotype was dissected to DRB1*04:01 and 04:04 in Finnish diabetics with the result that the 04:01 was associated with IAA being the first autoantibody to appear whereas the 04:04 was associated with GADA as the first autoantibody to appear (Mikk et al., 2020).

The HLA-DP genes (DPA1 and DPB1) have been associated with T1D as well, but LD between the DP and DR-DQ genes is strong and the results have been somewhat contradictory. However, based on analysis of high resolution genotyping data from a large number of samples in the Type 1 Diabetes Genetics Consortium (T1DGC), the DPB1*03:01 and DPB1*02:02 increase the disease risk whereas the DPB1*04:02 decreases the risk (Varney et al., 2010).

2.3.3 HLA class I association with type I diabetes

The first HLA association with T1D was actually discovered using HLA class I typing (Nerup, et al., 1974; Singal & Blajchman, 1973) but the found effect was later identified as a secondary association due to the dominant effect of the DR-DQ genes (Thomsen et al., 1979). Nevertheless, studies taking account the DR-DQ effect have demonstrated that some class I variants do propose DR-DQ-independent association with T1D, but this area has been less studied than class II. Across publications the most prominent HLA-A allele associated with T1D has been the HLA-A*24 (or 24:02 when genotyped on four-digit level). This allele has been shown to produce DR-DQ-independent risk for T1D and has been associated with younger age at onset (Howson et al., 2009; Nejentsev et al., 2007; Noble et al., 2002; Noble et al., 2010). Evidence for other HLA-A allele association have been presented as well, A*02 and A*30:02 for predisposition and A*01:01, A*03, A*11 and A*32:01 for protection (Howson, et al., 2009; Nejentsev, et al., 2007; Noble & Valdes, 2011; Noble, et al., 2002).

One of the most studied and acknowledged HLA-B allele has been the HLA-B*39 which, like the A*24, has been positively associated with the disease as well

as younger age at onset (Baschal et al., 2011; Howson, et al., 2009; Nejentsev, et al., 2007; Nejentsev et al., 1997; Noble, et al., 2010; Reijonen et al., 1997; Valdes et al., 2005). Studies where the B*39 allele has been genotyped on four-digit level two subtypes have been presented with disease association. The strongest evidence for disease association has been in the case of the B*39:06 allele (Baschal, et al., 2011; Noble, et al., 2010; Valdes, et al., 2005) but the B*39:01 allele risk has been noted in some cases as well (Baschal, et al., 2011; Valdes, et al., 2005). Other HLA-B alleles associated with disease predisposition have been the HLA-B*18 (or B*18:01) (Nejentsev, et al., 2007; Noble, et al., 2010; Valdes, et al., 2005) and with negative association the B*07:02, B*27, B*35:02 and B*44:03 (Nejentsev, et al., 2007; Noble, et al., 2010; Valdes, et al., 2005).

There are far less publications concerning DR-DQ independent HLA-C association with T1D than HLA-A or B but documentation for predisposing effect has been suggested at least for C*03:03, C*05:01, C*07:02 (also with younger age at onset), C*08:02 and C*16:01, and protective effect for C*07:01 (Noble, et al., 2010; Valdes, et al., 2005). However, the association of the HLA-C remains controversial since some studies have found the association to be due the DR-DQ (Howson, et al., 2009).

Although it has been acknowledged that the class I genes propose a class II independent risk, it seems that the effect is restricted to certain class II haplotypes and that class I can modify the risk of those haplotypes. For example the B*39:06 allele has been associated with elevated risk for DRB1*08:01-DQB1*04:02 and DRB1*01:01-DQB1*05:01 haplotypes but not for DRB1*04:01-DQB1*03:02 haplotype (Baschal, et al., 2011). The role of HLA class I in the islet autoimmunity has been addressed by follow-up studies but published data in this field is still scarce. The A*24, B*18 and B*39 have been associated with accelerated progression rate from the appearance of multiple autoantibodies to clinical disease (Balke et al., 2018; Lipponen et al., 2010; MbunweVan der Auwera, et al., 2013; Tait et al., 2003) indicating that class I affects the later stages of islet autoimmunity.

HLA class I genes have been shown to be hyperexpressed in the insulitis lesions of T1D patients (Arif et al., 2014; Foulis et al., 1986; In't Veld et al., 2007; Itoh et al., 1993; Richardson et al., 2016; Willcox et al., 2009) and several evidence implicate the importance of HLA class I and CD8+ T cells in the pathogenesis of T1D. Within insulitis lesion, the CD8+ T cells are the most predominant population followed by macrophages, CD4+ T cells, B lymphocytes and plasma cells, whereas FOXP3+ cells and natural killer cells are rare (Arif, et al., 2014; Willcox, et al., 2009). The involvement of CD8+ T cells in β-cell death is probable since CD8+ T cells with specificity for β-cell antigens have been shown to be present in the islets (and blood) of T1D patients (Babon et al., 2016; Coppieters, Dotta, et al., 2012;

Kronenberg et al., 2012; Pinkse et al., 2005; Skowera, et al., 2015) and because these β-cell specific CD8+ T cells have been reported to be able to kill isolated β-cells *in vitro* (Kronenberg, et al., 2012; Skowera et al., 2008). Moreover, effector CD8+ T cell targeted immunotherapy seems to delay β-cell death in patients with newly diagnosed T1D (Rigby et al., 2013) which also indicates direct involvement of CD8+ T cells.

2.3.4 Other genetic associations with type 1 diabetes

Decades of extensive research and recent genome-wide association studies (GWAS) have identified more than 60 loci associated with T1D. Associations with T1D not conferred by the classical HLA class I or II have been localized to other regions in the HLA and other areas in the human genome. Most of these loci are involved in the immune system or β-cell function and many of them have been associated with other autoimmune disorders as well (Pociot & Lernmark, 2016).

The HLA III region resides between HLA class I and II but holds no genes encoding classical HLA molecules. Instead, this gene-dense region encodes several genes of immunological relevance such as genes encoding components of the complement system, cytokines and heat shock proteins. Some of these have been associated with T1D, such as complement genes *C4A* and *C4B*, *TNFA* gene encoding the cytokine tumor necrosis factor alpha and MHC class I chain-related gene A *MICA* (a stress-induced gut epithelium antigen), but the results have been conflicting probably due to LD with HLA class II (Ajjan & Schroeder, 2019; Alizadeh et al., 2007; Noble, 2015).

Before the age of GWAS only few non-HLA determinants for T1D susceptibility were identified, of which the strongest associations conferred the insulin gene (*INS*), protein tyrosine phosphatase, non-receptor 22 (*PTPN22*) and cytotoxic T lymphocyte antigen 4 (*CTLA4*) (Bell et al., 1984; Bottini et al., 2004; Nistico et al., 1996). The insulin gene association comes from variable number of tandem repeats (VNTR) in the promoter region, and the predisposing genotype of the *INS* gene is associated with poor expression of insulin in the thymus (Pugliese et al., 1997; Vafiadis et al., 1997). This causes insulin-specific T cells not to be able to recognize HLA molecules loaded with preproinsulin antigens in proper scale, leading autoreactive T cells to escape the thymic selection. The *PTPN22* gene product is important in down-regulation of the immune response by affecting several immune cell populations, and is associated with multiple autoimmune diseases (Bottini & Peterson, 2014). The cytotoxic T lymphocyte antigen 4 is a protein receptor that also downregulates the immune system and has been associated with several autoimmune diseases (de Jong et al., 2016).

In the mid-2000s the GWA studies increased the number of T1D associated loci by a factor of 10. However, understanding the effects of these polymorphisms is challenging since many of them reside in non-coding areas. Moreover, the effects of these loci are often limited to certain cell populations and can have a cumulative effect with classical HLA variants (Bakay et al., 2019).

2.4 β-cell function and first-phase insulin response

Insulin is released from the β-cells in two phases in response of elevated plasma glucose level (Caumo & Luzi, 2004). The first-phase insulin secretion is a rapid response that happens within minutes after the β-cell is exposed to glucose. The second phase insulin secretion rises more gradually and is directly related to the degree and duration of the stimuli (Caumo & Luzi, 2004). The rapid first-phase insulin response (FPIR) is made possible by membrane-bound secretory granules in the β-cells that store active insulin (Daniel et al., 1999). The FPIR can be measured in an intravenous glucose tolerance test (IVGTT) that has widely been used to assess the capacity of β-cells to secrete insulin (Caumo & Luzi, 2004).

Declining FPIR (calculated as the sum of serum insulin concentrations at 1 and 3 minutes in the IVGTT) is characteristic to disease process leading to T1D. The FPIR has been shown to decrease as early as 6 years before diagnosis of T1D (Koskinen, et al., 2016) and an acceleration in the decline of FPIR has been detected during the last 2 years before diagnosis (Sosenko et al., 2013). Reduced FPIR has also been shown to predict the progression to T1D in children with multiple autoantibodies (Siljander, et al., 2013) and children who eventually progress to T1D have significantly lower mean FPIR throughout childhood compared to non-progressors (Koskinen, et al., 2016). The association of FPIR and genetic susceptibility to T1D has not been studied widely but in Childhood Diabetes in Finland (DiMe) study a declined FPIR was detected in siblings of newly diagnosed patients with DQB1*03:02/DQB1*02:01 risk genotype (Veijola et al., 1995). However, the association of declining FPIR and DR-DQ risk group was later shown to be secondary to the association of HLA and islet autoimmunity (Koskinen et al., 2018).

3 Aims

The purpose of this study was to investigate the heterogenic nature of type 1 diabetes by examining the T1D risk association of specific HLA class I alleles and their involvement in the diabetes associated autoimmunity in the Finnish population.

Specific aims were:

1. To assess the contribution of the HLA-A*24, B*39 and B*18 alleles on type 1 diabetes risk.
2. To investigate role the HLA-A*24 and B*39 allele in various phases of the autoimmune process leading to type 1 diabetes.
3. To establish weather HLA-B*39 genotyping would enhance the efficiency of HLA-DR-DQ genotype-based screening for type 1 diabetes risk.

4 Materials and Methods

4.1 Study population

4.1.1 Nuclear families from the Finnish Pediatric Diabetes Register (studies I and II)

The Finnish Pediatric Diabetes Register was established in 2002 with the purpose of collecting blood samples from children diagnosed with diabetes and their first-degree relatives. The samples are collected soon after diagnosis and analyzed for diabetes associated autoantibodies (ICA, IAA, GADA and IA-2A) and genotyped for HLA-DR-DQ genotypes (Parkkola, et al., 2013). According to the Pediatric Diabetes Research Group (PEDIA) website (Pediatric Diabetes Research Group, 2020) the register covers more than 90% of children diagnosed with type 1 diabetes and by the end of 2018 over 8774 children with type 1 diabetes and more than 26,000 family members were included in the Diabetes Register. Study I included samples from 1764 families and study II contained the same families, as well as, an additional 660 families. The families consisted of one affected index child and his/hers unaffected parents.

4.1.2 The follow-up cohort from the Finnish Type 1 Diabetes Prediction and Prevention Study (studies I, II and III)

Launched in 1994, the Finnish Type 1 Diabetes Prediction and Prevention Study investigates the risk factors and mechanisms involved in T1D aiming for discovering new treatment and prevention methods. In three Finnish university hospitals (Turku, Tampere, Oulu) new-born infants are genotyped for a panel of HLA-DR-DQ alleles. Children with defined risk-associated genotypes (Table 1) are invited to join a follow-up program with regular visits in clinical study centers. The children are monitored for diabetes-associated autoantibodies (ICA, IAA, GADA IA-2A and ZnT8A) and information about vaccinations, infections, diet and allergies is collected at every visit (Kupila et al., 2001). Originally, only ICA was tested in each visit, and if subject became ICA positive IAA, GADA

Table 1. HLA class II based genetic screening in DIPP study (modified from supplementary material from Ilonen et al. 2013)

Eligibility criteria for HLA based genetic screening	
Nov 1994 – Mar 1997	DQB1*03:02 present; DQB1*03:01, DQB1*06:02/3 not present
Apr 1997 – Aug 2004	DQB1*03:02 present; DQB1*03:01, DQB1*06:02 not present
Jan 1998 – Aug 2004	Boys with DQA1*05-DQB1*02 present also eligible in one of the three centers (Turku)
Sep 2004 – Feb 2010	DQA1*05-DQB1*02 / DRB1*04 (not *04:03/6)-DQB1*03:02
	DQA1*05-DQB1*02 / DQA1*05-DQB1*02
	DRB1*04 (not *04:03/6)-DB1*03:02 / DRB1*04 (not *04:03/6) – DQB1*03:02
	DRB1*04 (not *04:03/6)-DQB1*03:02 / DRB1*08-DQB1*04
	DRB1*04:01-DQB1*03:02 / DQB1*05:03, DQB1*06:02, DQA1*02:01-DQB1*03:03 not present
	DRB1*04:04-DQB1*03:02 / DQA1*02:01-DQB1*02
	DQA1*05-DQB1*02 / DQA1*03-DQB1*03:03
Mar 2010 –	DQA1*05-DQB1*02 / DRB1*04:01/2/4/5-DQB1*03:02
	DQA1*05-DQB1*02 / DQA1*05-DQB1*02
	DRB1*04:01/2/4/5-DQB1*03:02 / DRB1*04:01/2/4/5-DQB1*03:02
	DRB1*04:01/2/5-DQB1*03:02 / DQB1*04
	DRB1*04:01/2/5-DQB1*03:02 / DQB1*05:01
	DRB1*04:01/2/5-DQB1*03:02 / DQB1*06:04
	DRB1*04:01/2/5-DQB1*03:02 / DQA1*02:01-DQB1*02
	DRB1*04:01/2/5-DQB1*03:02 / DQA1*03-DQB1*03:03
	DQA1*05-DQB1*02 / DQA1*03-DQB1*03:03

and IA-2A were measured in following visits (Kupila, et al., 2001). Since 2003, the scheme has involved measurements of all four autoantibody specificities at each visit (Kukko et al., 2005). Later, the ZnT8A has been measured from individuals positive for other diabetes-associated autoantibodies (Salonen et. al., 2013). T1D was diagnosed according to the WHO criteria (World Health Organization, 1999). To date over 15 000 children with elevated genetic risk (Table 1) have been participating in the study and close to 400 of them have been diagnosed with T1D (Bauer et al., 2019).

In study I, a collection of 1071 samples from the DIPP study were used as an additional control series for the genotype analysis to gain more statistical power. The individuals were consecutively born infants with defined genotypes (Table 4). The second study (II) utilized the DIPP follow-up cohort by examining the role of HLA class I alleles in the autoimmune process leading to T1D. The cohort consisted of 528 children with at least two diabetes-associated autoantibodies as case series and 949 autoantibody negative children as controls for cases matched for sex, age and clinical center (Johanna Lempainen et al., 2015). The maximum follow-up time was 15 years. From the 528 children with at least two autoantibodies 294 developed diabetes during follow-up.

Study III was based on 438 children from the DIPP study who were tested for FPIR with an intravenous glucose tolerance test (IVGTT) at least one time (Koskinen, et al., 2018). The population was divided into two study groups. One group comprised of 195 children who had developed at least two biochemical autoantibodies during follow-up. The second group consisted of 243 children who had zero or one biochemical autoantibody at the time of the first IVGTT. The participating individuals were independent in each study except for the 195 children with at least two biochemical autoantibodies in study III who were also included in the 528 cases in study II.

4.1.3 Shared subjects in the FPDR and the DIPP study

Most of the children who participated in DIPP study and developed T1D during the follow-up have also given a sample to the FPDR. Therefore, there were some overlapping subjects in the DIPP and FPDR study populations used in the three studies. Of the 294 children with T1D in study II, 61 were included also in the FPDR nuclear families used in studies I and II. Of the 195 children with at least two biochemical autoantibodies in study III 28 were included also in the FPDR families in studies I and II.

4.1.4 Sample material

All of the samples used in HLA class I genotyping (studies I-III) were genomic DNA extracted from peripheral blood with salting out method (Olerup & Zetterquist, 1992) but the HLA class II genotyping (studies I and II) was mostly done using blood spots dried on sample collection paper (Kiviniemi et al., 2007). Blood serum or plasma was the sample material for autoantibody detection (studies II and III) (Siljander et al., 2009) and the FPIR (Study III) was measured from IVGTT blood samples (Bingley et al., 1992).

4.2 Genotyping assays (studies I-III)

4.2.1 HLA class I typing

4.2.1.1 HLA-B*39 assay

The HLA-B*39 assay was based on DELFIA principle described previously with HLA class II typing (Kiviniemi, et al., 2007; Nejentsev et al., 1999). In this method a biotinylated primer was used in PCR to allow the binding of the PCR

product to a streptavidin coated microtiter well. The product was then rendered single-stranded and lanthanide-labelled probes were used to identify the sequence of the PCR product by time resolved fluorometry detection. The HLA-B*39 primers were allele specific (Bunce et al., 1995) and are listed in Table 2 with the HLA-B*39 probe. The success of amplification was tested in each reaction by DQB1 primers and probe in study I and by β-actin primers and probe in study II and III (Table 2).

The 20 µl PCR reaction mix contained 1.0 U KAPATaq DNA polymerase (Roche), 0.8 x KAPA Taq Buffer A (Roche), 3.5 mmol/l $MgCl_2$, 0.2 mmol/l dNTPs, 1.5 mol/l betaine and 0.3 µmol/l B*39 5′ and 3’ primers, and 0.3 µmol/l control DQB1 (study I) primers or 0.2 µmol/l control β-actin (study II and III) primers. Then 20-40 ng of genomic DNA was added and following PCR protocol was used: an initial denaturation at 95 ˚C for 1 min was followed by 35 cycles of 20 seconds at 95 ˚C, 1 min at 65 ˚C and ramping of 0.5 ˚C/s to 72 ˚C and 1 min at 72 ˚C, final extension at 72 ˚C for 4 min. The hybridization was performed on streptavidin microtitration strips (PerkinElmer Life and Analytical Sciences Wallac, Turku, Finland). 5 µl of the PCR mix and 50 µl of Assay buffer (PerkinElmer Life and Analytical Sciences Wallac, Turku, Finland) were pipetted in duplicate to the streptavidin wells and incubated at room temperature with slow shaking for 30 min. The wells were then washed (DELFIA Platewash, PerkinElmer Life and Analytical Sciences Wallac) thrice with 150 µl of wash buffer containing 150 mmol/l NaCl, 5 mmol/l Tris–Cl (pH 7.75) and 0.00625% Tween 20. 150 µl of 50 mmol/l NaOH were added to the wells and after incubation at room temperature for 5 min the wells were washed thrice. 1.5 ng of the B*39 probe and 1 ng of the control probe were added in 100 µl of Assay buffer containing also 1 mol/l NaCl and 0.1% Tween 20. After one-hour incubation at 37 ˚C the wells were washed six times with wash buffer heated to 48 ˚C. 100 µl of DELFIA Enhancement solution (Kaivogen Oy, Turku, Finland) was added to the wells and after 30 min incubation with slow shaking the Eu-signals were measured with Victor 1420 Multilabel counter (PerkinElmer Life and Analytical Sciences Wallac) with the default settings. Then, 25 µl of DELFIA Enhancement was added to each well and after incubation with slow shaking at room temperature for 5 min the Tb-signal was measured on a Victor 1420 Multilabel counter with the default settings. Samples previously typed by the method developed by Bunce et al. (Bunce, et al., 1995) were used as positive and negative controls in each assay run and the performance of the new method was confirmed by testing a series of samples with both methods.

Table 2. Oligonucleotides in HLA assays

Oligonucleotides for HLA class I assays	Sequence	Label	
B*39-primers			
5' primer	CCG AGA GAG CCT GCG GAA		
3' primer	CGT GCC CTC CAG GTA GGT	5'-bio	
B*39-probes			
B*39	CGA CGG CAA GGA TTA CAT CG	5'-Eu	
B*39:06	CAC TTG GCA GAC		
A*24-primers			
5' primer	CAT GAA GTG TGA CGT GGA CAT CCG	5'-bio	
3' primer	CAC CTT GAT CTT CAT TGT GCT GGG		
A*24-probe	ACC CTC CAG ATG ATG TTT GG	5'-Eu	
Control primers			
DQB1 5' primer	GCA TGT GCT ACT TCA CCA ACG		
DQB1 3' primer	CCT TCT GGC TGT TCC AGT ACT	5'-bio	
β-actin 5' primer	GGC CGG AGT ATT GGG ACG A		
β-actin 3' primer	CCT CCA GGT AGG CTC TCT G	5'-bio	
Control probes			
DQB1 probe	CTT CGA CAG CGA CG	5'-Tb	
β-actin probe	AAT GCC AGG GTA CAT	5'-Tb	
Oligonucleotides for HLA class II assays	**Sequence**	**Label**	**Quencher sequence**
DQB1 probes			
*02	AAG AGA TCG TG	5'-Tb	CGC ACG ATC TCT
*03:01	TGG AGG TGT AC	5'-Eu	CGG TAC ACC TCC
*03:01/3	GCC GCC TGA CG	5'-Tb	CGT CAG GCG G
*03:02	GCC GCC TGC CG	5'-Eu	GGC AGG CGG
*04/5	TGC GGG GTG TGA C	5'-Tb	GTC ACA CCC CGC A
*04	AAC GGG ACC GAG C	5'-Eu	GTC ACA CCC CGC A
*05/6	GGG CGG CCT	5'-Tb	AGG CCG CCC
*05:01	ACC GGG CAG TGA	5'-Eu	TCA CTG CCC GGT
*06:02/3	TAC CGC GCG	5'-Tb	CGC GCG GTA
*06:03/4	TTG TAA CCA GAC AC	5'-Eu	GTG TCT GGT TAC A
control	CGC TTC GAC AG	5'-Tb	CTG TCG AAG CG
DQA1 probes			
*02:01	5`-CAC AGC AAC TTC CAG AC-3`	5'-Tb	
*03	5`-TCA TGG CTG TAC TG-3`	5'-Eu	
*05	5`-TAA TCA GAC TGT TCA-3`	5'-Sm	
DQB1 primers			
5' primer	GGGCATGTGCTACTTCACCAACG		
3' primer	CCTTCTGGCTGTTCCAGTACT		
DQA1 primers			
5' primer	5`-GGT AGC AGC GGT AGA GTT G-3`		
3' primer	5'-TAT GGT CTA AAC TTG TAC CAG T-3`	5'-bio	
DRB1 primers			
5' primer	GTT TCT TGG AGC AGG TTA AAC A		
3' primer	ACT CGC CGC TGC ACT GTG A	5`-bio	
DRB1 probes			
*04:03	GCC GAG GTG GA	5'-Eu	
*04:05	GCC TAG CGC CGA	5'-Eu	
*04:02	TGG AAG ACG AGC G	5'-Tb	
*04:01	GCA GAA GCG GGC	5'-Tb	
*04:02/3/4	GGT TGT GGA GAG C	5'-Eu	
control	GGG GAG TAC CGG GC	5'-Tb	

4.2.1.2 HLA-B*39:06 assay

The HLA-B*39:06 genotyping assay was essentially the same as the HLA-B*39 assay except that the probe was specific to HLA-B*39:06 allele (Table 2). More specifically, the probe could detect alleles B*39:06:01, B*39:06:02 as well as alleles B*39:33 and B*39:34, but the two latter have not been observed in the Finnish population (Haimila et al., 2013). To detect the presence of the B*39:06 allele a sample was first genotyped in the B*39 assay described in the previous section. If sample was positive for B*39 allele, the B*39:06 probe was used in DELFIA hybridization. If sample was positive, it was assigned as HLA-B*39:06.

4.2.1.3 HLA-B*39:01 assigning

A sample was assigned as HLA-B*39:01 when it was positive for the B*39 allele but negative for the B*39:06 allele. With this method it was not possible to separate the B*39:01 allele from other B*39 alleles except for the B*39:06. In the Finnish population only three B*39 alleles have been detected with the B*39:01 (freq. 0.0355) being the most abundant and the B*39:06 (freq. 0.0044) with lower frequency (Haimila, et al., 2013). The third one is the B*39:24 allele (freq. 0.0013) (Haimila, et al., 2013) which is considerably more infrequent than the B*39:01. Therefore, in this study, the frequency of B*39:24 alleles assigned as B*39:01 is remarkably low and the effect on results can be considered as negligible.

4.2.1.4 Verification for B*39 subtype assigning with Sanger sequencing

From a selection of samples, the hypervariable region of the 2nd exon of the HLA-B gene was sequenced for two purposes. In study I, where the HLA-B*39 allele was typed on two-digit level, there was a need to make a distinction between different B*39 alleles present in the Finnish population. Therefore, a total of 187 B*39 positive samples were sequenced. The same samples were used in assay verification for the HLA-B*39:06 (and B*39:01) typing assay in study II.

The sequencing protocol started with purification of the HLA-B*39 assay (section 4.3.1.1. HLA-B*39 assay) PCR product with NucleoSpin® Extract II kit (Macherey-Nagel, Düren, Germany) and the sequencing reaction was performed according to DYEnamic RT dye terminator kit (Amersham Biosciences, New Jersey, USA) using the 3′ B*39 primer (table 2). The product was then purified with NucleoSEQ® kit (Macherey-Nagel) and run in the MegaBACE 1000 (Amersham Biosciences). The sequence interpreted to present the B*39:01 allele was shared also by rare alleles B*39:02(04, 05, 07, 08, 10–13, 15, 17–19, 22, 23, 26, 27, 30–32, 35, 36, 38–41, 43) and the B*39:06 allele was shared by B*39:33(34).

4.2.1.5 HLA-A*24 assay

The HLA-A*24 typing was based on the DELFIA platform similar to the B*39 assays. The A*24 assay PCR was designed to amplify the A*24 allele with primers specified by Bunce et al. (Bunce, et al., 1995) and a probe was used to detect the A*24 allele with plate hybridization (Table 2). As an amplification control, primers and probe for β-actin gene were used in the assay (Table 2). The method detected alleles A*24:02-24:05 and A*24:07 of which the A*24:02 allele is the only one found in the Finnish population (Haimila, et al., 2013).

The 20 µl PCR reaction mix consisted of 1.0 x KAPATaq buffer A, 3.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM A*24:02 primers, 0.4 µM control (beta-actin) primers and 1 U KAPATaq DNA polymerase. Then a total of 20-40 ng of genomic DNA was added. The following thermal cycling protocol was used: The first denaturation at 96 ˚C for 1 min was followed by 4 rounds at 96 ˚C for 25 s, 70 ˚C for 45 s and 72 ˚C for 45 s. After this followed 20 rounds at 96 ˚C for 25 s, 65 ˚C for 50 s and 72 ˚C for 45 s. The protocol ended with 3 rounds at 96 ˚C for 25 s, 55 ˚C for 1 min and 72 ˚C for 2 min. The plate hybridization protocol was identical to the B*39 assay except all incubations and washes were at room temperature and A*24:02 specific probe was used.

4.2.1.6 HLA-B*18 assay

The HLA-B*18 allele was typed using allele-specific amplification and detection of the amplification product on agarose gel was performed as stated by Bunce et al. (Bunce, et al., 1995). The assay could detect both HLA-B*18 alleles (B*18:01 and B*18:03) found in the Finnish population (Haimila, et al., 2013) but no classification was made between them and thus in this study the alleles were referred to as B*18.

4.2.2 HLA class II typing

All of the samples used in studies I-III were typed for HLA-DRB1-DQA1-DQB1 genotype according to the FPDR and DIPP study protocol. The typing was tailored to define major haplotypes associated with risk or protection against type 1 diabetes. Typing was thus accordingly started with full-house typing for major DQB1 alleles using mostly four-digit resolution and was continued with a panel of probes defining three informative DQA1 alleles (DQA1*02:01, DQA1*03, DQA1*05) discriminating between major haplotypes containing DQB1*02, DQB1*03:01 and DQB1*03:03 alleles. DRB1*04 alleles associated with type 1 diabetes risk were further defined in DQB1*03:02 positive samples. The haplotypes are presented by allele names of actually typed alleles complemented

by DR specificities (in parenthesis) deduced based on known strong linkages in populations of European descent (Klitz et al., 2003). Samples with certain DQB1 genotypes were further sequenced for hypervariable region of the second exon to discern alternative genotypes.

The HLA-DQA1 and -DRB1 assays were performed using the DELFIA method (Kiviniemi, et al., 2007; Nejentsev, et al., 1999). The DQB1 assay was an in-house homogeneous assay (Kiviniemi, et al., 2007; Kiviniemi et al., 2005) where, in an asymmetric PCR, a section of the DQB1 gene was amplified and lanthanide labelled probes, as well as, complementary quencher oligonucleotides were included in the reaction. After the amplification the probes and quenchers were allowed to hybridize by cooling down the reaction. Time-resolved fluorometry was then used to measure the lanthanide signals to determine the genotype. All primer, probe and quencher sequences are listed in Table 1.

4.3 Islet autoantibody assays (studies II and III)

The DIPP study subjects included in studies II and III were monitored for diabetes associated autoantibodies prior to this study. The islet cell autoantibodies (ICAs) were detected with indirect immunofluorescence assay on human blood group O donor pancreas (Bottazzo, et al., 1974; Karjalainen, 1990) and radio-binding assays were used to detect autoantibodies to insulin (IAA) (Williams et al., 1997), glutamic acid decarboxylase 65 (GADA) (Savola, Sabbah, et al., 1998), insulinoma-associated protein-2 (IA-2A) (Savola, Bonifacio, et al., 1998) and zinc transporter 8 (Salonen et. al., 2013). The cut-off values were 2.5 Juvenile Diabetes Foundation units (JDFU) for ICA, 3.48 relative units (RU) for IAA, 5.36 RU for GADA, 0.43 RU for IA-2A (Siljander, et al., 2009) and 0.61 RU for ZnT8A (Salonen et. al., 2013). The disease sensitivities and specificities of the assays according to the 2002 to 2015 Diabetes Antibody Standardization Program/Islet Autoantibody Standardization Program workshops were 44% to 50% and 96% to 99% for IAA, 76% to 92% and 94% to 99% for GADA, and 64% to76% and 97% to 100% for IA-2A, respectively (Bauer, et al., 2019). For ZnT8A assay, according to the 2010 Diabetes Antibody Standardization Program the sensitivity was 60 and specificity 100 % (Salonen et. al., 2013). Persistent islet autoantibodies were defined by detection in two consecutive samples.

4.4 First-phase insulin response (study III)

The β-cell function was measured with first-phase insulin response in an intravenous glucose tolerance test. In the DIPP follow-up study, the IVGTTs were performed according to a standard protocol (Bingley, et al., 1992; Keskinen et al.,

2002) after detection of islet cell autoantibodies (ICA) and/or any biochemical autoantibodies (IAA, GADA, IA-2A or ZnT8A). In short, the test was carried out after overnight fasting. A glucose dose of 0.5 g/kg (maximum 35 g) in a 20 % solution was infused intravenously within 3 minutes (± 15 seconds). Blood samples were collected at 5 and 0 minutes before the start of the infusion and at 1, 3, 5, and 10 minutes after the infusion. The FPIR was calculated as the sum of insulin concentrations at 1 and 3 minutes during the IVGTT. The change in FPIR (ΔFPIR) was calculated as follows: The last FPIR measurement minus the first FPIR measurement divided by the time between the samples in years.

4.5 Statistical analyses

4.5.1 Affected Family-Based Association Control method (studies I and II)

The FPDR nuclear family data was analyzed with the Affected Family-Based Association Control (AFBAC) method which is a standard tool for family based association studies (Thomson, 1995). In studies I and II the FPDR families consisted of one child affected with T1D and his/hers unaffected parents. The alleles/haplotypes transmitted from the parents to the child (i.e. the child's genotype) were considered as case genotypes and the alleles/haplotypes not transmitted from the parents to the child constructed the artificial control genotypes. The formed genotypes were then treated as cases and controls and association was assessed based on odds ratio (OR), and significance was calculated with Fisher's exact test (2-sided) or the standard $\chi 2$ test when appropriate. The association was considered statistically significant when p value (two-tailed) was 0.05 or less. The analysis was performed with GraphPad Prism 5 software.

The HLA class I assays used in this study could only detect the presence of a given allele but not whether an individual was hetero/homozygote. Fortunately, in family data this status was deducible except in two cases. When a parent transmitted the studied HLA class I allele to an affected child it was impossible to know whether the parent was heterozygote or homozygote. The same problem arose when all members of a family were positive for the studied allele. Even though the HLA class I alleles studied in this project were quite rare and therefore the likelihood of a homozygote was small, it was decided to omit those uncertain non-transmitted alleles from the analysis (200 AFBACs in study I and 289 AFBACs in study II). This eliminated false positive associations that could have emerged if the unknown data had not been discarded.

4.5.2 Data adjustment (studies I and II)

In the case of T1D, locating associated variants (other than the HLA-DR-DQ) on the HLA gene area has proven to be difficult since the area is extremely heterogeneous, has a long and strong LD and is overpowered by the HLA-DR-DQ effect. Furthermore, the possible heterogeneity of the disease mechanism has yielded a need for more detailed association studies. In this study this problem was tackled by data stratification. More specifically, the HLA class I allele associations with T1D were tested in all HLA-DR-DQ haplotypes and genotypes that were detected in this data.

4.5.3 Survival analysis (study II)

The HLA class I association with diabetes associated autoimmunity was tested in study II with Kaplan-Meier survival analysis. The interest lied in the pre-clinical phase of T1D characterized by multiple persistent islet specific autoantibodies, as well as, in the time before the pre-clinical phase marked by the appearance of first autoantibody. The analysis included the A*24 and B*39:01 alleles. Because the frequency of the B*39:06 allele was low in this follow-up series it was excluded.

All the following analyses were performed with Kaplan-Meier survival analysis with log-rank test and results were considered statistically significant when p values were smaller than 0.05. The first survival analysis was the association of the A*24 and B*39:01 alleles with the first signs of the possible autoimmune process. In detail, the presence or lack of given allele was tested with autoantibody-free survival in timeline from birth to the time point of detecting the first biochemical autoantibody (IAA, GADA, IA-2A, ZnT8A) in the blood sera (maximum follow-up time 15 years). The results were then adjusted for specific HLA-DR/DQ genotypes: 1. DRB1*04:04-DQA1*03-DQB1*03:02 positive genotypes, 2. DRB1*04:04-DQA1*03-DQB1*03:02 / (DR3)-DQA1*05-DQB1*02 genotype and 3. DRB1*04:04-DQA1*03-DQB1*03:02 positive but (DR3)-DQA1*05-DQB1*02 negative genotypes.

The second survival analysis was the association of the A*24 and B*39:01 alleles with progression rate from positivity to multiple autoantibodies to clinical diabetes. The timeline was from the detection of second persistent biochemical autoantibody (IAA, GADA, IA-2A, ZnT8A) from blood sera to the time point of T1D diagnosis or to the age of 15. Identically to the first survival analysis, the log-rank test was used for significance and the results were stratified with specific HLA-DR-DQ genotypes. All the analyses were performed with SPSS 23 software.

4.5.4 Hierarchical linear model for FPIR (study III)

The association of the HLA-A*24 and B*39:01 alleles with the change in FPIR was tested in children with and without biochemically defined diabetes-associated autoantibodies (IAA, GADA, IA-2A, ZnT8A). Hierarchical linear models (Koskinen, et al., 2018) were applied to analyze the change in FPIR using ΔFPIR. Three types of models were applied (additive, recessive or dominant). The models were age-adjusted and included autoantibody status (0 or 1 autoantibody) in children without multiple autoantibodies and genotypes (positive or negative for studied HLA class I allele). The period of 0-5 years from the first IVGTT was examined. Analysis was performed with JMP Pro version 11.2 and p-values less than 0.05 (two-tailed) were considered statistically significant.

4.6 Ethical aspects

This study utilized samples collected in the framework of the Finnish Diabetes Prediction and Prevention study as well as the Finnish Pediatric Diabetes Register. The genetic analyses performed were included in the study plans approved by the ethics committees of participating University hospitals (DIPP study) and the Hospital District of Helsinki and Uusimaa (FPDR).

5 Results

5.1 HLA class I association with type 1 diabetes

5.1.1 HLA-B*39 (study I)

Assessing the HLA class I association with T1D started by genotyping the HLA-B*39 allele on two-digit level. A total of 311 B*39 positive parents were detected among 1764 FDPR nuclear families with the frequency of 0.088. In 202 families B*39 was transmitted to the affected child resulting in a significant association with T1D (OR=1.8, p<0.0001). The data was then stratified with HLA-DR-DQ haplotypes (Table 3a). The B*39 allele was found on 14 different DR-DQ haplotypes with unequal distribution. On eight of those, the number of B*39 alleles was less than ten. Almost 65% of the alleles resided on two haplotypes, namely (DR8)-DQB1*04 and DRB1*04:04-DQA1*03-DQB1*03:02. In both cases the B*39 allele was significantly associated with T1D after stratification (OR=1.79, p=0.0081 and OR=3.26, p=0.0001 for (DR8)-DQB1*04 and DRB1*04:04-DQA1*03-DQB1*03:02, respectively) indicating an independent predisposing effect. In this series, the (DR8)-DQB1*04 haplotype was associated with the disease only when the B*39 allele was present (OR=1.53, p=0.042 compared to B*39 negative OR=0.84, p=0.5) (Table 3a). Instead, the DRB1*04:04-DQA1*03-DQB1*03:02 haplotype was associated with the disease even when B*39 negative but the effect was considerably stronger when B*39 was present (OR=6.67, p<0.0001 when B*39 positive and OR=2.07, p<0.0001 when B*39 negative).

The association of the B*39 allele was also tested on genotype level by comparing the genotypes of the affected children to the affected family-based association control (AFBAC) genotypes which were formed from the parental haplotypes not transmitted to affected children. The presence of the B*39 allele among children with T1D was significantly increased compared to AFBACs (OR=1.86, p<0.0001) (Table 3b). The B*39 allele was most abundant on the DRB1*04:04-DQA1*03-DQB1*03:02 / (DR3)-DQA1*05-DQB1*02 genotype, with 36 diabetic children being positive for the allele. All of the AFBACs of the same genotype were negative. This yielded a very high odds ratio (OR=11.4) and

Table 3. The HLA-B*39 allele association with T1D in context of a) HLA class II haplotypes and b) HLA class II genotypes in 1764 trio families from the Finnish Pediatric Diabetes Register. Only statistically significant (or nearly) p values are displayed.

a) HLA class II haplotype	Transmitted			Non-transmitted			OR	CI (95%)	*p* value
	Total	B*39 +	%	Total	B*39 +	%			
(DR8)-DQB1*04	329	66	20.1	333	41	12.3	1.79	1.17-2.73	0.0081
DRB1*04:04-DQA1*03-DQB1*03:02	312	83	26.6	120	12	10.0	3.26	1.71-6.23	0.0001
(DR11/12/13)-DQA1*05-DQB1*03:01	75	6	8.0	273	18	6.6	1.23	0.47-3.22	
(DR1/10)-DQB1*05:01	489	10	2.0	629	13	2.1	0.99	0.43-2.27	
DRB1*04:01-DQA1*03-DQB1*03:02	1061	15	1.4	222	2	0.9	1.58	0.36-6.95	
(DR9)-DQA1*03-DQB1*03:03	136	10	7.4	130	6	4.6	1.64	0.58-4.65	
(DR15)-DQB1*06:02	23	1	4.3	461	10	2.2	2.05	0.25-16.7	
(DR3)-DQA1*05-DQB1*02	676	5	0.7	332	1	0.3	2.47	0.29-21.2	
(DR13)-DQB1*06:03	84	1	1.2	301	5	1.7	0.71	0.08-6.19	
(DR16)-DQB1*05:02	24	2	8.3	31	0		7.00	0.32-153	
DRB1*04:03-DQA1*03-DQB1*03:02	8	0		18	1	5.6			
(DR4)-DQA1*03-DQB1*03:01	62	1	1.6	103	0				
(DR7)-DQA1*02:01-DQB1*02	91	1	1.1	136	0				
(DR13)-DQB1*06:04	136	1	0.7	113	0				
Other haplotypes	22	0		126	0				
Total	3528	202	5.7	3328	109	3.7	1.72	1.41-2.28	< 0.0001

b) HLA class II genotype		Case			AFBAC			OR	CI (95%)	*p* value
		Total	B*39 +	%	Total	B*39 +	%			
DRB1*04:04-DQA1*03-DQB1*03:02 /	DRB1*04:04-DQA1*03-DQB1*03:02	11	7	63.6	2	1	50.0	1.75	0.08-36.3	
	DRB1*04:01-DQA1*03-DQB1*03:02	41	7	17.0	6	1	16.6	1.03	0.1-10.2	
	(DR8)-DQB1*04	36	13	36.1	10	2	20.0	2.26	0.41-12.2	
	(DR3)-DQA1*05-DQB1*02	103	36	34.9	10	0		11.4	0.64-199	0.0290
	(DR15)-DQB1*06:02	11	3	27.2	26	4	15.4	2.06	0.38-11.3	
	(DR1/10)-DQB1*05:01	47	15	31.9	21	2	9.5	4.45	0.91-21.6	0.0692
(DR8)-DQB1*04 /	DRB1*04:01-DQA1*03-DQB1*03:02	167	37	22.1	21	3	14.3	1.71	0.47-6.11	
	(DR9)-DQA1*03-DQB1*03:03	9	6	66.6	15	1	6.7	28.0	2.39-326	0.0037
	(DR8)-DQB1*04	7	2	28.5	21	7	33.3	0.80	0.12-5.21	
	(DR7)-DQA1*02:01-DQB1*02	31	6	19.3	8	1	12.5	1.68	0.17-16.4	
	(DR3)-DQA1*05-DQB1*02	27	9	33.3	31	2	6.5	7.75	1.4-37.4	0.0164
	(DR11/12/13)-DQA1*05-DQB1*03:01	2	0		24	7	29.2	0.47	0.02-10.9	
	(DR1/10)-DQB1*05:01	26	5	19.2	56	7	12.5	1.67	0.47- 5.85	
(DR1/10)-DQB1*05:01 /	(DR11/12/13)-DQA1*05-DQB1*03:01	8	1	12.5	50	7	14.0	0.94	0.10-8.83	
Other genotypes (B*39 <7)		1238	55	4.4	1363	63	4.6	0.96	0.66-1.39	
Total		1764	202	11.4	1664	108	6.5	1.86	1.46-2.38	< 0.0001

statistically significant association (p=0.0290) with the disease. The allele was also detected on genotypes where the DRB1*04:04-DQA1*03-DQB1*03:02 was associated with (DR1/10)-DQB1*05:01 or (DR8)-DQB1*04 (15 B*39 alleles on each) and also on the (DR8)-DQB1*04 / (DR3)-DQA1*05-DQB1*02 (11 B*39 alleles) genotype. Of these, the last genotype was the only one where the association of the B*39 allele with the disease was statistically significant (OR=7.25, p=0.0164) although a tendency was also seen on the DRB1*04:04-DQA1*03-DQB1*03:02 / (DR1/10)-DQB1*05:01 genotype (OR=4.45, p=0.069). The allele showed predisposing association (although the number of observed alleles was only 7) with the disease also on the (DR8)-DQB1*04 / (DR9)-DQA1*03-DQB1*03:03 genotype with high odds ratio (OR=28.0, p=0.0037). Even though the B*39 allele showed an independent association with T1D on both DRB1*04:04-DQA1*03-DQB1*03:02 and (DR8)-DQB1*04 haplotypes, no significant T1D association was seen with the DRB1*04:04-DQA1*03-DQB1*03:02 / (DR8)-DQB1*04 genotype. Of these, the last genotype was the only one where the association of B*39 allele with the disease was statistically significant (OR=7.25, p=0.0164) although a tendency was also seen on the DRB1*04:04-DQA1*03-DQB1*03:02 / (DR1/10)-DQB1*05:01 genotype (OR=4.45, p=0.069).

Additional control dataset was added to the genotype analysis for power gain. The B*39 allele was genotyped from 1071 samples with defined genotypes (Table 1 in methods section) identified from consecutively born infants genetically screened for the DIPP study as a control series. There were 156 B*39 alleles in this series (Table 4). The results were in line with the family data analysis and the added power yielded better statistical significance (Table 4). The strongest B*39 effect was on the (DR8)-DQB1*04 / (DR3)-DQA1*05-DQB1*02 genotype

Table 4. The HLA-B*39 allele association with T1D in context of selected HLA class II genotypes in children with T1D from the Finnish Pediatric Diabetes Register. 1071 consecutively born infants genetically screened in the frameworks of the DIPP study were use as population controls for power gain. Only statistically significant (or nearly) p values are displayed.

Class II genotype	T1D children			Population controls			OR	CI (95%)	*p* value
	Total	B*39 +	%	Total	B*39 +	%			
DRB1*04:04-DQA1*03-DQB1*03:02/									
(DR1/10)-DQB1*05:01	47	15	31.9	200	32	16.0	2.46	1.20-5.06	0.0214
(DR3)-DQA1*05-DQB1*02	103	36	35.0	201	27	13.4	3.46	1.95-6.14	< 0.0001
(DR7)-DQA1*02:01-DQB1*02	8	2	50.0	83	12	14.5	1.97	0.36-10.9	
(DR8)-DQB1*04	36	13	36.1	119	26	21.9	2.02	0.90-4.53	
(DR13)-DQB1*06:04	11	3	27.3	33	6	18.2	1.69	0.34-8.32	
(DR8)-DQB1*04 /									
(DR3)-DQA1*05 - DQB1*02	27	9	33.3	435	53	12.2	3.60	1.54-8.44	0.0052

(OR=3.60, p=0.0052) and on the DRB1*04:04-DQA1*03-DQB1*03:02 / (DR3)-DQA1*05-DQB1*02 genotype (OR=3.46, p<0.0001). Additionally, a clear independent effect was seen with the DRB1*04:04-DQA1*03-DQB1*03:02 / (DR1/10)-DQB1*05:01 genotype (OR=2.46, p=0.0214) which did not quite reach statistical significance in the family data. Even with this extra control set the B*39 effect was still not detected on the DRB1*04:04-DQA1*03-DQB1*03:02 / (DR8)-DQB1*04, although there were 26 B*39 alleles on this genotype in the DIPP data. Unfortunately, no data could be obtained from the interesting (DR8)-DQB1*04 / (DR9)-DQA1*03-DQB1*03:03 genotype since it was not included in the DIPP data.

5.1.2 HLA-B*39 subtypes (study II)

The B*39:01 and the B*30:06 alleles were genotyped from 2424 FPDR nuclear families. A total of 375 B*39:01 (frequency=0.074) and 58 B*39:06 (frequency=0.012) alleles were detected among parents (Tables 5 and 6). When no haplotype stratification was applied, the B*39:01 allele was associated with T1D (OR=1.77, p<0.0001) but the B*39:06 did not reach statistical significance (OR=1.57, p=0.078). After DR-DQ haplotype stratification the B*39:01 allele was mostly detected on both the DRB1*04:04-DQA1*03-DQB1*03:02 (126 B*39:01 alleles) and the (DR8)-DQB1*04 (101 B*39:01 alleles) haplotypes but the B*39:06 was almost exclusively detected on the (DR8)-DQB1*04 (39 B*39:06 alleles) haplotype. Moderate number of the B*39:01 allele was also detected on (DR1/10)-DQB1*05:01 (32) and (DR11/12/13)-DQA1*05-DQB1*03:01 (29) haplotypes. The B*39:01 allele was predisposing on the DRB1*04:04-DQA1*03-DQB1*03:02 haplotype (OR=2.54, p=0.0004) but not on the (DR8)-DQB1*04 haplotype or on any other haplotype. HLA-B*39:06 allele was, instead, associated with the disease on the (DR8)-DQB1*04 haplotype with high odds ratio (OR=4.25, p=0.0002). The second haplotype with more than 10 B*39:06 alleles detected was the protective (DR15)-DQB1*06:02 haplotype (11 alleles) with no transmissions to affected children and no association to T1D.

When examining the data on genotype level the results before stratification were in line with the haplotype data as the B*39:01 was associated with the disease but the B*39:06 did not quite reach statistical significance (Tables 5b and 6b). When genotype stratification was applied the B*39 subtypes were distributed unevenly between different genotypes. The B*39:01 was most abundant on the DRB1*04:04-DQA1*03-DQB1*03:02 / (DR3)-DQA1*05-DQB1*02 genotype (43 B*39:01 alleles, all in cases) where it was associated with the disease (OR=14.1,

Table 5a. The HLA-B*39:01 allele association with T1D in context of HLA class II haplotypes in 2424 trio families from the Finnish Pediatric Diabetes Register. Only statistically significant (or nearly) p values are displayed.

a) HLA class II haplotype	Transmitted			Non-transmitted			OR	CI (95%)	*p* value
	Total	B*39:01 +	%	Total	B*3901 +	%			
DRB1*04:04-DQA1*03-DQB1*03:02	438	108	24.7	158	18	11.4	2.54	1.49-4.35	0.0004
(DR8)-DQB1*04	466	59	12.7	411	42	10.2	1.27	0.84-1.94	
(DR1/10)-DQB1*05:01	675	16	2.3	849	16	1.8	1.26	0.63-2.55	
(DR11/12/13)-DQA1*05-DQB1*03:01	103	9	8.7	360	20	5.5	1.62	0.72-3.69	
(DR9)-DQA1*03-DQB1*03:03	191	10	5.2	168	10	5.9	0.87	0.35-2.15	
DRB1*04:01-DQA1*03-DQB1*03:02	1378	17	1.2	246	2	0.8	1.52	0.35-6.64	
(DR3)-DQA1*05-DQB1*02	943	9	0.9	393	2	0.5	1.88	0.41-8.76	
(DR13)-DQB1*06:03	117	2	1.7	396	5	1.2	1.36	0.26-7.10	
(DR13)-DQB1*06:04	203	5	2.4	146	0		8.12	0.44-148	
(DR15)-DQB1*06:02	31	0		623	3	0.4	2.81	0.14-55.7	
(DR7)-DQA1*02:01-DQB1*02	139	1	0.7	183	0				
(DR4)-DQA1*03-DQB1*03:01	80	0		107	1	0.9			
DRB1*0403-DQA1*03-DQB1*03:02	11	0		28	1	3.5			
(DR16)-DQB1*05:02	33	1	3.0	40	0				
Other haplotypes	40	0		162	0				
Total	4848	237	4.8	4270	120	2.8	1.77	1.42-2.22	< 0.0001

Table 5b. The HLA-B*39:01 allele association with T1D in context of HLA class II genotypes in 2424 trio families from the Finnish Pediatric Diabetes Register. Only statistically significant (or nearly) p values are displayed

b) HLA class II genotype		Case			AFBAC			OR	CI (95%)	*p* value
		Total	B*39:01 +	%	Total	B*39:01 +	%			
DRB1*04:04-DQA1*03-DQB1*03:02 /	DRB1*04:04-DQA1*03-DQB1*03:02	14	8	57.1	3	1	33.3	2.66	0.19-36.7	
	DRB1*04:01-DQA1*03-DQB1*03:02	56	10	17.8	7	1	14.2	1.30	0.14-12.1	
	(DR8)-DQB1*04	50	17	34.0	13	2	15.3	2.83	0.56-14.3	
	(DR3)-DQA1*05-DQB1*02	144	43	29.8	16	0		14.1	0.83-241	0.0067
	(DR15)-DQB1*06:02	14	3	21.4	29	4	13.7	1.70	0.33-8.93	
	(DR13)-DQB1*06:04	25	7	28.0	5	1	20	1.55	0.15-16.5	
	(DR1/10)-DQB1*05:01	69	19	27.5	33	3	9.1	3.80	1.04-13.9	0.0405
(DR8)-DQB1*04 /	DRB1*04:01-DQA1*03-DQB1*03:02	213	30	14.0	26	4	15.3	0.90	0.29-2.80	
	(DR9)-DQA1*03-DQB1*03:03	15	7	46.6	20	1	5.0	16.6	1.75-158	0.0111
	(DR8)-DQB1*04	11	3	27.2	24	7	29.1	0.91	0.19-4.48	
	(DR7)-DQA1*02:01-DQB1*02	49	7	14.2	15	3	20.0	0.66	0.15-2.98	
	(DR3)-DQA1*05-DQB1*02	47	5	10.6	34	3	8.8	1.23	0.27-5.54	
	(DR13)-DQB1*06:03	2	1	50.0	38	6	15.7	5.33	0.29-97.5	
	(DR1/10)-DQB1*05:01	44	4	9.1	76	8	10.5	0.85	0.24-3.00	
(DR1/10)-DQB1*05:01 /	DRB1*04:01-DQA1*03-DQB1*03:02	248	5	2.0	45	2	4.4	0.44	0.08-2.35	
	(DR9)-DQA1*03-DQB1*03:03	36	5	13.8	34	4	11.7	1.20	0.30-4.94	
	(DR11/12/13)-DQA1*05-DQB1*03:01	9	1	11.1	66	7	10.6	1.05	0.11-9.72	
Other genotypes (B*39:01<7)		1378	62	4.5	1651	63	3.8	1.15	0.81-1.65	
Total		2424	237	9.8	2135	120	5.6	1.82	1.45-2.29	< 0.0001

Table 6. The HLA-B*39:06 allele association with T1D in context of a) HLA class II haplotypes and b) HLA class II genotypes in 2424 trio families from the Finnish Pediatric Diabetes Register. Only statistically significant (or nearly) p values are displayed.

a) HLA class II haplotype	Transmitted			Non-transmitted			OR	CI (95%)	*p* value
	Total	B*39:06 +	%	Total	B*39:06 +	%			
(DR8)-DQB1*04	466	32	6.9	411	7	1.7	4.25	1.86-9.75	0.0002
(DR15)-DQB1*0602	31	0		623	11	1.8	0.85	0.05-14.7	
DRB1*0404-DQA1*03-DQB1*0302	438	2	0.5	158	0		1.82	0.09-38.0	
DRB1*0401-DQA1*03-DQB1*0302	1378	2	0.1	246	0		0.90	0.04-18.7	
(DR1/10)-DQB1*0501	675	0		849	1	0.1			
(DR11/12/13)-DQA1*05-DQB1*0301	103	0		360	1	0.3			
(DR4)-DQA1*03-DQB1*0301	80	1	3.0	107	0				
(DR16)-DQB1*0502	33	1	3.0	40	0				
Other haplotypes	1644	0		1476	0				
Total	4848	38	0.8	4270	20	0.5	1.68	0.98-2.90	0.0787

b) HLA class II genotype		Case			AFBAC			OR	CI (95%)	*p* value
		Total	B*39:06 +	%	Total	B*39:06 +	%			
(DR8)-DQB1*04	DRB1*0401-DQA1*03-DQB1*0302	213	12	5.6	26	0		3.29	0.19-57.2	
	(DR3)-DQA1*05-DQB1*02	47	8	17.0	34	0		14.9	0.82-267	0.0182
Other genotypes (B*39:06<7)		2164	18	0.8	2075	20	1.0	0.86	0.45-1.63	
Total		2424	38	1.6	2135	20	0.9	1.68	0.91-2.90	0.0777

p=0.0067). The second highest number of the allele (34 B*39:01 alleles) was on the DRB1*04:01-DQA1*03-DQB1*03:02 / (DR8)-DQB1*04 genotype but no disease association was detected. No effect was seen on the DRB1*04:04-DQA1*03-DQB1*03:02 / (DR8)-DQB1*04 genotype (19 B*39:01 alleles) either. Instead, with 22 B*39:01 alleles, a predisposing effect was detected on the DRB1*04:04-DQA1*03-DQB1*03:02 / (DR1/10)-DQB1*05:01 genotype (OR=3.80, p=0.0405) and with 8 B*39:01 alleles on the (DR8)-DQB1*04 / (DR9)-DQA1*03-DQB1*03:03 genotype with high odds ratio (OR=16.6, p=0.0111).

The B*39:06 allele was much rarer than the B*39:01 allele in this dataset and therefore the genotype analysis was hampered by lack of power. Still, the predisposing effect of the B*39:06 allele was detected on the (DR8)-DQB1*04 / (DR3)-DQA1*05-DQB1*02 genotype (8 B*39:06 alleles, all in cases) with high odds ratio (OR=14.8, p=0.0182). Even though the B*39:06 allele was most common on the DRB1*04:01-DQA1*03-DQB1*03:02 / (DR8)-DQB1*04 genotype (12 B*39:06 alleles, all in cases), no significant effect was detected.

5.1.3 HLA-B*18 (study II)

The effect of the B*18 allele was tested only in small scale. A set of families (149) from the FPDR positive for DRB1*04:04-DQA1*03-DQB1*03:02 haplotype but negative for B*39 allele were selected. A total of 52 B*18 alleles were detected on 159 DRB1*04:04-DQA1*03-DQB1*03:02 haplotypes. When transmitted and non-transmitted haplotypes were compared no association of the B*18 allele with T1D was detected in this haplotype (Table 7). Based on these results it was decided not to genotype the B*18 allele in a larger dataset.

Table 7. The association of the HLA-B*18 allele in 149 families positive for the DRB1*04:04-DQA1*03-DQB1*03:02 haplotype from the Finnish Pediatric Diabetes Register.

HLA class II haplotype	Transmitted			Non-transmitted			OR	CI (95%)	*p* value
	Total	B*18 +	%	Total	B*18 +	%			
DRB1*04:04-DQA1*03-DQB1*03:02	108	36	33.3	51	16	31.4	1.09	0.54-2.23	0.8578

5.1.4 HLA-A*24 (study II)

In the 2424 FPDR nuclear families a total of 794 A*24 alleles were detected in parents. The allele frequency (0.164) was thus higher than the frequency of the B*39 allele subtypes. The A*24 allele was transmitted to affected child 482 times and not transmitted 312 times, making it statistically significantly associated with T1D although the effect was quite weak (OR=1.22, p=0.010, Table 8a). When DR-DQ haplotype stratification was applied the A*24 allele was clearly more equally distributed among haplotypes compared to the B*39 subtypes. Similarly of the B*39:01 allele, the A*24 was most commonly detected on the DRB1*04:04-DQA1*03-DQB1*03:02 haplotype (142 A*24 alleles) where it was preferentially transmitted to the children with T1D (OR=1.98, p=0.0070). A similar number of the allele (139 A*24 alleles) was also seen on the (DR1/10)-DQB1*05:01 haplotype but no disease association was detected. Instead, with 94 detected alleles, the A*24 was associated with the disease on the (DR13)-DQB1*06:04 haplotype (OR=1.68, p=0.045) but not on the DRB1*04:01-DQA1*03-DQB1*03:02 (90 alleles), (DR8)-DQB1*04 (60 alleles) or (DR13)-DQB1*06:03 (60 alleles) haplotypes where it was also quite common.

Since the A*24 allele was more common than the B*39 subtypes the association analysis on genotype level yielded considerably more power than in the case of the B*39 alleles, and when no class II stratification was applied, the A*24 allele was associated with T1D (OR=1.25, p=0.0064). However, the A*24 allele was independently associated with T1D only on the (DR8)-DQB1*04 / (DR1/10)-DQB1*05:01 genotype (OR=3.01, p=0.0152), which comprises of none of those haplotypes where the A*24 effect was detected on haplotype level (Table 8b). Even though the allele was more common in absolute numbers on the DRB1*04:04-DQA1*03-DQB1*03:02 / (DR3)-DQA1*05-DQB1*02 (52 alleles), DRB1*04:01-DQA1*03-DQB1*03:02 / (DR1/10)-DQB1*05:01 (36 alleles), DRB1*04:01-DQA1*03-DQB1*03:02 / (DR8)-DQB1*04 (36 alleles), DRB1*04:04-DQA1*03-DQB1*03:02 / (DR1/10)-DQB1*05:01 (32 alleles) and DRB1*04:01-DQA1*03-DQB1*03:02 / (DR3)-DQA1*05-DQB1*02 (32 alleles) genotypes than on the (DR8)-DQB1*04 / (DR1/10)-DQB1*05:01 genotype (29 alleles), no association could be detected.

Table 8. The HLA-A*24 allele association with T1D in context of a) HLA class II haplotypes and b) HLA class II genotypes in 2350 trio families from the Finnish Pediatric Diabetes Register. Only statistically significant (or nearly) p values are displayed.

a) HLA class II haplotype	Transmitted			Non-transmitted			OR	CI (95%)	*p* value
	Total	A*24 +	%	Total	A*24 +	%			
DRB1*04:04-DQA1*03-DQB1*03:02	430	119	27.7	142	23	16.2	1.98	1.21-3.25	0.0070
(DR1/10)-DQB1*05:01	652	70	10.7	725	69	9.5	1.14	0.81-1.62	
(DR13)-DQB1*06:04	188	64	34.0	128	30	23.4	1.69	1.01-2.80	0.0457
DRB1*04:01-DQA1*03-DQB1*03:02	1344	76	5.6	216	15	6.9	0.80	0.45-1.43	
(DR8)-DQB1*04	450	38	8.4	354	22	6.2	1.40	0.81-2.40	
(DR13)-DQB1*06:03	111	20	18.0	323	40	12.4	1.56	0.86-2.80	
(DR9)-DQA1*03-DQB1*03:03	184	28	15.2	145	15	10.3	1.56	0.80-3.04	
(DR15)-DQB1*06:02	31	2	6.6	519	37	7.1	0.90	0.21-3.91	
(DR11/12/13)-DQA1*05-DQB1*03:01	101	12	11.9	307	23	7.5	1.67	0.80-3.48	
(DR3)-DQA1*05-DQB1*02	919	25	2.7	326	6	1.8	1.49	0.61-3.67	
(DR4)-DQA1*03-DQB1*03:01	77	13	16.9	90	6	6.7	2.84	1.03-7.89	0.0502
(DR7)-DQA1*02:01-DQB1*02	131	8	6.1	163	9	5.5	1.11	0.42-2.97	
(DR16)-DQB1*05:02	33	3	9.1	37	3	8.1	1.13	0.21-6.04	
(DR7)-DQA1*02:01-DQB1*03:03	6	0		82	6	7.3	0.90	0.05-17.9	
DRB1*0403-DQA1*03-DQB1*03:02	11	0		26	3	11.5	0.29	0.01-6.14	
DRB1*0402-DQA1*03-DQB1*03:02	6	2	33.3	2	1	50.0	0.50	0.02-12.9	
(DR14)-DQB1*05:03	1	0		39	3	7.7	3.48	0.12-102	
(DR4)-DQA1*03-DQB1*02	6	1	16.7	2	0				
DRB1*0408-DQA1*03-DQB1*03:04	1	1	100	0	0				
(DR13)-DQB1*06:09	4	0		10	1	10.0			
Other haplotypes	14	0		8	0				
Total	4700	482	10.3	3644	312	8.6	1.22	1.05-1.42	0.0100

b) HLA class II genotype		Case			AFBAC			OR	CI (95%)	*p* value
		Total	A*24 +	%	Total	A*24 +	%			
DRB1*04:04-DQA1*03-DQB1*03:02 /	DRB1*04:01-DQA1*03-DQB1*03:02	56	18	32.1	5	1	20.0	1.90	0.20-18.2	
	(DR8)-DQB1*04	50	17	34.0	12	3	25.0	1.55	0.37-6.47	
	(DR3)-DQA1*05-DQB1*02	142	47	33.1	14	5	35.7	0.89	0.28-2.81	
	(DR15)-DQB1*06:02	13	4	30.8	27	8	29.6	1.06	0.25-4.45	
	(DR13)-DQB1*06:04	23	14	60.9	4	1	25.0	4.67	0.42-52.2	
	(DR1/10)-DQB1*05:01	68	26	38.2	30	6	20.0	2.48	0.89-6.87	
DRB1*04:01-DQA1*03-DQB1*03:02 /	DRB1*04:01-DQA1*03-DQB1*03:02	102	9	8.8	8	1	12.5	0.68	0.07-6.14	
	(DR3)-DQA1*05-DQB1*02	358	31	8.7	20	1	5,0	1.80	0.23-13.9	
	(DR13)-DQB1*06:04	63	25	39.7	11	4	36.4	1.15	0.31-4.35	
	(DR13)-DQB1*06:03	73	19	26.0	25	5	20,0	1.41	0.46-4.28	
	(DR11/12/13)-DQA1*05-DQB1*03:01	40	10	25.0	13	1	7.7	4.00	0.46-34.8	
(DR8)-DQB1*04 /	DRB1*04:01-DQA1*03-DQB1*03:02	207	32	15.5	25	4	16.0	0.96	0.31-2.98	
	(DR1/10)-DQB1*05:01	41	17	41.5	63	12	19.0	3.01	1.24-7.29	0.0152
(DR1/10)-DQB1*05:01 /	DRB1*04:01-DQA1*03-DQB1*03:02	240	32	13.3	39	5	12.8	1.05	0.38-2.87	
	(DR9)-DQA1*03-DQB1*03:03	34	8	23.5	27	8	29.6	0.73	0.23-2.30	
	(DR7)-DQA1*02:01-DQB1*02	13	5	38.5	44	6	13.6	3.96	0.97-16.2	
	(DR4)-DQA1*03-DQB1*03:01	27	9	33.3	16	3	18.8	2.17	0.49-9.61	
	(DR3)-DQA1*05-DQB1*02	105	14	13.3	60	4	6.7	2.15	0.68-6.87	
	(DR15)-DQB1*06:02	5	2	40.0	106	19	17.9	3.05	0.48-19.6	
	(DR13)-DQB1*06:04	29	15	51.7	30	9	30.0	2.50	0.86-7.28	
	(DR13)-DQB1*06:03	5	1	20.0	59	10	16.9	1.23	0.12-12.2	
	(DR1/10)-DQB1*05:01	34	7	20.6	76	16	21.1	0.97	0.36-2.64	
(DR13)-DQB1*06:04 /	(DR3)-DQA1*05-DQB1*02	35	10	28.6	10	1	10.0	3.60	0.40-32.3	
	(DR15)-DQB1*06:02	0	27		0	10	37.0			
Other genotypes (A*24<10)		587	110	18.7	1071	169	15.8	1.23	0.94-1.60	
Total		2350	482	20.5	1822	312	17.1	1.25	1.07-1.46	0.0064

5.1.5 HLA-A*24 and B*39:01 alleles on the DRB1*04:04-DQA1*03-DQB1*03:02 haplotype (study II)

The HLA-A*24 and the B*39:01 alleles were both associated with T1D on the DRB1*04:04-DQA1*03-DQB1*03:02 haplotype when they were tested separately. Since these alleles can be on the same haplotype there was a need to test whether one of them or neither of them could be the actual variant of the independent association on this haplotype. This was performed by stratifying the haplotype with A*24 and B*39:01 allele (Table 9). There were 94 DRB1*04:04-DQA1*03-DQB1*03:02 haplotypes that were positive for both alleles, 29 positive for A*24 only and 20 positive for B*39:01 only. Neither of the alleles showed independent association over each other on this haplotype (Table 9). When the test was performed with the DRB1*04:04-DQA1*03-DQB1*03:02 haplotype positive for only A*24 or B*39:01 neither haplotype was associated with the disease (Table 9).

Table 9. The association of the HLA-A*24 and HLA-B*39:01 allele with T1D in the A*24-B*39:01-DRB1*04:04-DQA1*03-DQB1*03:02 haplotype, the association of the A*24 allele with T1D in B*39:01 negative DRB1*04:04-DQA1*03-DQB1*03:02 haplotype and the association of the B*39:01 allele with T1D in A*24 negative DRB1*04:04-DQA1*03-DQB1*03:02 haplotype. The DQA1*03-DQB1*03:02 is referred as DQ8 in the table

Haplotype	Transmitted			Non-transmitted			OR	CI (95%)	*p* value
	Total	HLA class I	%	Total	HLA class I	%			
		A*24+			**A*24+**				
HLA-B*39:01-DRB1*04:04-DQ8	98	82	83.6	16	12	75.0	1.70	0.48-5.97	0.4770
		B*39:01+			**B*39:01+**				
HLA-A*24-DRB1*04:04-DQ8	105	82	78.0	21	12	57.1	1.98	0.76-5.17	0.1948
		A*24+			**A*24+**				
DRB1*04:04-DQ8 (B*39:01 neg)	266	23	8.6	98	9	9.2	0.94	0.42-2.10	0.8373
		B*39:01+			**B*39:01+**				
DRB1*04:04-DQ8 (A*24 neg)	259	16	6.2	93	4		1.47	0.48-4.50	0.7930

5.2 The effect of HLA-B*39 allele on class II-based screening for type 1 diabetes risk (study I)

When the HLA-B*39 allele was first genotyped on two-digit level the family data results indicated a significant association with T1D in certain HLA-DR-DQ genotypes. It was decided to test whether adding the B*39 allele to the DIPP screening criteria would have any significant value. The current scheme's eligible genotypes are listed in Table 10 and it gives a sensitivity of 60.4% and specificity of 89.8%. Adding B*39 allele genotyping to the scheme in genotypes where B*39 positivity was able to identify risk ((DR3)-DQA1*05-DQB*02 / (DR8)-DQB1*04, (DR8)-DQB1*04 / (DR9)-DQA1*03-DQB1*03:03 and DRB1*04:04-DQA1*03-DQB1*03:02 / (DR1/10)-DQB1*05:01) increased the sensitivity of the screening scheme to 62.1% but decreased the specificity only to 89.5%.

Table 10. The effect of including HLA-B*39 allele to the current DIPP screening scheme on sensitivity and specificity of the screen.

HLA class II genotype		T1D children	AFBAC
Eligible genotypes in DIPP screening			
DRB1*04:01 - DQA1*03 - DQB1*03:02	(DR1/10) - DQB1*05:01	201	38
(DR3) - DQA1*05 - DQB1*02	(DR3) - DQA1*05 - DQB1*02	46	10
	(DR9) - DQA1*03 - DQB1*03:03	36	19
	DRB1*04:01 - DQA1*03 - DQB1*03:02	273	24
	DRB1*04:04 - DQA1*03 - DQB1*03:02	103	11
	DRB1*04:05 - DQA1*03 - DQB1*03:02	2	0
	DRB1*04:08 - DQA1*03 - DQB1*03:02	1	0
DRB1*04:01 - DQA1*03 - DQB1*03:02	(DR13) - DQB1*06:04	47	11
	(DR7) - DQA1*02:01 - DQB1*02	23	13
	(DR8) - DQB1*04	167	24
	(DR9) - DQA1*03 - DQB1*03:03	35	7
	DRB1*04:01 - DQA1*03 - DQB1*03:02	79	15
	DRB1*04:04 - DQA1*03 - DQB1*03:02	41	6
DRB1*04:04 - DQA1*03 - DQB1*03:02	DRB1*04:04 - DQA1*03 - DQB1*03:02	11	2
Total eligible genotypes		1065	180
Other genotypes		699	1584
Sensitivity		**60,4 %**	
Specificity		**89,8 %**	
Ineligible HLA-B*39 positive genotypes in DIPP screening			
DRB1*04:04 - DQA1*03 - DQB1*03:02	(DR1/10) - DQB1*05:01	15	2
(DR8) - DQB1*04	(DR9) - DQA1*03 - DQB1*03:03	6	1
	(DR3) - DQA1*05 - DQB1*02	9	2
Eligible genotypes		1065	180
Other genotypes		669	1579
Sensitivity		**62,1 %**	
Specificity		**89,5 %**	

5.3 HLA-A*24 and B*39 subtype association with autoimmunity and FPIR (studies II and III)

The association of the A*24 and B*39:01 alleles was tested with two stages of autoimmunity marked by biochemical autoantibodies against islet antigens (study II) and with the rate of β-cell destruction in pre-diabetes measured with change in first-phase insulin response (study III). The two stages of autoimmunity in study II were defined as: 1. the time from birth to the appearance of first persistent T1D associated biochemical autoantibody, and 2. progression time from two or more persistent biochemical autoantibodies to clinical diabetes.

In study II, 1477 subjects from the DIPP prospective follow-up cohort were genotyped for the A*24 (271 positive), B*39:01 (100 positive) and B*39:06 (7 positive) alleles. Since the frequency of the B*39:06 allele was low it had to be excluded from the survival analysis. All of the analyses were first performed with no stratification with the HLA-DR-DQ genotypes and then the results were tested with specifically selected genotype groups: the DRB1*04:04-DQA1*03-DQB1*03:02 positive genotypes, the DRB1*04:04-DQA1*03-DQB1*03:02 / (DR3)-DQA1*05-DQB1*02 genotype and the DRB1*04:04-DQA1*03-DQB1*03:02 positive but (DR3)-DQA1*05-DQB1*02 negative genotypes.

Study III included 438 children from the DIPP study who had at least two FPIR measurements and were genotyped for the A*24 (80), B*39:01 (31) and B*39:06 (4) alleles. Of those children 195 developed at least 2 biochemical autoantibodies during follow up. The remaining 243 children who had zero or one biochemical autoantibody were considered as control group. Because of low frequency, the B*39:06 allele was excluded from this analysis as well.

5.3.1 Emergence of islet autoimmunity defined by the first persistent autoantibody

The association of the A*24 and B*39:01 allele with T1D autoimmunity was first tested with time from birth to the point of first persistent autoantibody detection (Figures 4 and 5). No statistically significant association of the A*24 or B*39:01 allele with the emergence of autoantibodies was observed, not even after stratification.

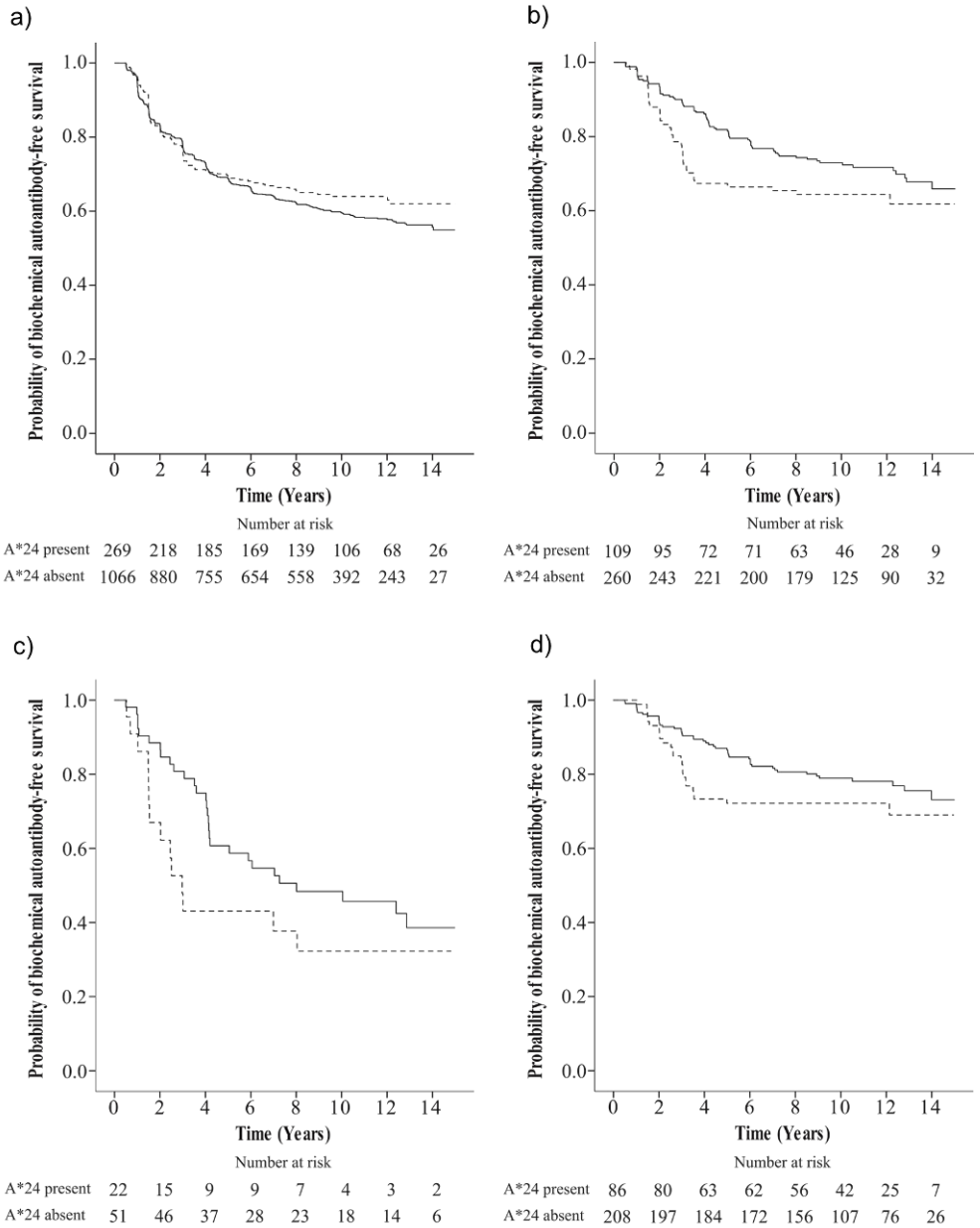

Figure 4. The effect of the HLA-A*24 allele (dashed line A*24 positive, solid line A*24 negative) on the emergence of first persistent biochemical diabetes-associated autoantibody with (a) no stratification (p=0.227), (b) among carriers of DRB1*04:04-DQA1*03-DQB1*03:02 positive genotypes (p=0.073), (c) among carriers of the DRB1*04:04-DQA1*03-DQB1*03:02 / (DR3)-DQA1*05-DQB1*02 positive genotype (p=0.107) and (d) among children with DRB1*04:04-DQA1*03-DQB1*03:02 positive and (DR3)-DQA1*05-DQB1*02 negative genotypes (p=0.158).

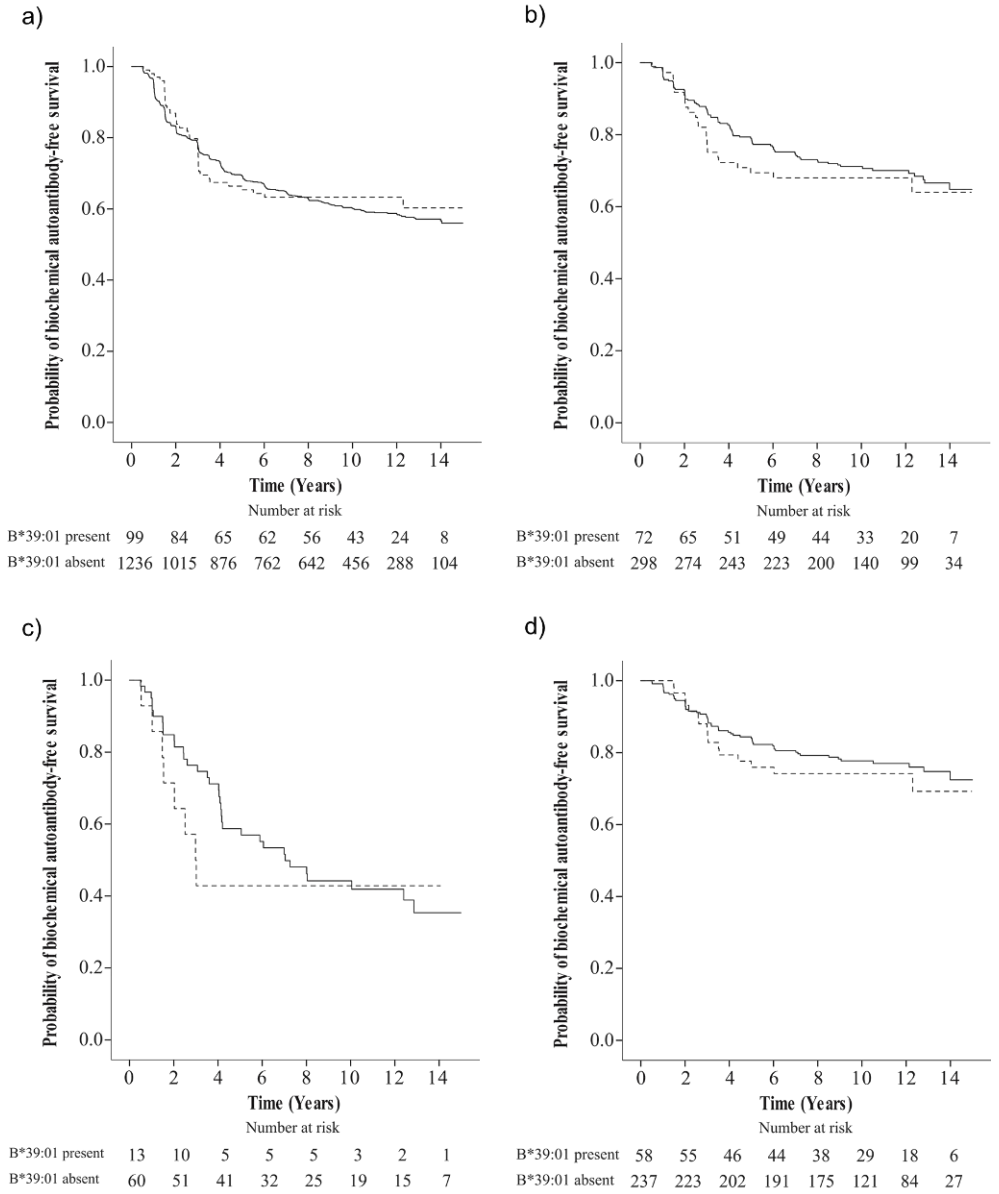

Figure 5. The effect of the HLA-B*39:01 allele (dashed line B*39:01 positive, solid line B*39:01 negative) on the emergence of first persistent biochemical diabetes-associated autoantibody with (a) no stratification (p=0.634), (b) among carriers of DRB1*04:04-DQA1*03-DQB1*03:02 positive genotypes (p=0.509), (c) among carriers of the DRB1*04:04-DQA1*03-DQB1*03:02 / (DR3)-DQA1*05-DQB1*02 positive genotype (p=0.542) and (d) among children with DRB1*04:04-DQA1*03-DQB1*03:02 positive and (DR3)-DQA1*05-DQB1*02 negative genotypes (p=0.499).

5.3.2 Progression rate from positivity to multiple biochemically defined autoantibodies to clinical T1D

The second test on autoimmunity was the A*24 and B*39:01 allele association with the time from seroconversion to clinical T1D (Figures 6 and 7). When no stratification was applied the A*24 allele was associated with shorter progression time (p=0.001). The effect was also detected in the DRB1*04:04-DQA1*03-DQB1*03:02 positive genotype group (p=0.017) and on the DRB1*04:04-DQA1*03-DQB1*03:02 / (DR3)-DQA1*05-DQB1*02 genotype (p=0.001) but not in the (DR3)-DQA1*05-DQB1*02 negative DRB1*04:04-DQA1*03-DQB1*03:02 genotype group. The B*39:01 allele showed no association without stratification, but it was associated with faster progression rate on the DRB1*04:04-DQA1*03-DQB1*03:02 / (DR3)-DQA1*05-DQB1*02 genotype (p=0.017).

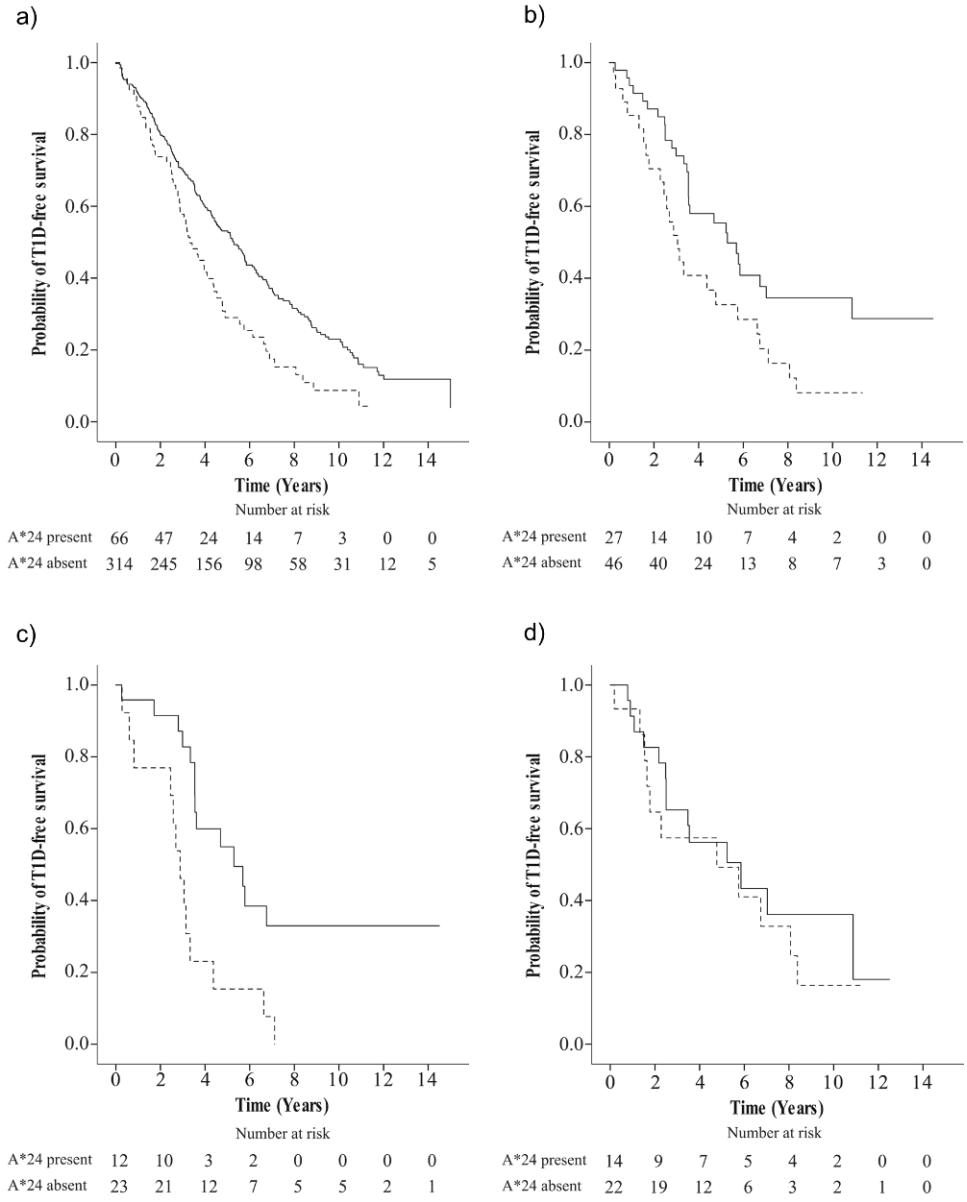

Figure 6. The effect of the HLA-A*24 allele (dashed line A*24 positive, solid line A*24 negative) on the progression time from the appearance of second biochemical autoantibody to type 1 diabetes with (a) no stratification (p=0.001), (b) among carriers of DRB1*04:04-DQA1*03-DQB1*03:02 positive genotypes (p=0.017), (c) among carriers of the DRB1*04:04-DQA1*03-DQB1*03:02 / (DR3)-DQA1*05-DQB1*02 positive genotype (p=0.001) and (d) among children with DRB1*04:04-DQA1*03-DQB1*03:02 positive and (DR3)-DQA1*05-DQB1*02 negative genotypes (p=0.561).

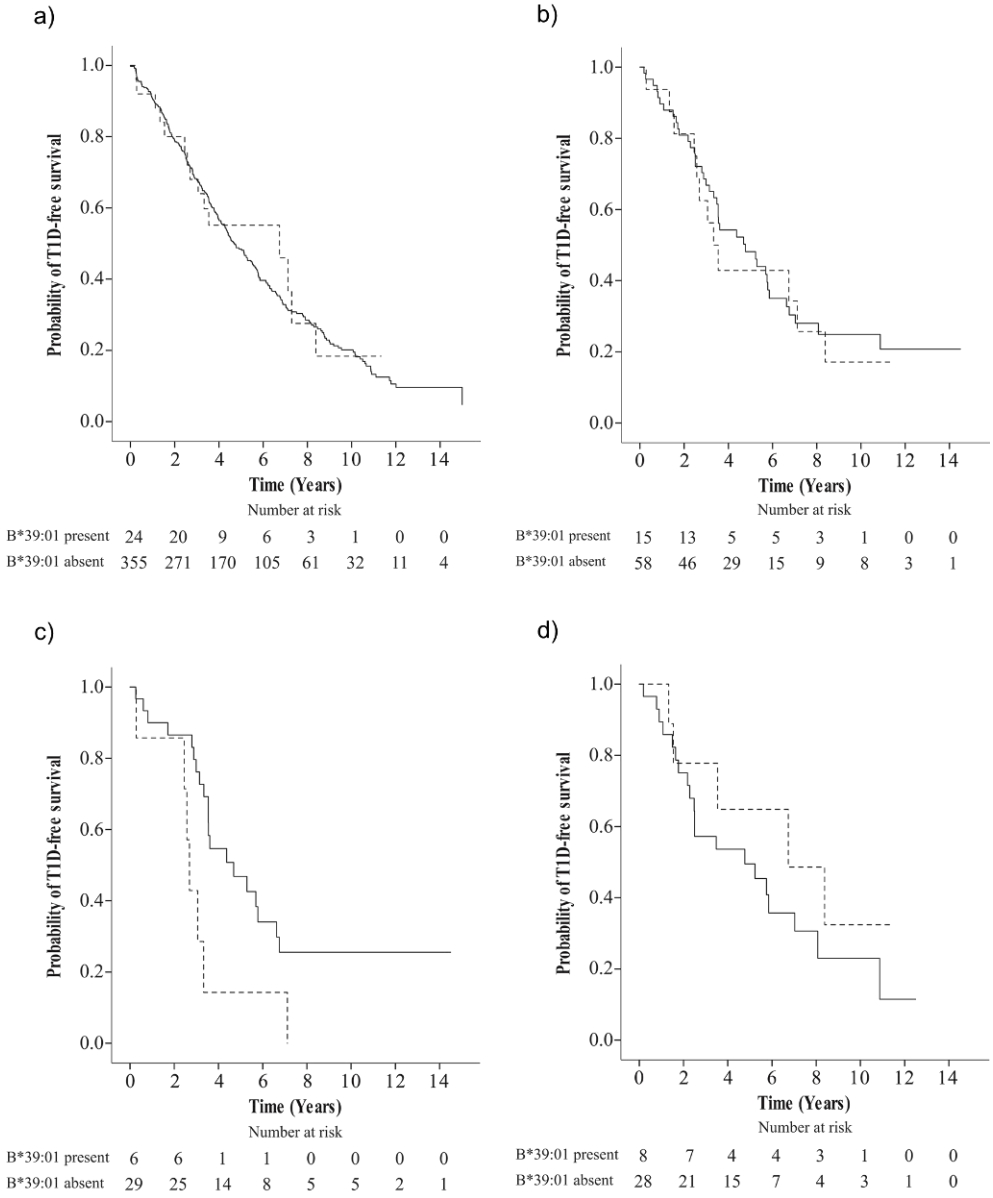

Figure 7. The effect of the HLA-B*39:01 allele (dashed line B*39:01 positive, solid line B*39:01 negative) on the progression time from the appearance of second biochemical autoantibody to type 1 diabetes with (a) no stratification (p=0.935), (b) among carriers of DRB1*04:04-DQA1*03-DQB1*03:02 positive genotypes (p=0.686), (c) among carriers of the DRB1*04:04-DQA1*03-DQB1*03:02 / (DR3)-DQA1*05-DQB1*02 positive genotype (p=0.017) and (d) among children with DRB1*04:04-DQA1*03-DQB1*03:02 positive and (DR3)-DQA1*05-DQB1*02 negative genotypes (p=0.321).

5.3.3 Longitudinal patterns of FPIR

The A*24 allele was associated with steeper decline of FPIR in children with multiple autoantibodies (p=0.037) but not in the control group (Table 11). No significant association was detected in the case of the B*39:01 allele in either groups.

Table 11. First phase insulin response (FPIR) as analyzed by a hierarchical linear mixed model adjusted for a) age in 243 children without multiple autoantibodies and b) age and number of autoantibodies from 195 children with multiple autoantibodies at the time of the first IVGTT. †P-value of individual coefficient indicates whether genotype influences FPIR (null hypothesis is that coefficient estimate equals zero).

a) Allele (n)	Status (n)	Coefficient estimate (SE)	P-value of individual coefficient estimate†	Model P-value
A*24 (233)	absent (185)	0.000187 (0.000054)	<0.0008	0.11
	present (48)	0.000327 (0.000103)	0.0011	
B*3901 (229)	absent (213)	0.000222 (0.000051)	<0.0001	0.28
	present (16)	0.000060 (0.000145)	0.68	

b) Allele (n)	Status (n)	Coefficient estimate (SE)	P-value of individual coefficient estimate†	Model P-value
A*24 (183)	present (32)	-0.00037 (0.000098)	0.0002	**0.037**
	absent (151)	-0.00015 (0.000035)	<0.0001	
B*3901 (186)	present (15)	0.000049 (0.000139)	0.73	0.10
	absent (171)	-0.00018 (0.000034)	<0.0001	

†P-value of individual coefficient indicates whether genotype influences FPIR (null hypothesis is that coefficient estimate equals zero)

6 Discussion

One of the main results of the study was that the HLA-A*24, B*39:01 and B*39:06 allele association with T1D was clearly restricted to certain DR-DQ haplotypes and genotypes. This underlines the heterogenic nature of the disease. The study also confirmed the involvement of the HLA class I in later stages of the diabetes associated autoimmunity as individuals with multiple autoantibodies progressed faster to clinical disease and showed a steeper decline in FPIR when positive for studied class I alleles.

6.1 HLA-B*39 association with type 1 diabetes

In study I, the HLA-B*39 allele was associated with T1D on two HLA-DR-DQ haplotypes. Then study II revealed that the DRB1*04:04-DQA1*03-DQB1*03:02 (DRB1*04:04-DQ8) association was accounted for the most common B*39 subtype in Finland, the B*39:01 allele, whereas the (DR8)-DQB1*04 (DR8-DQ4) association was due to the B*39:06 allele. The presence of the B*39:01 allele increased the risk of the DRB1*04:04-DQ8 haplotype but more strikingly, positivity for the B*39:06 changed the risk status of the DR8-DQ4 haplotype from neutral to susceptible. The OR of the B*39:06-DR8-DQ4 haplotype (4.05) was actually higher than the ORs of DRB1*04:04-DQ8 (2.58) or DR3-DQ2 (2.38) haplotypes in this family data demonstrating that this haplotype confers a high risk for T1D. Previous association studies that included the B*39:06 allele have presented similar results where the allele has been positively associated with the disease on the DR8-DQ4 haplotype (Baschal, et al., 2011; Noble, et al., 2010; Valdes, et al., 2005). In 2011, Baschal et al. noted the high-risk effect of the B*39:06-DR8-DQ4 haplotype as well by stating that the presence of the B*39:06 in the DR3-DQ2/DR8-DQ4 genotype had the absolute diabetes risk similar to the high risk DR3-DQ2/DR4-DQ8 genotype.

Previous studies have shown the B*39:06 association with T1D also on other DR-DQ haplotypes. In 2005, Valdes et al., and in 2011, Baschal et al. found that the allele was associated on DR1-DQB1*05:01 haplotype albeit the effect was considerably weaker than on DR8-DQ4. In addition to the DR8-DR4 and DR1-DQB1*05:01 haplotypes, Noble et al. (2010) reported the B*39:06 association also

on DR15-DQB1*06:02, DR4-DQ8 and possibly on DR3-DQ2 too. Our study was unable to determine any other DR-DQ haplotype effect simply because the B*39:06 was extremely rare on other than the DR8-DQ4 haplotype in our data set. The only other haplotype that was positive for the allele in more than 10 cases was the strongly protective (DR15)-DQB1*06:02 (or DR15-DQ6) haplotype on which none of the 11 B*39:06 alleles was transmitted to the affected child. The B*39:06 allele seems to be almost exclusively restricted to the DR8-DQ4 haplotype in the Finnish population whereas in many other Caucasian populations it is more widely spread among haplotypes. The study made by Valdes et al. (2005) was based on multiplex families collected from the North American Human Biological Data Interchange (HBDI, Philadelphia, PA) and the two others (Baschal, et al., 2011; Noble, et al., 2010) had multiplex families from the international Type 1 Diabetes Genetic Consortium (T1DGC) consisting of nine cohorts (Asia Pacific Network, European Network, North American Network, United Kingdom Network as well as collections from Denmark, HBDI, Joslin Diabetes Center and Sardinia) which emphasizes the population differences between these studies and study II.

The risk association of the B*39:01 allele on the DRB1*04:04-DQ8 haplotype was not as strong as the B*39:06 on DR8-DQ4 haplotype but significant, nevertheless. The allele was more common and more widely distributed among different haplotypes but no association on other haplotypes was detected. In fact, the number of B*39:01 positive DR8-DQ4 haplotypes was more than double of those that were B*39:06 positive suggesting that lack of power was not the reason why no B*39:01 association was detected. This either signifies that the B*39:01 allele association is restricted to only DRB1*04:04-DQ8 haplotype, at least in Finland, or that the association is due to LD with some other variant in that haplotype. It seems to be unlikely that other variants on the DRB1*04:04-DQ8 could account for the B*39:01 association since a large SNP study covering the whole HLA region by Nejentsev et al. in 2007 pinpointed the strongest susceptibility after class II to HLA-B and after that to the HLA-A. However, involvement of the A*24 allele in the association of the B*39:01 allele on this haplotype is possible and discussed in section 6.3.

The same three HLA class I studies mentioned in the B*39:06 discussion also included the B*39:01 allele but only one of them detected association with T1D which was restricted to DR16-DQB1*05:02 haplotype (Baschal, et al., 2011). This is somewhat uncommon haplotype in the Finnish population (Haimila, et al., 2013) and only one B*39:01 allele on this haplotype was detected in our family dataset, thus no association analysis could be conducted. As to why these three studies did not find the B*39:01-DRB1*04:04-DQ8 association is, again, probably because of allele frequency differences between populations. As can be seen in the paper of Baschal et al. (2011), the frequency of the B*39:01 allele was markedly lower than

the frequency of the B*39:06 allele in the T1DGC data which is quite the opposite to the Finnish population. Moreover, there were only two haplotypes (DR16-DQB1*05:02 and DR1-DQB1*05:01) on which the B*39:01 allele exceeded the number of ten meaning that no association could have been detected in other haplotypes.

Although there have been only few studies considering the HLA class I alleles on four-digit level, older publications have examined the class I associations on two-digit level. The effect of the B*39 allele on the DRB1*04:04-DQ8 haplotype was established by our group in the late 1990s in the Finnish population (Reijonen, et al., 1997), as well as, in Estonian and Russian populations (Nejentsev, et al., 1997). The same effect was detected, also by our group, in two studies examining these long haplotypes by microsatellite markers (Gombos et al., 2006; Nejentsev et al., 2000). The earlier one was a case-control study comparing microsatellite markers and A*24 and B*39 alleles between DR3-DQ2/DRB1*04:01-DQ8 and DR3-DQ2/DRB1*04:04-DQ8 genotype groups with the finding that two markers near the HLA-B conferred additional diabetes risk on the latter genotype but the B*39 effect was even stronger (Nejentsev, et al., 2000). The second study was based on a family data with either DRB1*04:04-DQ8 or DR8-DQ4 positivity in each family with the main finding that the B*39 allele and a C125 microsatellite marker contributed to the risk conferred by the DRB1*04:04-DQ8 haplotype (Gombos, et al., 2006). In light of the results presented in study II it can now be confirmed that the risk effect of the B*39 allele on the DRB1*04:04-DQ8 haplotype reported in these studies is conferred by the B*39:01 allele and not by the B*39:06.

The co-expressive nature of the classical HLA genes and the well-known example of how the diabetes risk of a heterozygous DR-DQ genotype exceeds risk of a homozygous genotype (DR4/DR3) are facts that imply the importance of studying HLA class I association in context of DR-DQ genotypes. However, considering the relatively low frequency of each class I allele and the large number of different DR-DQ genotypes, it is no surprise that for reliable results large datasets are crucial. For example, when considering the B*39 subtypes in study II, from 4559 case/AFBAC genotypes 169 different DR-DQ genotypes were detected with only 11 of them having n>100. This illustrates why studies considering separately all DR-DQ genotypes in class I disease risk association have been virtually non-existent. Only few publications have reported association data of class I allele in context of selected genotypes in special settings (such as autoimmunity) and will be discussed in appropriate sections.

In study II, the B*39:01 and the B*39:06 alleles were both associated with disease risk on DR-DQ genotypes combining the haplotype on which they were associated with the disease (DRB1*04:04-DQ8 and DR8-DQ4 respectively) and

the common susceptibility haplotype DR3-DQ2. In both cases the odds ratios were high (approx. 14) but the confidence intervals were extremely wide and overlapping number 1 which questions the significance of these results. However, all of the 43 B*39:01 and 8 B*39:06 alleles were in case genotypes (which also attributes to wide CIs) and the p-values were less than 0.05, and thus, these results can be considered statistically significant. As mentioned previously the risk effect of the B*39:06 allele on the DR8-DQ4/DR3-DQ2 genotype has been described before by Baschal et al. in 2011 and, in light of the results from study II, the B*39 association detected on the DRB1*04:04-DQ8/DR3-DQ2 genotype previously by our group (Nejentsev, et al., 2000) was most likely due to B*39:01 allele.

The involvement of the DR3-DQ2 haplotype in the genotype analysis of both the B*39:01 and B*39:06 allele (even though the B*39 alleles are rare in this haplotype) might suggest a common pathway to disease susceptibility for B*39 subtypes. It would have been interesting to see whether the B*39:01 allele would have been associated with the disease on the DR8-DQ4/DR3-DQ2 genotype since no association was detected in the DR8-DQ4 haplotype. Unfortunately, due to lack of power, no association analysis could be conducted. If there were an association, it could speak for interaction between the DR3-DQ2 haplotype and the B*39 alleles. This is a matter in need of further investigation. The B*39:01 and B*39:06 molecules are closely related with only two amino acid difference in the peptide binding groove meaning that a common mechanism in the disease etiology is plausible. However, this perspective is to be applied with caution since even small changes in the amino acid sequence of the peptide binding pocket of HLA molecules can affect antigen recognition drastically. For example, the A*24:02 and A*24:07 molecules differ only by one amino acid in the peptide binding groove (His and Gln, respectively) but the former allele is predisposing for T1D and the latter is not (Bugawan et al., 2002).

Even though the DR3-DQ2 might be a common nominator in the B*39 allele association it cannot be the only case. The B*39:01 was associated with the disease also on DRB1*04:04-DQ8 / (DR1/10)-DQB1*05:01 and DR8-DQ4 / DR9-DQB1*03:03 genotypes. The DRB1*04:04-DQ8 / (DR1/10)-DQB1*05:01 association was not surprising since the B*39:01 allele risk seem to be restricted to the DRB1*04:04-DQ8 haplotype in the Finnish population and the allele was third most common (but not very frequent) in the (DR1/10)-DQB1*05:01 haplotype. However, with the same logic the B*39:01 allele risk effect should have been detected on the DRB1*04:04-DQ8 / DR8-DQ4 genotype since 64 % of the B*39:01 alleles were detected on either DRB1*04:04-DQ8 or DR8-DQ4 haplotypes. The odds ratio of the DRB1*04:04-DQ8 / DR8-DQ4 genotype for B*39:01 allele was 2.83 but it was not statistically significant probably due to lack of power. The same non-significant effect was seen for the B*39:01 allele on the

DRB1*04:04-DQ8 homozygous genotype (OR=5.14) and for the B*39:06 allele on the DR8-DQ4 / DR8-DQ4 genotype (OR=5.1) indicting that the effect might be true but would need more power to be detected as significant.

The remarkably strong effect of the B*39:01 allele on the DR8-DQ4 / DR9-DQB1*03:03 genotype was surprising and highly interesting since the allele was not associated with the disease on either of these haplotypes. The odds ratios on both haplotypes were close to 1 and could not be attributable to lack of power. But because the frequency of the DR8-DQ4 / DR9-DQB1*03:03 genotype was quite small no definite conclusions can be made without further investigations. However, if the effect is true it could demonstrate heterogeneity in disease mechanisms and strongly state the importance of genotype level association studies for HLA class I.

6.2 HLA-A*24 association with type 1 diabetes

Several studies have reported the A*24 allele contribution to the T1D disease risk across populations. In 2002, Noble et al. (HBDI family data) reported that the A*24 allele association with T1D cannot be explained solely by LD with the DR-DQ and, in 2007, Nejentsev et al. (HBDI and British collections) published similar results depicting that the A*24 allele effect exists even after the DR-DQ is taken into account. Publications from the large T1DGC data (multiple populations) have stated the DR-DQ independent risk association as well (Howson, et al., 2009; Noble, et al., 2010). Similar results have been reported from Japanese and Filipino populations (Bugawan, et al., 2002; Nakanishi et al., 1999). In Finland, the A*24 allele risk effect has been shown to impact the DR4-DQ8 haplotype but not the DR3-DQ2 (Fennessy et al., 1994).

The results from study II confirm the A*24 allele association with T1D and define the effect seen on the DR4-DQ8 haplotype in the Finnish population (Fennessy, et al., 1994) to be specific to DRB1*04:04 and not DRB1*04:01. The A*24 allele effect was present also on neutral (DR13)-DQB1*06:04 and (DR4)-DQB1*03:01 haplotypes, neither of which have been depicted before. In addition to the A*24 risk association on the DRB1*04:04-DQ8 haplotype, Noble et al. (2010) described the effect on several DR-DQ haplotypes including DRB1*01-DRB1*05:01, DRB1*01-DQB1*05:04, DRB1*04:01-DQ8, DR3-DQ2, DR7-DQB1*03:03, DR8-DQ4 and DRB1*16-DQB1*05:02. Study II was unable to detect the A*24 effect on any of these additional haplotypes which is probably attributable to, as in the case of the B*39, population differences. This is supported by the fact that the A*24 allele is, compared to the B*39 allele subtypes, considerably more prevalent and thus lack of power is unlikely (with the exception of DR1-DQB1*05:04 and DR15-DQB1*05:02) to be the reason for not detecting an association. Compared to the B*39 allele subtypes, the effect of the A*24 allele

on different DR-DQ haplotypes was clearly weaker, which has been seen before (Howson, et al., 2009; Noble, et al., 2010). Because the A*24 allele association was seen after DR-DQ stratification on several haplotypes it would indicate an independent diabetes risk effect for the allele but restricted to specific haplotypes. This suggests a specific pathway to autoimmunity like in the case of the B*39 allele subtypes.

Even though the A*24 allele was more prevalent than B*39 subtypes, study II was able to detect only one significant association with T1D when the effect was considered on DR-DQ genotype level. The results were surprising since the association was on DR8-DQ4 / (DR1/10)-DQB1*05:01 genotype which could not have been expected on the grounds of haplotype analysis. The A*24 allele was not uncommon on either haplotype, but no risk effect was detected. The odds ratio of the A*24 allele on the DR8-DQ4 / (DR1/10)-DQB1*05:01 genotype was 3.0 (95% CI 1.24-7.28), which is a considerable risk effect. This emphasizes the importance of genotype analysis in HLA disease association studies since the prediction level of haplotype analysis might not be sufficient in all cases. The risk effect regarding the DR3-DQ2 haplotype seen in the B*39 subtype association on genotype level was not seen in the case of the A*24 allele. In fact, the odds ratio of the A*24 allele on the DRB1*04:04-DQ8 / DR3-DQ2 genotype was close to 1 despite the fact that the allele was most numerous on this genotype. This was surprising since the A*24 allele effect on progression from multiple autoantibody positivity to T1D was clearly restricted to the DRB1*04:04-DQ8 / DR3-DQ2 genotype. However, this can possibly be explained by the way the data was analyzed. Some of the data was lost due to uncertainty of homozygote/heterozygote calling of AFBAC haplotype (as discussed in section 4.6.1.) when A*24 allele was transmitted to child with T1D. The A*24 allele is considerably more prevalent than the B*39 allele and thus more data had to be discarded in the case of the A*24 allele.

6.3 HLA-A*24 and B*39:01 alleles and DRB1*04:04-DQ8 haplotype

On the DRB1*04:04-DQ8 haplotype, the A*24 and the B*39:01 alleles are often found together in the Finnish population and, according to the Finnish Red Cross Blood Service data, the haplotype is among 20 most common HLA-A-B-C-DRB1-DQA1-DQB1 haplotypes in Finland (Haimila, et al., 2013). In study II, a test was carried out to determine whether the A*24 or B*39:01 allele is the causative factor for the class I association on this haplotype but no indication for one allele having an effect over other was detected. When, in the same sample set, the effect of each allele was tested in the absence of the other allele (A* 24 allele association on B*39:01 negative DRB1*04:04-DQ8 haplotype and vice versa) neither the A*24

nor the B*39:01 was associated with T1D on DRB1*04:04-DQ8 haplotype. This suggests either an interaction between these alleles or that these alleles are simply markers for the association of some other (causative) variant. However, taking previous studies (discussed in sections 6.1. and 6.2.) into account it is unlikely (yet not impossible) that the association is caused by some other variant(s) in the HLA region than the B*39:01 or A*24 on this haplotype. Interactions between these alleles is possible and an additive effect cannot be ruled out.

6.4 HLA-B*18 association with type 1 diabetes

The B*18 allele has been shown to be associated with T1D independently from the DR-DQ in several publications (Howson, et al., 2009; Nejentsev, et al., 2007; Noble, et al., 2010; Valdes, et al., 2005). According to Noble et al. (2010) the predisposing effect of the B*18 allele was detected on DR3-DQ2 and DR4-DQ8 haplotypes but not on DR8-DQ4, DR1-DQ5 or DRB1*15:01-DQB1*06:02. In study II, the association was tested on DRB1*04:04-DQ8 haplotype but no effect was detected. This cannot be attributable to lack of power since the allele was fairly common on that haplotype, which leads to the conclusion that the B*18 allele is not a risk allele at least on the DRB1*04:04-DR8 haplotype in the Finnish population. The frequency of the DRB1*04:04 allele in the DR4-DQ8 group (all DR4 alleles excluding 04:03 and 04:06) in the Noble et al. (2010) paper is unknown and therefore the results cannot be directly compared. In addition, the results can be attributable to population differences as well. The DR3-DQ2 association was not included in study II since the B*18 allele is essentially missing and B*08 usually present on this haplotype in Finland (Haimila, et al., 2013; Ilonen et al., 1980; Tienari et al., 1992). Elsewhere, especially in Southern Europe, the B*18-DR3-DQ2 is fairly common and conserved extended haplotype and has been demonstrated to confer higher T1D risk than the B*08-DR3-DQ2 haplotype (Bilbao et al., 2006; Zavattari et al., 2001). A SNP (rs419434) upstream from HLA-DOA gene was confirmed to contribute to T1D risk on the B*18-DR3-DQ2 haplotype (Santin et al., 2009). HLA-DOA is an HLA class II α-chain paralogue expressed in B cell and is involved in HLA-DM-mediated peptide loading on MHC class II molecules. The contribution of this variant to the susceptibility conferred by the B*18-DR3-DQ2 haplotype, and whether lack of predisposing effect of the B*18 allele in the Finnish population could be attributable to this variant would be interesting to examine.

6.5 HLA-A*24 and B*39 alleles and autoimmunity

In study II, the effect of the A*24 and B*39:01 was investigated in two stages of subclinical diabetes-associated autoimmunity. The A*24 and B*39:01 alleles had no effect on the appearance autoantibodies but were associated with accelerated progression time from multiple autoantibodies to clinical disease. The effect was restricted to DRB1*04:04-DQ8 / DR3-DQ2 genotype in both alleles. The involvement of the A*24 allele in the later stages of the autoimmunity was confirmed in study III where it was associated with faster loss of β-cell function (measured as change in FPIR over time) in children with multiple autoantibodies but not in control group. This is in line with the general idea that the DR-DQ determines the initiation of the autoimmunity whereas the class I is involved in β-cell destruction after the autoimmune process has been set to motion. Studies have shown that the DR-DQ risk haplotypes are associated with appearance of autoantibodies, but after seroconversion the effect is lost (Bingley et al., 2016; Gorus, et al., 2017; Ilonen, et al., 2016; Krischer et al., 2017).

The involvement of the A*24 allele in diabetes associated autoimmunity was established already in the early 1990s in Japanese population (in which the A*24 allele is common) when the presence of the A*24 allele was shown to promote β-cell destruction in T1D patients (Nakanishi et al., 1993), and was associated with a group of patients who needed insulin therapy in less than 3 months after clinical onset (Kobayashi et al., 1993). In an Australian study, the A*24 allele was associated with ICA positive first-degree relatives (of T1D patients) who progressed to clinical disease in contrast to non-progressors (Honeyman et al., 1995). Later, the same group showed how the A*24 allele (along with A*30 and B*18) was associated with disease progression but not with autoantibody appearance (Tait, et al., 2003).

More recently and with longitudinal samples, two papers from the Belgian Diabetes Registry showed that the A*24 allele was associated with faster progression rate from autoantibody positivity to clinical disease especially in DR4-DQ8 positive individuals, and that additional screening of A*24 allele improved the detection of rapid progressors in autoantibody positive first degree relatives of T1D patients (Mbunwe, et al., 2013; Mbunwe, Van der Auwera, et al., 2013). However, the B*39 allele effect was not detected in that series (Mbunwe, et al., 2013). After study II, another publication from Belgium confirmed the results from their previous papers and noted that the A*24 association was restricted to DR4-DQ8 haplotype and positivity for IA-2A and ZnT8A (Balke, et al., 2018). Interestingly, this paper also described how the presence of the A*24 allele delayed the progression from single to multiple autoantibody positivity in GADA positive relatives. Study II did not analyze this step in the diabetes associated autoimmunity, but it would be worth to investigate whether this effect could be

detected in the DIPP series as well. The A*24 allele has also been linked to attenuated humoral response in T1D patients with negative association with IA-2A and ZnT8A (Howson et al., 2011; Long et al., 2013), as well as, in ICA positive first-degree relatives with negative association with GADA, ZnT8a and IA-2A (Ye et al., 2015). One plausible explanation for this reduced autoantibody spreading is that β-cell destruction might be more rapid in A*24 positive individuals, leaving less time for multiple-autoantibody profile to develop (Balke, et al., 2018; Long, et al., 2013).

Study II was the first publication to show the A*24 allele effect in T1D related autoimmunity in Finland. However, in a previous paper by our group the A*24 was investigated in DIPP follow-up cohort but no association was detected with progression rate to overt disease from the appearance of second persistent autoantibody (Lipponen, et al., 2010). Even though study II and the paper from Lipponen et al. used the same follow-up cohort from the DIPP study there were differences in the study frame that could account for the deviating results. Firstly, the study from Lipponen et al. had a considerably smaller cohort and secondly, autoantibodies against ZnT8 were not included as they were in study II. Also, Lipponen et al. used Cox-regression analysis whilst study II relied on Kaplan-Meier survival analysis.

However, Lipponen et al. did describe the B*39 allele association with accelerated progression rate from the appearance of two persistent autoantibodies to clinical T1D, which study II can now confirm to originate from the B*39:01 allele. The B*39 allele was also included in studies from the Belgian Diabetes Registry but no association with islet autoimmunity was detected (Balke, et al., 2018; Mbunwe, et al., 2013). This could be attributable to the fact that the B*39 allele frequency was fairly low in these studies and, as stated in section 6.1., population differences seem to have a significant role in the case of the B*39 allele association. The B*39:01 is more common in the Finnish population compared to other European derived populations whereas the B*39:06 predominates in many other populations. The B*39:01 effect might be more relevant in Finland (and in Estonia and Russia according to Nejentsev et al. 1997), specifically in combination of the DRB1*04:04-DQ8 haplotype, than elsewhere.

Study II was unable to analyze the role of B*39:06 allele in different stages of diabetes-related autoimmunity because the allele was rare in the DIPP cohort. The screening scheme has not specifically included the DR8-DQ4 haplotype which explains the near lack of the B*39:06 allele. This is very unfortunate since the allele has one of the strongest associations with T1D after DR-DQ but there are no reports considering specifically this allele and autoimmunity in a cohort study. However, Schloss et al. published an interesting study in 2018 where the group created a NOD class I knock-out model that expressed the human B*39:06 allele

(Schloss et al., 2018). They demonstrated that the B*39:06 allele was able to mediate the development of CD8+ T cells, support lymphocytic infiltration of the islets, and confer T1D susceptibility, suggesting that the B*39:06 allele has a distinct role in the autoimmunity leading to T1D.

In a very recent study, Yeo et al. (2020) reported the presence of B*39:06-restricted autoreactive CD8+ T cells in patients with newly diagnosed T1D. They found that compared to non-diabetics, B*39:06 positive newly diagnosed T1D patients had increased level of circulating preproinsulin (PPI)-specific transitional memory CD8+ T cells, and that in these children, the PPI-specific CD8+ T cells showed a significantly more antigen-experienced phenotype compared to polyclonal CD8+ T cells. The same study reported that in longitudinal follow-up, children who were positive for the A*24:02 allele had increased percentage of terminal effector cells within the insulin B (InsB)-specific CD8+ T cells in samples taken close to diagnosis compared to samples taken before the appearance of diabetes associated autoantibodies. It was noted that the transitional memory cells may be able to execute target cell killing and thus indicating direct involvement of the HLA class I molecules in β-cell death. In fact, the same group showed previously that A*24:02-restricted PPI-specific CD8+ T cell clones derived from patients with type 1 diabetes are able to kill isolated β-cells *in vitro* (Knight et al., 2013).

Another way to detect the autoimmune process leading to T1D is to examine the loss of β-cell function by first-phase insulin response, since the decline in FPIR, as well as, lower mean FPIR have been shown to predict progression to T1D several years before clinical symptoms (Koskinen, et al., 2016; Siljander, et al., 2013; Sosenko, et al., 2013). In study III, the A*24 allele was associated with steeper decline in FPIR over time in multiple autoantibody positive children compared to children with no autoantibodies. This further supports the involvement of the of HLA class I in β-cell killing, especially in later stages of autoimmunity. Unfortunately, no significant association was detected for the B*39:01 allele but the variant was somewhat infrequent in this series.

Study III also included eight T1D associated SNP markers (Floyel et al., 2014; Halminen et al., 1996; Ilonen, et al., 2018; J. Lempainen et al., 2015; Winkler et al., 2011) outside the HLA region, of which, variants of the *PTPN2*, *FUT2*, *CTSH* and *IKZF4* were associated with declining FPIR over time in multiple-autoantibody positive children compared to children with no autoantibodies. The CTSH has been shown to regulate β-cell function (Floyel, et al., 2014) and the *PTPN2* is involved in β-cell apoptosis (Moore et al., 2009) which makes the association with FPIR sensible. Also the *IKZF4* (Sharma et al., 2013) has an important role in lymphoid development (Sharma, et al., 2013) and *FUT2* is involved in host-pathogen

interactions (Smyth et al., 2011) highlighting the immune-mediated etiology of T1D.

The association of these four variants and the A*24 allele with steeper decline in FPIR in children with multiple autoantibodies instead of children with no autoantibodies indicates their involvement in the later stages of autoimmunity. Indeed, the *PTPN2* and *IKZF4* have been associated with faster progression to disease after seroconversion in the DIPP cohort before but not *FUT2* or *CTSH* (Lempainen, et al., 2015). The same DIPP study showed that SNPs representing *PTPN22*, *IFH1* and *INS*, that were also included in study III, were associated with development of β-cell autoimmunity (defined as the appearance of autoantibodies). It is therefore sensible that these markers are not associated with declining FPIR in children with multiple autoantibodies.

The exact mechanism behind all of these associations is certainly an area of future research. It seems that there are (at least) two critical steps for the diabetes associated autoimmunity to occur. Firstly, the genetic predisposition by HLA-DR-DQ is almost a necessity since vast majority of T1D patients have either the DR4-DQ8 or DR3-DQ2 haplotype or both, and because patients with a protective DR-DQ genotype are fairly rare (Erlich, et al., 2008; Hermann, et al., 2003; Ilonen, et al., 2016; Noble & Valdes, 2011). This indicates a critical role for the CD4+ T cells in triggering the autoimmunity. The fact that DR-DQ risk haplotypes are associated with the appearance of autoantibodies, suggest that autoreactive CD4+ T cells with specificity for MHC class II risk molecules (and islet autoantigens) are required for the activation of B cells becoming autoantibody producing plasma cells. With new epitope-specific HLA tetramer assays, β-cell autoantigen-specific CD4+ T cells have been detected in T1D patients and autoantibody positive children (Oling et al., 2012; Roep & Peakman, 2012). Interestingly, in addition to autoantigens, both CD4+ and CD8+ T cells recognize post-translationally modified peptides and hybrid epitopes (that are usually not targeted by autoantibodies) specific for β-cells (Delong et al., 2016; McLaughlin et al., 2016; van Lummel et al., 2014; Yang et al., 2014; Yang et al., 2013). This suggests that loss of tolerance to β-cell autoantigens might result from proteins developed in β-cell stress.

The second crucial step appears to be maintaining and enhancing the autoimmunity. The presence of one diabetes-associated autoantibody has shown to be of little predictive value suggesting that additional factors are needed for the execution of the autoimmune process. Although these factors are largely unknown, β-cell stress (by external cause) has been strongly implicated for being the reason for initiation of the autoimmune reaction as well as maintaining it. A subset of CD4+ T cells called regulatory T cells (Tregs) are a major component of peripheral tolerance (central tolerance takes place in thymus). These cells suppress the immune system and a lack of Treg-mediated control has been shown to play a role

in numerous autoimmune diseases (Grant et al., 2015). Their role in the pathogenesis of T1D has been studied widely with varying results, but there is evidence of altered function of Tregs (FOXP3+ and FOXP3- or Tr1 cells) in T1D patients, as well as, in individuals with HLA class II risk genotypes but without T1D (Hull et al., 2017). The involvement of Tregs in pathogenesis of T1D is supported by association of polymorphisms in genes that are likely involved in Treg function, such as *CTLA4*, *PTPN2*, *IL2RA*, *IL2* and *IL10*, with T1D (Todd 2010). Indeed, some of these polymorphisms (such as *PTPN2* and *IL2RA*) have been associated with reduced Treg IL-2 sensitivity in T1D patients, as well as, in healthy individuals (Hull, et al., 2017).

The role of other components of the immune system in the pathogenesis of T1D have not been considered of high importance. However, there is evidence that the complement system contributes to the development of T1D, and more specifically, the complement seems to have an important role in diabetic microvascular and macrovascular complications (Ajjan & Schroeder, 2019). Although diabetes-associated autoantibodies are not considered as major players in β-cell death, humoral immunity could have an enhancing effect in pathogenesis of T1D. For example, after depletion of B cells by rituximab (anti-CD20 monoclonal antibody) patients with newly diagnosed T1D showed reduced loss of endogenous insulin production (Pescovitz et al., 2014). Also, it has been shown that T1D patients have two types of insulitic profiles depending on whether insulitis lesions have high or low proportion of CD20+ B cells (Leete et al., 2016). Moreover, the insulitic profile with high proportion CD20+ B cells correlated strongly with lower age at disease onset, lower β-cell mass and lower proportion of residual insulin-containing islets indicating a more fulminant form of the disease in patients with this profile (Leete, et al., 2016). The B cells might enhance the survival of activated CD8+ T cells via cross-presentation of autoantigen peptides by HLA class I molecules (Hinman et al., 2014).

The hyperexpression of HLA class I and the predominance of CD8+ T cells in the insulitis lesion indicates a crucial role for HLA class I molecules and CD8+ T cells in β-cell death. Based on previous studies and the results presented here, it is acceptable to presume that the HLA class I and therefore CD8+ T cells are not key players in the initiation of the autoimmunity. Instead, the autoreactive CD8+ T cells are apparently crucial in β-cell killing after the autoimmune process has been set to motion. The HLA molecules encoded by the A*24 and B*39 alleles might, for example, have higher affinity for autoantigenic epitopes than molecules encoded by non-associated alleles and thus promote faster β-cell killing. Both the A*24 and B*39:06 allele have been shown to directly interact with preproinsulin and insulin B specific CD8+ T cells in T1D patients and that these T cells can probably directly kill β-cells (Yeo et al., 2020).

In the past it has been considered that (nearly) all β-cells die in pathogenesis of T1D. However, new evidence has emerged showing that β-cell loss can be highly variable among patients. It seems that especially people with older age at onset retain significant numbers of insulin-producing β-cells even long after diagnosis (Leete, et al., 2016). If the CD8+ T cells are indeed the major mechanism for β-cell destruction, then perhaps the HLA molecules encoded by diabetes-associated HLA class I alleles, such as the A*24 and B*39, present β-cells autoantigens to CD8+ T cells more efficiently. One could hypothesize that this might account for the islet phenotype with lower β-cell mass and younger age at onset, indicating a more aggressive form of diabetes-associated autoimmunity. However, this is merely a hypothesis and other genetic and external factors are more than likely to be involved as well. Still, this is a matter that would be worth to investigate. Although many genes have been associated with T1D, the association of the HLA class II and I variants have been the strongest ones implicating the crucial role of the molecules encoded by these variants in the T1D associated autoimmunity. However, the acknowledgement of other genetic variants associated with the disease is important since it is likely that specific combinations of different diabetes-associated alleles are required for disease onset, and that different combinations may account for differences in the level of aggressiveness of diabetes-associated autoimmunity.

6.6 Strengths and limitations of the study

A great strength of this study was the two large sample series essential to HLA association studies. The results from the FPDR families can be directly applied to the Finnish population since according to the PEDIA website the register represents over 90% of children with T1D in Finland (Pediatric Diabetes Research Group, 2020). The prospective follow-up cohort from the DIPP study is a decent representation of diabetics and subjects at risk in Finland since the follow-up cohort entails 60% of genotypes found in T1D patients. Also, the nature of the study has enabled to obtain well matched controls. However, unfortunately for this thesis work the HLA-DR-DQ included in the DIPP did not contain such haplotypes that were crucial for testing the B*39:06 effect in various stages of diabetes related autoimmunity.

The laboratory and statistical methods were well documented and described before. The HLA class I DELFIA assays could not distinguish between heterozygotes and homozygotes, but with rare alleles such as the B*39:01 and B*39:06 the frequency of homozygotes is low and the effect of false heterozygotes on association analysis results is exceedingly small. Nevertheless, in study II, it was decided to discard non-transmitted haplotypes with no definite class I allele

status. This resulted in small loss of power, but the results were essentially the same when those non-transmitted haplotypes (with class I allele status stated as negative) included in analysis (data not shown). For the DIPP data this was no issue since there were no artificial controls to construct and the subjects were simply positive or negative for a given allele.

6.7 General discussion and future prospects

Several factors make Finland an ideal working ground for type 1 diabetes study. First of all, Finland has the highest incidence of T1D in the world. Secondly, the genetic base of the Finnish population is relatively homogeneous and conserved which is helpful when studying the polymorphic HLA gene area. Thirdly, Finland has essentially free high-grade health care system and the Finns have a positive attitude towards research. This has enabled researchers to collect comprehensive sample registers such as The Finnish Pediatric Diabetes Register and the large follow-up cohort of the Finnish type 1 Diabetes Prediction and Prevention Study. These elements have given this doctoral thesis work a unique opportunity to study the complex genetic risk factors of T1D, as well as the involvement of these factors in the events leading to disease.

This was the first large-scale T1D study in Finland assessing the role of the A*24 allele and B*39 subtypes in context of DR-DQ haplotypes and genotypes as well as their role in the autoimmunity leading to disease. The A*24 and B*39:06 effect has been described in large scale in other populations before and can now be confirmed in the Finnish population as well. Combining previous results with the data presented here the B*39:01 allele seems to have a bigger role in T1D risk in Finland than some other populations. The interaction of the A*24 and B*39:01 alleles on the DRB1*04:04-DQ8 haplotype remained inconclusive and thus needs to be studied further. Another interesting matter for additional investigation is the role of the DR3-DQ2 haplotype in A*24, B*39:01 and B*39:06 positive genotypes since this association was not detected when haplotypes were analyzed. Finally, it would be fascinating to examine the class I genotype (in context of class II genotypes) association with T1D and with the autoimmunity behind the disease, but for conclusive results, this would need a huge dataset.

In future, HLA class I alleles could complement screening for people at T1D risk for studies and trials and help to reveal the heterogeneity within the disease. In light of what is already known about class I alleles, screening of certain class I variants would help to identify individuals with elevated risk for fast progression to disease. The specific role of these alleles in the disease process is still largely unknown and needs to be studied. This could establish the exact mechanisms

leading to disease which would most probably help in the goal of disease prevention.

7 Conclusions

The main conclusions of this study include:

1. The HLA-A*24, B*39:01 and B*39:06 alleles are associated with T1D, but the effect is restricted to certain haplotypes.
2. The role of the HLA class I in the T1D related autoimmunity was confirmed as the A*24 and B*39:01 alleles conferred faster progression from positivity of multiple autoantibodies to overt diabetes but had no effect on the first appearance of autoantibodies.
3. The effect on progression rate was restricted to the DRB1*04:04-DQ8/DR3-DQ2 genotype for both A*24 and B*39:01 alleles suggesting a common pathway in disease etiology.
4. In line with this, the A*24 allele was associated with steeper decline in FPIR in subjects with multiple autoantibodies but not in subjects with only one or no persistent autoantibody.
5. The HLA-B*18 is not associated with T1D in Finland, at least not in the context of the DRB1*04:04-DQ8 haplotype
6. The role of the HLA-B*39:01 in T1D association might be more substantial in Finns than in many other populations.
7. The results support disease heterogeneity in T1D.

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