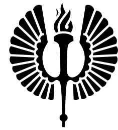
# POLYMORPHISMS AND GENETIC SUSCEPTIBILITY OF TYPE 1 DIABETES AND ADDISON'S DISEASE

Zsófia Gombos

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA - SER. D OSA – TOM. 1390 | MEDICA – ODONTOLOGICA | TURKU 2018




# POLYMORPHISMS AND GENETIC SUSCEPTIBILITY OF TYPE 1 DIABETES AND ADDISON'S DISEASE

Zsófia Gombos

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA - SER. D OSA - TOM. 1390 | MEDICA – ODONTOLOGICA | TURKU 2018

## University of Turku

Faculty of Medicine
Institute of Biomedicine
Medical Microbiology and Immunology
Turku Doctoral Programme of Molecular Medicine (TuDMM)
Immunogenetics Laboratory


## Supervised by


Professor Jorma Ilonen
University of Turku, Faculty of Medicine
Institute of Biomedicine
Immunogenetics Laboratory
Turku, Finland


## Reviewed by


Docent Päivi Saavalainen
Haartman Institute
Immunology Research
University of Helsinki
Helsinki, Finland

Associate professor Tiinamaija Tuomi
Haartman Institute
Immunobiology Research
University of Helsinki
Helsinki, Finland


## Opponent


Professor Francesco Cucca
Medical Genetics at Faculty of Medicine
University of Sassari
Sassari, Italy


The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.


ISBN 978-951-29-7428-3 (PRINT)
ISBN 978-951-29-7429-0 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Grano OY- Turku, Finland, 2018

*To my family*

# ABSTRACT

Zsófia Gombos
**Polymorphisms and genetic susceptibility of Type 1 diabetes and Addison's disease**

University of Turku, Faculty of Medicine, Institute of Biomedicine, Medical Microbiology and Immunology, Turku Doctoral Programme of Molecular Medicine (TuDMM), Immunogenetics Laboratory, Turku, Finland
Annales Universitatis Turkuensis
Turku, Finland, 2017

The HLA region accounts for approximately half of the genetic susceptibility in type 1 diabetes. The strongest genetic association with type 1 diabetes (T1D) is conferred by HLA class II alleles, where particular combinations of the DRB1, DQA1 and DQB1 define disease risk. Our aim was to investigate the effect of non-class II loci independently of the DQ-DR genes and to localize them using case-control study and transmission study in nuclear families. We explored the contribution of selected microsatellite markers in the HLA class III and I regions covering a 4Mb region telomeric to the DQB1 gene and also the HLA-A and -B gene alleles were analyzed for diabetes association. The effect of found markers in the various phases of progression of autoimmunity was further looked in children participating in the Finnish Type 1 Diabetes Prediction and Prevention study.

In our case-control study (stratified subjects for DR3/DRB1*04:04 and DR3/DRB1*04:01 genotypes) we found that the microsatellite markers located between C12A and C143 near the HLA-B gene confer a strong association for T1D. HLA-B*39 allele in linkage disequilibrium with the HLA-A*24 allele was associated with the highest risk on the DRB1*04:04 haplotype.

We extended the case-control study for Finnish nuclear families with parents carrying either the DRB1*08-DQB1*04 (DR8) or the DRB1*04:04-DQB1*03:02 (DR4) haplotypes. On the DRB1*04:04-DQB1*03:02 haplotype the D6S273 and C125 microsatellite markers showed independent disease association, the C125*200 allele appeared at an increased frequency on the HLA-B*39 positive DRB1*04:04-DQB1*03:02 haplotypes, suggesting an independent effect. In addition, presence of the D6S273*137 allele appeared to increase the disease predisposing effect of the DRB1*04:04-DQB1*03:02 haplotype but it was not possible dissect its effect on the HLA-B*39. On the DRB1*08-DQB1*04 haplotype the C143, C245 and MOGc microsatellite markers showed disease association. However, correcting for multiple comparisons, the disease association turned out not significant. The D6S273*135 and C143*417 alleles showed protective effect when haplotype method was used. HLA-B*39:01 and B*39:06 alleles did not shown any effect in DRB1*08-DQB1*04 haplotype although B*39:06 was too rare to demonstrate its disease association, needing larger series.

The association between the HLA-DR-DQ haplotypes and class I HLA-A and -B alleles during the progression from autoantibody seroconversion to clinical disease was studied in 249 children. The Cox-regression multivariate analysis demonstrated a significant promoting effect of HLA-B*39 allele after seroconversion for the second biochemical autoantibody, whereas the HLA-A*03 allele was associated with protection after the first- and second biochemical autoantibody appearances. The HLA-B*39 effect during the disease was mainly found in children carrying the DR3/DR4 genotype.

Addison's disease is an organ-specific autoimmune disease, which is often found together with T1D as part of a polyendocrinopathy syndrome. Haplotype analysis using the microsatellite markers did not provide statistical support to the importance of HLA regions other than HLA-DR-DQ loci in three different European populations (Finnish, Estonian, Russian). We found that Addison's disease was preferentially associated with DRB1*04:04 and also DRB1*04:03 alleles together with DQB1*03:02 contrasting thus with T1D where DRB1*04:01 was the most strongly associated allele in DQB1*03:02 positive haplotypes.

**Keywords:** type 1 diabetes, Addison's disease, HLA, DQB1, DQA1, DRB1, microsatellite markers, association

# TIIVISTELMÄ

Zsófia Gombos

**Perinnöllinen alttius ja polymorfismit tyypin 1 diabeteksessa ja Addisonin taudissa**

Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Lääketieteellinen Mikrobiologia ja Immunologia, Molekyylilääketieteen tohtoriohjelma (TuDMM), Immunogenetiikan laboratorio, Turku, Suomi

Annales Universitatis Turkuensis

Turku, Suomi, 2017

Noin puolet tyypin 1 diabeteksen geneettisestä alttiudesta tulee HLA-geenialueelta. Voimakkain assosiaatio on luokan II HLA -geeneillä DRB1, DQA1 ja DQB1, joiden alleeliyhdistelmät määrittävät sairausriskin. Tämän työn tavoitteena oli tutkia DQ ja DR geeneistä riippumattomien HLA alueen varianttien vaikutusta sairausriskiin ja lokalisoida ne käyttämällä tapaus-verrokkitutkimusta sekä ydinperheiden transmissiotutkimusta. Tutkimme valikoituja mikrosatelliittimarkkereita luokan II ja I HLA alueilla, sekä HLA-A ja -B alleelien assosiaatiota tyypin 1 diabetekseen. Löydettyjen markkereiden vaikutusta autoimmuniteetin etenemisen eri vaiheisiin tarkasteltiin lapsilla, jotka osallistuivat tyypin 1 diabeteksen ennustamista ja ehkäisyä tutkivaan projektiin.

Tapaus-verrokkitutkimuksessamme, jossa nämä kaltaistettiin DR3/DRB1*04:04 ja DR3/DRB1*04:01 genotyyppien suhteen, voimakkaimman assosiaation tuottivat C12A ja C143 mikrosatelliittien välissä olevat markkerit lähellä HLA-B geeniä. DRB1*04:04 haplotyypissä vahvin riski liittyi HLA-B*39 alleeliin, joka oli kytkentäepätasapainossa HLA-A*24 alleelin kanssa.

Tutkimusta laajennettiin DRB1*08-DQB1*04 (DR8) ja DRB1*04:04-DQB1*03:02 (DR4) positiivisiin suomalaisiin ydinperheisiin. Mikrosatelliittimarkkerit D6S273 ja C125 osoittivat itsenäistä assosiaatiota DRB1*04:04-DQB1*03:02 haplotyypissä. C125*200 -alleelin frekvenssi oli lisääntynyt HLA-B*39 positiivisessa DRB1*04:04-DQB1*03:02 haplotyypissä viitaten itsenäiseen efektiin. Lisäksi D6S273*137 alleeli vaikutti lisäävän DRB1*04:04-DQB1*03:02 haplotyypin altistavaa efektiä, mutta assosiaatiota ei pystytty erottamaan HLA-B*39 alleelin assosiaatiosta. DRB1*08-DQB1*04 haplotyypissä mikrosatelliittimarkkerit C143, C245 ja MOGc assosioituvat tautirskiin, mutta monivertailun korjauksen jälkeen assosiaatio ei enää ollut merkitsevä. Haplotyyppimenetelmän perusteella alleelit D6S273*135 ja C143*417 osoittivat suojaavaa vaikutusta. HLA-B*39:01 ja B*39:06 alleeleilla ei ollut merkitsevää vaikutusta DRB1*08-DQB1*04 haplotyypissä, mutta B*39:06 alleelin matala frekvenssi todennäköisesti selittää sen, miksi suuremmissa aineistoissa löydettyä assosiaatiota ei pystytty havaitsemaan.

HLA-DR-DQ haplotyyppien ja luokan I HLA-A ja -B -alleelien välistä yhteyttä taudin etenemiseen autovasta-aineiden serokonversiosta kliiniseen tautiin tutkittiin 249 lapsen avulla. Coxin regressioanalyysi osoitti HLA-B*39 alleelin nopeuttavan taudin kehittymistä, kun taas HLA-A*03 oli assosioitui suoja vaikutukseen ensimmäisen ja toisen autovasta-aineen ilmentymisen jälkeen. HLA-B*39 alleelin vaikutus taudin etenemiseen havaittiin pääasiassa lapsilla, joilla oli DR3/DR4-genotyyppi.

Addisonin tauti on elinspesifinen autoimmuunisairaus, joka esiintyy usein tyypin 1 diabeteksen kanssa osana polyendokrinopatiasyndroomaa. Tilastollista tukea HLA-DR-DQ lokuksen ulkopuolisten HLA alueen varianttien tärkeydelle ei löydetty kolmessa erilaisessa eurooppalaisessa populaatiossa (suomalaiset, virolaiset, venäläiset) mikrosatelliittimarkkereiden haplotyyppianalyysin perusteella. Havaitsimme, että Addisonin tauti assosioitui DRB1*04:04 ja DRB1*04:03 alleelien kanssa, erottuen tyypin 1 diabeteksesta, jossa DRB1*04:01 on voimakkaimmin assosiaatuva DRB1 -alleeli DQB1*03:02 positiivisissa haplotyypeissä.

**Avainsanat:** tyypin 1 diabetes, Addisonin tauti, HLA, DQB1, DQA1, DRB1, mikrosatelliitimarkkerit, assosiaatio

# TABLE OF CONTENTS

**ABSTRACT** ... 4
**TIIVISTELMÄ** ... 5
**TABLE OF CONTENTS** ... 6
**ABBREVIATIONS** ... 8
**LIST OF ORIGINAL PUBLICATIONS** ... 10
**1. INTRODUCTION** ... 11
**2. REVIEW OF LITERATURE** ... 12
2.1. Type 1 diabetes ... 12
2.1.1. Pathogenesis of type 1 diabetes ... 12
2.1.2. Environmental factors ... 15
2.1.3. Epidemiology ... 17
2.2. Genetics of type 1 diabetes ... 20
2.2.1. Human leukocyte antigen (HLA) ... 21
2.2.1.1. Structure and function of the HLA genes and molecules ... 21
2.2.1.2. HLA variation in T1D ... 26
2.2.1.3. The nomenclature of HLA alleles ... 27
2.2.2. HLA class II region and genetic risk of type 1 diabetes ... 28
2.2.3. Genetic risk for type 1 diabetes associated with polymorphism of MHC class I genes HLA-A, HLA-B and HLA-C ... 30
2.2.4. Genes in the “HLA class III” region ... 32
2.2.5. Microsatellite markers ... 32
2.2.6. T1D susceptibility genes outside the HLA region ... 36
2.2.6.1. Insulin gene polymorphism ... 36
2.2.6.2. CTLA-4 and PTPN22 gene polymorphisms ... 37
2.2.6.3. New generation of genetic studies in Type 1 Diabetes ... 38
2.3. Addison’s disease ... 40
2.3.1. Addison’s disease pathogenesis and etiology ... 40
2.3.2. Epidemiology ... 41
2.3.3. Autoantibodies in Addison’s disease ... 41
2.3.4. Genetic of Addison’s disease ... 42
**3. AIMS OF THE STUDY** ... 44
**4. MATERIALS AND METHODS** ... 45
4.1. Materials ... 45

4.2. Methods ........ 46
4.2.1. DNA ........ 46
4.2.2. HLA class II genotyping ........ 46
4.2.2.1. HLA-DQB1 genotyping ........ 47
4.2.2.2. HLA-DQA1 genotyping ........ 47
4.2.2.3. HLA-DR4 subtyping ........ 47
4.2.3. HLA class I genotyping ........ 47
4.2.4. Genotyping of microsatellites ........ 48
4.2.5. The autoantibody assays ........ 48
4.3. Statistical analysis and computer software ........ 48
**5. RESULTS ........ 50**
5.1. DRB1*04:04-DQB1*03:02 haplotype associated risk ........ 50
5.1.1. Localization of the class I and class III gene region genes affecting T1D susceptibility in HLA-DR/DQ matched case-control series (Publication I) ........ 50
5.1.2. Reconstruction of the extended haplotypes in families (Publication I-II) ........ 56
5.1.3. Localization of the independent disease association of the class I and class III gene region in Finnish nuclear families (Publication II) ........ 58
5.2. HLA-DR8-DQ4 haplotype associated risk (Publication II and Unpublished data) ........ 61
5.3. HLA class I region effect on the progression from diabetes associated Case-control series in three different populations (Publication IV) ........ 64
5.4. HLA class II and class I region effects on the Addison's disease susceptibility. Case-control series in three different populations (Publication IV) ........ 67
**6. DISCUSSION ........ 69**
6.1. Type 1 diabetes ........ 69
6.2. Addison's disease ........ 74
**7. CONCLUSIONS ........ 75**
**8. ACKNOWLEDGEMENTS ........ 76**
**9. REFERENCES ........ 78**
**10. ORIGINAL PUBLICATIONS I-IV ........ 99**

# ABBREVIATIONS

| | |
|---|---|
| ACAs | Adrenal cortex antibodies |
| ACTH | Adrenocorticotropic hormone |
| AAD | Autoimmune Addison’s disease |
| AD | Addison’s disease |
| AFBAC | Affected-Family-Based Artificial Control |
| APC | Antigen presenting cells |
| APS | Autoimmune polyendocrine syndrome |
| ASP | Affected sib-pair families |
| Asp | Aspartic acid |
| BB | BioBreeding |
| bp | Base pairs |
| CETDT | Conditional Extended Transmission Disequilibrium Test |
| cM | Centimorgan |
| CK | Control subjects |
| CM | Cow’s milk |
| CTLA-4 | Cytotoxic T-lymphocyte antigen-4 |
| CVB | Coxsackievirus B |
| DF | Degrees of Freedom |
| DIPP | Type 1 diabetes Prediction and Prevention study |
| Eu | Europium |
| GABA | γ-aminobutyric acid |
| GAD | Glutamic acid decarboxylase |
| GADA | Glutamic acid decarboxylase autoantibodies |
| GWAS | Genome-wide association studies |
| HBDI | Human Biological Data Interchange |
| HLA | Human leukocyte antigen |
| HM | Haplotype method |
| IAA | Insulin autoantibodies |
| IA-2A | Insulinoma-associated protein 2 autoantibodies |
| IAA | Insulin autoantibodies |
| ICA | Islet cell autoantibodies |
| INS | Insulin gene |

| | |
|---|---|
| JDFU | Juvenile Diabetes Foundation Units |
| kbp | Kilobase pair |
| kDa | kilodalton |
| LD | Linkage disequilibrium |
| LYP | Lymphoid tyrosine phosphatase |
| MMR | Mumps-Measles-Rubella |
| Mb | Mega base pairs=1000 000 base pairs |
| MHC | Major histocompatibility complex |
| Msats | Microsatellites |
| NKG2D | Natural Killer Group 2D |
| NOD | Non-obese diabetic |
| OR | Odds ratios |
| PIC | Polymorphism information content |
| PTPN22 | Protein tyrosine phosphatase 22 gene |
| RT-PCR | Reverse Transcription-Polymerase Chain Reaction |
| RU | Relative Unit |
| SNP | Single nucleotide polymorphism |
| Sm | Samarium |
| SSRs | Simple sequence repeats |
| SSP | Sequencing-specific primer |
| STR | Short tandem repeat |
| Tb | Terbium |
| T1DGC | Type 1 Diabetes Genetics Consortium |
| TDT | Transmission/Disequilibrium Test |
| T1D | Type 1 diabetes |
| TNF | Tumor necrosis factor |
| TNFα | Tumor necrosis factor alpha |
| TRF | Time-resolved fluorescence |
| TRIGR | Trial to Reduce IDDM in the Genetically at Risk |
| VNTR | Variable number tandem repeat |
| WES | Whole exome sequencing |
| ZnT8A | Zinc transporter 8 autoantibody |
| β2m | Beta2-microglobulin |

# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals I-IV.

I. Nejentsev S, Gombos Zs, Laine A-P, Veijola R, Knip M, Simell O, Vaarala O, Hans K. Åkerblom HK, Ilonen J. Non-class II HLA gene associated with type 1 diabetes maps to the 240-kb region near HLA-B. Diabetes. 49: 2217-2221, 2000

II. Gombos Zs, Wachowicz J, Veijola R, Åkerblom HK, Simell O, Knip M, Ilonen J, Hermann R. Human leukocyte antigen non-class II determinants for type 1 diabetes in the Finnish population. Human Immunology. 67: 714-721, 2006

III. Lipponen K, Gombos Zs, Kiviniemi M, Siljander H, Lempainen J, Hermann R, Veijola R, Simell O, Knip M, Ilonen J. Effect of HLA class I and class II alleles on progression from autoantibody positivity to overt type 1 diabetes in children with risk-associated class II genotypes. Diabetes. 59: 3253-3256, 2010

IV. Gombos Zs, Hermann R, Kiviniemi M, Nejentsev S, Reimand K, Fadeyev V, Peterson P, Uibo R, Ilonen J. Analysis of extended human leukocyte antigen haplotype association with Addison's disease in three populations. European Journal of Endocrinology. 157: 757-761, 2007

The original publications have been reproduced with the permission of the copyright holders.

# 1. INTRODUCTION

Autoimmune diseases affect up to 10% of the general Caucasian populations and include the organ-specific disorders such as type 1 diabetes (T1D) and Addison's disease (AD) with multifactorial etiology where both genetic and environmental components contribute to the disease risk (You, Henneberg 2016).

The human leukocyte antigen system (HLA; 6p21.3; 7.6Mb; 252 genes) consists of large number of genes (28%) with immune functions and confers the strongest genetic risk for type 1 diabetes and Addison's diseases. Among HLA region genes are the classical HLA class I (HLA-A, HLA-B, HLA-C) and class II (HLA-DRB, HLA-DQ, HLA-DP) genes, which encode antigen presenting molecules. HLA class III is a diverse collection of more than 20 genes including those encoding some complement proteins. Variations within HLA have been found to be associated with almost every autoimmune disease, with risk estimates exceeding by far those of other genetic susceptibility factors identified in these complex diseases. Strong linkage disequilibrium (LD) in the region has, however, complicated the identification of the causative variants. Population studies have shown that associations exist between different autoimmune diseases and particular peptide-presenting HLA class I or II molecules. Moreover, the HLA class I and class II genes are the most polymorphic loci in the human genome.

It should be noted that although HLA class I and class II show the strongest association in almost all autoimmune diseases, several other genes outside the classical HLA loci contribute to the diseases appearance.

The aim of the study was to investigate the effect of non-class II HLA loci in T1D and AD susceptibility by analyzing microsatellite markers in the HLA class III and class I region. In addition, our aim was to explore the effects of the HLA class I (common HLA-A and HLA-B) and class II alleles (HLA-DRB1, HLA-DQB1, HLA-DQA1) on progression from autoantibody positivity to overt T1D.

# 2. REVIEW OF LITERATURE

## 2.1. Type 1 diabetes

### 2.1.1. Pathogenesis of type 1 diabetes

Type 1 diabetes (T1D) is a T-cell mediated autoimmune disease involving the specific destruction of insulin-producing β-cells in the islets of Langerhans in pancreas. Immune-mediated T1D sometimes is referred to as type 1A. However, not all patients with type 1 diabetes have any evidence of autoimmunity. According to the recent American Diabetes Association classification this subgroup of autoantibody negative patients should be considered as type 1B (idiopathic) diabetes, reported mainly in African and Asian ancestry (American Diabetes Association 2012).

Both major classes of T cells (CD4+ and CD8+ T cells) are important in the autoimmune process. Destruction or loss of function of the β-cells is leading to loss of insulin secretion, and thus to an elevated blood glucose level and appearance of glucose in urine. The disease is most often diagnosed in children and adolescents, usually with classical symptoms, such as polydipsia, polyuria, fatigue, blurred vision and unexplained weight loss, finally ketoacidosis (Atkinson, Eisenbarth & Michels 2014, WHO-Type 1 diabetes). The duration of the asymptomatic preclinical period, the process leading to clinical symptoms, may last from few weeks up to several years. T1D appears when the function of most of the β-cells (85-90%) is lost and insulin produced by the remaining cells is not enough to control the blood-glucose level. Insulitis, chronic and uneven infiltration of inflammatory cells into islet, precedes the clinical disease (Figure 1) (Atkinson et al. 2015).

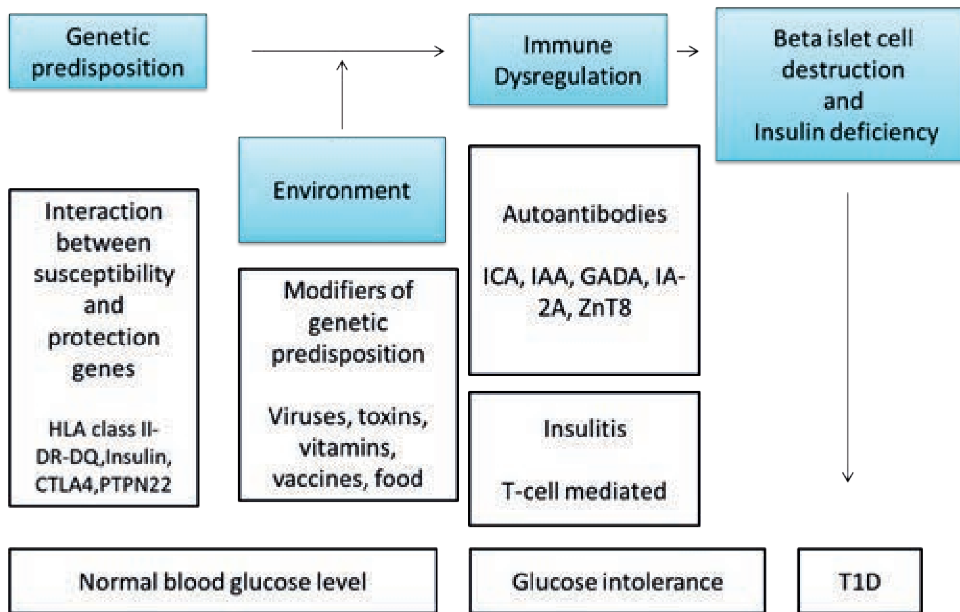



**Figure 1.** A simplified modern model for pathogenesis of type 1 diabetes (Modified from Atkinson et al. 2015).

The clinical manifestation of type 1 diabetes is the endpoint of a usually long-lasting immune-mediated destruction of the β-cells. At clinical diagnosis about 96-98% of patients with T1D have detectable titers of one or more autoantibodies against islet autoantigens in the peripheral blood, showing the autoimmune nature for the disease (Bingley 2010, Knip et al. 2016). Children with seroconversion to any two autoantibodies have a risk of >80% for the development of diabetes during childhood or adolescence at the 15-year follow-up (Ziegler et al. 2013). In contrast, 0.5% of the background population and 3-4% of first-degree relatives of T1D patients are positive for autoantibodies (Knip et al. 2010). Marker autoantibodies can appear as early as 6 months of age, with peak incidence before 2 years of age in the genetically susceptible children, and they are present months and years before manifestation of T1D (Atkinson, Eisenbarth & Michels 2014). These autoantibodies include islet cell autoantibodies (ICA), that were identified by indirect immunofluorescence assay using pancreatic tissue sections from blood group 0 organ donors as substrate to bind islet cell reacting antibodies in the studied sera (Bottazzo, Florin-Christensen & Doniach 1974). These antibodies represent a heterogeneous group which recognize various molecules like GADA (Baekkeskov et al. 1990), IA-2A (Gianani et al. 1995) and ZnT8A (Wenzlau et al. 2007). In addition islet specific autoantibodies include IAA, which are specific for β-cells

(Palmer et al. 1983). These four autoantibodies are antibodies to specific autoantigens and analyzed by specific radiobinding assays and therefore so-called biochemical autoantibodies. The general view is that the diabetes-associated autoantibodies are not directly involved in the β-cell destruction but function as biomarkers of an ongoing disease process. These diabetes-associated autoantibodies are the best characterized markers for future development of clinical T1D (Knip et al. 2016).

Presence of autoantibodies specific for only one protein have a low predictive value for the development of T1D, but combinations of multiple specific autoantibodies and high titers of them correlate strongly with risk of developing T1D and can be used as predictors (Knip et al. 2016, Ziegler et al. 2013). Approximately half of the children positive for multiple autoantibodies will develop T1D within 5 years whereas that proportion increased up to 84% during 15 years of follow-up. Younger children diagnosed with T1D are more often positive for IAA than older children or adults. In contrast, GADA, IA-2A and ZNT8A are found more evenly throughout childhood, GADA and ZNT8A being also common in adults diagnosed with T1D. IA-2A and ZnT8A autoantibodies common at diagnosis are rare as primary antibodies (Ilonen et al. 2018, Wenzlau et al. 2007). Persons with IA-2A and ZnT8A tend to progress more rapidly to T1D than persons without these antibodies (Regnell, Lernmark 2017)

Relationship between primary diabetes-associated autoantibodies and various HLA genotypes and alleles have been described in different studies. IAA is frequently the first autoantibody to appear in young children with appearance peak during the second year of life in the preclinical phase (Ilonen et al. 2013, Krischer et al. 2015). Only 75% of children positive for IAA autoantibody at diagnosis, in contrast, GADA, IA-2A or ZnT8A as the initial autoantibody were typically positive at diagnosis with 89.5%, 100% and 100%. That children remaining positive for IAA at diagnosis had a longer preclinical period duration as measured from seroconversion to diagnosis. IAA is associated with HLA-DRB1*04:01/2/4/5-DQB1*03:02 (commonly referred to as HLA-DR4-DQ8) risk haplotype, whereas primary GADA associated with DQA1*05-DQB1*02 (commonly referred to as HLA-DR3-DQ2) haplotype. The genetic associations of primary IAA- and primary GADA-association autoantibody predominate only in children diagnosed before the age of 10 years (Ilonen et al. 2018). Genes within the HLA-DQ gene region have been suggested to be primary denominators in the production of GADA association, whereas the HLA-DRB1 region might be more important in formation of IA-2A (Sanjeevi et al. 1998). This is supported by the observation that among DR4-DQ8 haplotypes DRB1*04:01 is strongly increased compared to the other common DR4 allele, DRB1*04:04, in patients with IA-2A as the only autoantibody present at diagnosis (Mäkinen et al. 2008).

### 2.1.2. Environmental factors

Environmental factors play an important role in the pathogenic process as demonstrated by the rapid increase in the disease incidence in most western countries during the last decades (DIAMOND Project Group 2006, Patterson et al. 2009). Some studies observations suggest that the proportion of patients with high-risk HLA genotypes has decreased, whereas the proportion of those with low-risk or protective HLA genotypes has increased over the last decades (Fourlanos et al. 2008, Gillespie et al. 2004, Hermann, Turpeinen et al. 2003, Vehik et al. 2008). These data are compatible with an increased environmental pressure. The roles of microbial infections including composition of human microbiota as well as partially intertwining nutritional factors have been targets of greatest interest (Knip, Simell 2012).

The development of T1D has 13 to 50% concordance in monozygotic twins which has been regarded to support the influence of environmental factors acting on genetic predisposition (Hyttinen et al. 2003, Redondo et al. 2001, Redondo et al. 2008). On the other hand, although monozygotic twins have the same or very similar DNA sequence and share the same environment, gene expression and DNA modification patterns in both cellular and humoral immunity, T-cell receptors and autoantibodies can differ significantly, meaning that monozygotic twins do not share similar immune repertoire. This challenges the conventional paradigm that monozygotic twins are identical genetic controls in which environment is the only differing variable (Haque, Gottesman & Wong 2009).

Some migration studies have suggested that moving from a low- to high incidence countries increase the incidence, emphasizing the influence of environmental conditions (Hussen, Persson & Moradi 2013, Oilinki et al. 2012, Sonigni, Lombardo 2010). Several studies among second-generation immigrants, adoptees from abroad and also some Sardinian immigrants appear to be in contrast with the environmental hypostasis and demonstrated original risk to remain in new environment (Ji et al. 2010, Sonigni, Lombardo 2010). This may indicate that both genetic and environmental factors can be responsible for differences in the incidence.

Several viruses like rotavirus, mumps virus and cytomegalovirus have been suggested to be associated with the development of T1D but currently the prime viral candidates are the enteroviruses such as Coxsackieviruses group B (CVB) (Rodriguez-Calvo et al. 2016). It has been shown in many different studies that antibodies against enteroviruses are more common in newly diagnosed T1D patients than in nondiabetic controls. Even though the enterovirus infections are less

frequent in Finland than in countries with lower incidence. Temporal association between seroconversion for the first diabetes-associated autoantibodies and enterovirus infections has been also reported. The long subclinical phase of the β-cell damaging process makes it difficult to identify a virus which could play role in the early stage of autoimmune process. Although it may be unlikely that T1D is caused by only the CVB1 serotype, whereas CBV3 and CBV6 infections may protective. The identification of this specific strain would be beneficial and would facilitate the design of an enterovirus vaccine (Knip, Simell 2012, Hyöty 2016).

Rotaviruses are very common gastrointestinal viruses that cause intestinal infection as well as viremia in young children. In the Australian BabyDiab Study, rotavirus infections were temporally associated with increases in islet autoantibodies (IAA, IA-2 and GAD65) in children before they developed diabetes (Rodriguez-Calvo et al. 2016).

In general, evidence from animal models suggests that viral infections can trigger the development of autoimmunity and diabetes caused by different mechanisms (Craig et al. 2013). Several mechanisms have been proposed for virus-mediated autoimmunity. Firstly, increasing evidence suggest that some virus infections can directly infect pancreatic tissues upregulating MHC class I molecules on β-cells or cause β-cells damage leading to the release of autoantigens and thus initiate the autoimmune process (Jun, Yoon 2003). Secondly, molecular mimicry hypothesis states that where viral proteins show close similarity to host tissue, they could induce cross reactivity responses against the self-antigen. For example, the P2-C protein sequence of Coxsackievirus B4 virus is partially similar to human GAD65 and within rotavirus VP7 protein there is high sequence similarity to T cell epitope peptides in the islet autoantigens like tyrosine phosphatase-like insulinoma antigen 2 and GAD65 which might be stimulating T cells to cross-react causing damage to host tissue (Rodriguez-Calvo et al. 2016).

Various dietary factors have also been linked to T1D during infancy and childhood. The duration of breastfeeding has shown inverse correlation with incidence of T1D (Lund-Blix et al. 2015) but early dietary exposure to supplementary cow's milk (CM) based formula and also other diet constituents like fruits, berries and roots have been associated with risk to T1D (Virtanen 2016). Cow's milk proteins, such as bovine serum albumin (Karjalainen et al. 1992), β-lactoglobulin (Vaarala et al. 1996), β-casein (Cavallo et al. 1996) and bovine insulin (Vaarala et al. 2012) obtained during infancy have been suggested as predisposing factors of T1D. CM proteins in infant nutrition do not cause diabetes directly. A possible explanation for this finding, higher cow's milk intake might promote progression to T1D in children with islet autoimmunity (Rewers, Ludvigsson 2016).

However, the large clinical TRIGR (Trial to Reduce IDDM in the Genetically at Risk) trial using hydrolyzed casein formula did not reduce the incidence of type 1 diabetes in the first seven years (Knip et al. 2014). Some chemicals in food, such as nitrates, nitrites, N-nitroso and low zinc in the drinking water have also been suggested to increase the risk for T1D (Samuelsson et al. 2011).

Lack of vitamin D supplementation in infancy has been shown to increase the risk of T1D in a European case-control study from seven centers (The EURODIAB Substudy 2 Group 1999). This study indicated that supplementation with vitamin D resulted in a reduced risk of T1D. Similar finding was seen in a Finnish birth cohort study where the daily high-dose D vitamin (2000 IU/day) supplementation decreased the risk when compared to no supplementation group (Hyppönen et al. 2001). A meta-analysis based on five studies concluded that vitamin D supplementation in early childhood may offer protection against the development of T1D (Zipitis, Akobeng 2008). On the other hand, prospective Diabetes Autoimmunity Study in the Young (DAISY) has found that neither the intake of D vitamin nor circulating plasma 25-hydroxyvitamin D concentration throughout childhood correlated with increased risk of β-cell autoimmunity or progression to T1D (Simpson et al. 2011). In addition, comparisons between neighboring low and high incidence countries did not find significant differences in the circulating vitamin D concentrations in pregnant women and schoolchildren (Viskari et al. 2006).

According to a widely accepted hypothesis, T1D is a multifactorial disease, clinical manifestation results from interaction between environmental and genetic factors and still poorly known environmental factors trigger an autoimmune process in a genetically susceptible individual leading to the destruction of the β-cells.

### 2.1.3. Epidemiology

According to the International Diabetes Federation, more than 132 600 children and adolescents under 19 years are estimated to be diagnosed with T1D annually worldwide. In 2017, altogether 1,106 500 (0-19 years) children and adolescents were estimated to have type 1 diabetes globally. Worldwide more than 20 million people are suffering with T1D (IDF Diabetes Atlas 2017). The epidemiologic patterns of the T1D regarding geographic distribution, gender, age of onset, as well seasonal changes and ethnic factors in populations are well studied. The incidence rates of childhood-onset T1D in the age group between 0-14 years varies considerably with geographic region (Galler et al. 2010).

Europe has by far the most informative and reliable data of T1D incidences (The EURODIAB ACE study: Europe and Diabetes). Etiology of Childhood Diabetes on an epidemiological basis from 1988 with 44 European centers participating (The EURODIAB ACE Study Group 2000). The WHO Multinational Project for Childhood Diabetes (DIAMOND) was started in 1990. The DIAMOND incidence study includes 114 populations from 57 countries from around the world, representing about 84 million children up to the year 2000 (DIAMOND Project Group 2006).

The incidence of T1D is increasing worldwide despite wide variations between continents, countries and regions (Diaz-Valencia, Bougneres & Valleron 2015). The incidence rates are commonly divided into five different groups: very low (<1/100 000/year), a low (1-4.9/100 000/year), an intermediate (5-9.99/100 000 per year), high (10-19.99/100 000 per year) and very high (≥20/100 000/year) (Karvonen et al. 2000). The highest incidence rates are found among Caucasians especially in Northern Europe (Finland, Sweden, Norway with ≥30/100 000/year), whereas the lowest ones found in Eastern Asia and South America (≤1/100 000/year). The worldwide incidence of T1D is described to vary at least 100- to 350-fold among 100 different countries (Borchers, Uibo & Gershwin 2010, Karvonen et al. 2000) being highest in Finland (>60/100 000 per in each year) (Harjutsalo et al. 2013) and Sardinia (around 50 cases/100 000 per in each year) (Bruno et al. 2013). By contrast, disease incidence is lowest in India (South Asia), Venezuela (South America) and China (Eastern Asia) with approximately 0.1 new cases in 100 000 children per each year (Borchers, Uibo & Gershwin 2010, http://www.diabetesatlas.org). Considerable differences in the frequency of genetic factors associated with T1D susceptibility and protection are found between various populations. The typical Caucasoid HLA susceptibility haplotypes and genotypes are rare among the Asians (Park, Eisenbarth 2001). This variation of risk and protective alleles might relate to the variable incidence rates among populations but considerable differences have also been found between populations with similar genetic background (Karvonen et al. 2000).

The general tendency in Europe is an increasing gradient from south to north with the lowest incidence countries such as Macedonia (3.6/100 000/year) and Greece (4.6/100 000/year) in south and very high incidence countries Finland, Sweden and Norway (≥60, 40 and 28 new cases/100 000 in yearly, respectively) in north (The EURODIAB ACE Study Group 2000, Ilonen et al. 2009). Incidence in many countries of the Eastern Central Europe is also relatively low but has been rapidly increasing (Patterson et al. 2009). Notably exception from this general model is the very high incidence Sardinia (≥50/100 000/year) (Bruno et al. 2013).

There are also sharp differences in neighboring regions. Ten-fold difference in the disease occurrence has been observed between different neighboring countries in Europe. For example, between Baltic countries (Estonia 10.5, Lithuania 7.4 and Latvia 5.9 new patients ≤14 years of age/ 100 000/year) and nearby in Finland, the difference in incidence is very large. One of the sharpest incidence differences also exists between Finland and the neighboring Karelian Republic of Russia (incidence 7.4/100 000/year) (Kondrashova et al. 2005). However, analyses of four diabetes-predictive autoantibodies found similar frequencies in both Finnish and Russian children in other islet autoantibodies than autoantibodies against IA-2A, which was of lower prevalence among Russians (Kondrashova et al. 2013). This suggests that the frequency of islet autoimmunity as such as common but progressive form of it often heralded by IA-2A is more common in Eastern Karelia. Similar findings have also been found in comparisons of autoantibody frequencies among other high and low incidence populations (Long et al. 2012, Padoa 2011). Reasons for incidence variations are still unclear; differences in both genetic and environmental factors have been suggested. Conspicuous differences in socioeconomic status often found between high and low incidence countries may associate with many additional factors related to different lifestyle and living conditions in the neighboring countries.

The incidence of T1D has increased worldwide since the beginning of statistics, and the increase was particularly large in the younger age (0-4 years) in Europe until 2005 (Harjutsalo, Sjöberg & Tuomilehto 2008). The change over time may be partly explained by changes in lifestyle causing rapid early growth and weight development (Harder et al. 2009, The EURODIAB Substudy 2 Study Group 2002). The mean of the annual increase in incidence is between 2.5%-3.6% worldwide although in Sweden from 2002 and later on in Finland between 2005 and 2011 a plateau in incidence rate has been reported. The first plateau was recorded in the 1980s in Finnish children aged between 10-14 years, after this incidence started to increase again (Berhan et al. 2011, Harjutsalo et al. 2008, Harjutsalo et al. 2013).

Although most autoimmune disorders more frequently affect females than males, the childhood-onset T1D is generally as common in boys and in girls. The male/female ratio is close to 1, although slight female or male predominance has been reported from some countries (Maahs et al. 2010). The peak of incidence in age of onset is about three years earlier in girls than in boys. Clear male predominance is found after the puberty (Harjutsalo et al. 2008, Wändell, Carlsson 2013).

Studies of diabetes seasonality distinguish between the seasonality of birth and seasonality of onset or diagnosis. Children born during the spring-summer (mainly in May) showed increased association for T1D in many studies (Kahn et al. 2009) but a German study showed that children and adolescents with T1D were more often born during the autumn-winter months (Neu et al. 2000). The seasonality of onset or diagnosis of T1D has been extensively studied and the results are conflicting. One third of analyzed centers had a peak in colder (October-January) and also one third had troughs in June to August in WHO DIAMOND Project. However, Finland has a clear trough in June and July and peaks in early spring and late autumn (Moltchanova et al. 2009).

Suggested explanations for seasonality in diagnosis include the fact that winter months bring more infections, and decrease in vitamin D levels during the darker months. Relationship between season of the birth and susceptibility for type 1 diabetes have attributed to for example intrauterine infections, dietary intake, short duration of breastfeeding and, early exposure to cow's milk proteins. However, the autoimmune process which lead to clinical diabetes is long, therefore the environmental factors associated with seasonality at diagnosis can be considered as disease precipitators acting at a late stage of prediabetes. The result of both could be higher autoimmune activity that causes β-cell destruction (Moltchanova et al. 2009, Patterson et al. 2015).

## 2.2. Genetics of type 1 diabetes

It is well known that T1D is a complex, multigenic disorder that is influenced by environmental factors. This means that gene polymorphisms affecting the disease risk can affect any phase of the disease process. To fully understand the genetics of T1D one should investigate the natural history from birth until clinical manifestation during the follow up.

First approach, follow newborns with family members affected type 1 diabetes. The risk of developing diabetes in first-degree relatives 8-15 fold higher and around 2 fold in second- and third-degree relatives with history of type 1 diabetes (Weires et al. 2007). Children with two affected first-degree family members have almost 30% higher risk for T1D. The risk of type 1 diabetes in offspring of diabetic parents is 5% by age 20 years (Bonifacio, Ziegler 2010), although they have different lifetime risk depending on whether the mother (~3%), father (~5%), or sibling (~8%) has the disease (Harjutsalo, Reunanen & Tuomilehto 2006). A young age of affected child at diagnosis, paternal young-onset diabetes, male sex, and older paternal age at delivery considerable increased the risk of type 1 diabetes for sibling (Harjutsalo, Podar & Tuomilehto 2005). On the contrary, the daughters of the mothers with T1D and young age at onset seem to be protected from diabetes in

Finnish longitudinal population-based study (Harjutsalo et al. 2010). The strong genetic contribution to T1D is illustrated with prospective long-term follow-up, by cumulative incidence among monozygotic twins. This is reported to be around 80% both had developed T1D by 60 years of age among monozygotic twins who were initially discordant (only one twin affected) for diabetes (Redondo et al. 2008). Dizygotic twin siblings have instead a 5-6% concordance rate for T1D similar to that in non-twin siblings (Morran et al. 2015).

Second approach, type 1 diabetes is known to be strongly associated with DRB1*03-DQA1*05:01-DQB1*02:01 (DR3-DQ2) and DRB1*04-DQA1*03-DQB1*03:02 (DR4-DQ8) haplotypes, alone or in combination. Approximately 90% of young children with T1D carry either HLA-DR3 or HLA-DR4 haplotype. On the other hand, some haplotypes are strongly protective like DR15-DQ6 (DRB1*15:01-DQA1*01:02-DQB1*06:02/*06:01). Newborn screening has been used to identify children at increased genetic risk who could be followed up for the appearance of β-cell autoantibodies that are known to be strongly associated with an increased risk for T1D. In children long-term followed up studies from birth such as in the Finnish Diabetes Prediction and Prevention (DIPP), The Environmental Determinants of Diabetes in the Young study (TEDDY), German Baby DIAB and in the Diabetes Autoimmunity Study in Young (DAISY) in the USA (Pociot, Lernmark 2016, Ziegler et al. 2013). Children without affected family members have a 0.3% risk for type 1 diabetes (Bonifacio, Ziegler 2010) but this is population specific and in Finland 0.9% when calculated based on yearly incidence of 60 cases/100 000 children.

The major genetic risk factors are the HLA class II region. In addition, around 50 loci outside HLA region contribute to the disease susceptibility, most of them revealed by genome-wide association studies (GWAS) (Pociot et al. 2010).

### 2.2.1. Human leukocyte antigen (HLA)

#### 2.2.1.1. Structure and function of the HLA genes and molecules

The major genetic determinants of type 1 diabetes reside in the HLA region within the major histocompatibility complex (MHC), containing many genes related to the functions of the immune system. The first report on genetic association between the MHC and T1D was published in 1973 (Singal, Blajchman 1973) and HLA studies have thereafter contributed much to the understanding of this disease. The HLA complex is located on the short arm of chromosome 6 and approximately 3.6 million base pairs (Mb) long. Molecular products encoded by HLA genes are involved in

immune regulation and cellular differentiation. The region contains more than 200 identified genes, over half of which predicted to be expressed. Of these, an estimated 40% are involved in immune responses, with a tendency for clustering by function. The HLA region is divided in three groups of genes: class I, class II and class III, with the class II loci at the centromeric end of the region and the class I loci at the telomeric end (Bodmer 1987). The class III genes are structurally and functionally different from two other classes, but class III name is often used due to their location on chromosome 6 between class I and class II region (Figure 2). While HLA class I molecules are present as transmembrane glycoproteins and expressed on the surface of all nucleated cells but to varying degrees. Class II molecule expression is restricted to B lymphocytes, dendritic cells, macrophages and activated T lymphocytes. Both HLA class I and class II molecules are involved in the presentation of antigens to T cells. Class I molecules present cytosolically derived intracellular peptides to the T cell receptor of CD8+ (cytotoxic) T cells. When the peptides are pathogen derived, this can lead to an immune response resulting in the killing of the infected cell. The class I molecules are additionally involved in innate immune responses by operating as ligand for natural killer cell receptors including inhibitory receptors. In contrast, class II genes are normally expressed by a subgroup of immune cells called antigen presenting cells (APC) that includes B cells, macrophages, dendritic cells and thymic epithelial cells, where they present peptide-fragments from exogenous antigens to $CD4^+$ T-cells. The HLA proteins are more polymorphic that any other protein in the human genome, having the ability to bind a wide range of peptides. Different HLA molecules have slightly different peptide-binding structure and different HLA molecule thus bind to and present different peptides to T cells (Morran et al. 2015).

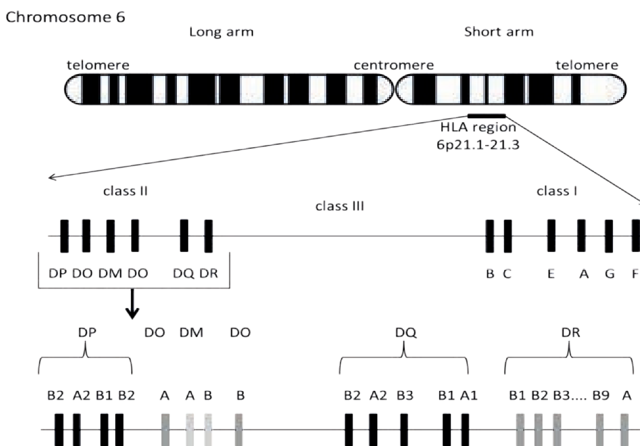



**Figure 2.** A schematic map of the HLA region with limited number of genes. Distance between loci are approximate.

The normal function of class I proteins is a presentation of peptides from expired or defective. The proteins encoded in the class I region include the classical molecules HLA-A, -B, -C which encode the α-polypeptide chains of the class I molecules. The heavy; α chain (about 45 kDa) is anchored to the cell membrane. The HLA class I α-chains have typical structure: different domains of the protein are encoded by different exons, thus α1, α2, α3 extracellular domains are encoded by exons 2, 3 and 4, a transmembrane region and cytoplasmic chain (Figure 3). The water-soluble light β-chain (12kDa) of the class I molecules is the non-polymorphic beta2-microgobulin (β2m) encoded by a gene outside the HLA region in chromosome 15. Typically, 8-10 amino acids length peptides can bind into the groove (Figure 5). The high polymorphism in the HLA-A, -B and -C region arises mostly of the exons 2 and 3 nucleotide substitutions (Klein, Sato 2000, Marsh, Parham 2001).

By contrast, non-classical HLA-E (HLA-E), -F (HLA-F) and -G (HLA-G) genes are oligomorphic in their coding sequences (Table 1).

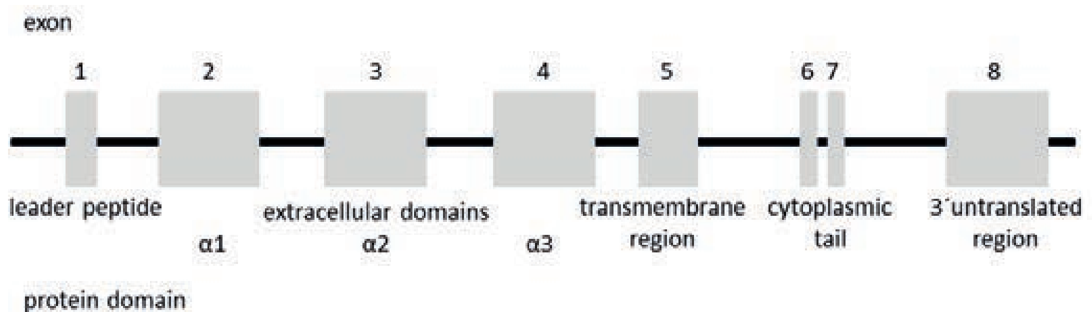



**Figure 3.** Schematic pictures of the HLA class I gene (Modified from Marsh, Parham 2001).

The HLA class II locus is located in the 6p21.3 region and contains approximately 700 kb. It consists of over 30 genes loci including the major class II structural genes encoding DP, DQ and DR molecules. There is strong linkage disequilibrium between the different HLA alleles, especially between the DR and DQ loci. Specific alleles of especially these two loci show strong associations with various autoimmune diseases.

Class II molecules consists of two polypeptide chains, α (about 33-35kDa) and β (about 26-28kDa), encoded by the A and B genes, respectively.

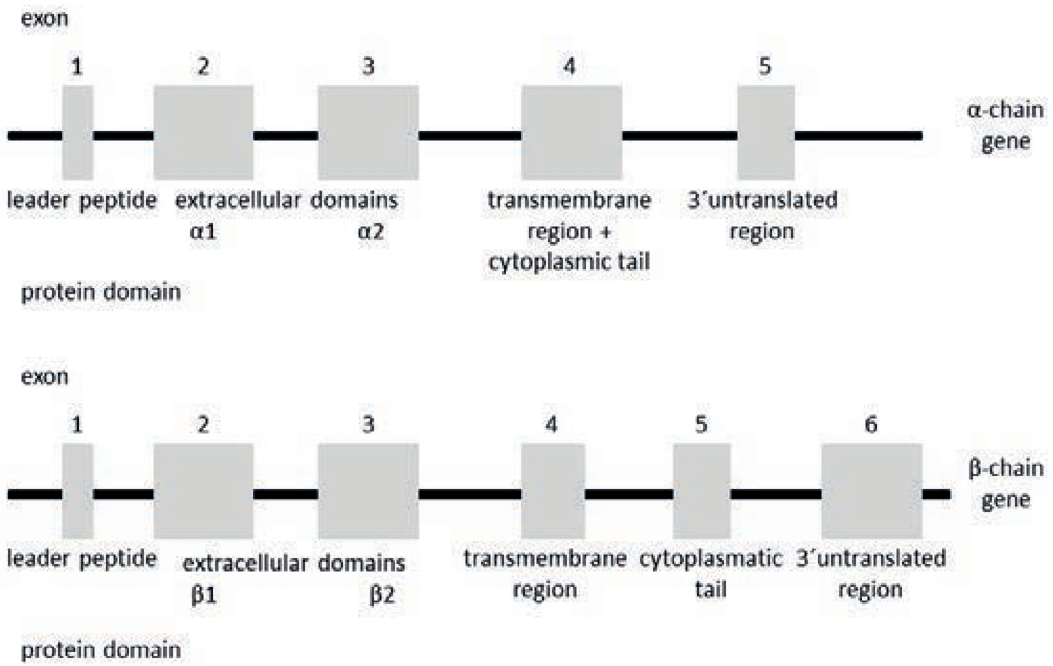



**Figure 4.** Schematic pictures of a HLA class II gene (Modified from Marsh, Parham 2001).

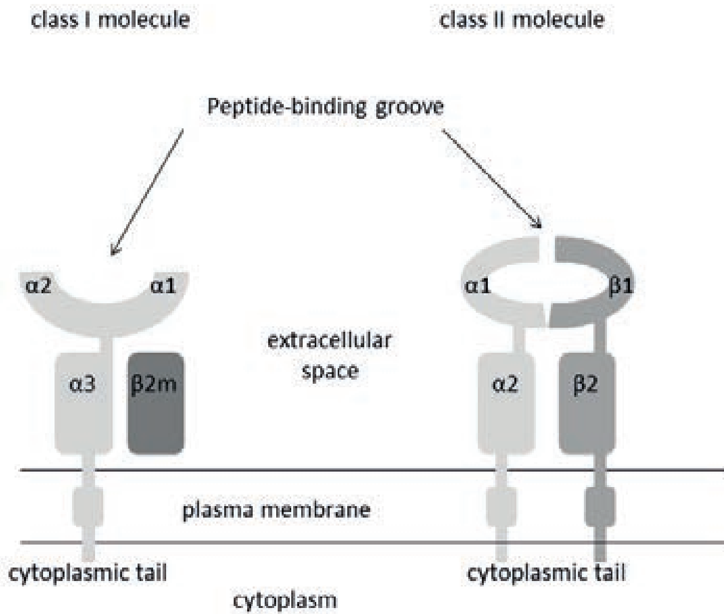



**Figure 5.** Structure of HLA class I and II molecules (Modified from Marsh, Parham 2001).

The α and β chains have two extracellular domains (α1, α2 and β1, β2), a transmembrane region and a cytoplasmic tail (Figure 4 and 5). The polymorphism of HLA class II originates from the α and the β chains, which are non-covalently bound together. The peptide binding-groove is formed by α1 and β1 domains together, in DQ and DP molecules the domains of both chains are polymorphic but in the DR molecule the α chain is not polymorphic in the peptide binding region (Williams 2001). The groove is open at both ends, which allows longer peptides to bind. Peptides bound by HLA class II molecules are thus generally longer than peptide bound by HLA class I molecules. The amino acid changes modify the peptide-binding specificity by altering the shapes of the antigen-binding groove. In contrast to DR, DQ and DP the polymorphism found in the HLA-DM and HLA-DO region is limited. In addition to the functional class II genes there are also class II pseudogenes, such as DQA2 and DQB2 within the region. The number of different alleles discovered for the highly polymorphic HLA loci has been increasing steadily. According to the IMGT/HLA database (http://www.ebi.ac.uk/imgt/hla/stats.html) over 18 000 unique allele sequences have been reported in HLA in 2018, over 13 000 alleles in class I and over 4800 class II alleles has been described (Table 1).

**Table 1.** Extent of polymorphism in HLA class I and class II (data content from the IMGT/HLA 2018 database).

| Locus | Number of alleles | Number of proteins |
|---|---|---|
| A | 4200 | 2923 |
| B | 5091 | 3664 |
| C | 3854 | 2644 |
| E+F+G | 27+30+60 | 8+5+19 |
| DRA | 7 | 2 |
| DRB | 2464 | 1793 |
| DQA1 | 94 | 35 |
| DQB1 | 1196 | 806 |
| DPA1+DPA2 | 65+5 | 27+2 |
| DPB1+DPB2 | 975+6 | 675+3 |
| DMA+DMB | 7+13 | 4+7 |
| DOA+DOB | 12+13 | 3+5 |

HLA-DR is rather complex in structure as it consists of several functional β-chain genes as well as pseudogenes (HLA-DRB2, -DRB6, -DRB7, -DRB8 and -DRB9). HLA-DRB3, -DRB4, and -DRB5 all produce a functional DRβ chain that can dimerize with the product of DRA1 gene. The DRB1 is found on all copies of chromosome 6, but the other three (as second locus DRB3, DRB4 and DRB5) functional genes are only present on some chromosome, dependent on the DRB1 gene on the chromosome. Allelic variants of DRB1 are linked with either none or one of the genes DRB3, DRB4 and DRB5 (Noble 2015).

The class III region is the most gene-dense part of the human genome which contains about 60 expressed genes. Many of these are also participating the innate immune system, encoding inflammatory cytokines, stress response proteins and complement factors (Klein, Sato 2000, Turner 2004).

#### 2.2.1.2. HLA variation in T1D

Diversity of HLA molecules may arise partly due to point mutations and also more frequently due to mechanisms as gene conversion. Many polymorphism in HLA genes leads to nonsynonymous amino acid changes in the peptide-binding groove of HLA molecules, that indicates a strong selection pressure on the peptide-binding groove and the importance of the HLA-peptide interaction. Interestingly, in people who are heterozygous at each of the six class I and class II HLA loci, it has been estimated that an APC could theoretically present over $10^{12}$ different peptides. The HLA-peptide-T cell receptor binding has been of particular interest in the context of different autoimmune diseases including T1D. A common feature of these diseases is tissue damage related to the presence of autoreactive T cells that escape both negative selections in the thymus and peripheral tolerance mechanisms. However, it remains to be determined whether autoreactive T cells are directly generated and activated by the mechanisms such as atypical HLA-peptide- T cell receptor binding orientation; low affinity peptide binding that facilitates thymic escape, T cell receptor-mediated stabilization of weak peptide-HLA interaction and presentation of peptides in a different binding register. Other mechanisms that may generate and activate autoreactive T cells are likely to be driven by epitope variation, including molecular mimicry; post-translational epitope modification and generation of hybrid peptides, and HLA stability may play an important role in T1D (Dendrou et al. 2018).

### 2.2.1.3. The nomenclature of HLA alleles

Two system of the nomenclature are applied to HLA. The first system was formed in 1965 and was based on serological specificity. Antigens were eventually assigned letters and numbers. The modern HLA nomenclature started in 1987 with HLA- and locus name, then separator ʿ*ʾand specification of the allele. The first two digits specify the group of alleles as corresponding to the serological antigen. The older system stopped in this level, not completely differentiating alleles. The third and fourth digits identified the specific allele. Digits five through six denote any synonymous mutations within the coding frame of the gene. The seventh and eight digits differentiate the mutations outside the coding region. In addition, the last change in nomenclature system was in 2010, thence number of suffixes were used to identify expression level that were such as L (low expression), N (not expressed), Q (questionable expression), S (secreted molecule but is not present on the cell surface), A (aberrant expression) and those found only in the cytoplasm (C). From 2017, no alleles have been named with the ʿCʾ or ʿAʾ suffices. Thence 2010 colons ʿ:ʾ were introduced as separators between pairs and digits. The completely described allele may be up to nine digits long, not including the HLA-prefix and locus notation to designate a specific allele at a given HLA locus (Figure 6) (Torres, Moraes 2011).

All new and confirmatory sequences should now be submitted directly to the WHO Nomenclature Committee for Factors of the HLA System via the IMGT/HLA database using the sequence submission tool provided (http://www.ebi.ac.uk/ipd/imgt/hla/).

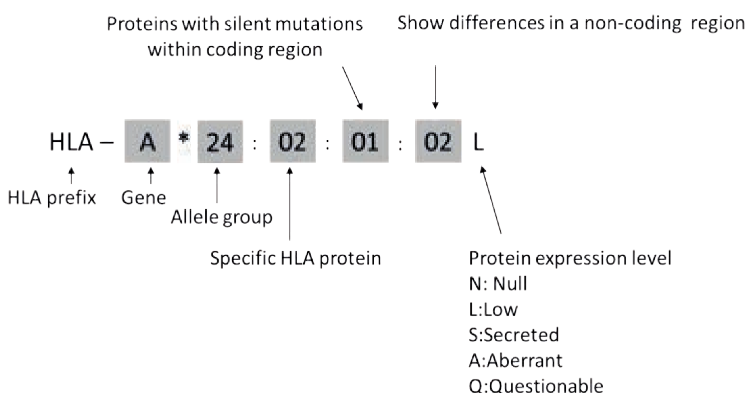



**Figure 6.** HLA nomenclature (Modified from Torres, Moraes 2011).

Fortunately, in most disease association studies the first two fields (four digits) such as allelic resolution or high resolution carrying the information on the amino acid sequence of the encoded protein also involve the important disease association (Kiviniemi et al. 2007). By contrast low resolution in DNA-based typing result at the level of the first filed (two digits) in the DNA based nomenclature (Nunes et al. 2011). Paradoxically, increased resolution of HLA genotyping can decrease statistical power of HLA disease association analysis by increasing the number of HLA subtypes (Noble, Erlich 2012).

### 2.2.2. HLA class II region and genetic risk of type 1 diabetes

An important aspect of the HLA region is the linkage disequilibrium (LD); it means nonrandom association of markers in various HLA loci. The strong LD means for example two specific alleles at two loci to be found together more often than expected based on their frequencies. The strong linkage disequilibrium complicates identification of the independent effects in the HLA region.

The strongest association between T1D and HLA region is seen with haplotypes and genotypes found in the DRB1-DQA1-DQB1 loci where individual alleles are often found only in one or a few haplotypic combinations (Noble 2015, Noble, Valdes 2011). For instance the DRB1*03:01 is found linked to DQA1*05:01 and DQB1*02:01, to create the haplotype as DRB1*03:01-DQA1*05:01-DQB1*02:01 often mentioned as DR3 haplotype and known to be risk haplotype for T1D.

The high- and low-risk DR4 haplotypes differed at the DQB1 locus such as high-risk DQB1*03:02 (DQ8) and low-risk DQB1*03:01 (DQ7). DQB1*03:02 encodes an Alanine whereas DQB1*03:01 encodes an Aspartic acid (Asp) at codon 57 on exon 2. This lead to structural difference between DQB1 molecules and e.g. Asp at position 57 confers protection. This residue in pocket of the peptide-binding groove, contributes to a salt-bridge which is missing in the neutral or susceptible amino acids at same position (Noble, Erlich 2012, Todd, Bell & McDevitt 1987). The two most common DR4-DQ8 haplotypes the DRB1*04:01-DQA1*03:01-DQB1*03:02 and DRB1*04:04-DQA1*03:01-DQB1*03:02 differ only at 71 and 86 amino acid positions (Lysine-Glycine vs Arginine-Valine) of DRB1 (Erlich et al. 2008). Position 86 contributes to pocket 1 while position 71 contributes to pocket 4 and 7 of the peptide-binding groove. Among the DR4 haplotypes the DRB1*04:03-DQA1*03:01-DQB1*03:02 was found to protect against T1D, by contrast the DRB1*04:01-DQA1*03:01-DQB1*03:02 haplotype shows a high disease risk and DRB1*04:04-DQA1*03:01-DQB1*03:02 a moderate one (Hermann, Knip et al. 2003, Hermann, Turpeinen et al. 2003, Ilonen 2016, Noble 2015).

Four haplotypes such as DRB1*15:01-DQA1*01:02-DQB1*06:02, DRB1*15:01-DQA1*01:02-DQB1*06:01, DRB1*14:01-DQA1*01:01-DQB1*05:03 and DRB1*07:01-DQA1*02:01-DQB1*03:03 were associated with strong protection from T1D in white Caucasian population. The DRB1*15:01-DQA1*01:02-DQB1*06:02 haplotype is associated with strong protection from T1D, even in the presence of high-risk HLA alleles and/or T1D-associated autoantibodies. The DRB1*15:01-DQA1*01:02-DQB1*06:01 haplotype was very rare in Finnish population (Erlich et al. 2008, Ilonen et al. 2016, Thomson et al. 2007). The disease association of various HLA-DR2 haplotypes expressing diverse linkage patterns of DRB1 and DQA1/DQB1 alleles or unusual DQA1/DQB1alleles in *cis* with the DRB1*15:01 allele suggests that protection from T1D maps largely to the DQA1*01:02 and DQB1*06:02 alleles, which together encode for the HLA-DQ6 heterodimer. In contrast DRB1*14:01-DQA1*01:01-DQB1*05:03 was rather common in some Central European populations and Italians (approximately 11% in controls) and could this contributed to the low incidence of T1D in these countries (Hermann, Turpeinen et al. 2003). It is not known at which stages in the natural history of T1D development this haplotype affords protection (Pugliese et al. 2016). The HLA-DRB1-DQA1-DQB1 haplotypes define the major genetic effect for T1D risk (Table 2).

**Table 2.** Selected susceptible and protective haplotype in the HLA class II region in the Finnish population (Ilonen 2016).

| Risk haplotypes in HLA region | Odds ratio (95% CI) | Risk factor |
|---|---|---|
| DRB1*04:01-DQA1*03-DQB1*03:02 | 10.11 (8.9-11.5) | Strong susceptibility |
| DRB1*04:05-DQA1*03-DQB1*03:02 (rare) | 3.01 (1.1-8.4) | Strong susceptibility |
| DRB1*04:04-DQA1*03-DQB1*03:02 | 2.8 (2.4-3.3) | Weak susceptibility |
| (DR3)-DQA1*05-DQB1*02 | 2.8 (2.5-3.2) | Weak susceptibility |
| **Protective haplotypes in HLA region** | **Odds ratio (95% CI)** | **Risk factor** |
| DRB1*04:03-DQA1*03-DQB1*03:02 | 0.4 (0.2-0.7) | Weak protection |
| (DR7)-DQA1*02:01-DQB1*03:03 | 0.08 (0.05-0.2) | Strong protection |
| (DR15)-DQB1*06:01 (rare) | 0.07 (0.01-0.5) | Strong protection |
| (DR15)-DQB1*06:02 (common) | 0.03 (0.03-0.05) | Strong protection |
| (DR14)-DQB1*05:03 | 0.03 (0.01-0.08) | Strong protection |

The individual risk is obviously affected by both inherited haplotypes in an additive manner without clear dominant or recessive effect although some specific interactions have been noted.

The DR3/DR4 haplotypes combinations or more specifically DRB1*03:01-DQA1*05-DQB1*02 and DRB1*04:01/02/04/05-DQA1*03:01-DQB1*03:02 heterozygous genotypes confer the greatest susceptibility to T1D, higher than homozygosity for either predisposing DR3 or DR4 haplotypes (Noble, Valdes 2011). The DR3/DR4 heterozygous genotypes can produce two heterodimers encoded in *cis* and two encoded in *trans* by product of DQA1*0301 from the DR4 haplotype combined with the product of DQB1*0201 from the DR3 haplotype, as well as the product of DQA1*0501 from the DR3 haplotype paired with the product of DQB1*0302 from DR4 haplotype. These *trans* molecules may be explanation for the high risk associated with heterozygous genotype. T-cell recognition of peptides bound to *trans*-encoded DQ heterodimers (i.e. DQA1*05:01 with DQB1*03:02 and DQA1*03:01 with DQB1*02:01) can differ significantly from the *cis*-encoded DQ (i.e. DQA1*05:01-DQB1*02:01 and DQA1*03:01-DQB1*03:02) molecules thus increasing the variability in T-cell repertoire (Erlich et al. 2008). In Caucasian populations up to 90% of T1D patients carries of DR3 or DR4 haplotype while DR3/DR4 is reported between 30-50% (Noble, Erlich 2012). However, DR4-DQ4 (DRB1*04:05-DQB1*04:01), DR9 (DRB1*09:01-DQB1*03:03) and DR8 (DRB1*08:02-DQB1*03:02) have been reported to be highly susceptible haplotypes among Japanese (Katahira et al. 2008). The absence of the DR3 and DR4-DQ8 haplotypes may contribute to lower incidence for T1D in Japan.

In fact, the T1D disease risk is most likely caused by combination of DR and DQ molecules (Erlich et al. 2008) and additionally the third type of classical HLA class II molecule DP encoded by the DPA1 and DPB1 genes seems to affect the disease risk. Both HLA DPA1 and DPB1 loci are highly polymorphic (Table 1). The DPA1*01:03-DPB1*03:01 and DPA1*01:03-DPB1*02:02 haplotypes have been associated with susceptibility and DPA1*01:03-DPB1*04:02 with a protective effect with T1D (Varney et al. 2010).

### 2.2.3. Genetic risk for type 1 diabetes associated with polymorphism of MHC class I genes HLA-A, HLA-B and HLA-C

While the alleles of class II HLA genes appear to have strongest association with T1D, several studies have shown additional associations between T1D and HLA class I gene alleles (HLA-A at 30Mb, HLA-B at 31.4Mb) independently from the HLA class II regions (HLA-DRB1 and HLA-

DQB1) which modify the susceptibility to the disease (Nejentsev et al. 2007, Noble et al. 2002, Noble et al. 2010, Mikk et al. 2014, Mikk et al. 2017, Valdes, Erlich & Noble 2005).

Three loci (HLA-A, HLA-B and HLA-C) in the HLA class I region are extremely polymorphic (Table 1) in the exons 2 and 3 encoding the α1 and α2 domains of the protein (Figure 3 and 5), which form the peptide-binding groove.

Some alleles of the HLA class I region have shown the association both at serological and at allele level with T1D, including the A*24, B*18 and B*39, with and also without connection to the HLA DR-DQ region. The B*39:06 allele appears to be the most highly predisposing (exclusively on DR8 haplotype) and B*57:01 alleles with greatest protective effect of class I alleles with T1D. Other significantly T1D associated alleles include as predisposing A*24:02, A*02:01, A*02:02, B*18:01, and C*05:01 and with protective effect A*11:01, A*32:01, A*66:01, B*07:02, B*44:03, B*35:02, C*16:01 and C*04:01 (Baschal et al. 2011, Mikk et al. 2014, Nejentsev et al. 2007, Noble et al. 2002, Noble et al. 2010, Reijonen et al. 1997, Valdes, Erlich & Noble 2005).

HLA class II molecules are involved in the initial antigen presentation event and in triggering the immune response. These may thus affect the initial events in development of the T1D. On the other hand, HLA class I molecules, given the role in target-cell recognition by CD8+ cytotoxic T lymphocytes, play a role in ongoing immune response and might affect the rate of pancreatic beta-cell destruction (Noble et al. 2002). This is supported by the significant association of the HLA-A*24:02, HLA-B*18:01 and HLA-B*39:06 with early disease onset in patients with highest risk DR3/DR4 genotype (Noble et al. 2002, Valdes, Erlich & Noble 2005). The HLA-A*24 has been reported to be linked with total beta-cell destruction (Nakanishi et al. 1993) and also with rapid progression of autoimmunity to clinical disease (Nakanishi et al. 1999). By contrast the HLA-A*03 and HLA-B*44:03 have been associated with protection in different Caucasian populations. The HLA-B*44:03 allele was associated also with older age at disease onset (age>15.1 years) (Noble et al. 2002, Valdes, Erlich & Noble 2005).

HLA-B*39 has been found predisposing on DRB1*04:04-DQA1*03-DQB1*03:02 and (DR8)-DQB1*04 haplotypes in Finland. Most significant effect was found in the (DR3)-DQA1*05-DQB1*02/DRB1*04:04-DQA1*03-DQB1*03:02 and (DR3)-DQA1*05-DQB1*02/(DR8)-DQB1*04 genotypes. The HLA-B*39 increased the DRB1*04:04-DQA1*03-DQB1*03:02 haplotype risk and changed the risk level associated with (DR8)-DQB1*04 haplotype from neutral/slightly protective to a predisposing one. In other haplotypes B*39 alleles were rare

(Mikk et al. 2017, Reijonen et al. 1997). The increasing effect of B*39 on DRB1*04:04-DQB1*03:02 conferred risk was also found in other populations in Baltic region (Nejentsev et al. 1997).

In the C locus eight alleles remain significantly associated with T1D in Caucasian multiplex families such as C*03:03, C*03:04 and C*07:01 with predisposing effect and C*04:01, C*06:02, C*07:02, C*08:02 and C*16:01 alleles with protection. The C*07:01 alleles also found on DRB1*04:04-DQB1*03:02 risk haplotype with strong LD (Valdes, Erlich & Noble 2005).

### 2.2.4. Genes in the "HLA class III" region

The HLA class III region is a 700 kb sequence located between the centromeric class II (HLA-DRA) and the telomeric class I regions (MICB). This region does not encode HLA molecules but is extremely gene-dense region encoding over 60 genes, resulting multiple molecules in the human genome. These include several secreted proteins with immune functions: components of the complement system (such as C2, C4, factor B), cytokines (such as TNFα, LTA and LTB), hormonal synthesis (steroid 21-hydoxylase, CYP21), heat shock proteins (HSPA1A, HSPA1B, HSPA1L) and extracellular matrix organization members (immunoglobulin superfamily) (Valdes, Thomson & Barcellos 2010). Encoded within the region is also BAT1, which belongs to the DEAD-box (aspartic acid-glutamic acid-alanine-aspartic acid) family of RNA-dependent ATPase (Price et al. 2004). Some polymorphisms are seen in the region that encodes a negative regulator of the inflammatory cytokines TNFα, which influences development of T1D (He et al. 2014). Nowadays, the HLA class III region has been completely defined and there is growing evidence that many of these genes are involved in susceptibility to a number of diseases (Milner, Campbell 2001).

### 2.2.5. Microsatellite markers

Microsatellites (Msats), or Simple Sequence Repeats (SSRs), consist of short tandem repeats (STRs); sort units of 1-10 base pairs in length of the DNA sequences, which are abundantly distributed across genomes and demonstrate high level of allele polymorphism. Msats are ubiquitous in prokaryotes and eukaryotes, present even in the smallest bacterial genomes. Msats can be found anywhere in the genome, both in protein-coding and noncoding regions. Because of their high mutability, microsatellites are thought to play a significant role in genome evolution by creating and maintaining quantitative genetic variation. In promoter regions, the length of Msats

may influence transcriptional activity.

Although the first microsatellite was characterized in 1984 by Jeffreys and colleagues as a polymorphic GGAT repeat in the human myoglobin gene the term “microsatellite” was introduced in 1989, by Litt and Luty, where “satellite” was used due to the fact that density gradient centrifugation separates DNA fragments with repetitive sequences into the upper “satellite” fraction with less density (Abdurakhmonov 2016). As genetic markers, microsatellites have been extensively studied in DNA-based genetic analyses in the past 34 years. In Pubmed database search with the keyword “microsatellite” found almost 55 000 research publication. However, searching for microsatellite and type 1 diabetes-related articles, only 282 articles has been published, the last publication in human studies by Steck and coworkers was in 2014 (Steck et al. 2014). Microsatellite markers are inherited from both parents, making them useful for parentage analysis and population genetic studies.

Microsatellite repeats typically exist between twenty and a few hundred bases. There are more than one million Msats loci (approximately 3% of the human genome) in the human genome and majority of them are dinucleotide repeats (~30%) (Sawaya et al. 2013). Seventy six percent of the human microsatellites repeats are A, CA, AAAN, AAN or AG. The most frequently appearing repeat is $(CA)_n$, which occur every 30 kbp around the hole genome in humans; both in protein-encoding and noncoding DNA, thereby this is the most commonly used microsatellite for the analysis, followed by $(AT)_n$, $(GA)_n$, $(GC)_n$, the last type being rare (Ellegren 2004). Polymorphism of a microsatellite depends on variation of the numbers of repetitions, numbers of alleles and also their distribution in the different populations. Parameters as the polymorphism information content (PIC) or percentage of the heterozygosity, calculated from the number of alleles and their frequencies in a population, have been used to classify microsatellites. A marker is considered as reasonably informative when the number of alleles is greater than five and the PIC or heterozygosity value above 0.75 (Foissac, Cambon-Thomsen 1998).

Number of known microsatellites in the HLA region is over than 1500 (846 in HLA class I, 295 in HLA class III and 386 in class II) and still increasing. Of them, more than 280 microsatellites were developed as genetic markers (146 in HLA class I, 61 in HLA class III and 61 in HLA class II). Allele sizes of the microsatellites in the HLA region has been varying between 79-473bp (Foissac, Cambon-Thomsen 1998, Foissac, Salhi & Cambon-Thomsen 2000, Shiina et al. 2009). The polymorphic microsatellite markers through the HLA region have been applied for exact mapping of disease-related genes within the HLA region in linkage and in disease susceptibility studies, and

also population, transplantation, and recombination studies (Foissac, Cambon-Thomsen 1998). Names of microsatellites in the MHC region were reorganized and renamed by Gourraud (Gourraud et al. 2004, Gourraud et al. 2006) by sequence based analyses. Detailed microsatellite marker information is available at the dbMHC database of the NCBI: (http://www.ncbi.nlm.nih.gov/gv/mhc/xslcgi.cgi?cmd=mssearch).

TNFα is a major proinflammatory cytokine encoded within HLA class III region which is involved in the islet β-cell destruction (Milner, Campbell 2001). A functional single nucleotide polymorphism (SNP) (TNFα -308 G/A) was identified in the promoter region of the human TNF gene. The TNFα -308A allele has been reported to associate with higher promoter activity (Green, Trial & Birdsall 1998) and also higher TNF expression in peripheral blood mononuclear cells (Das, Baniasadi & Kapuria 2006). Linkage disequilibrium between HLA genes and tumor necrosis factor (TNF) microsatellite was described in 1991 (Jongeneel et al. 1991) and the D6S273-TNFα region was found to be in stronger linkage disequilibrium in T1D patients with carrying high-risk haplotype on DR-DQ region than in control population (Hanifi-Moghaddam et al. 1998). The studies on association of TNFα polymorphism with T1D have, however, produced contradictory results. The TNFα -308 G/A was associated with T1D in several, but not in all studied populations (Deng et al. 1996). TNFα was found to be in LD with HLA DR3-DQ2 haplotypes that confer susceptibility to T1D among Caucasians in several populations (Javor et al. 2010, Törn et al. 2006,) and also among Brazilian patients (Patente et al. 2015). On the other hand, in North Indians (Kumar et al. 2007) and Moroccans (Bouqbis et al. 2003) divergent results were published. In addition THNFα*106 also TNFc microsatellite allele 167 has been found to define disease risk in HLA-DR3-DQ2 haplotypes independently of other markers in Sardinian population (Zavattari et al. 2001).

A novel family of the human MHC class I genes termed MICA has been located telomeric to the TNFα, centromeric of HLA-B gene and ~2Mb telomeric of HLA-DRB1. MIC proteins are considered to be markers of “stress” and expressed on the on the freshly isolated gastric epithelium, endothelial cells, fibroblasts and found many cells within the immune system but not present on $CD4^{+}$ and $CD8^{+}$ T cells or B cells. The highly polymorphic MIC genes only with one polymorphic position (alleles with methionine at codon 129) have 10-50 fold higher capacity to complex NKG2D than a valine at the same position. Only two of seven MIC loci MICA and MICB encode expressed transcripts, other five are pseudogenes (MICC, MICD, MICE, MICF and MICG). The MICA locus encodes membrane-bound polypeptides of 383-389 amino acids (length depend of number of alanine repeats in the transmembrane region, within humans more than 50 different

alleles). MICA molecules show homology with HLA class I molecules, but they do not combine with β2 microglobulin, do not bind peptides and are not expressed on the normal circulating lymphocytes. MICB is less polymorphic with 17 different alleles (Collins 2004). The most studied polymorphism is a short tandem repeat (STR) in the transmembrane domain of MICA (STR MICA). STR MICA is a GCT repeat microsatellite in MICA exon 5 and named based by the number of repeats (4, 5, 6, 7, 8, 9 and 10 repetitions). The STR MICA allele 5.1 is the most common allele (approximate frequency 0.5) in European populations which contains five GCT repeats and a G insertion (GGCT) that causes a frame shift, leading to a premature stop codon and cut of the cytoplasmic tail. MICA alleles have been reported to influence the risk for the T1D (Gambelunghe et al. 2007, Gupta et al. 2003). The STR MICA*5 and STR MICA*5.1 alleles were transmitted more frequently together with DQ8 and MICA*5.1 also with DQ2 haplotype in families samples (Nikitina-Zake et al. 2004). In several Caucasian populations like Swedish, Belgians and Latvians and also Asian Indians MICA*5 allele was found associated with T1D and MICA*6 negatively associated with the disease (Gupta et al. 2003, Roach et al. 2006, Sanjeevi et al. 2002, Shtauvere-Brameus et al. 2002, Van Autreve et al. 2006). In Koreans and Japanese patients MICA*4 alleles was present at a higher frequencies, whereas allele 6 was present at a lower frequency (Kawabata et al. 2000, Park et al. 2001).

The microsatellite locus D6S273 centromeric of TNF, and it is ~200 kb in size. Result of a case-control study suggested an association between marker D6S273 and type 1 diabetes. D6S273*2 (130bp length) allele has been conferred susceptibility on the DRB1*04:01 haplotype (Hanifi-Moghaddam et al. 1998, Lie et al. 1999) however Johansson and coworkers found also the D6S273*2 (130 bp length) are associated with T1D in family materials otherwise these association was independent of HLA DQ2-DR3 contributions (Johansson et al. 2003). The D6S273*138 and has been found to define disease risk in HLA-DR3-DQ2 haplotypes independently in Sardinian population (Zavattari et al. 2001).

The MIB microsatellite near the HLA-B locus (~25kb centromeric to HLA-B) has been shown to be in linkage disequilibrium with TNF microsatellites (Grimaldi et al. 1996).

The D6S2223 is located ~5.5Mb telomeric of HLA class II locus. D6S2223*3 associated with a reduction of T1D risk of DRB1*03-DQA1*05:01-DQB1*02:01 (DR3-DQ2) haplotypes in family analysis in different populations (Johansson et al. 2003, Lie et al. 1999).

Alleles of microsatellite loci D6S105 and D6S265 on the telomeric side of chromosome 6 are in

linkage disequilibrium with HLA class I alleles in the common European population haplotypes (Worwood, Raha-Chowdhury & Darke 1994).

### 2.2.6. T1D susceptibility genes outside the HLA region

#### 2.2.6.1. Insulin gene polymorphism

In the early 1980s, the insulin gene (INS) was identified as the first non-HLA locus linked to T1D susceptibility in numerous studies and different populations. INS is located on chromosome 11p15.5 designated as IDDM2, encompasses 1430 base pairs (bp), consists of three exons and two introns and encodes preproinsulin, the precursor of mature insulin. Preproinsulin is processed to proinsulin by removal of the signal peptide and then to mature biologically active insulin by removal of the C-peptide. A variable number of tandem repeat minisatellite (VNTR) region consist of 14 to 15 bp consensus sequence (5′-ACAGGGGTGTGGGG-3′) located 596 bp upstream of the INS gene, contains three classes of alleles according to the number of repeats involved. The shorter class I VNTR alleles contains 26-63 repeats, class II with 64-140 repeats and long class III alleles 141-209 repeats (Barratt et al. 2004, Durinovic-Bello et al. 2010). About 80% of Caucasoid alleles are between 30-44 repeats (class I) and rest are longer than 110 repeats in class III. Haplotypes of the VNTR class III alleles and neighboring variants have been divided into two protective lineages as protective haplotype and very protective haplotype, which correspond to VNTR class IIIA and IIIB subclasses (Stead et al. 2000). Intermediated lengths (class II) alleles are very rare in Europeans derived populations (Bennett et al. 1995). The class I alleles of the INS VNTR associated with the T1D risk with lower insulin mRNA and protein expression in the thymus, compared with the dominant protective class III alleles (Stead et al. 2000, Vafiadis et al. 2001). The VNTR regulates transcription rates of insulin and its precursors. Class I and class III alleles differentially affect transcription of insulin in the thymus and pancreas. Class III alleles result in 20% increased INS transcription of insulin in thymus and may thereby be associated with more efficient negative selection of insulin reactive T cells and less susceptibility to T1D as compared to class I alleles providing an attractive model for the role of the insulin gene in susceptibility to T1D.

The −23HphI (rs689) SNP with +1140A/T alleles are in linkage disequilibrium with VNTR, therefore A allele with the short class I and T allele with the long class III can be used as alternative markers for VNTR classes. In addition, the C allele of −2221MspI (rs3842729) is in LD with the VNTR class I and subclass IIIB, whereas the T alleles represents subclass IIIA. Individuals with T allele of these two SNPs have a reduced risk of T1D (Zhang et al. 2015).

### 2.2.6.2. CTLA-4 and PTPN22 gene polymorphisms

The cytotoxic T-lymphocyte antigen-4 gene (CTLA-4) known as IDDM12 is located with CD28 on the long arm of chromosome at 2q33. CTLA-4 consists of four exons and three introns and encodes a costimulatory molecule, which is expressed on the surface of activated T cells. CD28 involved in the regulation process of the activation of T cells by antigen-presenting cells and subsequent cellular immunity (Magistrelli et al. 1999). The CTLA-4 bound with higher affinity for the B7 on the antigen presenting cells than CD28. Common polymorphisms have been described both in the coding and promoter regions of the CTLA-4 gene. The most common polymorphism described in the coding region in 1$^{st}$ exon which leads to an alanine→threonine substitution (+G49A, rs231775) in the single peptide. The disease associated G allele is shown to reduce the CTLA-4 driven negative regulation of T-cell activation (Wang et al. 2014). The association between the CTLA-4 gene region and T1D was reported first by Nistico et al. in 1996 (Nistico et al. 1996). In addition to T1D, the CTLA-4 +49A/G polymorphisms have been associated with other autoimmune diseases as Addison's disease and rheumatoid arthritis, celiac- and Grave´s diseases (Xuan et al. 2013). Another common polymorphism located at 3´-UTR (G6230A, C60T, rs3087243) has also been found associated with T1D susceptibility. These two common polymorphisms were studied in meta-analysis, involved 58 different studies with more than 30 000 cases and more than 40 000 controls. The result demonstrated that the G49A and C60T polymorphisms of the CTLA-4 gene being in strong linkage are a risk factor for developing T1D in Caucasians and Middle Eastern populations, but no association was found in Africans (Wang et al. 2014).

The protein tyrosine phosphatase 22 gene (PTPN22) is located at 1p13.3-p13.1 and it encodes the lymphoid tyrosine phosphatase (LYP), which is important down-regulation of the immune response. Protein tyrosine phosphatases such as LYP are responsible preventing spontaneous T cell activation and they have the ability to prevent the response to antigen by dephosphorylating and inactivating T cell receptors. It has been demonstrated that single nucleotide polymorphism (SNP) in the PTPN22 gene can lead to susceptibility to autoimmune diseases such as T1D, Addison´s disease, rheumatoid arthritis, Grave's disease by decreasing in negative regulation of hyper-reactive T cells (Hermann et al. 2006, Noble, Erlich 2012). The association between the PTPN22 and T1D was described at first by Bottini and coworkers in 2004 (Bottini et al. 2004). They described and analyzed SNP at position 1858 (rs2476601) at codon 620 of LYP, which encodes arginine (1858 allele C) or tryptophan (1858 allele T) (Xuan et al. 2013). The 1858T allele frequencies showed geographic differences in different populations. In European countries, there is a south to north gradient in allele frequencies, from 2-3% in Italian and Sardinian populations (Saccucci et al. 2008),

7-8% Western populations (Zhernakova et al. 2005) up to 10-15% among Scandinavians (Nielsen et al. 2007) and Finns (Hermann et al. 2006).

#### 2.2.6.3. New generation of genetic studies in Type 1 Diabetes

A new generation of genetic studies is in progress. By the year 2000, a draft of the human genome sequence was completed. At that time the genome-wide association studies (GWAS) was provided for better resolution using high-density single-nucleotide-polymorphism (SNPs) throughout the genome. These have been widely used to define genes affecting different complex disorders, including inflammatory bowel disease, multiple sclerosis, Type 2 diabetes and Type 1 diabetes in a hypothesis-free context. GWAS compare common genetic variants in large numbers of affected cases to those in unaffected controls to determine whether an association with disease exists. Due to the big number of studied polymorphisms a very high p values for genome wide significance are needed. For example the GWAS performed by the Immunochip genotyping p value less than $5x10^{-8}$ is needed to identify new T1D-associated loci (Pociot 2017).

Nowadays, more than 3000 publications are catalogued by the National Human Genome Research Institute and the European Bioinformatics Institute (GWAS catalog http://www.genome.gov/gwastudies). The Catalog is a quality controlled, manually curated and literature-derived collection of all published GWAS assaying at least 100 000 SNPs (p values$<10^{-5}$). The GWAS studies predominantly test for association of common SNPs, with minor allele frequency higher than 5 %. The GWAS Catalogue reports 64 SNPs associations for T1D, but notably the disease-causing variants and genes are still largely unknown. The leading role in these studies belongs to International Consortia, which process individual DNA samples from various cohorts; among the main leaders have been the International Type 1 Diabetes Genetics Consortium and the Welcome Trust Case Control Consortium, all study populations were of European ancestry (Pociot 2017).

In 2007, primary result of the first successful GWAS in seven different multifactorial diseases, included 14 000 affected individuals from United Kingdom, 2000 with each disease. For comparison, two sets of control groups, each containing 1500 individuals were used. All study subject were genotyped using the same Affymetrix GeneChip 500K array. Results of analyzed data showed significant association between T1D and six regions on four different chromosomes (12q24, 12q13, 16p13, 18p11, 12p13 and 4q24) by the Wellcome Trust Case Control Consortium (Wellcome Trust Case Control Consortium 2007).

The largest of these studies, completed in 2010, was the Type 1 Diabetes Genetics Consortium (TIDGC). T1DGC is an international, multicenter research program established in 2002, by adding about 4000 affected sib-pair families (ASP) and more than 2600 controls from the same collections and used the Illumina 550K platforms in these studies. The main goals of T1DGC are to identify genomic regions and candidate genes whose variants modify the individual risk of T1D and help explain the clustering of the disease in families and expand the genetic resources for T1D research. Therefore four major research projects have been performed: particular examination of the HLA region by SNPs genotyping and high-resolution HLA typing; detailed investigation of published candidate genes; genome wide linkage scan and a GWAS and meta-analysis (Pociot et al. 2010).

The GWAS has inherent limitations. GWAS applies a non-candidate gene approach, and it is hypothesis-free and thus false positive results also easily arise. Most of the disease-associated SNPs map within the non-coding regions of the genome with high linkage disequilibrium with the functional SNPs. In addition, many significant SNPs identified by GWAS have relatively moderate or low risk in themselves, with the odds ratio between 1.1-1.3. However, despite the relatively weak effects, in combination with other clinical and pathological predictors, genotyping these susceptibility SNPs could be useful addition to assess disease risk and progression.

The next generation of genome-wide studies is underway, identifying additional variants at both known and novel loci. These studies based on high-density genotyping arrays, fine-mapping of known loci, sequencing of whole genomes and whole exomes, and integrating sequence results with functional studies.

In contrast to GWAS, studies in individuals with extreme phenotypes have often detected rare variants in coding regions within large functional effects. Since, 2005, next generation DNA sequencing platform such as whole exome sequencing (WES) is a highly effective approach in discovering genes underlying multifactorial disease. More than 95% of the analyzed 20 000 single nucleotide variants are already known as polymorphism in human population (Mohlke, Scott 2012).

Therefore, follow-up deep sequencing and functional studies are required to ascertain the biologic mechanisms.

## 2.3. Addison's disease

### 2.3.1. Addison's disease pathogenesis and etiology

Addison's disease (AD) or primary adrenal insufficiency is a disorder characterized by the damage of the adrenal cortex and deficiency in corticosteroid production as glucocorticoid (primarily cortisol) and mineralocorticoid (primarily aldosterone) hormones (Oelkers 1996). Addison's disease was described at first by Thomas Addison in his classical paper "On the Constitutional and Local Effects of Disease of the Supra-Renal Capsules" in 1855 (Løvås, Husebye 2005). In 1949, the synthesis of cortisone allowed the treatment of the condition (Brandão Neto, de Carvalho 2014).

Humoral and cellular immunity play roles in autoimmune AD pathogenesis. Environmental triggers, such a viral infections, drugs, smoking, food and stress could play role in genetically predisposed individuals (Brandão Neto, de Carvalho 2014).

The symptoms of the primary adrenal insufficiency develop when 90 percent of the adrenal cortex has been damaged. These are fatigue, reduced appetite with weight loss, joint and back pain and also most of the patients' experience salt craving and dizziness. Increased excretion of water can lead to dehydration and low blood pressure. The most conspicuous sign is the hyperpigmented skin, especially in sun-exposed areas, also in palmar creases and oral mucosal surfaces, which is caused by the elevated adrenocorticotropic hormone (ACTH) and renin levels. Typically, the serum cortisol level is below the normal range (Mitchell, Pearce 2012). Low serum aldosterone level, elevated plasma renin concentration or activity, and low dehydroepiandrosterone sulphate levels can further verify the diagnosis. The presence of the anti-21-hydroxylase antibodies is diagnostic for the autoimmune etiology (Falorni et al. 1995). Most of the patients have uncertain symptoms for many years before the diagnosis, by contrast, others progress more acutely (Myhre et al. 2002).

Treatment involves replacement of the deficient hormone. The untreated AD leads to death, hence the early recognition is extremely important, because many patients die undiagnosed. The well-treated patients may live close to a normal life and they have a normal survival rate (Bergthorsdottir et al. 2006).

Autoimmune AD is a part of an autoimmune polyendocrine syndrome (APS) in 60% of patients. APS-I is identified in only 5-10 % of patients with autoimmune AD in most populations. However, APS-I has been found to be more common in Finland and Sardinia (Napier, Pearce 2012). APS-II is defined as a combination of AD with autoimmune thyroid disease (Hashimoto´s thyroiditis or Graves´ disease) and/or type 1 diabetes and presents in approximately 50 % of individuals with

primary adrenal failure (Falorni et al. 2004, Myhre et al. 2002). The cross-sectional studies suggested that 50-60% of the patients with autoimmune AD have other one or more additional autoimmune disorder, most commonly autoimmune thyroid disease (about 30% of the AD cohort) and/or type 1 diabetes (10% of the AD patients develop) (Falorni et al. 2004, Myhre et al. 2002). Several other autoimmune diseases may also be present in the syndrome.

### 2.3.2. Epidemiology

In 1968 the prevalence of the Addison's disease was 39 in a million (Mason et al. 1968). The overall prevalence estimated to be between 40-60 people per million of the general population in worldwide. Nowadays the prevalence of the AD disease is increasing, reported incidence between 110-140 cases per million in European populations, making it 30-fold less prevalent than T1D (Mitchell, Pearce 2012). Prevalence of the AD is in various population is different: lowest in New Zealand with 0.45 per 100 000 inhabitants (Eason et al. 1982), in United States and in Japan 5 cases per 100 000 (Jacobson et al. 1997, Takayanagi et al. 2000) and 11.7 per 100 000 in Italy (Laureti et al. 1999) and 4-11 cases in 100 000 in Northern Europe (Willis, Vince 1997) with lowest with 4 in Finnish population and highest in Norway with 14.4 cases per 100 000 inhabitants (Erichsen et al. 2009).

Addison's disease is typically affecting young and middle aged (between 30-50 years) individuals and more often strikes women than men (ratio 1.5-3.5:1), although men with AD were significantly younger than the women with AD (aged 42±18.4 years versus aged 60±20.2 years) (Løvås, Husebye 2002, Myhre et al. 2002).

There is also a month-of-birth effect in autoimmune Addison's disease manifestation, with an increased risk for those born in during the winter months (December-January) and decreased in those born in the summer (Pazderska et al. 2016). This has been speculated to be related e.g. to numbers of infections in the critical period in infancy.

### 2.3.3. Autoantibodies in Addison's disease

The first autoantibodies associated with disease were detected with immunofluorescence using tissue sections from adrenal cortex in 1957 by Anderson et al. (Anderson et al. 1957). Immunofluorescence with human adrenal has become a useful diagnostic test for adrenal cortex antibodies (ACAs); these ACAs were of the immunoglobulin subclasses as IgG1, IgG2 and IgG4.

Winqvist and co-workers identified the steroidogenic enzyme 21-hydroxylase as the main target of the ACAs (Winqvist, Karlsson & Kampe 1992). The immunofluorescence assay (Betterle et al. 1999) is still in use, but largely replaced by highly sensitive and specific radiobinding assays for 21OH autoantibodies (Tanaka et al. 1997). Autoantibodies against 21-hydroxylase (CYP21A2 or PC450c17) are present in 70-90% AD cases compared to less than 0.5% frequency in general population (Myhre et al. 2002). Around 1% of first-degree relatives of patient with AD are positive for the anti-21-hydroxylase antibody in their blood. Of these, about 15 % will develop overt AD during an observation period of 6 years (Coco et al. 2006). Antibodies to steroid 17-α-hydroxylase are less sensitive markers for the disease, only 20-30% present in AD patients. These autoantibodies are often associated with gonadal failure in women (Falorni et al. 2002) and measured by indirect immunofluorescence assay (Betterle et al. 1999).

Nowadays there is no preventive therapy to halt or reverse the progressive destruction of adrenal cortex.

### 2.3.4. Genetics of Addison's disease

Large-scale genetic analysis in humans with autoimmune AD has not been done because of the rarity of the disease. The investigations carried out so far have been candidate-gene association studies with case-control design in small cohorts of patients; only few susceptibility loci have been identified to contribute to autoimmune AD (Mitchell, Pearce 2012).

The major genetic susceptibility for Addison's disease has been found in several populations conferred by HLA DR3-DQ2 (DRB1*03:01-DQA1*05-DQB1*02) and HLA DR4-DQ8 (DRB1*04:04-DQA1*03-DQB1*03:02) haplotypes, highest risk associated with a heterozygous combination of these haplotypes. The DRB1 (DRB1*03:01 and DRB1*04:04) seem to have stronger predisposing effect to AAD than DQB1 region (Mitchell, Pearce 2012). DRB1*0404 has shown disease association in United Sates and Norway, but not in Italian population where DRB1*04:03 was strongly protective (Gambelunghe et al. 2005). Combination of the two haplotypes, DR3-DQ2/DR4-DQ8 genotype, has also shown earlier disease manifestation than the other risk genotypes (Erichsen et al. 2009, Gambelunghe et al. 2005, Skinningsrud et al. 2011). By contrast DRB1*01-DQA1*01:01-DQB1*05:01 (DR1-DQ5), and DRB1*13:01-DQB1*06:03-DQA1*01:03, DRB1*13:02-DQB1*06:04-DQA1*01:02, and DRB1*07-DQB1*02:01-DQA1*02:01 conferred protection against Addison's disease (Erichsen et al. 2009, Myhre et al. 2002). Conserved extended haplotype including DR3 and HLA-B8 (without HLA-A1) together

confer the highest risk for Addison's disease, while the HLA-B15 is associated with protection from progression to clinical manifestation (Baker et al. 2010, Baker et al. 2011).

Several studies have reported associations with polymorphisms in HLA class I chain-related genes termed MIC, MICA and MICB, has been identified close to HLA-B locus. Park and coworkers reported increased risk associated with homozygosity for MICA5.1 as well as D6S273 microsatellite alleles. In DR3/DR4 genotypes, the D6S273*140 on the DR3 and D6S273*134 allele on the DR4 haplotype showed disease association (Park et al. 2002). The association of MICA5.1 with increased risk in DR3 haplotypes was confirmed by Skinningsrud et al. but they did not find any effect on DR404 haplotypes. The risk for Addison's disease was increased for DR3-D6S273*140-MICA5.1/DRB1*04:04-D6S273*134-MICA5.1 genotypes (Skinningsrud et al. 2011).

Allelic variants at several other genetic loci have been associated with autoimmune AD. The CTLA4 polymorphisms have also been associated with AD with A and G SNP in exon 1, and AT repeat in the 3' untranslated region of exon 3 and G or A alleles of the JO30 SNO downstream of this gene. Like CTLA4, PTPN22 gene encodes a negative regulator of T cell signaling. The same PTPN22 variant (T allele at rs2476601, associated with 1858C>T and Arg620Trp substitutions as in T1D has been implicated also in AD (Mitchell, Pearce 2012).

In the genome-wide association analysis, 17 SNPs (out of 64 analyzed SNPs), on eight different chromosomes (chromosome 2, 3, 6, 8, 13, 14, 18 and 21) were associated at a level of genome-wide significance ($p<5x10^{-7}$) in multiplex families and case-control cohorts from UK and Norway populations (Mitchell et al. 2015).

The number of the known risk genes is steadily increasing and will help understanding more clearly the pathogenesis of the Addison's disease together with new information on environmental factors involved.

# 3. AIMS OF THE STUDY

The strongest genetic association with different autoimmune disease is conferred by HLA class II alleles. The purpose of this study was to find additional polymorphisms within HLA region affecting the genetic susceptibility to type 1 diabetes and Addison's disease.

The specific aims were:

1, To localize the genes within class I and class III HLA region modifying the effect of class II HLA-DR/DQ loci on type 1 diabetes susceptibility.

2, To find whether risk alleles of the HLA-A and -B alleles within HLA class I region affect the progression from diabetes-associated autoimmunity to clinical disease.

3, To find whether the same HLA-B alleles within HLA class I region modify the risk conferred by HLA-DRB1*04:04-DQB1*03:02 haplotype in both type 1 diabetes and Addison's disease.

# 4. MATERIALS AND METHODS

## 4.1. Materials

In **Study I**, type 1 diabetic patients diagnosed before the age of 15 years were recruited mainly in university hospitals in Turku, Helsinki, Oulu and Tampere and control subjects were newborns who were screened for HLA-DR/DQ associated genetic susceptibility for T1D in context of the Finnish Diabetes Prediction and Prevention projects (DIPP) run in three University hospitals in Finland (Turku, Oulu and Tampere). Initially, we included more than 1400 type 1 diabetic patients and over 30,000 control subjects. After the HLA DR3/4(04:04) and DR3/4(04:01) genotype stratification, we analyzed 314 patients and 535 control subjects for ten selected ten microsatellite markers in non-class II HLA region (D6S273, TNFα, C12A, STR MICA, MIB, C125, C143, C245, C3211 and MOGc) and additionally, the presence of the HLA-A*24 and HLA-B*39 alleles were studied in the same sample groups.

In **Study II**, a total of 323 Finnish nuclear families typed for HLA-DR/DQ genotypes were involved in the analysis of eleven microsatellite markers within the HLA class III and class I region (D6S273, TNFα, C12A, STR MICA, MIB, C125, C143, C245, C3211, MOGc and D6S2223). Nuclear families were stratified into two separate groups: parents carrying the DRB1*08-DQB1*04 (referred as DR8 family set; n=188), parents with DRB1*04:04-DQB1*03:02 (referred as DR4 family set; n=135) haplotypes. Further genotyping was performed for the two main subtypes of HLA*B39 allele (B*39:01 and B*39:06). Each nuclear family consisted of one or two parent(s) and one affected child with T1D. Most of the families were recruited from four university hospitals in Finland (Turku, Oulu, Tampere and Helsinki).

**Study III**. Newborn infants with increased (HLA-DQB1*03:02/*02) or moderate risk (HLA-DQB1*03:02/x where x≠*02, *03:01 or *06:02) for type 1 diabetes were monitored for ICA; GADA, IAA and IA-2 autoantibodies at 3- to 6- month intervals in Turku and 3- to 12- month intervals in Tampere and Oulu. For further genotyping of class I alleles, we selected 249 samples, which were positive at least for one biochemically defined antibody besides ICA.

In **Study IV**, sixty-nine Addison's patients were collected from three different Eastern Baltic countries, namely Estonia (n=24), Finland (n=14) and Russia (n=31). Sixty-six out of sixty-nine patients were adults. None of the patients had type 1 polyendocrinopathy syndrome but 29.0% of the patients had type 2 autoimmune polyendocrinopathy syndrome. Seventy percent of the Estonian and Finnish patients were positive for the 21-hydroxylase antibodies, including seven out of eight

patients (Finnish n=6 and Estonian n=2) with type 2 autoimmune polyendocrinopathy syndrome. We have no data for further clinical information from Russian patients. As controls we used background populations which were collected from the same geographical regions (n=269 Estonian, n=1000 Finnish and n=413 Russian). The Finnish control samples were cord bloods collected from two university hospitals in Finland (Oulu; n=499 and Turku; n=501). HLA class II haplotypes were determined in control as well as in Addison's disease samples and also eleven microsatellites that were described in Study II, were determined in the patients' samples.

The diagnosis of T1D was based in the World Health Organization criteria.

The local Ethics Committees had approved all of the studies and informed consent was obtained from the participating subjects or from their parents.

## 4.2. Methods

### 4.2.1. DNA

In **Study I-IV**, genomic DNA was extracted from peripheral EDTA-blood using the salting-out method (Miller, Dykes & Polesky 1988) was used for all of the genetic analyses in this work. The regions of interests were amplified in a 96-well plate format in PCR in Hybraid Touchdown™ (Cambridge, UK) or MJ Research DNA Engine Tetrad™ (Massacgsetts, USA) thermal cyclers.

### 4.2.2. HLA class II genotyping

HLA-DQA1-DQB1 genotype and HLA-DRB1*04 subtypes were identified by PCR amplification combined with lanthanide-labelled oligonucleotide hybridization and time-resolved fluorometry detection. These methods were used in all four papers as follows. The polymorphic second exon was amplified using sequence-specific primer pairs biotinylated at the 5´-end. The biotinylated PCR products were transferred to the streptavidin-coated microtitration plates. After denaturation, DNA was hybridized to short allele-specific oligonucleotide probes labelled with either europium (Eu), terbium (Tb) or samarium (Sm) chelates. After the appropriate incubation time, enhancement and washing steps followed. Finally, the three-colour time-resolved fluorescence of lanthanide labels were detected in a 1234 DELFIA ™ Research Fluorometer (PerkinElmer, Wallac Oy). Results (counts/s and signal-to-background ratios) were analyzed with the Wallac Multicalc software.

#### 4.2.2.1. HLA-DQB1 genotyping

HLA-DQB1 gene proved to have the strongest association for T1D and Addison's disease. Therefore, all four studies were started with analyzing the major susceptibility (DQB1*03:02, *02,) and protective alleles (DQB1*03:01, *06:02, *06:03) of the HLA-DQB1 region (Sjöroos et al. 1995). HLA-DQB1 "full house" genotyping was performed with lanthanide-label hybridization assay (Kiviniemi et al. 2007, Laaksonen et al. 2002).

#### 4.2.2.2. HLA-DQA1 genotyping

The specific region of the second exon of the HLA-DQA1 gene was studied in **Paper I-IV** according to Sjöroos and co-workers publication (Sjöroos et al. 1998). We could differentiate the HLA-DQA1*02:01, *03 and *05 alleles with specific Eu labelled TRF hybridization assay.

#### 4.2.2.3. HLA-DR4 subtyping

Distribution of the DRB1 alleles of the DR4 group was analyzed among the HLA DQB1*03:02 positive subjects, because the DQB1*03:02 allele is in very strong linkage disequilibrium with DR4 alleles and various DRB1*04-DQB1*03:02 (DR4-DQ8) haplotypes differ in their T1D susceptibility depending of the specific DR4 allele. For DR4 subtyping, the high-resolution TRF-based technique was designed by Nejentsev and co-workers (Nejentsev et al. 1999). With this assay, the number of the tested alleles increased to 13 groups, these include 29 DR4 subtypes. This method enabled us to identify the most common Caucasian DRB1alleles.

### 4.2.3. HLA class I genotyping

The common HLA-A and HLA-B alleles in the Finnish population were typed using the sequence-specific primers (SSP) for amplification and detection of the PCR product on the agarose gels (Paper I, II and III) (Bunce et al. 1995, Bunce, Fanning & Welsh 1995). However the samples positive for HLA-B*39 were tested with the B*39:06 specific probe in nuclear families with HLA-DRB1*08-DQB1*04 haplotypes in unpublished results. This assay based on the DELFIA® principle (PerkinElmer Life and Analytic Sciences Wallac, Turku, Finland) used biotinylated primers in PCR to allow binding of the PCR product to a streptavidin coated microtiter well (Mikk et al. 2017).

### 4.2.4. Genotyping of microsatellites

In Paper I ten (D6S273, TNFα, C12A, STR MICA, MIB, C125, C143, C245, C3211, MOGc) and in Paper II and IV eleven microsatellites (D6S2223 added) covering approximately 4Mb over the HLA region were analyzed using the previously described genotyping technique by Foissac et al. in 1998 (Foissac, Cambon-Thomsen 1998). Each microsatellite region was amplified using primer pairs with one fluorescent-labelled (6-FAM, HEX or TET) primer. Fluorescent labels were attached to the 5´end of the forward primer as displayed the pseudo-colors blue, yellow and green. The lengths of the amplification products were determined on an automated multicapillary sequencer (MegaBace 1000; Amersham Pharmacia Biotech, Sunnyvale, CA, USA, ABI 310or on ABI 377 Applied Biosystems, Foster City, CA, USA). The data were analyzed by Genescan and Genetic Profiler software.

### 4.2.5. The autoantibody assays

Islet cell antibodies were detected with immunofluorescence assays as described earlier (Bottazzo, Florin-Christensen & Doniach 1974, Kimpimäki et al. 2002, Siljander et al. 2009). The detection limit for ICA was 2.5 Juvenile Diabetes Foundation Units (JDFU, sensitivity 100%, specificity 98%). Serum levels of IAA were quantified with micro-assay method, described by Williams et al. (Williams et al. 1997). GAD65 (GAD antibody GADA) and IA-2 antigen (IA-2A) were measured with a specific radiobinding assay (Savola, Bonifacio et al. 1998, Savola, Sabbah et al. 1998). The cut-off limits for IAA, GAD65 and IA-2 (respectively in Relative Unit; 1.56 RU, 5.36 RU and 0.43 RU) were based on representing the $99^{th}$ percentile for more than 350 Finnish non-diabetic subjects (Siljander et al. 2009).

## 4.3. Statistical analysis and computer software

Odds ratios were calculated according the Woolf's formula OR=ad/bc with weighted average (a=number of exposed cases, b= number of exposed controls, c=number of unexposed cases, d=number of unexposed controls). The homogeneity of different odds ratios were analyzed by Mantel-Haenszel method.

The allele frequencies were estimated by direct counting. For the calculation of the statistical significance the Chi-square test or Fisher's exact test when appropriate was used. Bonferroni

correction (mentioned as p cor.) was used Studies I, II and IV to reduce the chances of obtaining false-positive results due to multiple.

Ninety-five % confidence intervals were calculated to estimate the precision of the Chi-square test and the Odds ratios (Study I Study IV) using the Epistat software package. Genepop software package was (Raymond, Rousset 1995) utilized for the population differentiation test where the null hypothesis of similarity between the type 1 diabetes and controls populations was rejected, when $p<0.01$.

Transmission/Disequilibrium Test (TDT) is a useful method to detect the linkage and association between complex diseases- and analyzed genetic markers in nuclear families with heterozygous parents (Spielman, McGinnis & Ewens 1993). The TDT test is effective for any marker where data are available from parents and one or more affected offspring. In the study II TDT was performed using TDTPHASE program of the UNPHASED software package (Dudbridge et al. 2000). Conditional Extended TDT (CETDT) was used to estimate the disease association of non-class II microsatellite markers independent of HLA class II; DRB1-DQA1-DQB1 regions (Dudbridge 2003, Koeleman et al. 2000). Haplotypes analysis was done with GENEHUNTER 2.0 software in study II (Kruglyak et al. 1996). Data from 323 Finnish nuclear families allowed unambiguous assignment of alleles at three-locus haplotypes (DRB1-DQA1-DQB1) in HLA region. Control genotypes were determined by the affected-family-based artificial control (AFBAC) method where parental haplotypes not transmitted to the affected child in each trio family were used to form and artificial control (Falk, Rubinstein 1987, Thomson 1995).

In Study III the PASW 18.0 (SPSS Inc., Chicago, IL) statistical software was used for two different survival analyses; as non-parametric Kaplan-Meier method and for classical semi-parametric Cox-regression models. The effect of all typed HLA class I alleles and HLA class II haplotypes on progression to type 1diabetes after appearance of the biochemically defined autoantibodies was tested using Cox-regression.

# 5. RESULTS

## 5.1. DRB1*04:04-DQB1*03:02 haplotype associated risk

### 5.1.1. Localization of the class I and class III region genes affecting T1D susceptibility in HLA-DR/DQ matched case-control series (Publication I).

The effect of other than HLA-DR and -DQ genes within the HLA gene region on T1D susceptibility was analyzed using two case-control datasets matched for combination of DR3-DQ2 and DR4-DQ8 haplotypes but with different DR4 subtypes. DR3-DQ2 was deduced from the presence of DQA1*05-DQB1*02 and DR4 haplotype was either DRB1*04:01-DQB1*03:02 ((DR3/DR401), T1D n=241, controls n=354)) or DRB1*04:04-DQB1*03:02 ((DR3/DR404), T1D n=75, controls n=181)). Four selected class I gene alleles (HLA-B*39, HLA-B*62, HLA-A*02, HLA-A*24) and 10 microsatellite markers (D6S273, TNFα, C12A, STR MICA, MIB, C125, C143, C245, C3211 and MOGc) were analyzed from all samples. These cover a distance of ~2300 kb and include the whole HLA class I region and the borderline region between class I and class III (Figure 7).

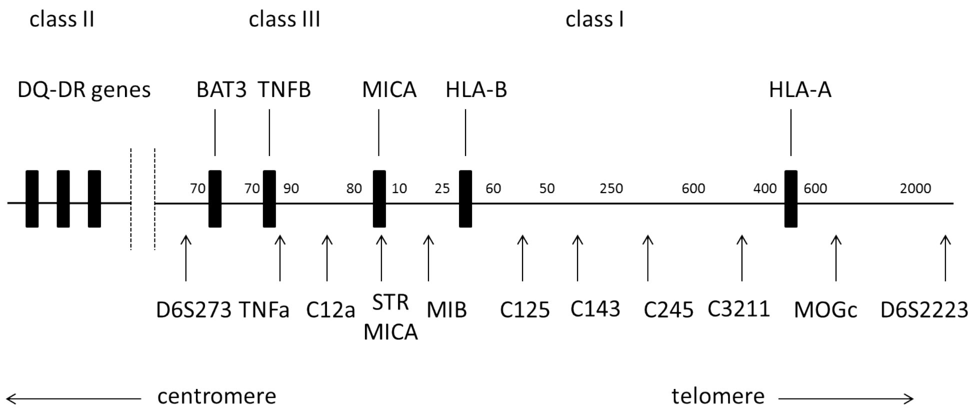



**Figure 7.** Schematic picture of markers in the HLA region with approximate distances (kb). ↑ microsatellite markers, ▌genes, TNFa- equal with TNFα (from original Publication I-II).

The distribution of the allele frequencies at ten microsatellite loci and presence of the tested HLA-A and HLA-B alleles was analyzed in the DR3/DR4 subgroups and the significance of differences between cases and controls tested using the Chi-square test. In addition, the population differentiation test was used as a complementary test. The distribution of analyzed markers in the region between the D6S273 and MOGc and their odds ratios (ORs) in the two groups of DR3/DR4 genotypes with DR4 allele being either DRB1*04:01 or DRB1*04:04 are shown in Table 3. In both

subgroups, a single allele of each microsatellite marker was found in great majority of both patients and controls without conferring any risk or protection effects. These alleles were interpreted to represent the extended DR3-DQ2 haplotype.

**Table 3.** The most common HLA markers in the DR3/DRB1*04:04 and DR3/DRB1*04:01 genotypes among the patient- and control groups (Modified from Publication I).

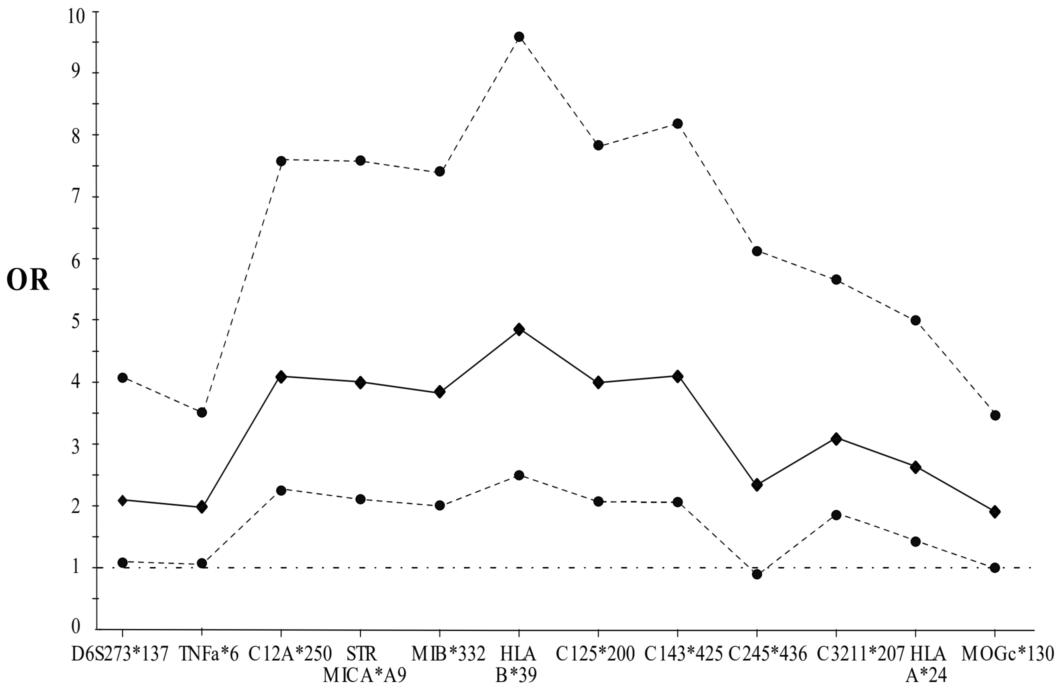
| **Markers allele sizes** | **Patients DR3/04:04 n=75 (%)** | **Controls DR3/04:04 n=181 (%)** | **OR (95%CI)** | **p/pcor** | **Patients DR3/04:01 n=241 (%)** | **Controls DR3/04:01 n=354 (%)** | **OR (95% CI)** | **p** |
|---|---|---|---|---|---|---|---|---|
| D6S273 *135 | 19 (25.3) | 77 (42.5) | 0.5 (0.2-0.9) | 0.01/NS | 88 (36.5) | 141 (39.8) | 0.9 (0.6-1.2) | NS |
| *137 | 56 (74.7) | 105 (58.0) | 2.1 (1.1-4.1) | 0.02/NS | 123 (51.0) | 170 (48.0) | 1.2 (0.8-1.6) | NS |
| df =7 ***141** | **65 (86.7)** | **138 (76.2)** | **2.0 (0.9-4.6)** | **NS** | **201 (83.4)** | **296 (83.6)** | **1.0 (0.6-1.6)** | **NS** |
| TNFα ***2** | **66 (88.0)** | **150 (82.9)** | **1.5 (0.6-3.6)** | **NS** | **228 (94.6)** | **326 (92.1)** | **1.5 (0.7-3.1)** | **NS** |
| *6 | 38 (50.7) | 62 (34.3) | 2 (1.1-3.5) | 0.02/NS | 72 (29.9) | 93 (26.3) | 1.2 (0.8-1.7) | NS |
| df=12 *11 | 29 (38.7) | 87(48.1) | 0.7 (0.4-1.2) | NS | 9 (3.7) | 14 (3.95) | 2.4 (0.4-2.4) | NS |
| C12A *236 | 9 (12.0) | 52 (28.7) | 0.3 (0.15-0.8) | 0.007/NS | 78 (32.4) | 93 (26.3) | 1.3 (0.9-1.9) | NS |
| *250 | 37 (49.3) | 35 (19.3) | 4.1 (2.2-7.6) | $2x10^{-6}/2.2x10^{-5}$ | 113 (46.9) | 154 (43.5) | 1.1 (0.8-1.6) | NS |
| df=11 ***256** | **61 (81.3)** | **138 (76.2)** | **1.4 (0.7-2.8)** | **NS** | **187 (77.6)** | **283 (79.9)** | **0.9 (0.6-1.3)** | NS |
| STR MICA *A4 | 9 (12.0) | 42 (23.2) | 0.5 (0.2-1.0) | NS | 40 (16.6) | 59 (16.7) | 1.0 (0.6-1.6) | NS |
| *A5 | 5 (6.7) | 30 (16.6) | 0.4 (0.1-1.0) | NS | 136 (56.4) | 191 (54.0) | 1.1 (0.8-1.6) | NS |
| ***A5.1** | **70 (93.3)** | **169 (93.4)** | **1.0 (0.3-3.4)** | **NS** | **205 (85.1)** | **304 (85.9)** | **0.9 (0.6-1.5)** | **NS** |
| df=4 *A9 | 34 (45.3) | 31 (17.1) | 4.0 (2.1-7.6) | $5x10^{-6}/2x10^{-5}$ | 32 (13.3) | 46 (12.9) | 1.0 (0.6-1.7) | NS |
| MIB *326 | 8 (10.7) | 32 (17.7) | 0.6 (0.2-1.3) | NS | 132 (54.8) | 181 (51.1) | 1.2 (0.8-1.6) | NS |
| *332 | 31 (41.3) | 28 (15.5) | 3.8 (2.0-7.4) | $2x10^{-5}/3x10^{-4}$ | 2 (0.83) | 7 (1.9) | 0.7 (0.1-3.8) | NS |
| df=15 ***350** | **65 (86.7)** | **164 (90.6)** | **0.7 (0.3-1.7)** | **NS** | **201 (83.4)** | **273 (77.1)** | **1.5 (0.9-2.6)** | **NS** |
| HLA B*39 | 31 (41.3) | 23 (12.7) | 4.8 (2.5-9.6) | $10^{-6}$ | | | | |
| B*62 | | | | | 133 (55.2) | 176 (49.7) | 1.3 (0.9-1.8) | NS |
| C125 ***194** | **59 (78.7)** | **135 (74.6)** | **1.3 (0.6-2.5)** | **NS** | **183 (76.0)** | **269(76.0)** | **1.0 (0.7-1.5)** | **NS** |
| *200 | 31 (41.3) | 27 (14.9) | 4.0 (2.1-7.8) | $9x10^{-6}/1.5x10^{-4}$ | 14 (5.8) | 20 (5.6) | 1.0 (0.5-2.2) | NS |
| *208 | 5 (6.7) | 29 (16.0) | 0.4 (0.1-1.1) | NS | 115 (47.7) | 152 (42.9) | 1.2 (0.7-1.7) | NS |
| df=17 *210 | 22 (29.3) | 61 (33.7) | 0.8 (0.4-1.5) | NS | 8 (3.3) | 19 (5.4) | 0.6 (0.2-1.5) | NS |
| C143 *425 | 28 (37.3) | 23 (12.7) | 4.1 (2.1-8.2) | $<2x10^{-5}/2.8x10^{-4}$ | 0 (0.0) | 1 (0.28) | 0.0 (0.0-25.5) | NS |
| *441 | 3 (4.0) | 10 (5.5) | 0.7 (0.2-2.9) | NS | 67 (27.8) | 84 (23.7) | 1.2 (0.8-1.8) | NS |
| *445 | 28 (37.3) | 89 (49.2) | 0.6 (0.3-1.1) | NS | 64 (26.6) | 92 (25.9) | 1.0 (0.7-1.5) | NS |
| df=14 ***449** | **54 (72.0)** | **127 (70.2)** | **1.1(0.6-2.1)** | **NS** | **196 (81.3)** | **285 (80.5)** | **1.1 (0.7-1.6)** | **NS** |
| C245 *428 | 18 (24.0) | 48 (26.5) | 0.9 (0.4-1.7) | NS | 110 (45.6) | 171 (48.3) | 0.9 (0.6-1.3) | NS |
| ***436** | **68 (90.7)** | **146 (80.7)** | **2.3 (0.93-6.1)** | **NS** | **201 (83.4)** | **296 (83.6)** | **1.0 (0.6-1.6)** | **NS** |
| *464 | 0 (0.0) | 0 (0.0) | | NS | 37 (15.4) | 41 (11.6) | 1.4 (0.8-2.3) | NS |
| *476 | 15 (20.0) | 61 (33.7) | 0.5 (0.2-1.0) | 0.04/NS | 5 (2.1) | 13 (3.7) | 0.6 (0.2-1.7) | NS |
| df=10 *436/*436 | 27 (36.0) | 38 (21.0) | 2.1 (1.1-4.0) | <0.02 | 47 (19.5) | 58 (16.4) | 1.2 (0.8-1.9) | NS |
| C3211 *205 | 11 (14.7) | 31 (17.1) | 0.8 (0.4-1.9) | NS | 92 (38.2) | 119(33.6) | 1.2 (0.8-1.7) | NS |
| *207 | 37 (49.3) | 43 (23.8) | 3.1 (1.7-5.7) | $10^{-4}/1.9x10^{-3}$ | 81 (33.6) | 130 (36.7) | 0.9 (0.6-1.2) | NS |
| *211 | 16 (21.3) | 64 (35.4) | 0.5 (0.3-1.0) | 0.04/NS | 24 (10.0) | 36 (10.2) | 1.0 (0.5-1.7) | NS |
| df=19 ***217** | **44 (58.7)** | **111 (61.3)** | **0.9 (0.5-1.6)** | **NS** | **150 (62.2)** | **216 (61.0)** | **1.1 (0.7-1.5)** | **NS** |
| HLA A*24 | 29 (38.7) | 35 (19.3) | 2.6 (1.4-5.0) | $<2x10^{-3}$ | | | | |
| A*2 | | | | | 148 (61.4) | 200 (56.5) | 1.2 (0.9-1.9) | NS |
| MOGc *130 | 50 (66.7) | 94 (51.9) | 1.9 (1.0-3.4) | 0.04/NS | 63 (26.14) | 95 (26.8) | 1.0 (0.7-1.4) | NS |
| *132 | 32 (42.7) | 89 (49.2) | 0.8 (0.4-1.4) | NS | 162 (67.2) | 213 (60.2) | 1.4 (0.9-1.9) | NS |
| df=14 *148 | 32 (42.7) | 75 (41.4) | 1.1 (0.6-1.9) | NS | 108 (44.8) | 183 (51.7) | 0.8 (0.6-1.1) | NS |

bold-alleles associated with the DR3 haplotype; df-degree of freedom, pcor-Bonferroni correction

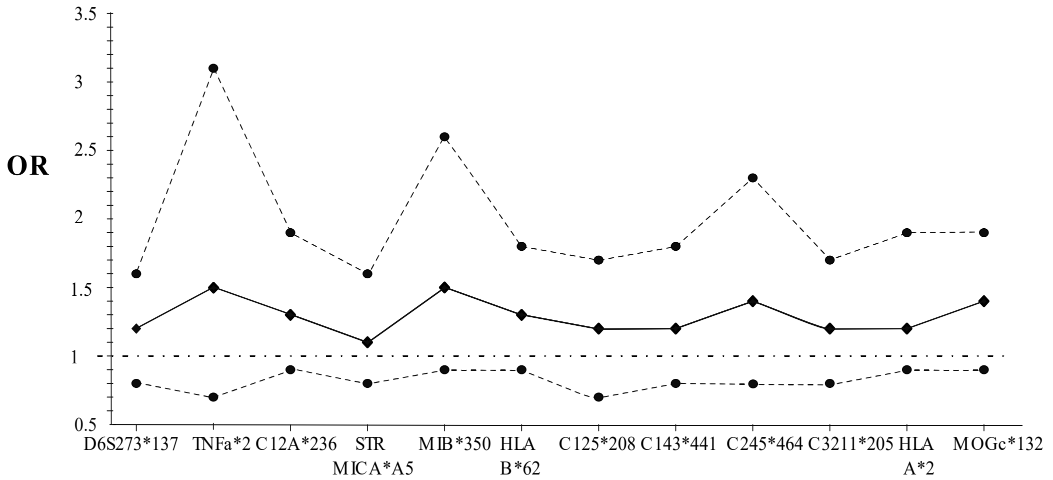
Markers with a significant difference between cases and controls were found in most analyzed loci in the DR3/DRB1*04:04 subgroup whereas no significant differences were found in the DR3/DRB1*04:01 subgroup of patients and control subjects. Figures 8A and 8B show OR values for T1D associated alleles in the two analyzed subgroups. We confirmed the previous findings that B*39 (Nejentsev et al. 1997, Reijonen et al. 1997) and A*24 (Honeyman et al. 1995) alleles have a strong disease association (respectively, OR 4.8, 95% CI 2.5-9.6 $p=10^{-6}$ and OR 2.6 95% CI 1.4-5.0 $p<2x10^{-3}$, respectively) on the DR3/DR04:04 genotype (Table 3).

None of the markers was associated with higher T1D risk than HLA-B*39 allele (OR 4.8) on the DR3/DRB1*04:04 genotype although the 95% CIs were overlapping in all comparisons of T1D associated alleles on the D6S273-TNFα-C12A-STR MICA-MIB-HLA B-C125-C143-C245-C3211-HLA A-MOGc segment of DRB1*04:04 (Table 3 and Fig 8A). The differences stayed statistically significant after Bonferroni correction in the following microsatellite alleles: C12A*250, STR MICA*A9, MIB*332 C125*200, C143*425 and C3211*207. Similar results were obtained by using the population differentiation test. C12A, STR MICA, C125 and C143 regions showed statistically significant differences between affected and background population with strong p values on the DR3/DR04:04stratified groups (Table 4).

In the DR3/DRB1*04:01 subgroup of patients and control subjects, statistical test did not reveal significant differences in any single allele on the D6S273-MOGc region (Table 3 and Figure 8B), although population differentiation result demonstrated a significant differentiation between patients and background population at MIB locus ($p= 4.63x10^{-3}$, threshold $p<10^{-2}$) and also the C12A marker was close to the significance level ($p=1.373x10^{-2}$) (Table 5).




A




B

**Figure 8.** Odds ratios for the strongest associated alleles in DR3/DRB1*04:04 (A) and DR3/DRB1*04:01 (B) groups (♦OR, ●95% CI for OR) (from original Publication I).

**Table 4.** Population differentiation test for comparison of T1D patients and controls with the stratified HLA genotype groups (from original Publication I). (significance level $p<0.01$; SE- standard error)

| Locus | DR3/DRB1*04:04<br>T1D n=75, Controls n=181 | | DR3/DRB1*04:01<br>T1D n=241, Controls n=354 | |
|---|---|---|---|---|
| | p values | SE | p values | SE |
| D6S273 | 0.02368 | 0.00040 | 0.09557 | 0.00114 |
| TNFα | 0.29543 | 0.00166 | 0.65175 | 0.00178 |
| C12A | **0.00326** | **0.00013** | 0.01373 | 0.00041 |
| STR MICA | **0.00018** | **0.00002** | 0.54007 | 0.00195 |
| MIB | 0.02614 | 0.00047 | **0.00463** | **0.00020** |
| C125 | **0.00239** | **0.00013** | 0.56609 | 0.00229 |
| C143 | **0.00013** | **0.00002** | 0.46173 | 0.00242 |
| C245 | 0.16287 | 0.00117 | 0.27557 | 0.00239 |
| C3211 | 0.02037 | 0.00042 | 0.14022 | 0.00189 |
| MOGc | 0.53826 | 0.00163 | 0.25469 | 0.00236 |

significant p values are bold

### 5.1.2. Reconstruction of the extended haplotypes in families (Publication I-II).

For reconstruction of extended haplotypes in families, CETDT (unphased package) was used to estimate the most frequently transmitted length at each microsatellite locus in 20 randomly selected heterozygous nuclear families with DR3/DRB1*04:04, 20 with DR3/DRB1*04:01 and 20 with DR3/(DR8)-DQB1*04 genotype (Table 5). The extended DR3 haplotype was DQB1*02-DQA1*05-D6S273*141-TNFα*2-C12A*256-STR MICA*A5.1-MIB*350-C125*194-C143*449-C245*436-C3211*217 (Table 3 and Table 5).

**Table 5.** Transmission disequilibrium analysis of selected HLA markers in DR3 haplotype with DR3/DRB1*04:04, DR3/DRB1*04:01 and DR3/(DR8)-DQB1*04 families (Unpublished data).

| Marker allele size | DR3/DRB1*04:04 | | | | DR3/DRB1*04:01 | | | | DR3/(DR8)-DQB1*04 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | T (n) | NT (n) | T (%) | p | T (n) | NT (n) | T (%) | p | T (n) | NT (n) | T (%) | p |
| D6S273*141 | 15 | 1 | 93.8 | $1.3x10^{-4}$ | 13 | 1 | 92.9 | $4.8x10^{-4}$ | 13 | 0 | 100 | $2.2x10^{-5}$ |
| TNFα*2 | 11 | 1 | 91.7 | $1.8x10^{-3}$ | 15 | 4 | 79.0 | $9.2x10^{-3}$ | 7 | 0 | 100 | $1.8x10^{-3}$ |
| C12A*256 | 14 | 1 | 93.3 | $2.4x10^{-4}$ | 14 | 3 | 82.4 | $5.5x10^{-3}$ | 4 | 0 | 100 | $1.8x10^{-2}$ |
| STR MICA*A5.1 | 16 | 2 | 88.9 | $4x10^{-4}$ | 15 | 3 | 83.3 | $3.1x10^{-3}$ | 10 | 0 | 100 | $1.9x10^{-4}$ |
| MIB*350 | 12 | 2 | 85.7 | $4.9x10^{-3}$ | 14 | 2 | 87.5 | $1.5x10^{-3}$ | 7 | 0 | 100 | $1.8x10^{-3}$ |
| C125*194 | 15 | 2 | 88.2 | $7.9x10^{-4}$ | 14 | 2 | 87.5 | $1.5x10^{-3}$ | 3 | 0 | 100 | $4.1x10^{-2}$ |
| C143*449 | 13 | 2 | 86.7 | $2.7x10^{-3}$ | 11 | 2 | 84.6 | $8.8x10^{-3}$ | 7 | 1 | 87.5 | $2.4x10^{-2}$ |
| C245*436 | 13 | 0 | 100 | $2.2x10^{-5}$ | 11 | 2 | 84.6 | $8.8x10^{-3}$ | 10 | 0 | 100 | $1.9x10^{-4}$ |
| C3211*217 | 10 | 1 | 90.9 | $3.5x10^{-3}$ | 9 | 2 | 81.8 | $2.8x10^{-2}$ | 3 | 0 | 100 | $4.1x10^{-2}$ |
| MOGc*148 | 3 | 1 | 11.1 | NS | 10 | 2 | 83.3 | $1.6x10^{-2}$ | 3 | 0 | 100 | $4.1x10^{-2}$ |

We could see in both family groups an extended DR3-DQ2 haplotype transmitted, which was identical with the most common microsatellite alleles in case control series up to C3211 in the telomeric end of the haplotype (Table 5). A common dominating MOGc locus allele could no more be detected. The finding demonstrates the high degree of homogeneity of the Finnish DR3-DQ2 haplotype.

Conditional extended TDT (CETDT) on the HLA region was used to estimate the most frequently transmitted length of each microsatellite also in the selected DRB1*04:04 and DRB1*04:01 haplotypes (unphased package). The extended DRB1*04:04 haplotype was DQB1*03:02-DRB1*04:04-D6S273*137-TNFα*6-C12A*250-STR MICA*A9-MIB*332-B*39-C125*200-

C143*425-C245*436-C3211*207-A*24-MOGc*130 corresponding to those markers increased in the case-control study (Table 3 and Table 6) whereas markers in the DRB1*04:01 haplotype was TNFa*2-C12A*236-STRMICA*A5-MIB*350-B*62-C125*208-C143*441-C245*464-C3211*205-A*2-MOGc*132 (Table 7).

**Table 6.** The most common with DRB1*04:04 haplotype transmitted allele (Unpublished data).

| Marker allele size | T (n) | T (%) | p value |
|---|---|---|---|
| D6S273*137 | 12 | 100 | $4.5x10^{-5}$ |
| TNFα*6 | 6 | 100 | $3.9x10^{-3}$ |
| C12A*250 | 7 | 100 | $1.8x10^{-3}$ |
| STR MICA*A9 | 7 | 100 | $1.8x10^{-3}$ |
| MIB*332 | 6 | 100 | $3.9x10^{-3}$ |
| HLA-B*39 | 7 | 100 | $1.8x10^{-3}$ |
| C125*200 | 6 | 100 | $3.9x10^{-3}$ |
| C143*425 | 6 | 100 | $3.9x10^{-3}$ |
| C245*436 | 7 | 100 | $1.8x10^{-3}$ |
| C3211*207 | 10 | 100 | $2x10^{-4}$ |
| HLA-A*24 | 5 | 100 | $8.5x10^{-3}$ |
| MOGc*130 | 5 | 100 | $8.5x10^{-3}$ |

**Table 7.** The most common with DRB1*04:01 haplotype transmitted allele (Unpublished data).

| Marker allele size | T (n) | NT (n) | T (%) | p value |
|---|---|---|---|---|
| D6S273*137 | 7 | 2 | 77.8 | NS |
| TNFα*2 | 8 | 2 | 80 | $4.9x10^{-2}$ |
| C12A*236 | 6 | 0 | 100 | $3.9x10^{-3}$ |
| STR MICA*A5 | 7 | 1 | 87.5 | $2.4x10^{-2}$ |
| MIB*350 | 7 | 1 | 87.5 | $2.4x10^{-2}$ |
| HLA-B*62 | 7 | 0 | 100 | $1.8x10^{-3}$ |
| C125*208 | 3 | 0 | 100 | $4.1x10^{-2}$ |
| C143*441 | 6 | 1 | 85.7 | $4.6x10^{-2}$ |
| C245*464 | 6 | 1 | 85.7 | $4.610^{-2}$ |
| C3211*205 | 6 | 0 | 100 | $3.9x10^{-3}$ |
| HLA-A*2 | 10 | 0 | 100 | $1.9x10^{-4}$ |
| MOGc*132 | 10 | 0 | 100 | $1.9x10^{-4}$ |

### 5.1.3. Localization of the independent disease association of the class I and class III gene region in Finnish nuclear families (Publication II).

We extended our study (Publication II.) for 135 Finnish nuclear families with DRB1*04:04-DQB1*03:02 (DR4) haplotype and also for 188 families with DRB1*08-DQB1*04 (DR8) haplotype. Both family sets were analyzed for HLA-B*39 allele and 11 microsatellite markers (D6S273, TNFα, C12A, STR MICA, MIB, C125, C143, C245, C3211, MOGc and D6S2223) covering a 4-Mb region telomeric to HLA-DQB1 gene (Figure 7). The HLA DRB1-DQA1-DQB1 region displayed strong disease association in both family sets using TDT and CETDT tests (DR4 $p=5x10^{-21}$, df=11 and DR8 $p=7x10^{-34}$ df=10) (Figure 9 and 10). Microsatellite alleles with parental frequency of less than 2% were pooled in both analyses.

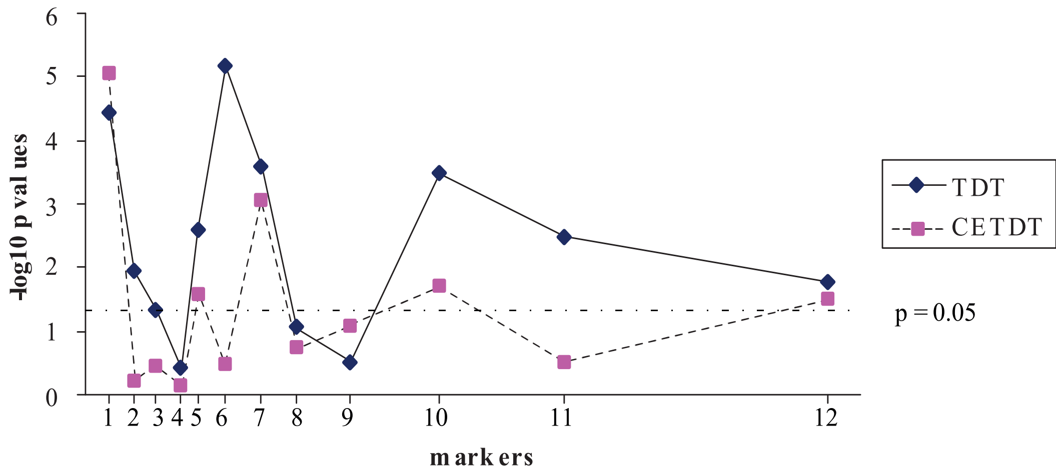



**Figure 9.** Selected microsatellite markers on 6p21 chromosome region in Finnish nuclear families with DRB1*04:04 haplotype. TDT and CETDT –log10p values shown separately for all analyzed markers (from original Publication II).

Markers are numbered as follows: 1, D6S273 2, TNFα 3, C12A 4, STR MICA 5, MIB 6, HLA-B*39 7, C125 8, C143 9, C245 10, C3211 11, MOGc 12, D6S2223

In the DRB1*04:04 families markers in three separate gene region as D6S273-TNFα, MIB-HLA B*39-C125 and C3211-MOGc-D6S2223 showed association with T1D both in the single locus TDT analysis and CETDT analysis conditioned for HLA haplotype (Table 8, Figure 9). Associated regions peaked at D6S273, HLA-B and C3211 loci in single locus TDT analysis.

**Table 8.** Transmission disequilibrium analysis with selected markers in the HLA region in HLA-DRB1*04:04-DQB1*03:02 haplotype families (Modified from Publication II). (p cor. calculated with Bonferroni correction)

| Marker | Allele | T (n) | NT (n) | T (%) | p value of allele in TDT | Global TDT p value | Global CETDT p value | Corrected global CETDT p value |
|---|---|---|---|---|---|---|---|---|
| D6S273<br>df = 7 | 135<br>137<br>139<br>Others | 34<br>70<br>0<br>69 | 63<br>41<br>11<br>58 | 35.1<br>63.1<br>0<br>54.3 | $3x10^{-3}$<br>$6x10^{-3}$<br>$9.4x10^{-5}$ | $3.5x10^{-5}$ | $9x10^{-6}$ | $10^{-4}$ |
| TNFα<br>df = 9 | 6<br>10<br>Others | 56<br>5<br>139 | 33<br>22<br>145 | 62.9<br>18.5<br>48.5 | $1.4x10^{-2}$<br>$7x10^{-4}$ | $9x10^{-3}$ | NS | NS |
| MIB<br>df = 12 | 332<br>342<br>348<br>350<br>Others | 30<br>2<br>6<br>53<br>100 | 13<br>10<br>15<br>30<br>123 | 69.8<br>16.7<br>28.6<br>63.9 | $9x10^{-3}$<br>$1.6x10^{-2}$<br>$4.6x10^{-2}$<br>$1.1x10^{-2}$ | $2x10^{-3}$ | $3.3x10^{-2}$ | NS |
| HLA-B*39 | 3901<br>negative | 46<br>12 | 12<br>46 | 79.3<br>20.7 | $3.9x10^{-6}$<br>$3.9x10^{-6}$ | $4x10^{-6}$ | NS | NS |
| C125<br>df = 12 | 194<br>200<br>208<br>Others | 39<br>36<br>12<br>129 | 19<br>12<br>35<br>150 | 67.2<br>75.0<br>25.5<br>46.2 | $8x10^{-3}$<br>$3.9x10^{-4}$<br>$6x10^{-4}$ | $2x10^{-4}$ | $8x10^{-4}$ | $9.5x10^{-3}$ |
| C3211<br>df = 11 | 195<br>207<br>209<br>213<br>Others | 15<br>46<br>13<br>4<br>120 | 30<br>25<br>3<br>13<br>127 | 33.3<br>64.8<br>81.2<br>23.5<br>48.5 | $2.4x10^{-2}$<br>$1.2x10^{-2}$<br>$9x10^{-3}$<br>$2.5x10^{-2}$ | $3x10^{-4}$ | $1.4x10^{-2}$ | NS |
| MOGc<br>df = 9 | 130<br>134<br>144<br>Others | 56<br>1<br>1<br>103 | 34<br>9<br>6<br>112 | 62.2<br>10.0<br>14.3<br>47.9 | $1.9x10^{-2}$<br>$7x10^{-3}$<br>$4.7x10^{-2}$ | $2x10^{-3}$ | NS | NS |
| D6S2223<br>df = 3 | 170<br>176<br>Others | 50<br>4<br>54 | 33<br>17<br>58 | 60.2<br>19.1<br>48.2 | NS<br>$3x10^{-3}$ | $1.6x10^{-2}$ | $3.7x10^{-2}$ | NS |

df-degree of freedom, OR-odds ratio, CI-confidence interval

To extend the analysis to determine the effect of non-class II markers on the fixed DRB1*04:04-DQB1*03:02 haplotype the conditional haplotype method (HM) was used (Figure 9, Table 9). The C125*200 allele was confirmed to have an independent disease association on the DRB1*04:04-DQB1*03:02 haplotype with OR 3.56 (p=0.039). The effect of the D6S273*137 and HLA-B*39 allele did not quite reach the statistical significance (Table 9). The C125*200 was similarly increased also on the extended HLA DRB1*04:04-DQB1*03:02-B*39 haplotype (31/40 transmitted, 77.5%) compared to AFBAC controls (3/7 transmitted, 42.8%) but, the difference was

not significant.

**Table 9.** Disease associated markers of the DRB1*04:04-DQB1*03:02 haplotypes in the Finnish families using the haplotype methods (from original Publication II).

| Marker | Marker length | Patients (n) | AFBAC (n) | p value | OR | 95% CI |
|---|---|---|---|---|---|---|
| D6S273 | 137 | 54 | 12 | 0.059 | 2.45 | 0.97-6.266 |
| | All others | 33 | 18 | | | |
| HLA-B*39 | 3901 | 45 | 8 | 0.054 | 2.57 | 0.987-6.852 |
| | All others | 57 | 26 | | | |
| C125 | 200 | 31 | 4 | 0.039 | 3.56 | 1.056-13.182 |
| | 208 | 1 | 4 | 0.021 | 0.077 | 0.003-0.783 |
| | All others | 60 | 24 | | | |

AFBAC-affected family-based artificial controls

### 5.2. HLA-DR8-DQ4 haplotype associated risk (Publication II and Unpublished data).

The same microsatellite selection was analyzed also in the family set with DR8-DQ4 haplotype. The D6S273, C143, C245, MOGc and D6S2223 microsatellites showed disease association in the TDT test and three of them, C143, C245, and MOGc remained significant also in CETDT analysis (Figure 10 and Table 10). The C143*445 and the C143*449 (p= 0.019, p=0.003, respectively) alleles in the C143 markers and the C245*436 (p=0.008) were significantly increased in the affected offspring. Microsatellite alleles with a parental frequency of less than 2% were pooled in the TDT and CETDT analysis. The haplotype method also revealed significant increase of the C143*449 allele and reciprocal decrease of C143*417. D6S273*135 allele was also decreased on transmitted haplotypes (Table 11).

Further HLA-B genotyping allowed us to distinguish between B*39:01 and B*39:06 alleles in selected B*39 positive samples. We observed 44 B*39:01 and 8 B*39:06 alleles in the 188 nuclear families with DRB1*08-DQB1*04 haplotype (Table 10). The B*39:06 allele appeared exclusively on the (DR8)-DQB1*04 haplotype. The results of conditional HM analysis of B*39 locus on DRB1*08-DQB1*04 haplotypes are displayed in Table 12. HLA-B*39:01 occurred less often in patient than control haplotypes but the rare B*39:06 was more common in patient haplotypes although neither one of these differences was significant.

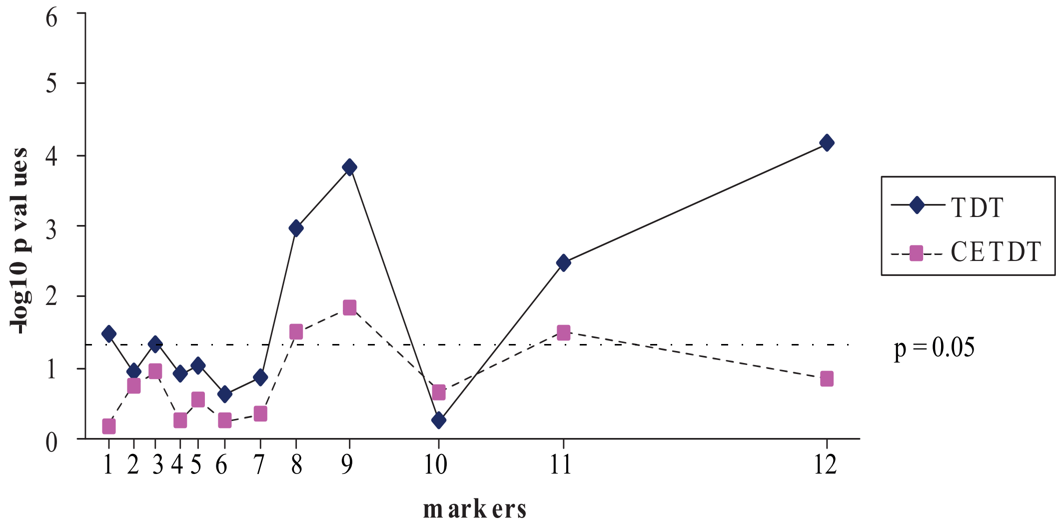



**Figure 10.** Selected microsatellite markers on 6p21 chromosome region with Finnish nuclear families with DR8-DQ4 haplotype. TDT and CETDT –log10p values shown separately in all analyzed markers (from original Publication II).

Markers are numbered as follows: 1, D6S273 2, TNF 3, C12A 4, STR MICA 5, MIB 6, HLA-B*39 7, C125 8, C143 9, C245 10, C3211 11, MOGc 12, D6S2223

**Table 10.** Transmission disequilibrium analysis with selected markers in the HLA region in HLA-DRB1*08-DQB1*04 haplotype families (Modified from Publication II with unpublished data).

| Marker | Allele | T (n) | NT (n) | T (%) | p value | Global TDT p value | Global CETDT p value |
|---|---|---|---|---|---|---|---|
| D6S273 df = 6 | 135 | 55 | 81 | 40.4 | 0.025 | 0.037 | NS |
| | 137 | 75 | 69 | 52.1 | NS | | |
| | 141 | 37 | 18 | 67.3 | 0.01 | | |
| | Others | 71 | 70 | NS | NS | | |
| HLA-B*39 df = 2 | 3901 | 19 | 25 | 43.2 | NS | NS | NS |
| | 3906 | 5 | 3 | 62.5 | NS | | |
| | Negative | 28 | 24 | 53.8 | NS | | |
| C143 df = 11 | 417 | 12 | 22 | 35.3 | NS | 0.001 | 0.036 |
| | 421 | 11 | 25 | 30.6 | 0.018 | | |
| | 433 | 6 | 19 | 24.0 | 0.008 | | |
| | 445 | 60 | 37 | 61.9 | 0.019 | | |
| | 449 | 75 | 43 | 63.6 | 0.003 | | |
| | Others | 88 | 106 | 45.4 | NS | | |
| C245 df = 9 | 436 | 74 | 45 | 62.2 | 0.008 | $10^{-4}$ | 0.014 |
| | 440 | 3 | 11 | 21.4 | 0.027 | | |
| | 444 | 1 | 7 | 12.5 | 0.024 | | |
| | 468 | 3 | 15 | 16.7 | 0.003 | | |
| | 476 | 2 | 10 | 16.7 | 0.016 | | |
| | Others | 96 | 91 | 51.3 | NS | | |
| MOGc df = 9 | 130 | 64 | 46 | 58.2 | NS | 0.006 | 0.033 |
| | 132 | 73 | 53 | 57.9 | NS | | |
| | 134 | 3 | 11 | 21.4 | 0.027 | | |
| | Others | 55 | 85 | 39.3 | NS | | |
| D6S2223 df = 3 | 168 | 12 | 39 | 23.5 | 0.0001 | $6.7 \times 10^{-5}$ | NS |
| | 170 | 90 | 47 | 65.7 | 0.0002 | | |
| | Others | 70 | 86 | 44.9 | NS | | |

Bonferroni correction on Global CETDT p value was NS in all analyzed markers

**Table 11.** Analysis of the DRB1*08-DQB1*04 extended haplotypes in nuclear families using the haplotype method (modified from Publication II with unpublished data).

| Marker | Marker length | Patients (n) | AFBAC (n) | p value | OR | 95% CI |
|---|---|---|---|---|---|---|
| D6S273 | 135 | 18 | 39 | 0.043 | 0.48 | 0.23-0.98 |
| | All others | 56 | 58 | | | |
| HLA-B*39 | 3901 | 4 | 14 | NS | NS | NS |
| | 3906 | 5 | 3 | NS | NS | NS |
| | Negative | 70 | 85 | | | |
| C143 | 417 | 1 | 12 | 0.005 | 0.08 | 0.004-0.62 |
| | 449 | 22 | 13 | 0.053 | 2.26 | 0.97-5.28 |
| | All others | 50 | 56 | | | |
| C245 | 436 | 27 | 26 | NS | NS | NS |
| | 440 | 1 | 5 | NS | NS | NS |
| | All others | 35 | 40 | | | |
| MOGc | 132 | 27 | 39 | NS | NS | NS |
| | All others | 38 | 46 | | | |
| D6S2223 | 170 | 43 | 41 | NS | NS | NS |
| | All others | 33 | 45 | | | |

AFBAC-affected family-based artificial controls

### 5.3. HLA class I region effect on the progression from diabetes associated autoimmunity to clinical disease (Publication III).

The effect of other than HLA-DR and -DQ genes within the HLA gene clas I region during the progression from autoantibody seroconversion to overt diabetes was analyzed in 249 children positive for ICA and at least one biochemical autoantibody (IAA, GADA and IA-2A). These children were analyzed for a panel of different HLA-A (A*01, -A*02, -A*03, -A*24, -A*28 and -A*32) and HLA-B (-B*08, -B*27, -B*35, -B*39, -B*56 -B*60 and -B*62) alleles. Out of them, 195 were positive for at least two biochemically defined autoantibodies. The median age of the children at the time of seroconversion to positivity for the first biochemically antibody was 1.8 years (range 0.3-9.9 years) and for the second biochemically defined autoantibody 2.0 years (0.8-9.9 years). During the follow-up 136 children developed type 1 diabetes.

When the effect of the HLA-DR-DQ haplotypes was tested by using the Cox-regression univariate analysis, the HLA-DR3/DR4 genotype ((DR3)-DQA1*05-DQB1*02 combined with DRB1*04:01-DQB1*03:02 or DRB1*04:04-DQB1*03:02) showed significiant association (p=0.01) with progression to disease after the appearance of the first biochemical autoantibody. When effect of the HLA-DR-DQ haplotypes was tested, the (DR3)-DQA1*05-DQB1*02 was found to be associated with progression to T1D (p value 0.015) and (DR1/10)-DQB1*05:01 (p value 0.03) haplotype with

protection againts overt disease. Similar associations were found when these two haplotypes were associated with DRB1*04:01-DQB1*03:02, suggesting that the association of (DR3)-DQA1*05-DQB1*02 and (DR1/10)-DQB1*05:01 haplotypes most probably were secondary to the DR3/DR4 ((DR3)-DQA1*05-DQB1*02/DRB1*04:01/04-DQB1*03:02) genotype association.

Multivariate analysis adjusted for the age at appearance of the first and second biochemically defined autoantibodies showed that class I alleles HLA-A*03, and HLA-B*39 affected the progress rate form seroconversion to T1D. B*39 was associated with more rapid progression whereas A*03 was protecting against progression (Table 12). These effects were more significant when measured from seroconversion for at least two biochemical autoantibodies. HLA-B*39 effect was significant only when analyzed after appearance of the second autoantibody (p value 0.014). HLA-A*24 allele did not have any significant effect with progression of autoimmunity.

**Table 12.** Cox-regression analysis of HLA class I region´s effects on progression to clinical diabetes in children with diabetes-associated autoantibodies. Adjusted according to the age at appearance of different autoantibodies (from original Publication III).

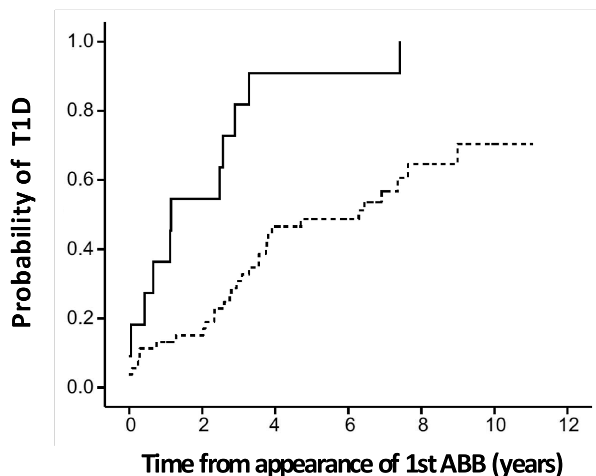
| **HLA class I-II region** | **After the first biochemically defined autoantibody** | | **After the second biochemically defined autoantibody** | |
|---|---|---|---|---|
| | **p value** | **Hazard ratio (95 % CI)** | **p value** | **Hazard ratio (95 % CI)** |
| A*03 | 0.042 | 0.611 (0.38-0.98) | 0.027 | 0.552 (0.33-0.93) |
| A*24 | 0.191 | 1.422 (0.84-2.41) | 0.598 | 1.176 (0.64-2.15) |
| B*39 | 0.159 | 1.549 (0.84-2.84) | 0.014 | 2.401 (1.2-4.82) |

The same analysis was also performed after dividing the children according to the presence of the HLA-DR3/DR4 genotype (Table 13). The strong effect of HLA-B*39 on the progression from β-cell autoimmunity to clinical disease was only seen in subject carrying the DR3/DR4 genotype combining both major class II risk haplotypes. The rate of disease development is also shown in the Kaplan-Meier curve in Figure 11. The number of children in follow-up at each time point is also given.

Contrary to the B*39 effect the protective effect of A*03 was seen in follow up children with other than DR3/4 genotype. HLA-A*24 allele did not have any significancy effect on progression to T1D.

**Table 13.** Multivariate Cox-regression analysis of HLA class I effects on progression to clinical diabetes. Adjusted according to age at appearance of first/second biochemically defined autoantibody (from original Publication III).

| **Progression after appearance of the first biochemically defined autoantibody** | | | | |
|---|---|---|---|---|
| **HLA-class I region** | **DR3/4 not present** | | **DR3/4 present** | |
| | **p value** | **Hazard ratio (95 % CI)** | **p value** | **Hazard ratio (95 % CI)** |
| A*03 | 0.015 | 0.49 (0.28-0.87) | 0.741 | 1.14 (0.52-2.52) |
| A*24 | 0.096 | 1.643 (0.91-2.95) | 0.142 | 0.372 (0.1-1.39) |
| B*39 | 0.660 | 1.21 (0.52-2.805) | 0.004 | 6.564 (1.80-23.93) |
| **Progression after appearance of the second biochemically defined autoantibody** | | | | |
| A*03 | 0.003 | 0.39 (0.21-0.72) | 0.554 | 1.31 (0.53-3.21) |
| A*24 | 0.252 | 1.455 (0.77-2.77) | 0.117 | 0.297 (0.06-1.36) |
| B*39 | 0.013 | 3.16 (1.28-7.81) | 0.007 | 7.45 (1.74-31.93) |




| | | | | | | | |
|---|---|---|---|---|---|---|---|
| B39* positive | 11 | 5 | 1 | 1 | 0 | 0 | 0 |
| B39* negative | 53 | 44 | 27 | 21 | 8 | 8 | 1 |

**Figure 11.** The effect of the HLA-B*39 allele on the progression to T1D in children with DR3/DR4 combination after development of the first biochemically-characterized autoantibody (p value 0.00007, log-rank test). B*39 positive children indicated by the solid and B*39 negative by dashed lines (from original Publication III).

### 5.4. HLA class II and class I region effects on the Addison's disease susceptibility. Case-control series in three different populations (Publication IV).

The effect of HLA-DR and -DQ genes within the HLA gene region on Addison's disease susceptibility was analyzed using case-control datasets in three populations: Finnish (patients n=14, control subjects n=1000), Russian (patients n=31, control subjects n=413) and Estonian (patients n=24, control subjects n =269). Most of the patients were adults (66 out of 69) and 70% of Estonian and Finnish patients were positive for antibodies against 21-hydroxylase including seven out of eight patients with type 2 autoimmune polyendocrinopathy syndrome. All samples were analyzed using genotyping of HLA class II genes HLA-DRB1, HLA-DQA1, HLA-DQB1 and 11 microsatellite markers (D6S273, TNFα, C12A, STR MICA, MIB, C125, C143, C245, C3211, MOGc and D6S2223) (Figure 7). In addition the presence of HLA-B*39 was tested in all subjects.

The HLA-(DR3)-DQA1*05-DQB1*02 haplotype was associated with Addison's disease in all three analyzed populations (Table 14), but the frequency of this haplotype showed significant variation. Eleven out of 14 Finns (78.6%), 12 out of 24 (50%) Estonians and only 11 out of 31 (35.5%)

Russian patients were positive for the haplotype. The HLA-DRB1*04:04-DQB1*03:02 haplotype was found also increased among the patients. This was highly significant among Estonian and Russian patients but did not quite reach statistical significance in the small group of Finnish patients. Instead, HLA-DRB1*04:01 haplotype was not increased among patients with Addison's disease.

The DRB1*04:03 allele was also significantly more common among the patients than background populations in Russians (6 out of 31 and 2 out of 413, respectively, p value $10^{-4}$, OR 49.3 and 95% CI 8.35-375.04). It was combined with DQB1*03:02 in three out of six patients and in three with DQB1*03:05 allele.

Analysis of 11 microsatellite markers covering ~4MB over the HLA region with haplotype method did not provide statistical support to the importance of loci other than HLA region in Addison's disease. The HLA-B*39 allele was observed only in 5 cases among 69 Addison's patients and the HLA-DRB1*04:04-DQB1*03:02-B*39 haplotype was detected only once in a Finnish patient.

**Table 14.** The main HLA haplotype frequencies in the three analyzed Addison's disease-associated population (Modified from Publication IV).

| Population | Haplotype | Patients N (%) | Controls n (%) | OR | p value | 95 % CI |
|---|---|---|---|---|---|---|
| Finns N = 14 n = 1000 | DQA1*05-DQB1*02-(DR3) | 11 (78.6) | 202 (20.2) | 14.5 | <0.0001 | 3.7 - 41.4 |
| | DQB1*03:02-DRB1*04:01-(DR4) | 2 (14.3) | 124 (12.4) | - | NS | - |
| | DQB1*03:02-DRB1*04:04-(DR4) | 3 (21.4) | 62 (6.2) | 4.1 | 0.078 | 0.9 -1 6.5 |
| Estonians N = 24 n = 269 | DQA1*05-DQB1*02-(DR3) | 12 (50.0) | 60 (22.3) | 3.5 | 0.0056 | 1.4 - 8.8 |
| | DQB1*03:02-DRB1*04:01-(DR4) | 0 (0) | 12 (4.5) | - | NS | - |
| | DQB1*03:02-DRB1*04:04-(DR4) | 7 (29.2) | 25 (9.3) | 4.0 | 0.008 | 1.4 - 11.6 |
| Russians N = 31 n = 413 | DQA1*05-DQB1*02-(DR3) | 11 (35.5) | 65 (15.7) | 2.9 | 0.0102 | 1.3 - 6.8 |
| | DQB1*03:02-DRB1*04:01-(DR4) | 3 (9.7) | 31 (7.5) | - | NS | - |
| | DQB1*03:02-DRB1*04:04-(DR4) | 7 (22.6) | 28 (6.8) | 4.0 | 0.0051 | 1.4 - 10.9 |

# 6. DISCUSSION

## 6.1. Type 1 diabetes

There are several loci within the HLA region responsible for the susceptibility to T1D. The major disease determinants are in class II gene cluster DRB1-DQA1-DQB1 and weaker effects are exerted by HLA class I region where identity of affecting genes is still unclear. Based also on data from the Type 1 Diabetes Genetics Consortium (T1DG) genome-wide association study the DRB1-DQA1-DQB1 haplotypes have the strongest predisposing effect with T1D, which may differ between populations (Noble, Erlich 2012). In this work the contribution of non-class II HLA loci to disease risk was explored using a set of microsatellite markers, which covered 4-Mb region telomeric to the DQB1 gene in Finnish population. This thesis aimed to further characterize extended HLA haplotypes and to identify non-class II loci modifying the genetic susceptibility to T1D using both case-control and nuclear family studies as well as follow up of autoantibody positive children.

In the case-control study datasets were matched for combination of DR3-DQ2 and DR4-DQ8 haplotypes and further for DQB1*03:02 haplotypes with different DR4 subtypes. DR3-DQ2 is an extended haplotype showing extremely small amount of variation in the studied region and one could thus deduce variation to be derived mainly from the various DR4-DQ8 haplotypes. Among the DR4 subtypes only DRB1*04:01 and DRB1*04:04 are frequent in Finnish populations (Hermann, Turpeinen et al. 2003) and these were analyzed in our case-control study. Chi-square statistic and population differentiation test were applied to map the marker of the strongest T1D association within the D6S273-MOGc segment. The same region was also analyzed using two nuclear family sets parents carrying DRB1*08-DQB1*04 (DR8) and DRB1*04:04-DQB1*03:02 (DR4) haplotypes. The DRB1*08-DQB1*04 haplotype is the third most predisposing haplotype after the high risk DR3 and DR4 in type 1 diabetes in the T1DGC series (Erlich et al. 2008) and also one of the three haplotypes in Finland sharing OR value around 1.0 after DR3 and DR4 risk haplotypes and being also of particular high frequency in Finland compared to other European populations (Ilonen 2016). TDT and CETDT were used to estimate disease association of non-class II markers of DR-DQ region and the independent disease association of the analyzed microsatellite markers was further confirmed using the haplotype methods.

We found that in the case-control and family studies in the DR3/DRB1*04:01, DR3/DRB1*04:04 and DR3/(DR8)-DQB1*04 stratified groups that there was only one allele at each microsatellite locus present in the majority of analyzed groups. Therefore we could determine the extended haplotype in DR3 and also in both DR4 subtypes. This finding implies that in our studies, the DR3

haplotype does not contain any additional disease associated marker which is in concordance of the strong linkage disequilibrium of the this extended haplotype, particularly A1, B8, DR3, DQ2 in Finland as the other common DR3-DQ2 haplotype associated with A30 and B18 is practically absent in the Finnish population (Haimila, Penttilä et al. 2013, Haimila, Peräsaari et al. 2013).

In the DR3-DRB1*04:04 stratified group we found that markers between C12A and C143 near the HLA-B region confer strong additional disease association, which, however, is exceeded by the B*39 effect suggesting that diabetes associated gene is located on this area and is probable B*39 itself. This finding was supported by the population differentiation test. None of the markers were associated with higher risk than HLA-B*39.

In the DR3/DRB1*04:01 stratified group of patients and control subjects, no single microsatellite allele was associated with the significant changes in the disease risk although the MIB locus showed a difference between the background and patients populations in the population differentiation test.

The present case-control study does not support the idea the polymorphism of transmembrane region with MICA protein (STR MICA) could independently be responsible for diabetes association (Van Autreve et al. 2006) although the STR MICA*A9 conferred higher risk on the DR3/DRB1*04:04 than DR3/DRB1*04:01 (OR 4.0 vs OR 1.03, respectively). Markers located in this region have also been associated with several other autoimmune diseases (Bilbao et al. 2003, Gambelunghe et al. 1999, Mizuki et al. 1997)

Several studies have tried to map a non-class II HLA gene associated with type 1 diabetes. At first, by analyzing the DR3-B8 and DR3-B18 haplotypes, it was predicted that the region between HLA-B and BAT3 should contain a gene associated with T1D (Degli-Esposti et al. 1992). Another study suggested that the non-class II HLA gene of type 1 diabetes susceptibility is located between the D6S273 and TNFα markers (Hanifi-Moghaddam et al. 1998). However, D6S265 marker next to the TNFα located ~1.3 Mb telomerically was used. Because no markers were tested between these two markers, to our mind, the telomeric boundary of the candidate region could not be specified exactly. A non-class II HLA gene associated with type 1 diabetes was mapped also on D6S2223, ~2.5 Mb telomeric to MOGc (Lie et al. 1999). Our data does not support this result, because we had a weak effect on MOGc-D6S2223 markers.

We also analyzed the same region using nuclear families with DRB1*08-DQB1*04 (DR8) or DRB1*04:04-DQB1*03:02 (DR4) haplotypes. In the DR4 family sets the D6S2223-TNFα, MIB-

B*39-C125 and C3211-MOGc-D6S2223 genomic regions showed association with disease in the single locus TDT. In the conditional transmission analysis where parents carried the DRB1*04:04-DQB1*03:02 haplotype, the D6S273 and C125 remained to show strong disease association independently from the DR-DQ haplotype. Using the haplotype method the predisposing effect of the C125*200 allele was confirmed. The earlier findings that B*39 modifies the risk effect of DRB1*04:04-DQB1*03:02 haplotypes (Nejentsev et al. 1997, Reijonen et al. 1997) was now confirmed to be due to the B*39:01 subtype but we observed multiple microsatellite markers which might also contribute to the disease risk.

In addition the D6S273 marker appeared to confer T1D risk in the TDT and CETDT analysis on the DRB1*04:04-DQB1*03:02 family sets. The D6S273*137 allele was more frequently transmitted to affected offspring, however did not differ significantly in the DRB1*04:04-DQB1*03:02 nuclear families. Similarly to our finding family studies in several countries observed that DRB1*03:01-DQB1*02-B*18:01 haplotypes carrying D6S273*2 (corresponding to our D6S273*131 allele size) (Johansson et al. 2003, Lie et al. 1999) and DRB1*03-DQB1*02-B*08:01 haplotypes carry allele D6S273*5 (corresponding to our D6S273*137 allele size), whereas no such phenomenon was observed on DR4 haplotypes (Lie et al. 1999, Valdes et al. 2005).

Lie and coworkers described that the D6S2223*3 allele appears with decreased frequency on the DRB1*03-DQA1*05:01-DQB1*02:01 haplotype (Lie et al. 1999). We did not analyze the DR3-DQ2 haplotype for this marker but no independent association of D6S2223 marker on the DR4 haplotype was seen.

In the DR8 family set the CETDT analysis produced also several significant associations although these did not remain significant after correcting p values by the number of comparisons made. This may, however, be too insensitive when markers in linkage disequilibrium were used. The haplotype method analysis also suggested that the D6S273*135 allele is decreased in patients with DRB1*08-DQB1*04 haplotypes, finding being similar to the tendency on DRB1*04:04-DQB1*03:02 haplotypes.

Also the C143*417 allele associated inversely and independently on the DR8 haplotype. No such data has been published before this finding.

Considering these data, it looks that detailed mapping of non-class II HLA loci modifying type 1 diabetes risk might improve possibilities for accurate disease prediction in different populations. Most studies have demonstrated DRB1*04:04-DQB1*03:02 haplotype is associated with lower risk

to T1D than the DRB1*04:01-DQB1*03:02 haplotype (Donner et al. 2000, Erlich et al. 2008, Hermann; Turpeinen et al. 2003,Thomson et al. 2007). However, importance of the HLA-B*39 marker within the DRB1*04:04-DQA1*03-DQB1*03:02 haplotype on the disease risk has been previously described in the Finnish, Estonian and Russian population by our group (Nejentsev et al. 1997, Reijonen et al. 1997) and it was shown that the risk of B*39 positive HLA-DRB1*04:04 haplotype was at the same level as that of HLA-DRB1*04:01 positive haplotype (Nejentsev et al. 1997). The risk of the B*39:01 allele with type 1 diabetes has not been detected in earlier studies, but the HLA-B*39:01-DRB1*04:04-DQA1*03-DQB1*03:02 haplotype is rare in many European and European derived populations (Noble et al. 2010, Valdes, Erlich & Noble 2005). The HLA-B*39:01 allele has been found also in other haplotypes than DRB1*04:04-DQA1*03-DQB1*03:02, it is common also in (DR8)-DQB1*04 haplotype, but in a big Finnish family trio analysis it was associated with T1D only on the DRB1*04:04-DQA1*03-DQB1*03:02 haplotype (Mikk et al. 2017).

This study now confirmed that the HLA-B*39:01 allele had the strongest predisposing effect among the analyzed markers on the DR3/DRB1*04:04 genotype in Finnish T1D patients. The HLA-B*39:01 allele has also a strong effect in the late phase of progression of type 1 diabetes-associated autoimmunity which was apparent from the appearance of the second instead of the first biochemical antibody. The strong effect of HLA-B*39:01 on the progression form β-cell autoimmunity to clinical disease was only seen in subject carrying the combination of both major class II haplotypes, the DR3/DR4 genotypes. Among the DR3/DR4 heterozygotes, 7 of 11 HLA-B*39 positive samples were DRB1*04:04 positive.

Independent risk of certain class I alleles has been established more recently using also dense SNP analysis and HLA-B*39 allele was also identified there as the strongest class I risk allele and also associated with lower age-at-diagnosis both in family and case-control trials (Nejentsev et al. 2007).

We now analyzed separately the HLA-B*39:06 and HLA-B*39:01 allele predisposing effect in 188 nuclear families with DRB1*08-DQB1*04 haplotype. The HLA-B*39:06 allele appeared exclusively on the (DR8)-DQB1*04 haplotype and absent on DRB1*04:04-DQB1*03:02 but was rare and did not significantly associate with increased T1D risk although many surrounding microsatellite markers did. A bigger family series still found a significant T1D association of HLA-B*39:06 on the (DR8)-DQB1*04 haplotype also in the Finnish population (Mikk et al. 2014, Mikk et al. 2017). It has also been described earlier in the Collections of Human Biological Data Interchange (HBDI), and type 1 Diabetes Genetics Consortium (T1DG), where the B*39:06 allele

had risk association on four DRB1-DQB1 haplotypes including DR8, DR1, DR2 and DR4 haplotypes (Noble et al. 2010). B*39:06 showed the strongest association on DRB1*08:01-DQB1*04:02 (DR8) and DRB1*01:01-DQB1*05:01 (DR1) haplotypes and also associated with younger age at onset (Baschal et al. 2011, Noble et al. 2010, Valdes, Erlich & Noble 2005). However the B*39:06 allele is extremely rare on the DRB1*01:01-DQB1*05:01 in Finnish population (Mikk et al. 2017).

The HLA-A*24 allele often presents in haplotype LD with B*39 alleles (Haimila, Peräsaari et al. 2013, Mikk et al. 2014, Reijonen et al. 1997). In this study, disease risk association of A*24 allele was confirmed in the DR3/DRB1*04:04 group using case-control analyzes. Previously, the A*24 allele has been reported to increase risk in type 1 diabetes populations (Honeyman et al. 1995). The A*24 allele was associated with DRB1*04:04-DQB1*03:02 haplotype but the association was weaker than that of HLA-B*39:01 allele. In the follow up DIPP cohort a survival analysis was used to test the HLA-A*24 allele effect on the seroconversion for persistent islet autoantibodies, and further on the progression rate to clinical disease after the appearance of multiple (at least 2) biochemically defined islet autoantibodies. The A*24 allele was not associated in Cox-regression with disease risk or with progression of autoimmunity on our stratified samples. However, later on with extended analysis by Mikk and coworkers the HLA-A*24 allele was found associated with faster disease progression especially in the (DR3)-DQA1*05-DQB1*02/DRB1*04:04-DQA1*03-DQB1*03:02 genotype (Mikk et al. 2017). In the analysis of autoantibody positive subjects from the Belgian Diabetes Register the A*24 allele was associated with faster progression independently as well as in the presence of DQ8 haplotype to clinical diabetes (Mbunwe et al. 2013).

The finding on the effects of class II alleles on the autoantibody appearance and class I alleles on the disease progress after established autoimmunity has created a hypothesis that HLA class II molecules are important in the initiation of the autoimmunity via CD4 positive T-helper cells by recognizing the initial antigen whereas the class I molecules affect the progression to clinical disease after the initial triggering via CD8 positive cytotoxic T cells.

## 6.2. Addison's disease

Addison's disease is also a polygenic autoimmune disease with HLA region as the most important determinant of the disease risk (Myhre et al. 2002, Skinningsrud et al. 2011). The HLA class II region encoding DR and DQ molecules is also the most strongly associated gene locus in Addison's disease. (Baker et al. 2010, Erichsen et al. 2009).

The result obtained in this study confirms the association in patients with Addison's disease with two class II HLA haplotypes. HLA DQA1*05-DQB1*02-(DR3) and DQB1*03:02-DRB1*04:04-(DR4) were significantly increased although considerable odds ratio differences were found between the Estonian, Russian and Finnish populations. The DR3-DQ2 haplotype association was strongest among Finns, whereas the effect of the DQB1*03:02-DRB1*04:04 haplotype was stronger among Estonians and Russians. The susceptible alleles are more diverse in Estonians and Russians. These differences may be generated by the patient selection but might also be caused by the genetic differences between the three populations.

Haplotype analysis using microsatellite markers did not provide statistical support to the significance of other loci than HLA-DR-DQ loci. The B*39 allele increasing Type 1 diabetes risk of DQB1*03:02-DRB1*04:04-(DR4) haplotype was present only in one Finnish patient of three ones with the haplotype and no one in the 14 patients in other populations.

The found diversity of DRB1*04:04-DQB1*03:02 positive haplotypes and the fact that DRB1*04:01-DQB1*03:02 does not shown any association with Addison's disease emphasized the role of DRB1*04:04 molecules itself. In this respect, the increased frequency of the DRB1*04:03 haplotype in the Russian patients was also interesting. The DRB1*04:01 and DRB1*04:04 haplotypes were common DR4 alleles, the DRB1*04:03 was present very rarely among Finns and Estonians. This is also the case in many populations where the DRB1*04:04 association of Addison's disease has been described (Myhre et al. 2002, Erichsen et al. 2009).

Few studies have suggested the importance MICA5.1 allele in Addison's disease (Gambelunghe et al. 1999, Park et al. 2002). The present results also found that the MICA5.1 allele is very common in all patients groups but it was not genotyped in controls thus preventing analysis of its independent effect. Despite strong LD between MICA5.1 and DR-DQ region it was anyway not found in all disease associated DR3 haplotypes, which indicates that it does not explain DR3-DQ2 association.

# 7. CONCLUSIONS

After decades of research and thousands of reports; HLA remains the strongest predictor of type 1 diabetes and Addison's disease risks. However, the genes involved in the region are multiple and not yet completely known. The accumulated data demonstrate the difficulties involved with the identification of complex autoimmune diseases genes in various European populations.

The clear associations of the HLA-B*39:01 and the A*24 alleles with type 1 diabetes in case-control and progression studies were confirmed, whether they act independent remains inconclusive. HLA-B locus with B*39:01 appears to associate with the peak risk in HLA-DRB1*04:04-DQB1*03:02 haplotype, although effect on neighboring regions cannot be excluded. Notably, the B*39:01 does not increase the disease risk in (DR8)-DQB1*04 haplotype, although it is common also with this haplotype.

The HLA-B*39:06 allele was rare and although it has been shown in larger studies to increase T1D risk it cannot explain the risk modifying effects of microsatellites surrounding the B-locus. Further studies will be needed to clarify these effects. Our results emphasize the importance of haplotypes, but also genotype analyses in HLA studies are important to reveal interactions, which can otherwise remain undetected. The identified class I alleles and probably new markers in the region associated with increased risk on the certain class II haplotypes and genotypes provide additional biomarkers for screening susceptibility to type 1 diabetes and for predicting the rate of progression to clinical disease after the development of autoantibodies, which will be important e.g. for selection of cases and controls in secondary prevention studies aiming at hindering the progression to clinical disease.

Contrary to T1D we could not find any effect of class I region of DRB1*04:04-DQB1*03:02 haplotypes on Addison's disease which finding supports the significance of DRB1*04:04 molecule itself in disease risk as the more common DRB1*04:01-DQB1*03:02 haplotype does not increase the risk. Interestingly also the DRB1*04:03 allele, rare among Finnish and Estonians, seemed to increase the risk in Russian population.

There are still many regions of smaller effect or low frequency to be discovered, and better exploitation of existing type 1 diabetes and Addison's cohorts with more powerful and targeted methods is an informative step forward.

## 8. ACKNOWLEDGEMENTS

This work was carried out at the Immunogenetics Laboratory, Institute of Biomedicine and the Medicity Research Laboratory, Faculty of Medicine, University of Turku in affiliation with the Turku Doctoral Programme of Molecular Medicine (TuDMM).

I wish to express my deepest gratitude to my supervisor, Professor Jorma Ilonen for this excellent guidance, continuous encouragement, and patience. The importance of your optimistic attitude and the innumerable discussions and advice given during these years cannot be described.

I also wish to thank Docent Robert Hermann (†) for introducing me to the fascinating researcher world. The importance of your optimistic attitude and innumerable discussions and advice given during these years cannot be described. I miss you so much. Emléked örökké szívemben él!

I wish to thank the official reviewers of my thesis, Docent Päivi Saavalainen and Associate Professor Tiinamaija Tuomi, for their valuable comments, constructive criticism and suggestions to improve this thesis.

I warmly thank the member of my supervising committee, Docent Kálmán Büki for his optimistic and encouraging attitude and valuable comments during different stages of this project. Special thanks to you for all the tips about writing and for revising the language of the thesis.

I have had pleasure of working with many talented people in our laboratory and I want to collectively thank all the past and present members of our laboratory. I am deeply grateful to Antti-Pekka Laine, Ilse Ekman, Johanna Lempainen, Mari-Liis Mikk, Milla Valta, Minna Kiviniemi, Sirpa Pahkuri and Tanja Kakko for their friendship and the pleasant company in the office. Thanks to you it was an enjoyable time.

I wish to thank Ritva Suominen, Mia Karlsson, Terhi Laakso, Piia Nurmi, Anne Suominen, Eija Salo and Terttu Laurén for the warm atmosphere they have created in the lab.

I would like to thank all my Hungarian friends and relatives for the many enjoyable moments spent together. I am especially grateful to Andrea Kozák-Bárány, Bogáta Kovács, Csilla Sipeky and Petya Valtakari for the warm friendship lasting through the years. Thank you for being here whenever I need you!

I am also fortunate to have friends who have no idea what I actually do for living, but who still have always patiently listened to all my complaints about all matters relating to work issues. Thank you for everything Eszter Virág and Tünde Gombos.

Szeretném külön megköszönni szüleimnek a belém vetett hitüket és a feltétel nélküli szeretetüket.

Finally, with all my heart, I thank my dear husband Jenő for his never-ending patience, love and support. I thank to my two lovely children Balázs and Máté for reminding me of the things that matter most in life. Szeretlek Titeket!

Turku, 2018

Zsófia Gombos

# 9. REFERENCES

Abdurakhmonov, I.Y. (2016), "Introduction to Microsatellites: Basics, Trends and Highlights", *Microsatellite Markers book,* chapter 1, pp. 3-16.

American Diabetes Association. (2012), "Diagnosis and classification of diabetes mellitus", *Diabetes Care,* vol. 35, Supplement 1, s64-71.

Anderson, J.R., Goudie, R.B., Gray, K.G. & Timbury, G.C. (1957), "Auto-antibodies in Addison's disease", *Lancet (London, England),* vol. 272, no. 6979, pp. 1123-1124.

Atkinson, M.A., Eisenbarth, G.S. & Michels, A.W. (2014), "Type 1 diabetes", *Lancet (London, England),* vol. 383, no. 9911, pp. 69-82.

Atkinson, M.A., von Herrath, M., Powers, A.C. & Clare-Salzler, M. (2015), "Current concepts on the pathogenesis of type 1 diabetes-considerations for attempts to prevent and reverse the disease", *Diabetes care,* vol. 38, no. 6, pp. 979-988.

Baekkeskov, S., Aanstoot, H.J., Christgau, S., Reetz, A., Solimena, M., Cascalho, M., Folli, F., Richter-Olesen, H. & De Camilli, P. (1990), "Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase", *Nature,* vol. 347, no. 6289, pp. 151-156.

Baker, P.R., Baschal, E.E., Fain, P.R., Nanduri, P., Triolo, T.M., Siebert, J.C., Armstrong, T.K., Babu, S.R., Rewers, M.J., Gottlieb, P.A., Barker, J.M. & Eisenbarth, G.S. (2011), "Dominant suppression of Addison's disease associated with HLA-B15", *The Journal of clinical endocrinology and metabolism,* vol. 96, no. 7, pp. 2154-2162.

Baker, P.R., Baschal, E.E., Fain, P.R., Triolo, T.M., Nanduri, P., Siebert, J.C., Armstrong, T.K., Babu, S.R., Rewers, M.J., Gottlieb, P.A., Barker, J.M. & Eisenbarth, G.S. (2010), "Haplotype analysis discriminates genetic risk for DR3-associated endocrine autoimmunity and helps define extreme risk for Addison's disease", *The Journal of clinical endocrinology and metabolism,* vol. 95, no. 10, pp. E263-70.

Barratt, B.J., Payne, F., Lowe, C.E., Hermann, R., Healy, B.C., Harold, D., Concannon, P., Gharani, N., McCarthy, M.I., Olavesen, M.G., McCormack, R., Guja, C., Ionescu-Tirgoviste, C., Undlien, D.E., Ronningen, K.S., Gillespie, K.M., Tuomilehto-Wolf, E., Tuomilehto, J., Bennett, S.T., Clayton, D.G., Cordell, H.J. & Todd, J.A. (2004), "Remapping the insulin gene/IDDM2 locus in type 1 diabetes", *Diabetes,* vol. 53, no. 7, pp. 1884-1889.

Baschal, E.E., Baker, P.R., Eyring, K.R., Siebert, J.C., Jasinski, J.M. & Eisenbarth, G.S. (2011), "The HLA-B 3906 allele imparts a high risk of diabetes only on specific HLA-DR/DQ haplotypes", *Diabetologia,* vol. 54, no. 7, pp. 1702-1709.

Bennett, S.T., Lucassen, A.M., Gough, S.C., Powell, E.E., Undlien, D.E., Pritchard, L.E., Merriman, M.E., Kawaguchi, Y., Dronsfield, M.J. & Pociot, F. (1995), "Susceptibility to

human type 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulin gene minisatellite locus", *Nature genetics,* vol. 9, no. 3, pp. 284-292.

Bergthorsdottir, R., Leonsson-Zachrisson, M., Oden, A. & Johannsson, G. (2006), "Premature mortality in patients with Addison's disease: a population-based study", *The Journal of clinical endocrinology and metabolism,* vol. 91, no. 12, pp. 4849-4853.

Berhan, Y., Waernbaum, I., Lind, T., Mollsten, A., Dahlquist, G. & Swedish Childhood Diabetes Study Group (2011), "Thirty years of prospective nationwide incidence of childhood type 1 diabetes: the accelerating increase by time tends to level off in Sweden", *Diabetes,* vol. 60, no. 2, pp. 577-581.

Betterle, C., Volpato, M., Pedini, B., Chen, S., Smith, B.R. & Furmaniak, J. (1999), "Adrenal-cortex autoantibodies and steroid-producing cells autoantibodies in patients with Addison's disease: comparison of immunofluorescence and immunoprecipitation assays", *The Journal of clinical endocrinology and metabolism,* vol. 84, no. 2, pp. 618-622.

Bilbao, J.R., Martin-Pagola, A., Perez De Nanclares, G., Calvo, B., Vitoria, J.C., Vazquez, F. & Castano, L. (2003), "HLA-DRB1 and MICA in autoimmunity: common associated alleles in autoimmune disorders", *Annals of the New York Academy of Sciences,* vol. 1005, pp. 314-318.

Bingley, P.J. (2010), "Clinical applications of diabetes antibody testing", *The Journal of clinical endocrinology and metabolism,* vol. 95, no. 1, pp. 25-33.

Bodmer, W.F. (1987), "The HLA system: structure and function", *Journal of clinical pathology,* vol. 40, no. 9, pp. 948-958.

Bonifacio, E. & Ziegler, A.G. (2010), "Advances in the prediction and natural history of type 1 diabetes", *Endocrinology and metabolism clinics of North America,* vol. 39, no. 3, pp. 513-525.

Borchers, A.T., Uibo, R. & Gershwin, M.E. (2010), "The geoepidemiology of type 1 diabetes", *Autoimmunity reviews,* vol. 9, no. 5, pp. A355-65.

Bottazzo, G.F., Florin-Christensen, A. & Doniach, D. (1974), "Islet-cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies", *Lancet (London, England),* vol. 2, no. 7892, pp. 1279-1283.

Bottini, N., Musumeci, L., Alonso, A., Rahmouni, S., Nika, K., Rostamkhani, M., MacMurray, J., Meloni, G.F., Lucarelli, P., Pellecchia, M., Eisenbarth, G.S., Comings, D. & Mustelin, T. (2004), "A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes", *Nature genetics,* vol. 36, no. 4, pp. 337-338.

Bouqbis, L., Akhayat, O., Garchon, H.J., Calafell, F. & Izaabel, H. (2003), "TNFA-TNFB haplotypes modify susceptibility to type I diabetes mellitus independently of HLA class II in a Moroccan population", *Tissue antigens,* vol. 61, no. 1, pp. 72-79.

Brandão Neto, R.A., de Carvalho, J.F. (2014), "Diagnosis and classification of Addison´s disease (autoimmune adrenalitis)", *Autoimmunity Reviews,* vol. 13, no. 4-5, pp. 408-411.

Bruno, G., Maule, M., Biggeri, A., Ledda, A., Mannu, C., Merletti, F., Songini, M. & Sardinian Group for Diabetes Epidemiology (2013), "More than 20 years of registration of type 1 diabetes in Sardinian children: temporal variations of incidence with age, period of diagnosis, and year of birth", *Diabetes,* vol. 62, no. 10, pp. 3542-3546.

Bunce, M., Fanning, G.C. & Welsh, K.I. (1995), "Comprehensive, serologically equivalent DNA typing for HLA-B by PCR using sequence-specific primers (PCR-SSP)", *Tissue antigens,* vol. 45, no. 2, pp. 81-90.

Bunce, M., O'Neill, C.M., Barnardo, M.C., Krausa, P., Browning, M.J., Morris, P.J. & Welsh, K.I. (1995), "Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP)", *Tissue antigens,* vol. 46, no. 5, pp. 355-367.

Cavallo, M.G., Fava, D., Monetini, L., Barone, F. & Pozzilli, P. (1996), "Cell-mediated immune response to beta casein in recent-onset insulin-dependent diabetes: implications for disease pathogenesis", *Lancet (London, England),* vol. 348, no. 9032, pp. 926-928.

Coco, G., Dal Pra, C., Presotto, F., Albergoni, M.P., Canova, C., Pedini, B., Zanchetta, R., Chen, S., Furmaniak, J., Rees Smith, B., Mantero, F. & Betterle, C. (2006), "Estimated risk for developing autoimmune Addison's disease in patients with adrenal cortex autoantibodies", *The Journal of clinical endocrinology and metabolism,* vol. 91, no. 5, pp. 1637-1645.

Collins, R.W. (2004), "Human MHC class I chain related (MIC) genes: their biological function and relevance to disease and transplantation", *European journal of immunogenetics : official journal of the British Society for Histocompatibility and Immunogenetics,* vol. 31, no. 3, pp. 105-114.

Craig, M.E., Nair, S., Stein, H. & Rawlinson, W.D. (2013), "Viruses and type 1 diabetes: a new look at an old story", *Pediatric diabetes,* vol. 14, no. 3, pp. 149-158.

Das, S.N., Baniasadi, V. & Kapuria, V. (2006), "Association of -308 TNF-alpha promoter polymorphism with type 1 diabetes in North Indians", *International journal of immunogenetics,* vol. 33, no. 6, pp. 411-416.

dbMHC database of the NCBI http://www.ncbi.nlm.nih.gov/gv/mhc/xslcgi.cgi?cmd=mssearch

Degli-Esposti, M.A., Abraham, L.J., McCann, V., Spies, T., Christiansen, F.T. & Dawkins, R.L. (1992), "Ancestral haplotypes reveal the role of the central MHC in the immunogenetics of IDDM", *Immunogenetics,* vol. 36, no. 6, pp. 345-356.

Dendrou, C.A., Petersen, J., Rossjohn, J. & Fugger, L. (2018), "HLA variation and disease", *Nature Reviews Immunology,* vol. 18, no. 5, pp. 325-339.

Deng, G.Y., Maclaren, N.K., Huang, H.S., Zhang, L.P. & She, J.X. (1996), "No primary association between the 308 polymorphism in the tumor necrosis factor alpha promoter region and insulin-dependent diabetes mellitus", *Human immunology,* vol. 45, no. 2, pp. 137-142.

DIAMOND Project Group (2006), "Incidence and trends of childhood Type 1 diabetes worldwide 1990-1999", *Diabetic medicine : a journal of the British Diabetic Association,* vol. 23, no. 8, pp. 857-866.

Diaz-Valencia, P.A., Bougneres, P. & Valleron, A.J. (2015), "Global epidemiology of type 1 diabetes in young adults and adults: a systematic review", *BMC public health,* vol. 15, pp. 255-015-1591-y.

Donner, H., Seidl, C., Van der Auwera, B., Braun, J., Siegmund, T., Herwig, J., Weets, I., Usadel, K.H. & Badenhoop, K. (2000), "HLA-DRB1*04 and susceptibility to type 1 diabetes mellitus in a German/Belgian family and German case-control study. The Belgian Diabetes Registry", *Tissue antigens,* vol. 55, no. 3, pp. 271-274.

Dudbridge, F. (2003), "Pedigree disequilibrium tests for multilocus haplotypes", *Genetic epidemiology,* vol. 25, no. 2, pp. 115-121.

Dudbridge, F., Koeleman, B.P., Todd, J.A. & Clayton, D.G. (2000), "Unbiased application of the transmission/disequilibrium test to multilocus haplotypes", *American Journal of Human Genetics,* vol. 66, no. 6, pp. 2009-2012.

Durinovic-Bello, I., Wu, R.P., Gersuk, V.H., Sanda, S., Shilling, H.G. & Nepom, G.T. (2010), "Insulin gene VNTR genotype associates with frequency and phenotype of the autoimmune response to proinsulin", *Genes and immunity,* vol. 11, no. 2, pp. 188-193.

Eason, R.J., Croxson, M.S., Perry, M.C. & Somerfield, S.D. (1982), "Addison's disease, adrenal autoantibodies and computerised adrenal tomography", *The New Zealand medical journal,* vol. 95, no. 714, pp. 569-573.

Ellegren, H. (2004), "Microsatellites: simple sequences with complex evolution", *Nature reviews.Genetics,* vol. 5, no. 6, pp. 435-445.

Erichsen, M.M., Lovas, K., Skinningsrud, B., Wolff, A.B., Undlien, D.E., Svartberg, J., Fougner, K.J., Berg, T.J., Bollerslev, J., Mella, B., Carlson, J.A., Erlich, H. & Husebye, E.S. (2009), "Clinical, immunological, and genetic features of autoimmune primary adrenal insufficiency: observations from a Norwegian registry", *The Journal of clinical endocrinology and metabolism,* vol. 94, no. 12, pp. 4882-4890.

Erlich, H., Valdes, A.M., Noble, J., Carlson, J.A., Varney, M., Concannon, P., Mychaleckyj, J.C., Todd, J.A., Bonella, P., Fear, A.L., Lavant, E., Louey, A., Moonsamy, P. & Type 1 Diabetes Genetics Consortium (2008), "HLA DR-DQ haplotypes and genotypes and type 1 diabetes risk: analysis of the type 1 diabetes genetics consortium families", *Diabetes,* vol. 57, no. 4, pp. 1084-1092.

Falk, C.T. & Rubinstein, P. (1987), "Haplotype relative risks: an easy reliable way to construct a proper control sample for risk calculations", *Ann. of Hum. Genetics,* vol. 51, no. 3, pp. 227-233.

Falorni, A., Laureti, S., Candeloro, P., Perrino, S., Coronella, C., Bizzarro, A., Bellastella, A.,

Santeusanio, F. & De Bellis, A. (2002), "Steroid-cell autoantibodies are preferentially expressed in women with premature ovarian failure who have adrenal autoimmunity", *Fertility and sterility,* vol. 78, no. 2, pp. 270-279.

Falorni, A., Laureti, S., De Bellis, A., Zanchetta, R., Tiberti, C., Arnaldi, G., Bini, V., Beck-Peccoz, P., Bizzarro, A., Dotta, F., Mantero, F., Bellastella, A., Betterle, C., Santeusanio, F. & SIE Addison Study Group (2004), "Italian addison network study: update of diagnostic criteria for the etiological classification of primary adrenal insufficiency", *The Journal of clinical endocrinology and metabolism,* vol. 89, no. 4, pp. 1598-1604.

Falorni, A., Nikoshkov, A., Laureti, S., Grenback, E., Hulting, A.L., Casucci, G., Santeusanio, F., Brunetti, P., Luthman, H. & Lernmark, A. (1995), "High diagnostic accuracy for idiopathic Addison's disease with a sensitive radiobinding assay for autoantibodies against recombinant human 21-hydroxylase", *The Journal of clinical endocrinology and metabolism,* vol. 80, no. 9, pp. 2752-2755.

Foissac, A. & Cambon-Thomsen, A. (1998), "Microsatellites in the HLA region: 1998 update", *Tissue antigens,* vol. 52, no. 4, pp. 318-352.

Foissac, A., Salhi, M. & Cambon-Thomsen, A. (2000), "Microsatellites in the HLA region: 1999 update", *Tissue antigens,* vol. 55, no. 6, pp. 477-509.

Fourlanos, S., Varney, M.D., Tait, B.D., Morahan, G., Honeyman, M.C., Colman, P.G. & Harrison, L.C. (2008), "The rising incidence of type 1 diabetes is accounted for by cases with lower-risk human leukocyte antigen genotypes", *Diabetes Care,* vol. 32, no. 8, pp. 1546-1549.

Galler, A., Stange, T., Muller, G., Nake, A., Vogel, C., Kapellen, T., Bartelt, H., Kunath, H., Koch, R., Kiess, W., Rothe, U. & Childhood Diabetes Registry in Saxony, Germany (2010), "Incidence of childhood diabetes in children aged less than 15 years and its clinical and metabolic characteristics at the time of diagnosis: data from the Childhood Diabetes Registry of Saxony, Germany", *Hormone research in paediatrics,* vol. 74, no. 4, pp. 285-291.

Gambelunghe, G., Brozzetti, A., Ghaderi, M., Candeloro, P., Tortoioli, C. & Falorni, A. (2007),"MICA gene polymorphism in the pathogenesis of type 1 diabetes", *Annals of the New York Academy of Sciences,* vol. 1110, pp. 92-98.

Gambelunghe, G., Falorni, A., Ghaderi, M., Laureti, S., Tortoioli, C., Santeusanio, F., Brunetti, P. & Sanjeevi, C.B. (1999), "Microsatellite polymorphism of the MHC class I chain-related (MIC-A and MIC-B) genes marks the risk for autoimmune Addison's disease", *The Journal of clinical endocrinology and metabolism,* vol. 84, no. 10, pp. 3701-3707.

Gambelunghe, G., Kockum, I., Bini, V., De Giorgi, G., Celi, F., Betterle, C., Giordano, R., Libe, R., Falorni, A., Umbria Type 1 Diabetes Registry & Italian Addison Network (2005), "Retrovirus-like long-terminal repeat DQ-LTR13 and genetic susceptibility to type 1 diabetes and autoimmune Addison's disease", *Diabetes,* vol. 54, no. 3, pp. 900-905.

Gianani, R., Rabin, D.U., Verge, C.F., Yu, L., Babu, S.R., Pietropaolo, M. & Eisenbarth, G.S.

(1995), "ICA512 autoantibody radioassay", *Diabetes,* vol. 44, no. 11, pp. 1340-1344.

Gillespie, K.M., Bain, S.C., Barnett, A.H., Bingley, P.J., Christie, M.R., Gill, G.V. & Gale, E.A. (2004), "The rising incidence of childhood type 1 diabetes and reduced contribution of high-risk HLA haplotypes", *Lancet,* vol. 364, no. 9446, pp. 1699-1700.

Gourraud, P.A., Feolo, M., Hoffman, D., Helmberg, W. & Cambon-Thomsen, A. (2006), "The dbMHC microsatellite portal: a public resource for the storage and display of MHC microsatellite information", *Tissue antigens,* vol. 67, no. 5, pp. 395-401.

Gourraud, P.A., Mano, S., Barnetche, T., Carrington, M., Inoko, H. & Cambon-Thomsen, A. (2004), "Integration of microsatellite characteristics in the MHC region: a literature and sequence based analysis", *Tissue antigens,* vol. 64, no. 5, pp. 543-555.

Green, D.M., Trial, J. & Birdsall, H.H. (1998), "TNF-alpha released by comigrating monocytes promotes transendothelial migration of activated lymphocytes", *Journal of immunology (Baltimore, Md.: 1950),* vol. 161, no. 5, pp. 2481-2489.

Grimaldi, M.C., Clayton, J., Pontarotti, P., Cambon-Thomsen, A. & Crouau-Roy, B. (1996), "New highly polymorphic microsatellite marker in linkage disequilibrium with HLA-B", *Human immunology,* vol. 51, no. 2, pp. 89-94.

Gupta, M., Nikitina-Zake, L., Zarghami, M., Landin-Olsson, M., Kockum, I., Lernmark, A. & Sanjeevi, C.B. (2003), "Association between the transmembrane region polymorphism of MHC class I chain related gene-A and type 1 diabetes mellitus in Sweden", *Human immunology,* vol. 64, no. 5, pp. 553-561.

GWAS catalog http://www.genome.gov/gwastudies

Haimila, K., Penttilä, A., Arvola, A., Auvinen, M.K. & Korhonen, M. (2013), "Analysis of the adequate size of a cord blood bank and comparison of HLA haplotype distributions between four populations", *Human immunology,* vol. 74, no. 2, pp. 189-195.

Haimila, K., Peräsaari, J., Linjama, T., Koskela, S., Saarinenl, T., Lauronen, J., Auvinen, M.K. & Jaatinen, T. (2013), "HLA antigen, allele and haplotype frequencies and their use in virtual panel reactive antigen calculations in the Finnish population", *Tissue antigens,* vol. 81, no. 1, pp. 35-43.

Hanifi-Moghaddam, P., de Knijf, P., Roep, B.O., Van der Auwera, B., Naipal, A., Gorus, F., Schuit, F. & Giphart, M.J. (1998), "Genetic structure of IDDM1: two separate regions in the major histocompatibility complex contribute to susceptibility or protection. Belgian Diabetes Registry", *Diabetes,* vol. 47, no. 2, pp. 263-269.

Haque, F.N., Gottesman, I.I., Wong, A.H. (2009), "Not really identical:epigenetic differences in monozygotic twins and implications for twin studies in psychiatry", *American journal of medical genetics,* vol. 151C, no. 2, pp 136-141.

Harder, T., Roepke, K., Diller, N., Stechling, Y., Dudenhausen, J.W. & Plagemann, A. (2009),

"Birth weight, early weight gain, and subsequent risk of type 1 diabetes: systematic review and meta-analysis", *American Journal of Epidemiology,* vol. 169, no. 12, pp. 1428-1436.

Harjutsalo, V., Podar, T. & Tuomilehto, J. (2005), "Cumulative incidence of type 1 diabetes in 10,168 siblings of Finnish young-onset type 1 diabetic patients", *Diabetes,* vol. 54, no. 2, pp. 563-569.

Harjutsalo, V., Reunanen, A. & Tuomilehto, J. (2006), "Differential transmission of type 1 diabetes from diabetic fathers and mothers to their offspring", *Diabetes,* vol. 55, no. 5, pp. 1517-1524.

Harjutsalo, V., Sjöberg, L. & Tuomilehto, J. (2008), "Time trends in the incidence of type 1 diabetes in Finnish children: a cohort study", *Lancet (London, England),* vol. 371, no. 9626, pp. 1777-1782.

Harjutsalo, V., Lammi, N., Karvonen, M. & Groop, P.H. (2010), "Age at onset of type 1 diabetes in parents and recurrence risk in ofspring", *Diabetes,* vol. 59, no. 1, pp. 210-214.

Harjutsalo, V., Sund, R., Knip, M. & Groop, P.H. (2013), "Incidence of type 1 diabetes in Finland",*Jama,* vol. 310, no. 4, pp. 427-428.

He, J.S., Xie, P.S., Luo, D.S., Sun, C.J., Zhang, Y.G. & Liu, F.X. (2014), "Role of immune dysfunction in pathogenesis of type 1 diabetes mellitus in children", *Asian Pacific journal of tropical medicine,* vol. 7, no. 10, pp. 823-826.

Hermann, R., Knip, M., Veijola, R., Simell, O., Laine, A.P., Akerblom, H.K., Groop, P.H., Forsblom, C., Pettersson-Fernholm, K., Ilonen, J. & FinnDiane Study Group (2003), "Temporal changes in the frequencies of HLA genotypes in patients with Type 1 diabetes-indication of an increased environmental pressure?", *Diabetologia,* vol. 46, no. 3, pp. 420-425.

Hermann, R., Lipponen, K., Kiviniemi, M., Kakko, T., Veijola, R., Simell, O., Knip, M. & Ilonen, J. (2006), "Lymphoid tyrosine phosphatase (LYP/PTPN22) Arg620Trp variant regulates insulin autoimmunity and progression to type 1 diabetes", *Diabetologia,* vol. 49, no. 6, pp. 1198-1208.

Hermann, R., Turpeinen, H., Laine, A.P., Veijola, R., Knip, M., Simell, O., Sipila, I., Akerblom, H.K. & Ilonen, J. (2003), "HLA DR-DQ-encoded genetic determinants of childhood-onset type 1 diabetes in Finland: an analysis of 622 nuclear families", *Tissue antigens,* vol. 62, no. 2, pp. 162-169.

Honeyman, M.C., Harrison, L.C., Drummond, B., Colman, P.G. & Tait, B.D. (1995), "Analysis of families at risk for insulin-dependent diabetes mellitus reveals that HLA antigens influence progression to clinical disease", *Molecular medicine (Cambridge, Mass.),* vol. 1, no. 5, pp. 576-582.

Hussen, H.I., Persson, M. & Moradi, T. (2013), "The trends and the risk of type 1 diabetes over the past 40 years: an analysis by birth cohorts and by parental migration background in Sweden", *BMJ open,* vol. 3, no. 10, pp. e003418-2013-003418.

Hyöty, H. (2016), "Viruses in the type 1 diabetes", *Pediatric Diabetes,* vol. 17. no. 22, pp. 56-64.

Hyppönen, E., Läärä, E., Reunanen, A., Järvelin, M.R. & Virtanen, S.M. (2001), "Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study", *Lancet (London, England),* vol. 358, no. 9292, pp. 1500-1503.

Hyttinen, V., Kaprio, J., Kinnunen, L., Koskenvuo, M. & Tuomilehto, J. (2003), "Genetic liability of type 1 diabetes and the onset age among 22,650 young Finnish twin pairs: a nationwide follow-up study", *Diabetes,* vol. 52, no. 4, pp. 1052-1055.

International Diabetes Federation (IDF) Diabetes Atlas, Eighth Edition, (2017), http://www.diabetesatlas.org

Ilonen, J. (2016), "Genetic susceptibility to type 1 diabetes in childhood-estimation of HLA class II associated disease risk and class II effect in various phases of islet autoimmunity", *Pediatr.Diabetes,* vol. 17, no. 22, pp. 8-16.

Ilonen, J., Hammais, A., Laine, A.P., Lempainen, J., Vaarala, O., Veijola, R., Simell, O. & Knip, M. (2013), "Patterns of β-cell autoantibody appearance and genetic association during the first years of life", *Diabetes,* vol. 62, no. 10, pp. 3636-3640.

Ilonen, J., Kocova, M., Lipponen, K., Sukarova-Angelovska, E., Jovanovska, A. & Knip, M. (2009), "HLA-DR-DQ haplotypes and type 1 diabetes in Macedonia", *Human immunology,* vol. 70, no. 6, pp. 461-463.

Ilonen, J., Lempainen, J., Hammais, A., Laine, A.P., Härkönen, T., Toppari, J., Veijola, R., Knip, M., The Finnish Pediatric Diabetes Register. (2018), "Primary islet autoantibody at initial seroconversion and autoantibodies at diagnosis of type 1 diabetes as markers of disease heterogeneity", *Pediatric Diabetes,* vol. 19, no. 2, pp. 284-292.

IMGT/HLA database (2018), http://www.ebi.ac.uk/ipd/imgt/hla/

Jacobson, D.L., Gange, S.J., Rose, N.R. & Graham, N.M. (1997), "Epidemiology and estimated population burden of selected autoimmune diseases in the United States", *Clinical immunology and immunopathology,* vol. 84, no. 3, pp. 223-243.

Javor, J., Ferencik, S., Bucova, M., Stuchlikova, M., Martinka, E., Barak, L., Strbova, L., Grosse-Wilde, H. & Buc, M. (2010), "Polymorphisms in the genes encoding TGF-beta1, TNF-alpha, and IL-6 show association with type 1 diabetes mellitus in the Slovak population", *Archivum Immunologiae et Therapiae Experimentalis,* vol. 58, no. 5, pp. 385-393.

Ji, J., Hemminki, K., Sundquist, J. & Sundquist, K. (2010), "Ethnic differences in incidence of type 1 diabetes among second-generation immigrants and adoptees from abroad", *The Journal of Clinical Endocrinology and Metabolism,* vol. 95, no. 2, pp. 847-850.

Johansson, S., Lie, B.A., Todd, J.A., Pociot, F., Nerup, J., Cambon-Thomsen, A., Kockum, I., Akselsen, H.E., Thorsby, E. & Undlien, D.E. (2003), "Evidence of at least two type 1 diabetes susceptibility genes in the HLA complex distinct from HLA-DQB1, -DQA1 and -DRB1",

*Genes and immunity,* vol. 4, no. 1, pp. 46-53.

Jongeneel, C.V., Briant, L., Udalova, I.A., Sevin, A., Nedospasov, S.A. & Cambon-Thomsen, A. (1991), "Extensive genetic polymorphism in the human tumor necrosis factor region and relation to extended HLA haplotypes", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 88, no. 21, pp. 9717-9721.

Jun, H.S. & Yoon, J.W. (2003), "A new look at viruses in type 1 diabetes", *Diabetes/metabolism research and reviews,* vol. 19, no. 1, pp. 8-31.

Kahn, H.S., Morgan, T.M., Case, L.D., Dabelea, D., Mayer-Davis, E.J., Lawrence, J.M., Marcovina, S.M., Imperatore, G. & SEARCH for Diabetes in Youth Study Group (2009), "Association of type 1 diabetes with month of birth among U.S. youth: The SEARCH for Diabetes in Youth Study", *Diabetes care,* vol. 32, no. 11, pp. 2010-2015.

Karjalainen, J., Martin, J.M., Knip, M., Ilonen, J., Robinson, B.H., Savilahti, E., Åkerblom, H.K. & Dosch, H.M. (1992), "A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus", *The New England journal of medicine,* vol. 327, no. 5, pp. 302-307.

Karvonen, M., Viik-Kajander, M., Moltchanova, E., Libman, I., LaPorte, R. & Tuomilehto, J. (2000), "Incidence of childhood type 1 diabetes worldwide. Diabetes Mondiale (DiaMond) Project Group", *Diabetes care,* vol. 23, no. 10, pp. 1516-1526.

Katahira, M., Ishiguro, T., Segawa, S., Kuzuya-Nagao, K., Hara, I. & Nishisaki, T. (2008), "Reevaluation of human leukocyte antigen DR-DQ haplotype and genotype in type 1 diabetes in the Japanese population", *Hormone research,* vol. 69, no. 5, pp. 284-289.

Kawabata, Y., Ikegami, H., Kawaguchi, Y., Fujisawa, T., Hotta, M., Ueda, H., Shintani, M., Nojima, K., Ono, M., Nishino, M., Taniguchi, H., Noso, S., Yamada, K., Babaya, N. & Ogihara, T. (2000), "Age-related association of MHC class I chain-related gene A (MICA) with type 1 (insulin-dependent) diabetes mellitus", *Human immunology,* vol. 61, no. 6, pp. 624-629.

Kimpimäki, T., Kulmala, P., Savola, K., Kupila, A., Korhonen, S., Simell, T., Ilonen, J., Simell, O. & Knip, M. (2002), "Natural history of beta-cell autoimmunity in young children with increased genetic susceptibility to type 1 diabetes recruited from the general population", *The Journal of clinical endocrinology and metabolism,* vol. 87, no. 10, pp. 4572-4579.

Kiviniemi, M., Hermann, R., Nurmi, J., Ziegler, A.G., Knip, M., Simell, O., Veijola, R., Lövgren, T., Ilonen, J. & TEDDY Study Group (2007), "A high-throughput population screening system for the estimation of genetic risk for type 1 diabetes: an application for the TEDDY (the Environmental Determinants of Diabetes in the Young) study", *Diabetes technology & therapeutics,* vol. 9, no. 5, pp. 460-472.

Klein, J. & Sato, A. (2000), "The HLA system. First of two parts", *The New England journal of medicine,* vol. 343, no. 10, pp. 702-709.

Knip, M., Åkerblom, H.K., Becker, D., Dosch, H.M., Dupre, J., Fraser, W., Howard, N., Ilonen, J.,

Krischer, J.P., Kordonouri, O., Lawson, M.L., Palmer, J.P., Savilahti, E., Vaarala, O., Virtanen, S.M. & TRIGR Study Group (2014), "Hydrolyzed infant formula and early beta-cell autoimmunity: a randomized clinical trial", *Jama,* vol. 311, no. 22, pp. 2279-2287.

Knip, M., Korhonen, S., Kulmala, P., Veijola, R., Reunanen, A., Raitakari, O.T., Viikari, J. & Akerblom, H.K. (2010), "Prediction of type 1 diabetes in the general population", *Diabetes care,* vol. 33, no. 6, pp. 1206-1212.

Knip, M., Siljander, H., Ilonen, J., Simell, O. & Veijola, R. (2016), "Role of humoral beta-cell autoimmunity in type 1 diabetes", *Pediatric diabetes,* vol. 17 Suppl 22, pp. 17-24.

Knip, M. & Simell, O. (2012), "Environmental triggers of type 1 diabetes", *Cold Spring Harbor perspectives in medicine,* vol. 2, no. 7, pp. a007690.

Koeleman, B.P., Dudbridge, F., Cordell, H.J. & Todd, J.A. (2000), "Adaptation of the extended transmission/disequilibrium test to distinguish disease associations of multiple loci: the Conditional Extended Transmission/Disequilibrium Test", *Annals of Human Genetics,* vol. 64, no. Pt 3, pp. 207-213.

Kondrashova, A., Reunanen, A., Romanov, A., Karvonen, A., Viskari, H., Vesikari, T., Ilonen, J., Knip, M. & Hyoty, H. (2005), "A six-fold gradient in the incidence of type 1 diabetes at the eastern border of Finland", *Annals of Medicine,* vol. 37, no. 1, pp. 67-72.

Kondrashova, A., Seiskari, T., Ilonen, J., Knip, M. & Hyoty, H. (2013), "The 'Hygiene hypothesis' and the sharp gradient in the incidence of autoimmune and allergic diseases between Russian Karelia and Finland", *APMIS : Acta Pathologica, Microbiologica, et Immunologica Scandinavica,* vol. 121, no. 6, pp. 478-493.

Krischer, J.P., Lynch, K.F., Schatz, D.A., Ilonen J., Lernmark, Å., Hagopian, W.A., Rewers, M.J., She, J.X., Simell, O.G., Toppari, J., Ziegler, A.G., Akolkar, B., Bonifacio, E., TEDDY study Group, (2015), "The 6 year incidence of diabetes-associated autoantibodies in genetically at-risk children:the TEDDY study", *Diabetologia,*vol.58, no. 5, pp. 980-987.

Kruglyak, L., Daly, M.J., Reeve-Daly, M.P. & Lander, E.S. (1996), "Parametric and nonparametric linkage analysis: a unified multipoint approach", *American Journal of Human Genetics,* vol. 58, no. 6, pp. 1347-1363.

Kumar, R., Goswami, R., Agarwal, S., Israni, N., Singh, S.K., & Rani, R. (2007), "Association and interaction of the TNF-alpha gene with other pro- and anti-inflammatory cytokine genes and HLA genes in patients with type 1 diabetes from North India", *Tissue Antigens,* vol 69, no. 6, pp. 557-567.

Laaksonen, M., Pastinen, T., Sjöroos, M., Kuokkanen, S., Ruutiainen, J., Sumelahti, M.L., Reijonen, H., Salonen, R., Wikström, J., Panelius, M., Partanen, J., Tienari, P.J. & Ilonen, J. (2002), "HLA class II associated risk and protection against multiple sclerosis-a Finnish family study", *Journal of neuroimmunology,* vol. 122, no. 1-2, pp. 140-145.

Laureti, S., Vecchi, L., Santeusanio, F. & Falorni, A. (1999), "Is the prevalence of Addison's

disease underestimated?", *The Journal of clinical endocrinology and metabolism,* vol. 84, no. 5, pp. 1762.

Lie, B.A., Todd, J.A., Pociot, F., Nerup, J., Akselsen, H.E., Joner, G., Dahl-Jorgensen, K., Ronningen, K.S., Thorsby, E. & Undlien, D.E. (1999), "The predisposition to type 1 diabetes linked to the human leukocyte antigen complex includes at least one non-class II gene", *American Journal of Human Genetics,* vol. 64, no. 3, pp. 793-800.

Long, A.E., Gillespie, K.M., Rokni, S., Bingley, P.J. & Williams, A.J. (2012), "Rising incidence of type 1 diabetes is associated with altered immunophenotype at diagnosis", *Diabetes,* vol. 61, no. 3, pp. 683-686.

Løvås, K. & Husebye, E.S. (2005), "Addison's disease", *Lancet (London, England),* vol. 365, no. 9476, pp. 2058-2061.

Løvås, K. & Husebye, E.S. (2002), "High prevalence and increasing incidence of Addison's disease in western Norway", *Clinical endocrinology,* vol. 56, no. 6, pp. 787-791.

Lund-Blix, N.A., Stene, L.C., Rasmussen, T., Torjesen, P.A., Andersen, L.F. & Ronningen, K.S. (2015), "Infant feeding in relation to islet autoimmunity and type 1 diabetes in genetically susceptible children: the MIDIA Study", *Diabetes care,* vol. 38, no. 2, pp. 257-263.

Maash, D.M., West, N.A., Lawrence, J.M. & Mayer-Davis, E.J. (2010), "Epidemiology of Type 1 Diabetes", *Endocrinology and Metabolism Clinics of North America,* vol. 39, no. 3, pp. 481-497.

Magistrelli, G., Jeannin, P., Herbault, N., Benoit De Coignac, A., Gauchat, J.F., Bonnefoy, J.Y. & Delneste, Y. (1999), "A soluble form of CTLA-4 generated by alternative splicing is expressed by nonstimulated human T cells", *European journal of immunology,* vol. 29, no. 11, pp. 3596-3602.

Mäkinen, A., Härkönen, T., Ilonen, J., Knip, M. & Finnish Pediatric Diabetes Register (2008), "Characterization of the humoral immune response to islet antigen 2 in children with newly diagnosed type 1 diabetes", *European journal of endocrinology,* vol. 159, no. 1, pp. 19-26.

Marsh, S. & Parham, P.,Barber L. (2001), *THE HLA FactsBook,* 1st edn, Academic Press.

Mason, A.S., Meade, T.W., Lee, J.A. & Morris, J.N. (1968), "Epidemiological and clinical picture of Addison's disease", *Lancet (London, England),* vol. 2, no. 7571, pp. 744-747.

Mbunwe, E., Van der Auwera, B.J., Vermeulen, I., Demeester, S., Van Dalem, A., Balti, E.V., Van Aken, S., Derdelinckx, L., Dorchy, H., De Schepper, J., van Schravendijk, C., Wenzlau, J.M., Hutton, J.C., Pipeleers, D., Weets, I., Gorus, F.K. & Belgian Diabetes Registry (2013), "HLA-A*24 is an independent predictor of 5-year progression to diabetes in autoantibody-positive first-degree relatives of type 1 diabetic patients", *Diabetes,* vol. 62, no. 4, pp. 1345-1350.

Mikk, M.L., Heikkinen, T., El-Amir, M.I., Kiviniemi, M., Laine, A.P., Harkonen, T., Veijola, R., Toppari, J., Knip, M., Ilonen, J. & Finnish Paediatric Diabetes Register (2017), "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,* vol. 89, no. 4, pp. 215-224.

Mikk, M.L., Kiviniemi, M., Laine, A.P., Harkonen, T., Veijola, R., Simell, O., Knip, M., Ilonen, J. & Finnish Paediatric Diabetes Register (2014), "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,* vol. 75, no. 1, pp. 65-70.

Miller, S.A., Dykes, D.D. & Polesky, H.F. (1988), "A simple salting out procedure for extracting DNA from human nucleated cells", *Nucleic acids research,* vol. 16, no. 3, pp. 1215.

Milner, C.M. & Campbell, R.D. (2001), "Genetic organization of the human MHC class III region", *Frontiers in bioscience : a journal and virtual library,* vol. 6, pp. D914-26.

Mitchell, A.L. & Pearce, S.H. (2012), "Autoimmune Addison disease: pathophysiology and genetic complexity", *Nature reviews.Endocrinology,* vol. 8, no. 5, pp. 306-316.

Mitchell, A.L., Wolff, A.B., MacArthur, K., Weaver, J.U., Vaidya, B., Swedish Addison Registry Study Group, Erichsen, M.M., Darlay, R., Husebye, E.S., Cordell, H.J. & Pearce, S.H. (2015), "Linkage Analysis in Autoimmune Addison's Disease:NFATC1 as a Potential Novel Susceptibility Locus", *PloS One,* vol. 10, no. 6, pp. 1-13.

Mizuki, N., Ota, M., Kimura, M., Ohno, S., Ando, H., Katsuyama, Y., Yamazaki, M., Watanabe, K., Goto, K., Nakamura, S., Bahram, S. & Inoko, H. (1997), "Triplet repeat polymorphism in the transmembrane region of the MICA gene: a strong association of six GCT repetitions with Behcet disease", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 94, no. 4, pp. 1298-1303.

Mohlke, K.L. & Scott, L.J. (2012), "What Will Diabetes Genomes Tell Us?", *Current Diabetes Reports,* vol. 12, no 6, pp. 643-650.

Moltchanova, E.V., Schreier, N., Lammi, N. & Karvonen, M. (2009), "Seasonal variation of diagnosis of Type 1 diabetes mellitus in children worldwide", *Diabetic medicine : a journal of the British Diabetic Association,* vol. 26, no. 7, pp. 673-678.

Morran, M.P., Vonberg, A., Khadra, A. & Pietropaolo, M. (2015), "Immunogenetics of type 1 diabetes mellitus", *Molecular aspects of medicine,* vol. 42, pp. 42-60.

Myhre, A.G., Undlien, D.E., Lovas, K., Uhlving, S., Nedrebo, B.G., Fougner, K.J., Trovik, T., Sorheim, J.I. & Husebye, E.S. (2002), "Autoimmune adrenocortical failure in Norway autoantibodies and human leukocyte antigen class II associations related to clinical features", *The Journal of clinical endocrinology and metabolism,* vol. 87, no. 2, pp. 618-623.

Nakanishi, K., Kobayashi, T., Murase, T., Nakatsuji, T., Inoko, H., Tsuji, K. & Kosaka, K. (1993), "Association of HLA-A24 with complete beta-cell destruction in IDDM", *Diabetes,* vol. 42, no. 7, pp. 1086-1093.

Nakanishi, K., Kobayashi, T., Murase, T., Naruse, T., Nose, Y. & Inoko, H. (1999), "Human

leukocyte antigen-A24 and -DQA1*0301 in Japanese insulin-dependent diabetes mellitus: independent contributions to susceptibility to the disease and additive contributions to acceleration of beta-cell destruction", *The Journal of clinical endocrinology and metabolism,* vol. 84, no. 10, pp. 3721-3725.

Napier, C. & Pearce, S. H. (2012), "Autoimmune Addison's disease", *Presse Medicale,* vol. 12, no. 41,pp. 626-635.

Nejentsev, S., Howson, J.M., Walker, N.M., Szeszko, J., Field, S.F., Stevens, H.E., Reynolds, P., Hardy, M., King, E., Masters, J., Hulme, J., Maier, L.M., Smyth, D., Bailey, R., Cooper, J.D., Ribas, G., Campbell, R.D., Clayton, D.G., Todd, J.A. & Wellcome Trust Case Control Consortium (2007), "Localization of type 1 diabetes susceptibility to the MHC class I genes HLA-B and HLA-A", *Nature,* vol. 450, no. 7171, pp. 887-892.

Nejentsev, S., Reijonen, H., Adojaan, B., Kovalchuk, L., Sochnevs, A., Schwartz, E.I., Åkerblom, H.K. & Ilonen, J. (1997), "The effect of HLA-B allele on the IDDM risk defined by DRB1*04 subtypes and DQB1*0302", *Diabetes,* vol. 46, no. 11, pp. 1888-1892.

Nejentsev, S., Sjoroos, M., Soukka, T., Knip, M., Simell, O., Lovgren, T. & Ilonen, J. (1999), "Population-based genetic screening for the estimation of Type 1 diabetes mellitus risk in Finland: selective genotyping of markers in the HLA-DQB1, HLA-DQA1 and HLA-DRB1 loci", *Diabetic medicine : a journal of the British Diabetic Association,* vol. 16, no. 12, pp. 985-992.

Neu, A., Kehrer, M., Ashkenazi, I. & Laron, Z. (2000), "Seasonality of birth in children (0-14 years) with diabetes mellitus type 1 in Baden-Wuerttemberg, Germany", *Journal of pediatric endocrinology & metabolism : JPEM,* vol. 13, no. 8, pp. 1081-1085.

Nielsen, C., Hansen, D., Husby, S. & Lillevang, S.T. (2007), "Sex-specific association of the human PTPN22 1858T-allele with type 1 diabetes", *International journal of immunogenetics,* vol. 34, no. 6, pp. 469-473.

Nikitina-Zake, L., Ghaderi, M., Park, Y., Babu, S., Eisenbarth, G. & Sanjeevi, C.B. (2004), "MICA gene polymorphism in HBDI multiplex families", *Annals of the New York Academy of Sciences,* vol. 1037, pp. 150-156.

Nistico, L., Buzzetti, R., Pritchard, L.E., Van der Auwera, B., Giovannini, C., Bosi, E., Larrad, M.T., Rios, M.S., Chow, C.C., Cockram, C.S., Jacobs, K., Mijovic, C., Bain, S.C., Barnett, A.H., Vandewalle, C.L., Schuit, F., Gorus, F.K., Tosi, R., Pozzilli, P. & Todd, J.A. (1996), "The CTLA-4 gene region of chromosome 2q33 is linked to, and associated with, type 1 diabetes. Belgian Diabetes Registry", *Human molecular genetics,* vol. 5, no. 7, pp. 1075-1080.

Noble, J.A. (2015), "Immunogenetics of type 1 diabetes: A comprehensive review", *Journal of Autoimmunity,* vol. 64, pp. 101-112.

Noble, J.A. & Erlich, H.A. (2012), "Genetics of type 1 diabetes", *Cold Spring Harbor perspectives in medicine,* vol. 2, no. 1, pp. a007732.

Noble, J.A. & Valdes, A.M. (2011), "Genetics of the HLA region in the prediction of type 1 diabetes", *Current diabetes reports,* vol. 11, no. 6, pp. 533-542.

Noble, J.A., Valdes, A.M., Bugawan, T.L., Apple, R.J., Thomson, G. & Erlich, H.A. (2002), "The HLA class I A locus affects susceptibility to type 1 diabetes", *Human immunology,* vol. 63, no.8, pp. 657-664.

Noble, J.A., Valdes, A.M., Varney, M.D., Carlson, J.A., Moonsamy, P., Fear, A.L., Lane, J.A., Lavant, E., Rappner, R., Louey, A., Concannon, P., Mychaleckyj, J.C., Erlich, H.A. & Type 1 Diabetes Genetics Consortium (2010), "HLA class I and genetic susceptibility to type 1 diabetes: results from the Type 1 Diabetes Genetics Consortium", *Diabetes,* vol. 59, no. 11, pp. 2972-2979.

Nunes, E., Heslop, H., Fernandez-Vina, M., Taves, C., Wagenknecht, D.R., Eisenbrey, A.B., Fischer, G., Poulton, K., Wacker, K., Hurley, C.K., Noreen, H. & Sacchi, N. (2011), "Definitions of histocompatibility typing terms", *Blood,* vol. 118, no. 23, pp. 180-183.

Oelkers, W. (1996), "Adrenal insufficiency", *The New England journal of medicine,* vol. 335, no. 16, pp. 1206-1212.

Oilinki, T., Otonkoski, T., Ilonen, J., Knip, M. & Miettinen, P.J. (2012), "Prevalence and characteristics of diabetes among Somali children and adolescents living in Helsinki, Finland", *Pediatric diabetes,* vol. 13, no. 2, pp. 176-180.

Padoa, C. (2011), "The epidemiology and pathogenesis of type 1 diabetes mellitus in Africa.", *Journal of Endocrinology,Metabolism and Diabetes od South Africa,* vol. 16, no. 3, pp. 130-136.

Palmer, J.P., Asplin, C.M., Clemons, P., Lyen, K., Tatpati, O., Raghu, P.K. & Paquette, T.L. (1983), "Insulin antibodies in insulin-dependent diabetics before insulin treatment", *Science (New York, N.Y.),* vol. 222, no. 4630, pp. 1337-1339.

Park, Y. & Eisenbarth, G.S. (2001), "Genetic susceptibility factors of Type 1 diabetes in Asians", *Diabetes/metabolism research and reviews,* vol. 17, no. 1, pp. 2-11.

Park, Y., Lee, H., Sanjeevi, C.B. & Eisenbarth, G.S. (2001), "MICA polymorphism is associated with type 1 diabetes in the Korean population", *Diabetes care,* vol. 24, no. 1, pp. 33-38.

Park, Y.S., Sanjeevi, C.B., Robles, D., Yu, L., Rewers, M., Gottlieb, P.A., Fain, P. & Eisenbarth, G.S. (2002), "Additional association of intra-MHC genes, MICA and D6S273, with Addison's disease", *Tissue antigens,* vol. 60, no. 2, pp. 155-163.

Patente, T.A., Monteiro, M.B., Vieira, S.M., Rossi da Silva, M.E., Nery, M., Queiroz, M., Azevedo, M.J., Canani, L.H., Parisi, M.C., Pavin, E.J., Mainardi, D., Javor, J., Velho, G., Coimbra, C.N. & Correa-Giannella, M.L. (2015), "Linkage disequilibrium with HLA-DRB1-DQB1 haplotypes explains the association of TNF-308G>A variant with type 1 diabetes in a Brazilian cohort", *Gene,* vol. 568, no. 1, pp. 50-54.

Patterson, C.C., Dahlquist, G.G., Gyürüs, É., Green, A., Soltész, G. & EURODIAB Study Group (2009), "Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 2005-20: a multicentre prospective registration study", *Lancet (London, England),* vol. 373, no. 9680, pp. 2027-2033.

Patterson, C.C., Gyürüs, É., Rosenbauer, J., Cinek, O., Neu, A., Schober, E., Parslow, R.C., Joner, G., Svensson, J., Castell, C., Bingley, P.J., Schoenle, E., Jarosz-Chobot, P., Urbonaité, B., Rothe, U., Krzisnik, C., Jonescu-Tirgoviste, C., Weets, I., Kocova, M., Stipancic, G., Samardzic, M., de Beaufort, C.E., Green, A., Soltész, G. & Dahlquist, G.G. (2015), "Seasonal variation in month of diagnosis in children with type 1 diabetes registered in 23 European centres during 1989-2008: little short-term influence of sunshine hours or average temperature", *Pediatric Diabetes,* vol. 16, no. 8, pp. 573-580.

Pazderska, A., Fichna, M., Mitchell, A.L., Napier, C.M., Gan, E., Ruchala, M., Santibanez-Koref, M. & Pearce, S.H. (2016), "Impact of Month of Birth on the Risk of Development of Autoimmune Addison's Disease", *The Journal of clinical endocrinology and metabolism,* vol. 101, no. 11, pp. 4214-4218.

Pociot, F. (2017), "Type 1 diabetes genome-wide association studies: not to be lost in translation", *Clinical and Translational Immunology,* vol. 6, no. 12, pp. 01-07.

Pociot, F., Akolkar, B., Concannon, P., Erlich, H.A., Julier, C., Morahan, G., Nierras, C.R., Todd, J.A., Rich, S.S. & Nerup, J. (2010), "Genetics of Type 1 diabetes:What's Next?", *Diabetes,* vol. 59, no. 7, pp. 1561-1571.

Pociot, F. & Lernmark, Å. (2016), "Genetic risk factors for type 1 diabetes", *Lancet (London, England),* vol. 387, no. 10035, pp. 2331-2339.

Price, P., Wong, A.M., Williamson, D., Voon, D., Baltic, S., Allcock, R.J., Boodhoo, A. & Christiansen, F.T. (2004), "Polymorphisms at positions -22 and -348 in the promoter of the BAT1 gene affect transcription and the binding of nuclear factors", *Human molecular genetics,* vol. 13, no. 9, pp. 967-974.

Pugliese, A., Boulware, D., Yu, L., Babu, S., Steck, A.K., Becker, D., Rodriguez, H., DiMeglio, L., Evans-Molina, C., Harrison, L.C., Schartz, D., Palmer, J.P., Greenbaum, C., Eisenbarth, G.S, Sosenko, J.M., Type 1 Diabetes TrialNet Study Group. (2016), "HLA-DRB1*15:01-DQA1*01:02-DQB1*06:02 haplotype protects autoantibody-positive relatives from type 1 diabetes throughout the stages of disease progression", *Diabetes,* vol. 65, no. 4, pp1109-1119.

Raymond M., Rousset F. (1995), "An exact test for population differentiation", *Evolution,* vol. 49, no. 6, pp. 1280-1283.

Redondo, M.J., Jeffrey, J., Fain, P.R., Eisenbarth, G.S. & Orban, T. (2008), "Concordance for islet autoimmunity among monozygotic twins", *The New England journal of medicine,* vol. 359, no. 26, pp. 2849-2850.

Redondo, M.J., Yu, L., Hawa, M., Mackenzie, T., Pyke, D.A., Eisenbarth, G.S. & Leslie, R.D. (2001), "Heterogeneity of type I diabetes: analysis of monozygotic twins in Great Britain and the United States", *Diabetologia,* vol. 44, no. 3, pp. 354-362.

Reijonen, H., Nejentsev, S., Tuokko, J., Koskinen, S., Tuomilehto-Wolf, E., Åkerblom, H.K. & Ilonen, J. (1997), "HLA-DR4 subtype and -B alleles in DQB1*0302-positive haplotypes associated with IDDM. The Childhood Diabetes in Finland Study Group", *European journal of immunogenetics : official journal of the British Society for Histocompatibility and Immunogenetics,* vol. 24, no. 5, pp. 357-363.

Regnell, S.E. & Lernmark Å. (2017), "Early prediction of autoimmune (type 1) diabetes", *Diabetologia,* vol. 60, no. 8, pp. 1370-1381.

Rewers, M. & Ludvigsson, J. (2016), "Environmental risk factors for type 1 diabetes", *Lancet (London, England),* vol. 387, no. 10035, pp. 2340-2348.

Roach, J.C., Deutsch, K., Li, S., Siegel, A.F., Bekris, L.M., Einhaus, D.C., Sheridan, C.M., Glusman, G., Hood, L., Lernmark, A., Janer, M., Swedish Childhood Diabetes Study Group & Diabetes Incidence in Sweden Study Group (2006), "Genetic mapping at 3-kilobase resolution reveals inositol 1,4,5-triphosphate receptor 3 as a risk factor for type 1 diabetes in Sweden", *American Journal of Human Genetics,* vol. 79, no. 4, pp. 614-627.

Rodriguez-Calvo, T., Sabouri, S., Anquetil, F. & von Herrath, M.G. (2016), "The viral paradigm in type 1 diabetes: Who are the main suspects?", *Autoimmunity reviews,* vol. 15, no. 10, pp. 964-969.

Saccucci, P., Del Duca, E., Rapini, N., Verrotti, A., Piccinini, S., Maccari, A., Canu, G., Angelini, F., Fontana, L., Giannini, C., Chiarelli, F., Manca Bitti, M.L. & Bottini, N. (2008), "Association between PTPN22 C1858T and type 1 diabetes: a replication in continental Italy", *Tissue antigens,* vol. 71, no. 3, pp. 234-237.

Samuelsson, U., Oikarinen, S., Hyöty, H. & Ludvigsson, J. (2011), "Low zinc in drinking water is associated with the risk of type 1 diabetes in children", *Pediatric diabetes,* vol. 12, no. 3 Pt 1, pp. 156-164.

Sanjeevi, C.B., Hagopian, W.A., Landin-Olsson, M., Kockum, I., Woo, W., Palmer, J.P., Lernmark, Å. & Dahlquist, G. (1998), "Association between autoantibody markers and subtypes of DR4 and DR4-DQ in Swedish children with insulin-dependent diabetes reveals closer association of tyrosine pyrophosphatase autoimmunity with DR4 than DQ8", *Tissue antigens,* vol. 51, no. 3, pp. 281-286.

Sanjeevi, C.B., Kanungo, A., Berzina, L., Shtauvere-Brameus, A., Ghaderi, M. & Samal, K.C. (2002), "MHC class I chain-related gene a alleles distinguish malnutrition-modulated diabetes, insulin-dependent diabetes, and non-insulin- dependent diabetes mellitus patients from eastern India", *Annals of the New York Academy of Sciences,* vol. 958, pp. 341-344.

Savola, K., Bonifacio, E., Sabbah, E., Kulmala, P., Vähäsalo, P., Karjalainen, J., Tuomilehto-

marker of IDDM with clinical onset in childhood and adolescence. Childhood Diabetes in Finland Study Group", *Diabetologia,* vol. 41, no. 4, pp. 424-429.

Savola, K., Sabbah, E., Kulmala, P., Vähäsalo, P., Ilonen, J. & Knip, M. (1998), "Autoantibodies associated with Type I diabetes mellitus persist after diagnosis in children", *Diabetologia,* vol. 41, no. 11, pp. 1293-1297.

Sawaya, S., Bagshaw, A., Buschiazzo, E., Kumar, P., Chowdhury, S., Black, M.A. & Gemmell, N. (2013), "Microsatellite tandem repeats are abundant in human promoters and are associated with regulatory elements", *PloS one,* vol. 8, no. 2, pp. e54710.

Shiina, T., Hosomichi, K., Inoko, H. & Kulski, J.K. (2009), "The HLA genomic loci map: expression, interaction, diversity and disease", *Journal of human genetics,* vol. 54, no. 1, pp. 15-39.

Shtauvere-Brameus, A., Ghaderi, M., Rumba, I. & Sanjeevi, C.B. (2002), "Microsatellite allele 5 of MHC class I chain-related gene a increases the risk for insulin-dependent diabetes mellitus in latvians", *Annals of the New York Academy of Sciences,* vol. 958, pp. 349-352.

Siljander, H.T., Simell, S., Hekkala, A., Lähde, J., Simell, T., Vähäsalo, P., Veijola, R., Ilonen, J., Simell, O. & Knip, M. (2009), "Predictive characteristics of diabetes-associated autoantibodies among children with HLA-conferred disease susceptibility in the general population", *Diabetes,* vol. 58, no. 12, pp. 2835-2842.

Simpson, M., Brady, H., Yin, X., Seifert, J., Barriga, K., Hoffman, M., Bugawan, T., Baron, A.E., Sokol, R.J., Eisenbarth, G., Erlich, H., Rewers, M. & Norris, J.M. (2011), "No association of vitamin D intake or 25-hydroxyvitamin D levels in childhood with risk of islet autoimmunity and type 1 diabetes: the Diabetes Autoimmunity Study in the Young (DAISY)", *Diabetologia,* vol. 54, no. 11, pp. 2779-2788.

Singal, D.P. & Blajchman, M.A. (1973), "Histocompatibility (HL-A) antigens, lymphocytotoxic antibodies and tissue antibodies in patients with diabetes mellitus", *Diabetes,* vol. 22, no. 6, pp. 429-432.

Sjöroos, M., Iitia, A., Ilonen, J., Reijonen, H. & Lovgren, T. (1995), "Triple-label hybridization assay for type-1 diabetes-related HLA alleles", *BioTechniques,* vol. 18, no. 5, pp. 870-877.

Sjöroos, M., Ilonen, J., Reijonen, H. & Lovgren, T. (1998), "Time-resolved fluorometry based sandwich hybridisation assay for HLA-DQA1 typing", *Disease markers,* vol. 14, no. 1, pp. 9-19.

Skinningsrud, B., Lie, B.A., Lavant, E., Carlson, J.A., Erlich, H., Akselsen, H.E., Gervin, K., Wolff, A.B., Erichsen, M.M., Lovas, K., Husebye, E.S. & Undlien, D.E. (2011), "Multiple loci in the HLA complex are associated with Addison's disease", *The Journal of clinical endocrinology and metabolism,* vol. 96, no. 10, pp. 1703-1708.

Sonigni, M., Lombardo, C. (2010), "The Sardinian Way to Type 1 Diabetes", *Diabetes Technology Society,* vol. 4, no. 5, pp. 1248-1255.

Spielman, R.S., McGinnis, R.E. & Ewens, W.J. (1993), "Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM)", *American Journal of Human Genetics,* vol. 52, no. 3, pp. 506-516.

Stead, J.D., Buard, J., Todd, J.A. & Jeffreys, A.J. (2000), "Influence of allele lineage on the role of the insulin minisatellite in susceptibility to type 1 diabetes", *Human molecular genetics,* vol. 9, no. 20, pp. 2929-2935.

Steck, A.K., Dong, F., Wong, R., Fouts, A., Liu, E., Romanos, J., Wiljmenga, C., Norris, J.M. & Rewers, M.J. (2014), "Improving prediction of type 1 diabetes by testing non-HLA genetic variants in addition to HLA markers", *Pediatric Diabetes,* vol. 15, no. 5, pp. 255-362.

Takayanagi, R., Miura, K., Nakagawa, H. & Nawata, H. (2000), "Epidemiologic study of adrenal gland disorders in Japan", *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie,* vol. 54 Suppl 1, pp. 164-168.

Tanaka, H., Perez, M.S., Powell, M., Sanders, J.F., Sawicka, J., Chen, S., Prentice, L., Asawa, T., Betterle, C., Volpato, M., Smith, B.R. & Furmaniak, J. (1997), "Steroid 21-hydroxylase autoantibodies: measurements with a new immunoprecipitation assay", *The Journal of clinical endocrinology and metabolism,* vol. 82, no. 5, pp. 1440-1446.

The EURODIAB ACE Study Group (2000), "Variation and trends in incidence of childhood diabetes in Europe. EURODIAB ACE Study Group", *Lancet (London, England),* vol. 355, no. 9207, pp. 873-876.

The EURODIAB Substudy 2 Study Group (2002), "Rapid early growth is associated with increased risk of childhood type 1 diabetes in various European populations", *Diabetes care,* vol. 25, no. 10, pp. 1755-1760.

The EURODIAB Substudy 2 Group (1999), "Vitamin D supplement in early childhood and risk for Type I (insulin-dependent) diabetes mellitus. The EURODIAB Substudy 2 Study Group", *Diabetologia,* vol. 42, no. 1, pp. 51-54.

Thomson, G. (1995), "Mapping disease genes: family-based association studies", *American Journal of Human Genetics,* vol. 57, no. 2, pp. 487-498.

Thomson, G., Valdes, A.M., Noble, J.A., Kockum, I., Grote, M.N., Najman, J., Erlich, H.A., Cucca, F., Pugliese, A., Steenkiste, A., Dorman, J.S., Caillat-Zucman, S., Hermann, R., Ilonen, J., Lambert, A.P., Bingley, P.J., Gillespie, K.M., Lernmark, Å., Sanjeevi, C.B., Ronningen, K.S., Undlien, D.E., Thorsby, E., Petrone, A., Buzzetti, R., Koeleman, B.P., Roep, B.O., Saruhan-Direskeneli, G., Uyar, F.A., Gunoz, H., Gorodezky, C., Alaez, C., Boehm, B.O., Mlynarski, W., Ikegami, H., Berrino, M., Fasano, M.E., Dametto, E., Israel, S., Brautbar, C., Santiago-Cortes, A., Frazer de Llado, T., She, J.X., Bugawan, T.L., Rotter, J.I., Raffel, L., Zeidler, A., Leyva-Cobian, F., Hawkins, B.R., Chan, S.H., Castano, L., Pociot, F. & Nerup, J. (2007), "Relative predispositional effects of HLA class II DRB1-DQB1 haplotypes and genotypes on type 1 diabetes: a meta-analysis", *Tissue antigens,* vol. 70, no. 2, pp. 110-127.

Todd, J.A., Bell, J.I. & McDevitt, H.O. (1987), "HLA-DQ beta gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus", *Nature,* vol. 329, no. 6140, pp. 599-604.

Törn, C., Hillman, M., Sanjeevi, C.B. & Landin-Olsson, M. (2006), "Polymorphisms of TNF microsatellite marker a and HLA-DR-DQ in diabetes mellitus-a study in 609 Swedish subjects", *Human immunology,* vol. 67, no. 7, pp. 527-534.

Torres, M.A. & Moraes, M.E. (2011), "Nomenclature for factors of the HLA system", *Einstein (Sao Paulo, Brazil),* vol. 9, no. 2, pp. 249-251.

Turner, D. (2004), "The human leucocyte antigen (HLA) system", *Vox sanguinis,* vol. 87 Suppl1, pp. 87-90.

Vaarala, O., Ilonen, J., Ruohtula, T., Pesola, J., Virtanen, S.M., Harkonen, T., Koski, M., Kallioinen, H., Tossavainen, O., Poussa, T., Järvenpää, A.L., Komulainen, J., Lounamaa, R., Åkerblom, H.K. & Knip, M. (2012), "Removal of Bovine Insulin From Cow's Milk Formula and Early Initiation of Beta-Cell Autoimmunity in the FINDIA Pilot Study", *Archives of Pediatrics & Adolescent Medicine,* vol. 166, no. 7, pp. 608-614.

Vaarala, O., Klemetti, P., Savilahti, E., Reijonen, H., Ilonen, J. & Åkerblom, H.K. (1996), "Cellular immune response to cow's milk beta-lactoglobulin in patients with newly diagnosed IDDM", *Diabetes,* vol. 45, no. 2, pp. 178-182.

Vafiadis, P., Ounissi-Benkalha, H., Palumbo, M., Grabs, R., Rousseau, M., Goodyer, C.G. & Polychronakos, C. (2001), "Class III alleles of the variable number of tandem repeat insulin polymorphism associated with silencing of thymic insulin predispose to type 1 diabetes", *The Journal of clinical endocrinology and metabolism,* vol. 86, no. 8, pp. 3705-3710.

Valdes, A.M., Erlich, H.A. & Noble, J.A. (2005), "Human leukocyte antigen class I B and C loci contribute to Type 1 Diabetes (T1D) susceptibility and age at T1D onset", *Human immunology,* vol. 66, no. 3, pp. 301-313.

Valdes, A.M., Thomson, G. & Barcellos, L.F. (2010), "Genetic variation within the HLA class III influences T1D susceptibility conferred by high-risk HLA haplotypes", *Genes and immunity,* vol. 11, no. 3, pp. 209-218.

Valdes, A.M., Wapelhorst, B., Concannon, P., Erlich, H.A., Thomson, G. & Noble, J.A. (2005), "Extended DR3-D6S273-HLA-B haplotypes are associated with increased susceptibility to type 1 diabetes in US Caucasians", *Tissue antigens,* vol. 65, no. 1, pp. 115-119.

Van Autreve, J.E., Koeleman, B.P., Quartier, E., Aminkeng, F., Weets, I., Gorus, F.K., Van der Auwera, B.J. & Belgian Diabetes Registry (2006), "MICA is associated with type 1 diabetes in the Belgian population, independent of HLA-DQ", *Human immunology,* vol. 67, no. 1-2, pp. 94-101.

Varney, M.D., Valdes, A.M., Carlson, J.A., Noble, J.A., Tait, B.D., Bonella, P., Lavant, E., Fear, A.L., Louey, A., Moonsamy, P., Mychaleckyj, J.C., Erlich, H. & Type 1 Diabetes Genetics

Consortium (2010), "HLA DPA1, DPB1 alleles and haplotypes contribute to the risk associated with type 1 diabetes: analysis of the type 1 diabetes genetics consortium families", *Diabetes,* vol. 59, no. 8, pp. 2055-2062.

Vehik, K., Hamman, R.F., Lezotte, D., Norris, J.M., Klingensmith, G.J., Rewers, M. & Dabelea, D. (2008), "Trends in high-risk HLA susceptibility genes among Colorado youth with type 1 diabetes", *Diabetes Care,* vol. 31, no. 7, pp. 1392-1396.

Virtanen, S.M. (2016), "Dietary factors in the development of type 1 diabetes", *Pediatric diabetes,* vol. 17 Suppl 22, pp. 49-55.

Viskari, H., Kondrashova, A., Koskela, P., Knip, M. & Hyöty, H. (2006), "Circulating vitamin D concentrations in two neighboring populations with markedly different incidence of type 1 diabetes", *Diabetes care,* vol. 29, no. 6, pp. 1458-1459.

Wändell, P.E. & Carlsson, A.C. (2013), "Time trends and gender differences in incidence and prevalence of type 1 diabetes in Sweden", *Current diabetes reviews,* vol. 9, no. 4, pp. 342-349.

Wang, J., Liu, L., Ma, J., Sun, F., Zhao, Z. & Gu, M. (2014), "Common variants on cytotoxic T lymphocyte antigen-4 polymorphisms contributes to type 1 diabetes susceptibility: evidence based on 58 studies", *PloS one,* vol. 9, no. 1, pp. 859-882.

Weires, M.B., Tausch, B., Haug, P.J., Edwards, C.Q., Wetter, T. & Cannon-Albright, L.A. (2007), "Familiality of diabetes mellitus", *Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association,* vol. 115, no. 10, pp. 634-640.

Wellcome Trust Case Control Consortium (2007), "Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls", *Nature,* vol. 447, no. 7145, pp. 661-678.

Wenzlau, J.M., Juhl, K., Yu, L., Moua, O., Sarkar, S.A., Gottlieb, P., Rewers, M., Eisenbarth, G.S., Jensen, J., Davidson, H.W. & Hutton, J.C. (2007), "The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 104, no. 43, pp. 17040-17045.

WHO-Type 1 diabetes http://www.who.int/diabetes/action_online/basics/en/index1.html

Williams, A.J., Bingley, P.J., Bonifacio, E., Palmer, J.P. & Gale, E.A. (1997), "A novel micro-assay for insulin autoantibodies", *Journal of Autoimmunity,* vol. 10, no. 5, pp. 473-478.

Williams, T.M. (2001), "Human leukocyte antigen gene polymorphism and the histocompatibility laboratory", *The Journal of molecular diagnostics:JMD,* vol. 3, no. 3, pp. 98-104.

Willis, A.C. & Vince, F.P. (1997), "The prevalence of Addison's disease in Coventry, UK", *Postgraduate medical journal,* vol. 73, no. 859, pp. 286-288.

Winqvist, O., Karlsson, F.A. & Kampe, O. (1992), "21-Hydroxylase, a major autoantigen in idiopathic Addison's disease", *Lancet (London, England),* vol. 339, no. 8809, pp. 1559-1562.

Worwood, M., Raha-Chowdhury, R. & Darke, C. (1994), "Distribution of alleles at D6S105 and D6S265 with possible HLA haplotype associations", *Tissue antigens,* vol. 44, no. 5, pp. 322-325.

Xuan, C., Lun, L.M., Zhao, J.X., Wang, H.W., Zhu, B.Z., Yu, S., Liu, Z. & He, G.W. (2013), "PTPN22 gene polymorphism (C1858T) is associated with susceptibility to type 1 diabetes: a meta-analysis of 19,495 cases and 25,341 controls", *Annals of Human Genetics,* vol. 77, no. 3, pp. 191-203.

You, W.P. & Henneberg, M. (2016), "Type 1 diabetes prevalence increasing globally and regionally:the role of natural selection and life expectancy at birth", *BMJ open diabetes research & care,* vol. 4, no. 1, pp.1-7.

Zavattari, P., Lampis, R., Motzo, C., Loddo, M., Mulargia, A., Whalen, M., Maioli, M., Angius, E., Todd, J.A. & Cucca, F. (2001), "Conditional linkage disequilibrium analysis of a complex disease superlocus, IDDM1 in the HLA region, reveals the presence of independent modifying gene effects influencing the type 1 diabetes risk encoded by the major HLA-DQB1, -DRB1 disease loci", *Human molecular genetics,* vol. 10, no. 8, pp. 881-889.

Zhang, N., Huang, W., Dong, F., Liu, Y., Zhang, B., Jing, L., Wang, M., Yang, G. & Jing, C. (2015), "Insulin gene VNTR polymorphisms −2221MspI and −23HphI are associated with type 1 diabetes and latent autoimmune diabetes in adults: a meta-analysis", *Acta Diabetologica,* vol. 52, no. 6, pp. 1143-1153.

Zhernakova, A., Eerligh, P., Wijmenga, C., Barrera, P., Roep, B.O. & Koeleman, B.P. (2005), "Differential association of the PTPN22 coding variant with autoimmune diseases in a Dutch population", *Genes and immunity,* vol. 6, no. 6, pp. 459-461.

Ziegler, A.G., Rewers, M., Simell, O., Simell, T., Lempainen, J., Steck, A., Winkler, C., Ilonen, J., Veijola, R., Knip, M., Bonifacio, E. & Eisenbarth, G.S. (2013), "Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children", *Jama,* vol. 309, no. 23, pp. 2473-2479.

Zipitis, C.S. & Akobeng, A.K. (2008), "Vitamin D supplementation in early childhood and risk of type 1 diabetes: a systematic review and meta-analysis", *Archives of Disease in Childhood,* vol.93, no. 6, pp. 512-517.

Annales Universitatis Turkuensis

UNIVERSITY OF TURKU

Grano Oy, Turku , Finland 2018

ISBN 978-951-29-7428-3 (PRINT)
ISBN 978-951-29-7429-0 (PDF)
ISSN 0355-9483 (Print) ISSN 2343-3213 (Online)