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# Immunogenetic studies on autoimmune diabetes mellitus

*(PhD Thesis)*

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My special thanks go to my family, whose love enabled me to complete this work.

# Contents

# List of abbreviations

| | |
|---|---|
| APC | Antigen presentig cells |
| ATP | Adenosine triphosphate |
| *CTLA-4* | Cytotoxic T-lymphocyte associated protein $ |
| GAD | Glutamic decarboxylase |
| GDM | Gestational diabetes mellitus |
| HLA | Human leukocyte antigen |
| IA-2 | Protein tyrosine phophatase |
| IAA | Insulin autoantibodies |
| ICA | Islet cells autoantibodies |
| IDDM | Insulin dependent diabetes mellitus |
| IL | Interleukin |
| TNF | Tumor necrosis factor |
| INS | Insulin gene |
| INS-VNTR | Insulin gene – variable number of tandem repeats |
| LADA | Latent autoimmune diabetes in adults |
| MHC | major histocompatibility complex |
| *MIC-A* | MHC class I chain - related protein A |
| MRDM | Malnutrition diabetes mellitus |
| *NFKB1* | Nuclear factor kappa B, subunit 1 |
| *NFKBIA* | Nuclear factor of kappa light chain gene enhancer in B – cells inhibitor, alpha |
| NIDDM | Noninsulin-dependent diabetes mellitus |
| NK cell | Natural killer cell |
| NKG2D | C - type lectin like NK receptor |
| OSTDM | Other specific types of diabetes mellitus |
| STR | Short tandem repeats |
| T1D | Type 1 diabetes mellitus |
| T2D | Type 2 diabetes mellitus |
| WHO | World Health Organisation |

# 1. INTRODUCTION TO DIABETES MELLITUS

## 1.1. Diagnosis, Classification and Definition of Diabetes Mellitus

Diabetes mellitus associates a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both.

Several pathogenic processes are causing the development of diabetes. These range from autoimmune destruction of the $\beta$-cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action. The basis of the changes in carbohydrate, fat, and protein metabolism in diabetes is deficient action of insulin on target tissues.

The majority of diabetes cases fall into two etiopathogenetic categories (discussed in greater detail below). Type 1 diabetes is caused by an absolute deficiency of insulin secretion. The cause of type 2 diabetes is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response (see Fig 1). The etiologic classifications follow this section (Table 1, 2)

Table 1 **Classification of diabetes mellitus**

| Classification of diabetes mellitus | | |
|---|---|---|
| **WHO -1985*** | **WHO -2002**** | **ADA -2002***** |
| IDDM | T1DM | T1D |
| NIDDM | T2DM | T2D |
| MRDM | Not specified | Not specified |
| Not specified | GDM | GDM |
| Not specified | OSTDM | OSTDM |

* World Health Organization: Diabetes Mellitus. Geneva, WHO, Technical
** World Health Organization: Laboratory Diagnosis and Monitoring of Diabetes Mellitus. Geneva, WHO, 2002
***American Diabetes Asocciation : Diagnosis and Classification of Diabetes Mellitus, Diabetes Care 2004: 27, S5-S10

medicine? Risk prediction and gene therapy are often mentioned. However, risk prediction without prevention is of little benefit to the patient and may do more harm than good [3].

**Fig. 1** - Disorders of glycemia: etiologic types and stages.

| | **Normoglycemia** | **Hyperglycemia** | | | |
|---|---|---|---|---|---|
| **STAGES** | | | | | |
| **TYPES** | Normal glucose tolerance | Impaired Glucose Tolerance or | Diabetes Mellitus | | |
| | | Impaired Fasting Glucose | | | |
| | | (Pre-Diabetes) | Not insulin requiring | Insulin for control | Insulin for survival |
| **Type 1*** | | | | | |
| **Type 2** | | | | | |
| **Other Specific Types **** | | | | | |
| **Gestational Diabetes **** | | | | | |

- *Even after presenting in ketoacidosis, these patients can briefly return to normoglycemia without requiring continuous therapy (i.e., "honeymoon" remission);
- ** in rare instances, patients in these categories (e.g., Vacor toxicity, type 1 diabetes presenting in pregnancy) may require insulin for survival.

A. **Immune-mediated diabetes.** This group of diabetics accounts for only 5–10% of those with diabetes, previously described with the terms insulin dependent diabetes, type I diabetes, or juvenile- onset diabetes, results from a cellular-mediated autoimmune destruction of the $\beta$-cells of the pancreas [4]. Specific markers of the immune destruction of the $\beta$-cell include islet cell autoantibodies, autoantibodies to insulin, autoantibodies to glutamic acid decarboxylase (GAD65), and autoantibodies to the tyrosine phosphatases IA-2 and IA-2$\alpha$. Usually one of these autoantibodies is present in 85–90% of individuals when fasting hyperglycemia is initially detected [5].

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A. **Immune-mediated diabetes.** This group of diabetics accounts for only 5–10% of those with diabetes, previously described with the terms insulin dependent diabetes, type I diabetes, or juvenile- onset diabetes, results from a cellular-mediated autoimmune destruction of the $\beta$-cells of the pancreas [4]. Specific markers of the immune destruction of the $\beta$-cell include islet cell autoantibodies, autoantibodies to insulin, autoantibodies to glutamic acid decarboxylase (GAD65), and autoantibodies to the tyrosine phosphatases IA-2 and IA-2α. Usually one of these autoantibodies is present in 85–90% of individuals when fasting hyperglycemia is initially detected [5].

The disease has strong HLA associations, with linkage to the DQA and DQB genes, and it is influenced by the DRB genes. These *HLA-DR/DQ* alleles can be either predisposing or protective. The $\beta$-cell destruction is quite variable in this case of T1D, being rapid in some individuals (mainly infants and children) and slow in others (mainly adults). Immune T1D commonly occurs in childhood and adolescence, but was described also at any age, even in the 8th and 9th decades of life. The destruction of $\beta$-cells has multiple genetic causes and is also related to environmental factors that are still unsufficiently defined. Children and adolescents may present with ketoacidosis as the first sign of the disease. Others may present modest fasting hyperglycemia that can rapidly change to hyperglycemia and/or ketoacidosis in the presence of infection or other stress. Still others may retain residual $\beta$-cell function sufficient to prevent ketoacidosis for several years; such individuals eventually become dependent on insulin for survival and are at risk. Later there is little or no insulin secretion, as manifested by low or undetectable levels of plasma C-peptide in T1D patients.

B. **Idiopathic diabetes.** This category encopasses forms of type 1 diabetes with unknown etiologies. These patients may have permanent insulinopenia and are prone to ketoacidosis, but have no evidence of autoimmunity. Only a minority of patients with type 1 diabetes fall into this category, of those who do, most are of African or Asian ancestry. Individuals with this form of diabetes suffer from episodic ketoacidosis and exhibit varying degrees of insulin deficiency between episodes.

This form of diabetes is strongly inherited, lacks immunological evidence for $\beta$- cell autoimmunity, and is not HLA associated. An absolute requirement for insulin replacement therapy in affected patients may come and go [6-8].

### 1.1.2. Type 2 diabetes (T2D)

T2D accounts for 90–95% of those with diabetes, previously referred to as non-insulin – dependent diabetes, type II diabetes, or adult-onset diabetes, matching individuals who have insulin resistance and usually have relative (rather than absolute) insulin deficiency. Patients do not need insulin treatment to survive usually. Autoimmune destruction of $\beta$-cells does not occur, and patients do not have any of the other causes of diabetes listed in the part of T1D.

Obesity itself causes some degree of insulin resistance that is why the most patients with T2D are obese or may have an increased percentage of body fat distributed predominantly in the abdominal region. Ketoacidosis seldom occurs spontaneously in this T2D; usually it arises only in association with the stress of another illness such as infection. T2D frequently goes undiagnosed for many years because the hyperglycemia develops gradually. Patients are because of this at increased risk of developing macrovascular and microvascular complications.

T2D patients may have insulin levels appearing normal or elevated, the higher blood glucose levels in these diabetic patients would be expected to result in even higher insulin values if their $\beta$-cell function been normal. Insulin secretion is oftendefective in these patients and insufficient to compensate for insulin resistance. Weight reduction and/or pharmacological treatment of hyperglycemia can rapidly improve the insulin sensitivity but the blood glucose level is seldom restored to normal. The risk of developing T2D increases with age, obesity, and lack of physical activity.

Women with prior Gestational Diabetes Mellitus (GDM) are more affected as well as individuals with hypertension or dyslipidemia, and its frequency varies in different racial ethnic subgroups. The association with a strong genetic predisposition is bigger than is for the autoimmune form of T1D. However, the genetics of this form of diabetes are complex and not clearly defined[9-11].

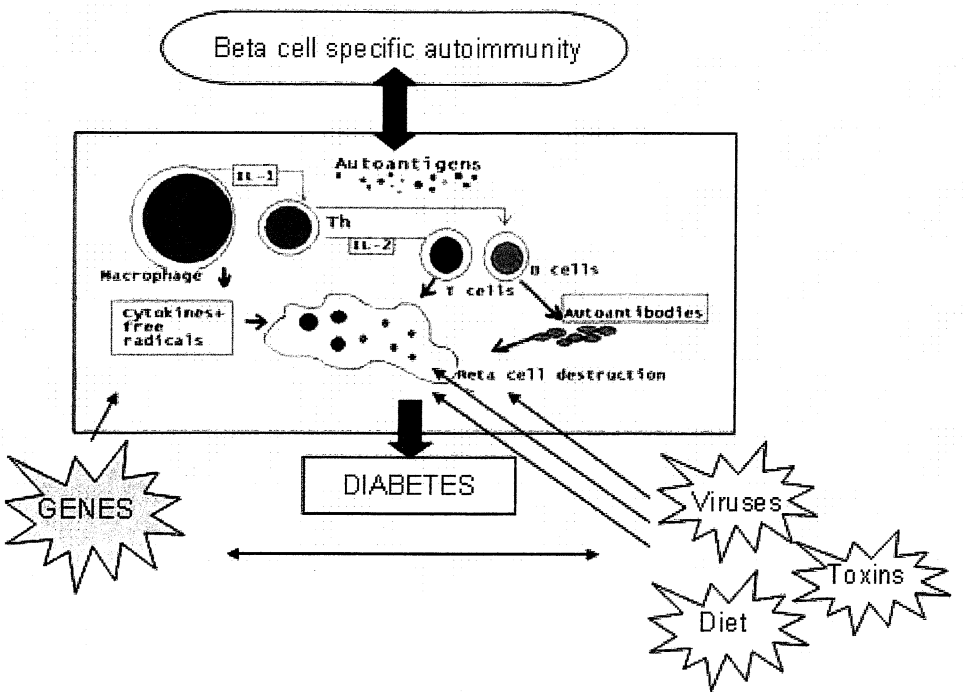


**Fig 3** Causes of Hyperglycemia in Type 2 Diabetes: an imbalance between the insulin production capacity of the islet $\beta$- cell and the requirement for insulin action in insulin target tissues such a liver, adipose tissue and skeletal muscles [12].

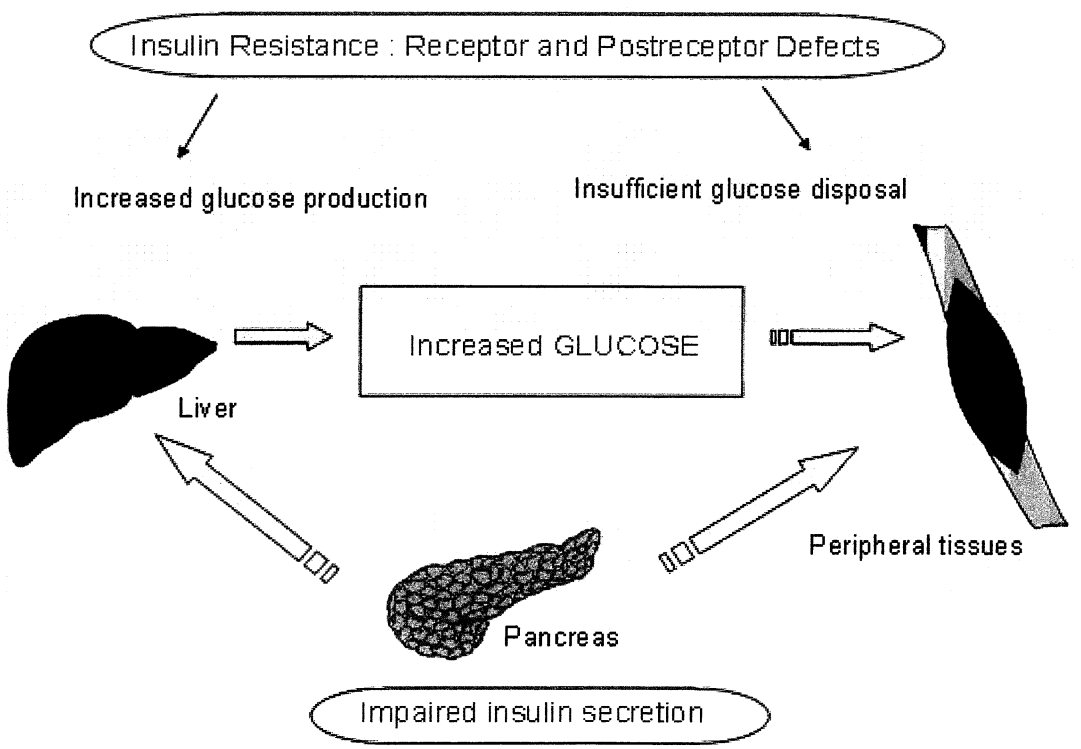


Table 2 — **Etiologic classification of diabetes mellitus**

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| |
|---|
| **I. Type 1 diabetes** (*β*-cell destruction, usually leading to absolute insulin deficiency) |
| A. Immune mediated |
| B. Idiopathic |
| |
| **II. Type 2 diabetes** (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance) |
| |
| **III. Other specific types** |
| ***A. Genetic defects of β-cell function*** |
| 1. Chromosome 12, HNF-1α (MODY3) |
| 2. Chromosome 7, glucokinase (MODY2) |
| 3. Chromosome 20, HNF-4α (MODY1) |
| 4. Chromosome 13, insulin promoter factor-1 (IPF-1; MODY4) |
| 5. Chromosome 17, HNF-1*β*- (MODY5) |
| 6. Chromosome 2, *NeuroD1* (MODY6) |
| 7. Mitochondrial DNA |
| 8. Others |
| ***B. Genetic defects in insulin action*** |
| 1. Type A insulin resistance |
| 2. Leprechaunism |
| 3. Rabson-Mendenhall syndrome |
| 4. Lipoatrophic diabetes |
| 5. Others |
| ***C. Diseases of the exocrine pancreas*** |
| 1. Pancreatitis |
| 2. Trauma/pancreatectomy |
| 3. Neoplasia |
| 4. Cystic fibrosis |
| 5. Hemochromatosis |
| 6. Fibrocalculous pancreatopathy |
| 7. Others |
| ***D. Endocrinopathies*** |
| 1. Acromegaly |
| 2. Cushing's syndrome |
| 3. Glucagonoma |
| 4. Pheochromocytoma |
| 5. Hyperthyroidism |
| 6. Somatostatinoma |
| 7. Aldosteronoma |
| 8. Others |
| ***E. Drug- or chemical-induced*** |
| 1. Vacor |
| 2. Pentamidine |
| 3. Nicotinic acid |
| 4. Glucocorticoids |
| 5. Thyroid hormone |
| 6. Diazoxide |

| |
|---|
| 7. $\beta$-adrenergic agonists |
| 8. Thiazides |
| 9. Dilantin |
| 10. α -Interferon |
| 11. Others |
| ***F. Infections*** |
| 1. Congenital rubella |
| 2. Cytomegalovirus |
| 3. Others |
| ***G. Uncommon forms of immune-mediated diabetes*** |
| 1. "Stiff-man" syndrome |
| 2. Anti–insulin receptor antibodies |
| 3. Others |
| ***H. Other genetic syndromes sometimes associated with diabetes*** |
| 1. Down's syndrome |
| 2. Klinefelter's syndrome |
| 3. Turner's syndrome |
| 4. Wolfram's syndrome |
| 5. Friedreich's ataxia |
| 6. Huntington's chorea |
| 7. Laurence-Moon-Biedl syndrome |
| 8. Myotonic dystrophy |
| 9. Porphyria |
| 10. Prader-Willi syndrome |
| 11. Others |
| |
| **IV. Gestational diabetes mellitus (GDM)** |

* Patients with any form of diabetes may require insulin treatment at some stage of their disease. Such use of insulin does not, of itself, classify the patient.

## 1.1.3. Other specific types of Diabetes Mellitus

### A. Genetic defects of β-cell function

Some forms of diabetes are connected to the impaired function of in *β*-cell function due to monogenetic defect. It is typical for these cases that the onset of hyperglycemia is observed at an early age (generally before age 25 years).

They referee to maturity onset diabetes of the young (MODY) and are characterized by impaired insulin secretion with minimal or no defects in insulin action and an autosomal dominant inheritance pattern. Mutations at six genetic loci on different chromosomes have been identified to date. The most common form is associated with mutations on chromosome 12 referred to as hepatocyte nuclear factor ***(HNF)-1α***. A second form is associated with mutations in the glucokinase gene on chromosome 7p. Glucokinase converts glucose to glucose-6-phosphate, the metabolism of which, in turn, stimulates insulin secretion by the *β*-cell. Thus, glucokinase serves as the "glucose sensor" for the *β*-cell. Because of defects in the glucokinase gene, increased plasma levels of glucose are necessary to elicit normal levels of insulin secretion. The less common forms result from mutations in other transcription factors, including ***HNF-4α, HNF-1β*** , insulin promoter factor ***(IPF)-1***, and ***NeuroD1***.

Point mutations in mitochondrial DNA have been found to be associated with diabetes mellitus and deafness. The most common mutation occurs at position 3243 in the tRNA leucine gene, leading to an A-to-G transition. An identical lesion occurs in the MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like syndrome); however, diabetes is not part of this syndrome, suggesting different phenotypic expressions of this genetic lesion.

Genetic abnormalities that result in the inability to convert proinsulin to insulin have been identified in a few families with an autosomal dominant pattern, too. Similarly, the production of mutant insulin molecules with resultant impaired receptor binding has also been identified with an autosomal inheritance in a few families with a mildly glucose elevation or even normal glucose metabolism [13-15].

## B.Genetic defects in insulin action

Mutations of the insulin receptor may cause abnormalities in glucose matabolism ranging from hyperinsulinemia and modest hyperglycemia to severe diabetes. A group of these patients with these mutations may have acanthosis nigricans. Women may be virilized and have enlarged, cystic ovaries. This syndrome was termed previously as type A insulin resistance.

Leprechaunism and the Rabson-Mendenhall syndrome are two pediatric syndromes that have mutations in the insulin receptor gene with subsequent alterations in insulin receptor function and extreme insulin resistance.

Alterations in the structure and function of the insulin receptor cannot be demonstrated in patients with insulin resistant lipoatrophic diabetes. It is assumed that the lesion(s) must reside in the postreceptor signal transduction pathways[16-18].

## C. Diseases of the exocrine pancreas

Pancreatitis, trauma, infection, pancreatectomy, and pancreatic carcinoma, may cause damage to the pancreas due to the extensivity of the damage diabetes occur. If extensive enough, cystic fibrosis and hemochromatosis will also destroy $\beta$-cells and impair insulin secretion.. Pancreatic fibrosis and calcium stones in the exocrine ducts have been found at autopsy of diabetic patients[19-21].

## D. Endocrinopathies

Hormones (e.g., growth hormone, cortisol, glucagon, and epinephrine) could antagonize insulin action. Excess amounts of these hormones (typical for e.g., acromegaly, Cushing's syndrome, glucagonoma, pheochromocytoma, respectively) can cause diabetes in individuals with preexisting defects in insulin secretion. Hyperglycemia disappears when the hormone excess is resolved. Somatostatinoma- and aldosteronoma -induced hypokalemia can cause diabetes, at least in part, by inhibiting insulin secretion. Hyperglycemia generally resolves after successful removal of the tumor [22].

## E. Drug or chemical induced

The sequence or relative importance of $\beta$-cell dysfunction and insulin resistance in this type of diabetes is unknown. Certain toxins such as Vacor (a rat

poison) and intravenous pentamidine can permanently destroy pancreatic β-cells. There are also many drugs that can impair insulin action. Examples include nicotinic acid and glucocorticoids (α-interferon) The list shown in Table 2 is not all-inclusive, but reflects the more commonly recognized drug-, hormone-, or toxin-induced forms of diabetes [23].

## F. Infections

Diabetes may occur following congenital rubella and other viral infections, although most of these patients have HLA and immune markers characteristic of T1D. In addition, coxsackievirus B, cytomegalovirus, adenovirus, and mumps have been implicated in inducing certain cases of the disease as β-cell destruction [24-26].

## G. Uncommon forms of immune –mediated diabetes

The Stiff-man syndrome is an autoimmune disorder of the central nervous system. Patients have high titers of the GAD autoantibodies, and approximately one-third will develop diabetes.

Anti–insulin receptor antibodies can cause diabetes by binding to the insulin receptor, thereby blocking the binding of insulin to its receptor in target tissues. In some cases, these antibodies can act as an insulin agonist after binding to the receptor and can thereby cause hypoglycemia.

Anti–insulin receptor antibodies are occasionally found in patients with systemic lupus erythematosus and other autoimmune diseases. As in other states of extreme insulin resistance, patients with anti–insulin receptor antibodies often have acanthosis nigricans. In the past, this syndrome was termed type B insulin resistance [27].

## H. Other genetic syndromes sometimes associated with diabetes

Many genetic syndromes are accompanied by an increased incidence of diabetes mellitus. These include the chromosomal abnormalities of Down's syndrome, Klinefelter's syndrome, and Turner's syndrome.

Wolfram's syndrome is an autosomal recessive disorder characterized by insulin-deficient diabetes and the absence of β-cells at autopsy. Additional

manifestations include diabetes insipidus, hypogonadism, optic atrophy, and neural deafness. Other syndromes are listed in Table 2 [28].

## I. Gestational diabetes mellitus

GDM is defined as any degree of glucose intolerance with onset or the first recognition during pregnancy. It does not exclude the possibility that unrecognized glucose intolerance may have antedated or begun concomitantly with the pregnancy. The prevalence may range from 1 to 14% of pregnancies, depending on the population studied. GDM represents nearly 90% of all pregnancies complicated by diabetes, occurs normally during pregnancy, particularly in the 3$^{rd}$ trimester [29,30].

## 1.2. Immunology of Type 1 Diabetes Mellitus

It is believed that T1D develops as genetically encoded susceptibility to T cell mediated autoimmunity.The cellular autoimmunity in T1D responsible for the destruction of pancreatic $\beta$- cells is mediated by T cells. B cells produce autoantibodies to $\beta$-cell antigens, and in turn present these self-antigens to T cells, but $\beta$-cells and autoantibodies are not strictly required in this autoimmune process [31].

In most cases a preclinical period marked by the presence of autoantibodies to pancreatic $\beta$-cell antigens precedes the onset of hyperglycemia by a few years. This long pre-clinical period provides an opportunity for prevention. Known autoantigens include insulin, glutamic acid decarboxylase (GAD), and antibodies against the islet cell antigen 512 phosphatase (IA-2), of which only insulin is cell specific (see Table 3).

Insulin autoantibodies occur more in DR4 haplotype patients and are useful if measured prior to administering exogenous insulin. Glutamic acid decarboxylase antibodies persist the longest time following diagnosis and are useful in confirming autoimmune etiology in long-standing cases. The presence of more than one type of antibody is highly predictive of disease, and occurs years before clinical manifestations [32].

Specific class II and class I histocompatibility alleles determine peptides that are more or less likely to be the target of autoimmunity when tolerance mechanisms fail. Targeting of islet specific peptides by CD4 and CD8 T cells leads to specific islet $\beta$-cell destruction for both human and rodent models. Anti-islet autoantibodies are excellent predictors of T1D, but most likely have only a small but potentially important role in pathogenesis. The specific target organ, the insulin-producing $\beta$—cell of the islet can be manipulated to profoundly alter diabetes propensity, but it is likely that these manipulations (e.g. co stimulatory molecules such as B7.1 expression in the islets, insulin gene knockouts) produce their effects by indirect influence on immune targeting [33]

Table 3 **Autoantigens in type 1 diabetes mellitus**

| **Autoantigens in type 1 diabetes mellitus [34-36]** | |
|---|---|
| **Major autoantigens** | Glutamic acid decaorboxylase (GAD 65) |
| | Protein thyrosine phosphatase (ICA512/IA-2) |
| | Insulin (IAA) |
| **Minor autoantigens** | ICA 12 (SOX13) |
| | ICA 69 |
| | Carboxypeptidase H |
| | Gangliosides (GM-1) |
| | Sulfatides |
| **Shared autoantigens** | Tissue transglutaminase (TTG) |
| | 21 – hydroxylase (21- OH) |
| | Other antigens |

The trigger that leads to the pathogenesis of type 1 diabetes is currently unknown. It is well established that the pathophysiology of the disease is biphasic. In the first stage, leukocytes infiltrate the pancreatic islets in response that does not cause damage. In the second phase, which occurs only in diabetes – prone individuals and strains, autoreactive T cells aquire aggressive potential and destroy the majority of the pancreatic islets [37].

Rodents and humans exhibit a physiological ripple of apoptic $\beta$- cells death shortly after birth, which induces an adaptive autoimmune response towards islet-antigens, both in diabetes/prone non-obese diabetic mice (NOD) and in mice that do not developed diabetes. It was proposed that early T-cell mediated autoimmune response towards islet-antigens is physiological, purposeful and beneficial [38].

Numerous immunostimulatory protocols prevent the development of diabetes, including the treatment with the non-specific immunostimulatory agents, complete Freud's adjuvants or bacillus Calmette –Guerin vaccine or with autoantigen specific vaccine (see Table 4). It is demonstrated that the ability of these immunostimulatory agents to inhibit diabetes development in NOD mice is dependent not on Th1 or Th2 cytokine shift but on the presence of the Th1 cytokine IFN$\gamma$. Minor islets damage in young mice stimulates a protective autoimmune response, which results in prevention diabetes. Similarly, in line with observations that active immunisation with self-antigens that are associated with damage to a specific tissue promotes the recovery of this tissue from injury.

Table 4 **Immunostimulatory treatments that prevent diabetes**

| **Immunostimulatory treatments that prevent diabetes** | | | |
|---|---|---|---|
| **Autoantigen non-specific** | **Ref** | **Autoantigen - specific** | **Ref** |
| Virus infection | [39] | Immunization with STZ-treated islets | [40] |
| Schistosoma mansoni infection | [41] | DC vaccination with insulin B chain | (55) |
| Mycobacterium avium or Mycobacterium bovis infection | [42] | DC vaccination with GAD65 | [43] |
| BCG vaccination | [44] | Immunisation with insulin or GAD65 in incomplete Fruend's adjuvant | 57 |
| CFA vaccination | [45] | Vaccination with insulin or pro-insulin derived peptides | [46] |
| Empty plastid DNA or CpG oligonucleotide vaccination | [47] | Passive transfer of GAD65-reactive T cells | [48] |
| CD3 antibody | [49] | | |
| Vitamin D3 | [50] | | |
| Heat shock protein 60 (P277) peptide vaccination | [51,52] | | |

## 1.3. Genetics of Type 1 Diabetes Mellitus

The main goal for understanding the genetics of T1D is a complete understanding of disease pathophysiology, necessary for the development of more conventional interventions to prevent $\beta$-cell distruction. The T-cell mediated autoimmune process that destroys pancreatic $\beta$-cells in T1D is a complex phenotype influenced by multiple genetic and environmental factors. Human leukocyte antigen (HLA) accounts for about half of the genetic susceptibility, through a large variety of protective and predisposing haplotypes. Other important loci associated with T1D, with much smaller effects than HLA, include the insulin variable number of tandem repeats *(INS-VNTR)*, *PTPN22*, and *CTLA-4* etc.

Type 1 diabetes is becoming one of the best-studied genetically complex autoimmune disorders. Most of the genes involved in T1D susceptibility, however, remain unknown. To date, all known genetic associations have been detected on the basis of the candidate gene approach. The alternative is the positional approach, in which the gene is identified without prior functional knowledge, on the basis of its location in the genome. This location is identified on the basis of coinheritance of the corresponding chromosomal segment with the disease phenotype: pairs of diabetic siblings are genotyped at arbitrary polymorphic markers, equally spaced throughout the genome, to identify regions shared by them at a frequency higher than the expected 50%. This positional approach, so successful with monogenic or Mendelian disorders, has resulted in the naming of eighteen loci *( IDDM1–IDDM18 )[53]*, of which almost all have turned out to be statistical artifacts due to underestimates of the sample size required for meaningful statistical power [54].

The identification of additional loci and functional analysis of known ones, no matter how small each individual effect is, will provide: (1) pathophysiological insights necessary for the development of preventive interventions; (2) risk prediction to identify individuals that can benefit from them, and (3) potentially, identification of distinct subgenotypes, with different immune dysregulation pathways leading to the common disease phenotype that may respond to different preventive interventions.

### 1.3.1. HLA clas II – IDDM1 locus

The first candidate locus studied and found to be strongly associated with T1D was the human leukocyte antigen (HLA) region on chromosome 6p21.3 [55,56]. This cluster of homologous cell-surface proteins is divided into class I (A, B, C) and class II (DP, DQ, DR). These proteins are unique in that they are more than an order of magnitude, more polymorphic than any other protein in the human genome. This diversity is driven by the positive selection of new alleles that confer the advantage of heterozygosity. This increases the ability to optimally bind a broader range of epitopes and remain current with the evolution of pathogens. The single-chain class I molecules are ubiquitously expressed and present intracellular antigen to CD8+ cells. Class II molecules are composed of α and β- chains and are responsible for presenting extracellular antigen to CD4+ cells, via specialized antigen-presenting cells. Genetically, the class II region has been found to contribute strongly to T1D susceptibility, attributable mostly to the DR and DQ genes. Linkage diaequilibrium refers to the strong correlation between alleles at neighboring single nucleotide polymorphisms (SNPs) that are inherited as a block [57]. The association is, therefore, mapped to whole clusters of adjacent alleles (i.e., haplotypes) which encompass more than one gene, rather than to individual alleles.

Alleles are designated with a number that follows an asterisk. Most T1D-relevant polymorphisms are amino acid changes in exon 2 of the α-chain of DR and both α and β- chains of DQ. Thus, the most common T1D-predisposing haplotypes in Caucasians are *DRB1*0301-DQA1*0501-DQB1*0201* and *DRB1*0401-DQA1*0301-DQB1*0302*. These are abbreviated as *DR3-DQ2* and *DR4-DQ8*. Interestingly, heterozygosity for *DQ2/DQ8* (which, because of linkage disequilibrium almost always implies *DR3/DR4* heterozygosity) confers the highest T1D risk in Caucasians.

The risk is higher than homozygosity for either haplotype, indicating qualitative rather than merely quantitative interactions between alleles. This genotype is found in 3% of the general population, but in 30% of T1D patients, conferring a 15-fold relative risk and an earlier onset of disease. Most of the remaining Caucasians with T1D have at least one of these two haplotypes.

**Table 5** Some known gene associated with diabetes (published in 2 studies*) [7]
* with the exception of MODY 4 and MODY 6

| Type | Gene | Gene name | Function | SNP, allele or locus marker | Refs. |
|---|---|---|---|---|---|
| T1D | ***HLA*** | Human leukocyte antigen | Immune system regulation | Variants in multiple genes | 16,27 |
| T1D | ***INS*** | Insulin | Involved in numerous aspects of metabolism | VNTR | 16,28 |
| T1D | ***CTLA4*** | Cytotoxic T-Lymphocyte-associated protein 4 | Immune system regulation | T17A | 29,30 |
| T1D | ***PTPN22*** | Protein thyrosine phosphate non-receptor type 22 | Immune system regulation | SNP C1858T | 31,32 |
| T2D | ***ABCC8*** | ATP-binding casette subfamily CV, sylfonylurea receptor | Regulator of potassium channels | SNPs in various exons | 33,34 |
| T2D | ***CAPN10*** | Calpain 10 | Protease | Various intronic SNP haplotypes | 35,36 |
| T2D | ***GCGR*** | Glucagon receptor | Controls hepatic glucose production and insulin secretion | G40S | 37,38 |
| T2D | ***GCK*** | Glucokinase | Glucose metabolism | Microsatellite in 3′end of gene | 39,40 |
| T2D | ***KCNJ11*** | Potassium inwardly-rectifying channel, subfamily J, member 11 | Regulation of insulin secretion | E23K | 41,42 |
| T2D | ***PPARG*** | Peroxisome proliferator –activated receptor γ | Transciption factor | Pro12Ala | 43,44 |
| T2D | ***HNF4A*** | Hepatocyte nuclear factor 4 α | Transciption factor | P2 promoter SNPs | 45,46 |
| T2D | ***SLC2A1*** | Glut 1 | Glucose transporter | XbaI (-) restriction site | 47,48 |
| MODY 1 | ***HNF4A*** | Hepatocyte nuclear factor 4 α | Transciption factor | Mutation in 13 families | 18,19 |
| MODY 2 | ***GCK*** | Glucokinase | Glucose metabolism | 130 different mutation descridbed | 20,21 |
| MODY 3 | ***TCF1*** | Hepatocyte nuclear factor 1 α | Transciption factor | 120 different mutations described – in all racial ethnic bacgrounds | 20,22 |
| MODY 4 | ***IPF1*** | Insulin promoter factor 1 | Transciption factor | Rare mutations – 1 family described | 23 |
| MODY 5 | ***TCF2*** | Hepatocyte nuclear factor 1β | Transciption factor | Rare mutations | 24,25 |
| MODY 6 | ***NEUROD1*** | Neurogenic differentiation 1 | Transciption factor | Mutations described in 2 families with autosomal dominant form | 26 |

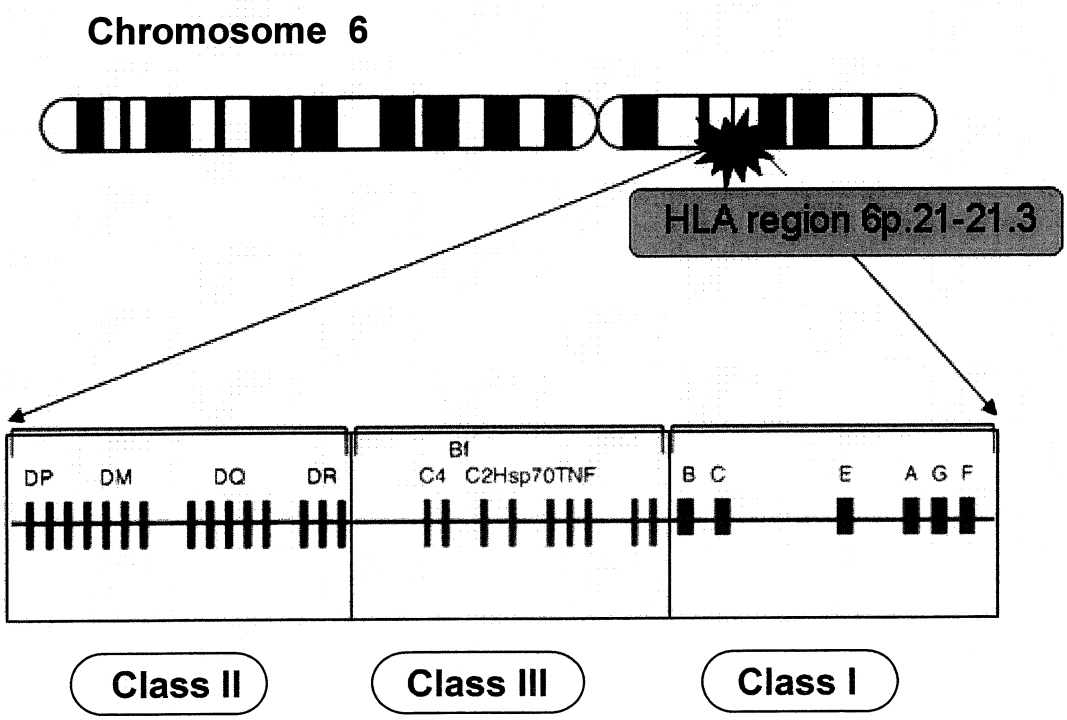



**Fig. 4. Gene map of the human leukocyte antigen (HLA) region.** The HLA region spans 4 x $10^6$ nucleotides on chromosome 6p21.1 to p21.3, with class II, class III and class I genes located from the centromeric (Cen) to the telomeric (Tel) end. HLA class I molecules restrict $CD8^+$ cytotoxic T lymphocyte function and mediate immune responses against 'endogenous' antigens and virally infected targets, whereas HLA class II molecules are involved in the presentation of 'exogenous' antigens to T helper cells. The HLA class III region contains many genes encoding proteins that are unrelated to cell-mediated immunity but that nevertheless modulate or regulate immune responses in some way, including tumour necrosis factor (TNF), heat shock proteins (Hsps) and complement proteins (C2, C4)

Conversely, the *HLA-DQ6* haplotype, *DRB1*1501-DQA1*0102-DQB1*0602*, has a protective association with T1D. It is found in <1% of diabetic children and 20% of the general population [93] . If it is in combination with a predisposing haplotype, the individual remains at low risk [94].

At a molecular level, risk alleles differ structurally from protective alleles. Most characteristic is the absence of an aspartic acid molecule at position 57 of the $\beta$-chain of the DQ molecule. This reverses the electric charge of the peptide-binding groove of the HLA-DQ8 molecule, thereby possibly altering the binding of insulin epitopes [95].

The role of HLA in T1D was further studied in animal models. The equivalent to HLA in mice is the mice major histocompatibility complex - the H-2 system. Work on autoimmune-prone nonobese diabetic (NOD) mice expressing a diabetogenic human HLA class II gene in the presence of a mouse diabetes-resistant

MHC class II genotype failed to develop diabetes [96-99]. In fact, in NOD mice with a transgene expressing higher levels of its own diabetogenic MHC class II molecules, there was a decrease in diabetes frequency [100]. This indicates that MHC alleles predispose to diabetes through loss of desirable function, rather than gain of undesirable function. It is therefore speculated that weak binding of some crucial T1D-related autoepitope(s) by predisposing class II alleles fails to generate sufficient tolerance, either thymic or peripheral. This could result in the targeting of the autoepitope in the periphery, under conditions of aberrant immune function, as created by other genetic loci or environmental inputs.

At present it is possible to identify at birth individuals at risk by HLA typing, with a risk of approximately one in fifteen for *DQ2/DQ8* heterozygotes in the general population, and, a risk of one in two for *DQ2/DQ8* heterozygotes among first degree relatives [101,102]. Identifying populations with high risk might be of benefit to studies addressing the implication of environmental influences, and environmental or genetic-based preventive therapies. In addition, individuals with T1D, depending upon HLA alleles, are also at high risk for celiac disease (one in three of *DQ2* homozygous type 1 patients have anti-transglutaminase autoantibodies) [103], Addison's disease [104,105], or other autoimmune conditions. Thus, understanding the genetics of type 1 diabetes will certainly contribute to our knowledge of autoimmunity in general. [106]

Approximately 50% of the familial aggregation of the juvenile type 1 diabetes is determined by the major histocompatibility locus, and in particular class II or immune response alleles. There is hierarchy of risk associated with specific class II haplotypes and genotypes (for HLA nomenclature see Immunology of T1D book Teaching Slides at www.barbaradaviscenter.org). The relative risk provided by such genotypes is remarkably similar across populations [107]. While 2.4% of the general population of Colorado is DQ2/DQ8 heterozygotes, 50% of young children developing the disease (age <5) are heterozygotes as well as 30–40% of older children. In addition to DQ alleles, DR4 subtypes influence risk (detailed on Table 6). The crystal structure of DQ8 with an insulin peptide B: 9–23 has been published and it is thought that these class II alleles primarily influence diabetes risk by the peptides they bind and present to T lymphocytes [108]. Of note *DQ8* introduced to a mouse model increases diabetes risk. A subset of HLA alleles provides dominant protection against diabetes, the most common of which (*DQA1*0102, DQB1*0602*)

is associated with *DR2* (*DRB1*1501*). Another dramatically protective allele is *DRB1*1401*, associated with *DQA1*0101, DQB1*0503*. Though the protection is dramatic, with less than 1% of children with T1D having *DQB1*0602* compared to 20% of the general population, it is not absolute, particularly in individuals who develop diabetes at older ages among whom 5% have *DQB1*0602*. It appears that both DQ and DR alleles can provide dominant protection, as evidenced by relatively rare haplotypes where alternative DR and DQ alleles are present in families of patients with T1D.

In animal models dominant protection may be determined by deletion of specific autoreactive T lymphocytes by multiple non-diabetogenic class II alleles. Other genes within the major histocompatibility complex may also influence age of onset of diabetes. Class I HLA alleles, such as *A24* have been reported to be associated with a younger age of onset of T1D. In a small series, the *HLA-A2* allele was present in 75% of children developing anti-islet autoimmunity before age 3 [101]. In addition there is an extended HLA haplotype, which is conserved over a very long distance, with *HLA DQ2, DR3, MIC-A5.1, B8, and A1,* which is associated with T1D, myasthenia gravis, Addison's disease, and other autoimmune disorders. The extensive linkage disequilibrium for this haplotype makes it difficult to pinpoint specific nucleotides that contribute to diabetes susceptibility.

**Table 6** Class II HLA haplotypes associated with susceptibility for or protection against type 1 diabetes

| **DRB1** | | **DQA1** | **DQB1** | | **Diabetes association** |
|---|---|---|---|---|---|
| ***0301** | DR3 | ***0501** | ***0201** | DQ2 | **Strongly positive** |
| ***0401** | DR4 | ***0301** | ***0302** | DQ8 | **Strongly positive** |
| ***0405** | DR4 | ***0301** | ***0302** | DQ8 | **Strongly positive** |
| ***0801** | DR8 | ***0401** | ***0402** | DQ4 | **Positive** |
| ***1601** | DR2 | ***0102** | ***0502** | DQ5 | **Positive** |
| ***0101** | DR1 | ***0101** | ***0501** | DQ5 | **Positive** |
| ***0403** | DR4 | ***0301** | ***0302** | DQ8 | **Neutral or weakly negative** |
| ***0701** | DR7 | ***0201** | ***0201** | DQ2 | **negative** |
| ***1501** | DR2 | ***0102** | ***0602** | DQ6 | **Strongly negative** |
| ***1401** | DR6 | ***0101** | ***0503** | DQ5 | **Strongly negative** |
| ***0701** | DR7 | ***0201** | ***0303** | DQ9 | **Strongly negative** |

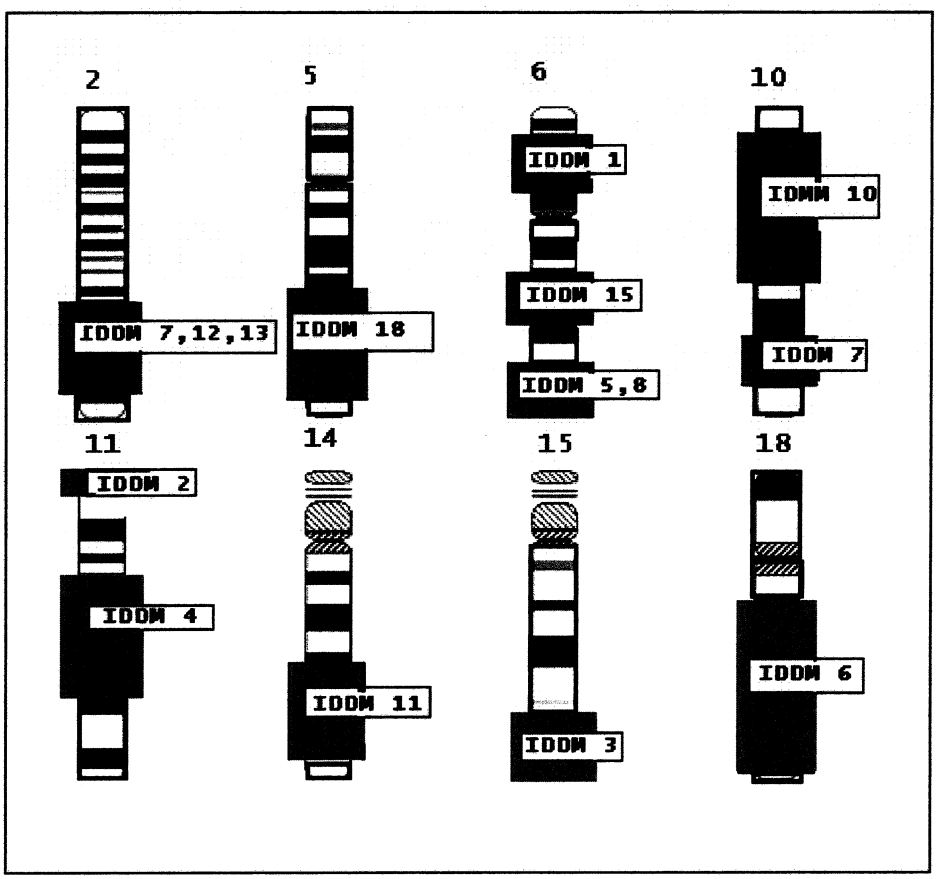
### 1.3.2. NON- HLA class II – other IDDM loci

The HLA haplotypes of highest risk are found commonly in the general population, but only a fraction of these carriers develop T1D. This low penetrance indicates that HLA cannot account for all of the genetic susceptibility to T1D. One quarter of all siblings of T1D patients share no HLA haplotype with their affected sibling, yet these individuals have a T1D risk 7-fold higher than the general population. On this basis it was calculated that HLA accounts for about 40–50% of the genetic risk [109]. Linkage studies have clearly shown that there is no other T1D locus of amplitude of effect approaching this. Discovering the numerous other loci responsible for the rest of the genetic susceptibility, no matter how small the individual effects may be, is important in understanding the disease (see Table 7 and Fig 4). We describe shortly only IDDM2 and IDDM12.

**Table 7** List of IDDM loci

| Locus | Chromosome | Gene |
|---|---|---|
| **IDDM 1** | 6p21 | HLA |
| **IDDM 2** | 11p21 | INS |
| **IDDM 3** | 15q | |
| **IDDM 4** | 11q13 | ZFM1 (Zinc finger protein 162) |
| **IDDM 5** | 6q25 | SOD2 (superoxid dismutase) |
| **IDDM 6** | 18q | DCC associated gene<br>ZNF236<br>bcl-2 |
| **IDDM 7** | 2q31 | NEUROD1<br>IGPR (islet specific glucose 6 – phosphatase)<br>IL-1 gene cluster, HOXD8, GAD1, GALNT3 |
| **IDDM 8** | 6q27 | |
| **IDDM 9** | 3q21-q25 | |
| **IDDM 10** | 10p11.2-q11.2 | GAD2 |
| **IDDM 11** | 14q24.3-q31 | ENSA (alpha-endosulphine) |
| **IDDM 12** | 2q33 | *CTLA-4*<br>CD28<br>ICOS |
| **IDDM 13** | 2q34 | |
| **IDDM 14** | | |
| **IDDM 15** | 6q21 | Transient neonatal diabetes |
| **IDDM 16** | | Ig heavy chain |
| **IDDM 17** | 10q25 | |
| **IDDM 18** | 5q31.1-33.1 | ILB12 |

**Fig. 5** IDDM loci associated with diabetes mellitus - chromosome localisation




## IDDM 2 locus

The second confirmed locus contributing to T1D risk is the insulin gene locus *(INS)*. In human, there is a *VNTR* (Variable Nucleotide Tandem Repeat) of the insulin gene .The longer *VNTR* is associated with "minor" protection from T1D and with increased insulin mRNA in the thymus [110].

In the NOD mouse model linkage studies have not implicated either the insulin 1 or 2 (mice have two insulin genes, humans one insulin gene) loci in diabetes risk, but recent studies of insulin gene knockouts suggest that loss of either gene can dramatically alter the development of diabetes [111,112].

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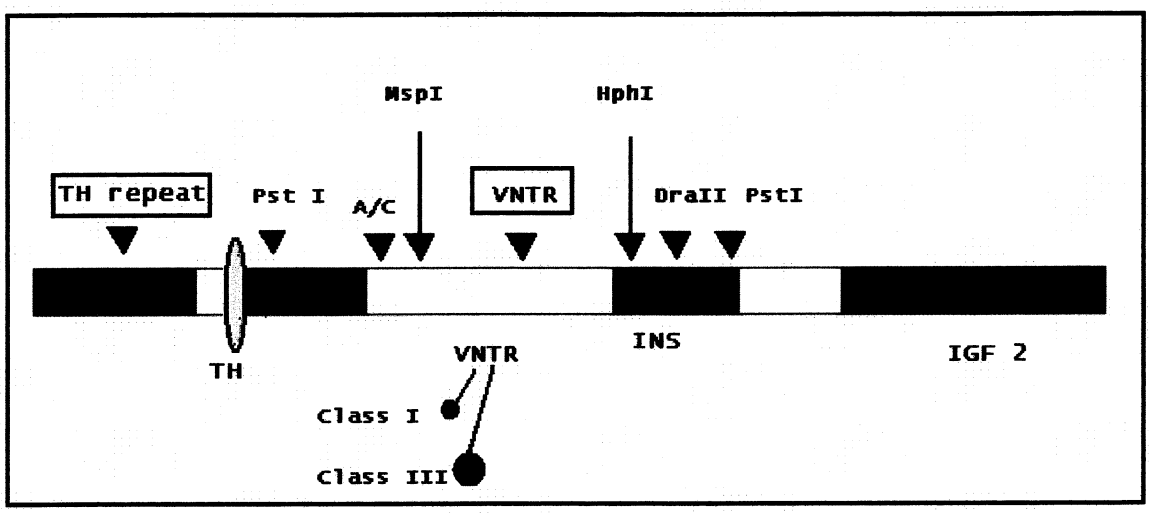


**Fig 6.** Insulin *VNTR* minisatellite repeats. The *INS -VNTR* is located 365 bp upstream from the insulin gene *(INS)* promoter. It is also 5 kb upstream from a second potential target for regulation, insulin-like growth factor II *(IGF2)* and 10 kb downstream of the tyrosine hydroxylase gene *(TH )*. Class I and class III alleles differ by the number of tandem repeats of the *VNTR* consensus sequence. Class I alleles predispose to and class III alleles protect from type 1 diabetes. Adapted from [113] .

## IDDM12

Cytotoxic T-lymphocyte associated protein 4 (*CTLA-4*) and CD28 on chromosome 2q33 are likely candidate genes for T1D and other autoimmune diseases because of their important role in the T-cell proliferative response. The *CTLA-4* gene encodes a receptor expressed by activated T-cells. *CTLA-4* binds B7 molecules, and limits the proliferative response of activated T-cells, some of which could be autoreactive. Mutations or polymorphism leading to altered activity of *CTLA-4* are believed to play an important role in risk of developing autoimmunity. Nistico et al. [114] reported linkage and association evidence suggesting a susceptibility locus (designated as IDDM12) in the *CTLA4/CD28* region. In T1D, the effect of IDDM12 seems to be independent of HLA alleles or the VNTR risk genotype [115]. Polymorphism A/G in the first exon of *CTLA-4* results in amino acid change (Thr/Ala). The presence of alanine at codon 17 of *CTLA-4* has been associated with susceptibility to T1D. Strong effect of IDDM12 on T1D risk has been observed in three Mediterranean –European populations (Italian, Spanish, French), in the Mexican-American population and the population of Korea and a weak transmission deviation

It is likely that polymorphisms of the *CTLA-4* gene have a measurable influence, but it has been very difficult to confirm loci or to pinpoint specific genes [116]. This is a*PPAR*ent even when large families with multiple affected (e.g. IDDM17 on chromosome 10) individuals give strong evidence of linkage (e.g. IDDM17 on chromosome 10). Whether T1D is oligogenic with susceptibility alleles at different loci segregating in different families or polygenic, multiple approaches and large family resources are likely to be required to identify specific susceptibility genes. [117]

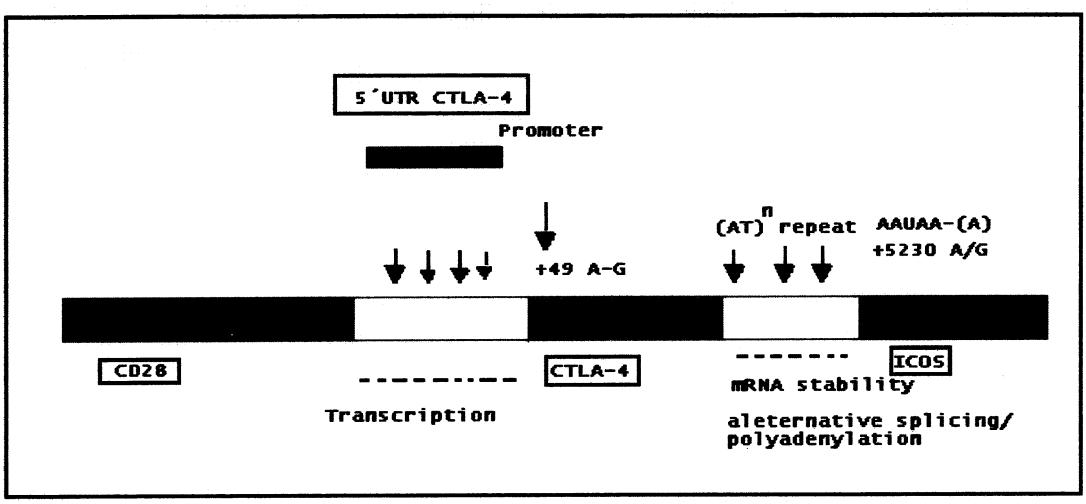


**Fig 7.** *CTLA-4* on chromosome 2q33. *CTLA-4* and its flanking 5´UTR and 3´ UTR regions lay within type 1 diabetes (T1D)-associated LD block. *CD28* and *ICOS* , two other candidate genes important in immune function, lie outsidethe haplotype block. The *A6230G* single nucleotide polymorphism (SNP) in the 3´UTR flanking region of *CTLA-4* exhibits a strong association with Graves' disease and T1D, through an unknown mechanism. Effects from the 5´UTR end of the region also cannot be ruled out, with either a promoter polymorphism or an amino acid substitution in the signal peptide. Various SNPs and their potential mechanism of action are noted. Adapted from [118] .

Although the NOD mouse has been extensively studied as a diabetes model, none of the genes responsible, with the exception of the MHC, have been yet identified. This may be due to extensive heterogeneity of gene assortment required for the disease [119]. Of note, subphenotypes such as expression of insulin antibodies and insulitis can be separated genetically from progression to diabetes [120]. Recently embryonic stem cells carrying NOD genome have been developed [121]. This will facilitate the analysis of targeted gene mutations without the need of backcrossing. Thus the animal models provide compelling evidence of MHC restriction plus non-MHC genes that influence immune function. This combination,

together with the knowledge that bone marrow transplants transfer susceptibility and resistance depending on the donor, is a common theme towards the comprehension of the disease [122].

### 1.3.3. Non IDDM loci

Multiple other loci have been implicated in risk for T1D, especially with the analysis of hundreds of sibling pairs with disease [123]

#### *1.3.3.1. MIC-A*

**Major histocompatibility complex (MHC) class I chain –related gene A *(MIC-A)*** is also among the many other loci that have been associated with susceptibility to T1D. The *MIC-A* is located within the MHC class I region of the chromosome 6. It contains the long open reading frame encoding three distinct extracellular domains (α1, α2 and α3) a transmembrane segment, and a cytoplasmatic tail. It has been reported that *MIC-A* may be recognized by a subpopulation of intestinal γδ T cells [124] and may play a role in the activation of a subpopulation of NK cells that express the *NKG2D* receptor [125].

Sequence analysis of the exon 5 encoding the transmembrane segment has showed a trinucleotide repeat (GCT) microsatellite polymorphism within this region [126,127]. So far, six alleles of the exon 5 of the *MIC-A* gene, which differ in number of GCT repetitions at position 296, have been identified. These alleles contain 4, 5, 6, 9, 10 repetitions of GCT with an additional nucleotide insertion GGCT. The *MIC-A* alleles have been accordingly named *A4, A5, A6, A9, A10* and *A5.1* respectively [128,129]. Due to the proximity of the *MIC-A* locus to HLA-B and HLA-C, *MIC-A* polymorphisms have been investigated for associations with a variety of HLA-associated diseases. Some of these studies have shown a positive association of *MIC-A* with the diseases. It was thoughtthese observations are probably due to the linkage disequilibrium between *MIC-A* and HLA-B. However, most disease associations have been aimed to the *MIC-A* transmembrane region and the six identified variants. Several recent studies of the *MIC–A* transmembrane polymorphism have shown an association of the *A5* allele with the susceptibility to young onset T1D [130,131] but this allele has not been found to be a risk for adult-onset T1D [132]

### *1.3.3.2. NFKB1* and *NFKBIA*

Cytokine-induced β-cell death is an important factor in the pathogenesis of T1D. Since the nuclear factor kappa-β (NFκB) plays an important role in cytokine-induced gene activation, it is a possible candidate gene for T1D predisposition. NF-kappa is a transcription factor responsible for modulating the expression of many genes involved in cell proliferation, differentiation, apoptosis and metastasis. NFκB interacts with kappa inhibitory proteins (IκB) to regulate gene expression [133].

The transcription factor nuclear factor-κB (NF-κB) (8,9) has an important role in cytokine induced β-cell apoptosis, since apoptosis is prevented in human and rat primary β-cells, and in insulin-producing cell lines, by inhibition of NF-κB activation [134,135]. NF-κB blocking by an IκBα (S/A)2 super repressor also prevents rat β-cell apoptosis induced by IFN-γ + dsRNA (a byproduct of viral infection) [136]. Furthermore, conditional and specific NF-κB blockade *in vivo* protects pancreatic β-cells against toxic/immuno-mediated diabetes following multiple low doses of streptozotocin [137]. Paradoxically, NF-κB-regulated genes have been shown to inhibit apoptosis in diverse cell types [138-142] The development of diabetes has been also correlated with elevated levels of proinflammatory cytokines and enhanced antigen presentation function in DC of NOD mice [143]. That is why it is noteworthy to look into the polymorphisms of genes connected to NFκB signaling pathway.

NF-κB is formed by homo or heterodimers of five NF-κB family members [144]. These dimers are usually present in an inactive form in the cytoplasm, where they remain bound to a group of related inhibitory κB (IκB) proteins [145,146]. Stimulation by pro-inflammatory cytokines, such as IL-1β- and TNF-α, results in NF-κB activation through the classical pathway [147], leading to degradation of the IκB proteins, and consequent translocation of NF-κB to the nucleus [148,149]. The different isoforms of IκB play distinct and complementary roles in the regulation of specific NF-κB dimers (15,16,22,26), resulting in 4 both quantitative and qualitative changes in gene expression [150,151]. These characteristics are specific for different cell types, and might vary in a particular cell type exposed to different stimuli [152,153]. Since no information is available regarding these parameters in β- cells, it

remains to be determined whether NF-κB activation in these cells has specific activation characteristics that make it to exert a pro-apoptotic effect, while it exerts an antiapoptotic effect in most other cell types.

The investigation of common variants within the genes coding for NFκB and IκB, *NFKB1* [4q24] and *NFKBIA* [14q13], for involvement in autoimmune diabetes and T2D complications was performed in previous studies. A polymorphic (CA) dinucleotide repeats microsatellite has been identified near the *NFKB1* gene. *NFKBIA* genotypes (A/G in 3'UTR) have been determined as wild-type 424 bp; variant 306 and 118 bp; and heterozygote 424, 306 and 118 bp after restriction digestion [154].

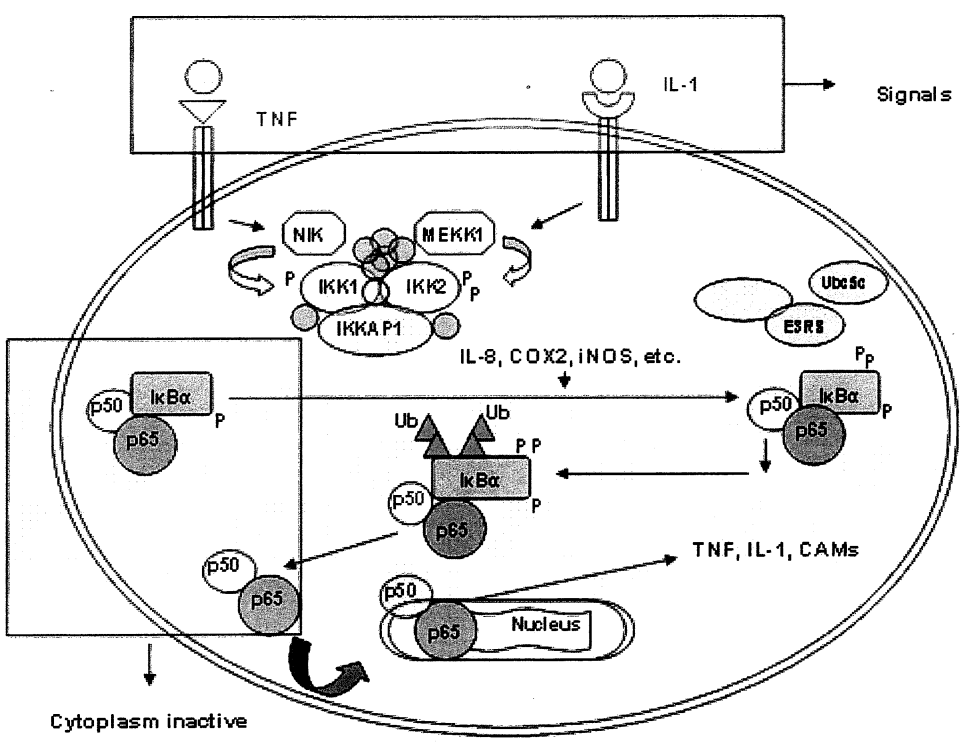


**Fig. 7**: Molecular mechanisms of NFkB signaling – regulation of gene expression via p50-p65 complex

### 1.3.3.3. Interleukin-18

The interleukin -18 *(IL-18)* belongs to the group of Th1 cytokines and is a member of IL-1 superfamily, located on chromosome 11q22.2.-q22.3. Beside other

biological activities it is initiating and promoting host defense and inflammation. This pleiotropic cytokine is produced predominantly by activated monocytes/macrophages involved in the regulation of innate and acquired immune response and plays a key role in autoimmune inflammatory and infection diseases [155,156]. *IL-18* interacts with IL-12 inducting together the interferon – γ (IFN- γ), modulating aktivity of natural killer cells, increasing tumor necrosis factor – α (TNF-α ) and IL-1 production by macorphages, up regulating the expression of adhesion molecules, and inducing nitric oxide production in the area of chronic inflammation.

## 1.4. Epidemiology of Type 1 Diabetes Mellitus

Type 1 diabetes risk varies dramatically between populations with incidence rates in Asia approaching 1/100,000 versus more than 40/100,000 in Finland [157].There has been a general trend of increasing incidence with an approximate doubling over the past two decades in Scandinavia. This provides the best evidence that environmental factors influence the development of diabetes at a population level, either decreasing or increasing diabetes risk.

Studies of monozygotic and dizygotic twins provide risk estimates for monozygotic twins ranging from 20 to 50%. Despite the wide range in the risk obtained from different studies, the estimates of heritability of diabetes susceptibility, defined as the proportion of the variation in susceptibility attributed to genetic factors and obtained by comparing risks in monozygous twins and dizygous twins, were very similar (0.73 and 0.72) in two large population-based studies [158,159]. With long-term follow up the risk is at least 50% based on analyses of combined cohorts of initially discordant monozygotic twins followed up to 50 years from the onset of diabetes in the first twin. Increasing risk for diabetes with longer follow-up has also been reported for non-twin siblings and offspring of affected patients [160].

More important it is likely that groups of monozygotic twins differ in concordance depending on genetic etiology of their T1D. It has been observed that if the first twin of a monozygotic twin pair develops diabetes prior to age 5, the risk for the second twin to develop diabetes within 20 years of follow up is 50%, while if the first twin develops diabetes after age 20, the risk for the co-twin is less than 5% [161]. HLA class II alleles probably influence risk as does insulin gene

polymorphisms. In contrast to monozygotic twins, the risk for developing diabetes or anti-islet autoantibodies does not appear to differ between dizygotic twins and siblings of patients with T1D. Early reports of increased prevalence of anti-islet autoantibodies in dizygotic twins have been retracted [162], and a combined series indicates a relatively low prevalence of autoantibody-positivity in dizygotic twins. For monozygotic twins, expression of any of the "biochemical" anti-islet autoantibodies (GAD65, ICA512 (IA-2) or insulin autoantibodies) is associated with a very high risk of progression to T1D [163]. Based on decades of follow up, the development of diabetes in autoantibody-positive monozygotic twins is preceded by progressive loss of insulin secretion [164].

## 1.5. Environmental risk factors of Type 1 Diabetes Mellitus

Viruses and nutritional factors (mostly cow's milk protein) have been incriminated [165-167], but definitive proof in the form of multiple confirmations is lacking for any of them. Once these factors are identified, their interaction with the other components of genetic susceptibility will lead to a full understanding of disease etiology.

Currently, the only proven, but rare, infectious cause of T1D is congenital rubella infection. While universal rubella infection has perhaps eliminated a few cases of T1D, there are currently no trials attempting to prevent T1D through modification of other candidate infectious factors.

Both autoimmunity and T1D may be associated with enteroviral (EV) infections. People with T1D or autoimmunity may have a stronger humoral response to infection due to their particular genotypes or they can be in a nonspecific hyper-immune state marked by elevation of antibody levels to a variety of antigens. Rotavirus and herpes viruses have been also associated with $\beta$ cell autoimmunity and T1D [168]. It is plausible that early infection with a non-diabetogenic strain of a virus can induce immunity to antigenetically similar diabetogenic strains and protects from T1D. These hypotheses are based on extensive studies in animal models, but have been extremely difficult to test in humans.

Non–specific infections in a susceptible host can induce transient autoimmunity that may or may not lead to cumulative $\beta$ cell damage over time [169].

Increased incidence of diabetes has been reported in susceptible animal delivered by c-section or kept in a sterile environment postnatal. On the other hand exposure of NOD mice or BB rats to specific rodent viruses reduces the risk of diabetes. These data may suggest that improvement in personal hygiene leading to reduction in exposure to microbial agents in early childhood may promote T1D in genetically susceptible children similar to childhood asthma [170,171].

Recent studies have not proven any association between childhood immunization and $\beta$-cell autoimmunity or clinical diabetes, but proposed protective effect of breast feeding on the incidence of T1D has attracted enormous interest. Cohort studies failed to find an association between infant diet exposures and $\beta$ cell autoimmunity.

# 2. Aims of the study and Methodology

- To characterize the genetic background of patients with different types of diabetes mellitus (T1D in children, T1D in adults, LADA, T2D, MODY)

- To test the gene expression of diabetes associated *HLA-DRB1*04* and *NFKB1* genes in Antigen Presenting Cells of T1D patients.
  Patients were divided into the tested subgroups in relation to the *HLA class II*, *NFKB1,* and *NFKBIA* genotypes and disease type (T1DM in children, T1DM in adults, and LADA)

Methodology to tested polymorphisms in following genes:

- *HLA-DRB1* - (human leukocyte antigen – gene DRB1) : PCR-SSP
- *HLA-DQB1* - (human leukocyte antigen – gene DQB1): PCR-SSP
- *NFKB1* - nuclear factor kappa –B, subunit 1: PCR – Fragment analysis
- IkB *(NFKBIA)* – nuclear factor of kappa light chain gene enhancer in B – cells inhibitor, alpha – PCR-RFLP
- *MIC-A* – major histocompatibility complex class one chain – related protein A: PCR – Fragment analysis
- *IL-18* – interleukin 18: PCR-RFLP
- *PPARγ* – peroxisome proliferator activated receptor – gamma: PCR-RFLP
- *GCK*– glucokinase : PCR-sequencing
- *mRNA expression testing was performed by qRT-PCR relative quantification (applying ΔΔCt method)*

# 3. Commentary to the original papers

## List of original papers

**Paper 1** : Kolostova K, Pinterova D, Novota P, Romzova M, Cejkova P, Pruhova S, Lebl J, Treslova L, Andel M, Cerna M: HLA, *NFKB1* and *NFKBIA* Gene Polymorphism Profile in Autoimmune Diabetes Mellitus Patients. Exp Clin Endocrinol Diabetes 115:124-129, 2007

**Paper 2:** Cerna M, **Kolostova K**, Novota P, Romzova M, Cejkova P, Pinterova D Pruhova S, Treslova L, Andel **M** Autoimmune Diabetes Mellitus with Adult Onset and Type 1 Diabetes Mellitus in Children Have Different Genetic Predispositions. Ann. N. Y.Acad. Sci 1-11 (2007)

**Paper 3:** Cerna M, Novota P, **Kolostova K**, Cejkova P, Zdarsky E, Novakova D, Kucera P, Novak J, Andel M: HLA in Czech adult patients with autoimmune diabetes mellitus: comparison with Czech children with type 1 diabetes and patients with type 2 diabetes. Eur J Immunogenet 30:401-407, 2003.

**Paper 4:** Novota P, Cerna M, **Kolostova K,** Cejkova P, Zdarsky E, Novakova D, Kucera P, Novak J, Andel M: Diabetes mellitus in adults: association of HLA DRB1 and DQB1 diabetes risk alleles with GADab presence and C-peptide secretion. Immunol Lett 95:229-232, 2004

**Paper 5:** Novota P, **Kolostova K**, Pinterova D, Novak J, Treslova L, Andel M, Cerna M: Interleukin *IL-18* gene promoter polymorphisms in adult patients with type 1 diabetes mellitus and latent autoimmune diabetes in adults. Immunol Lett 96:247-251, 2005

**Paper 6:** Novota P, **Kolostova K,** Pinterova D, Novak J, Weber P, Treslova L, Kovar J, Andel M, Cerna M: Association of MHC class I chain related gene-A microsatellite polymorphism with the susceptibility to T1DM and LADA in Czech adult patients. Int J Immunogenet 32:273-275, 2005

**Paper 7:** Romzova M, Hohenadel D, **Kolostova K,** Pinterova D, Fojtikova M, Ruzickova S, Dostal C, Bosak V, Rychlik I, Cerna M: NFkappaB and its inhibitor IkappaB in relation to type 2 diabetes and its microvascular and atherosclerotic complications. Hum Immunol 67:706-713, 2006

**Paper 8:** Pinterova D, Cerna M, **Kolostova K,** Novota P, Cimburova M, Romzova M, Kubena A, Andel M: The frequency of alleles of the Pro12Ala polymorphism in *PPARgamma2* is different between healthy controls and patients with type 2 diabetes. Folia Biol (Praha) 50:153-156, 2004.

**Paper 9:** Pinterova D, Ek J, **Kolostova K,** Pruhova S, Novota P, Romzova M, Feigerlova E, Cerna M, Lebl J, Pedersen O, Hansen T: Six novel mutations in the *GCK* gene in MODY patients. Clin Genet 71:95-96, 2007

**Paper 10:** Feigerlova E, Pruhova S, Dittertova L, Lebl J, Pinterova D, **Kolostova K,** Cerna M, Pedersen O, Hansen T: Aetiological heterogeneity of asymptomatic hyperglycaemia in children and adolescents. Eur J Pediatr 165:446-452, 2006

**Paper 11: Kolostova K**, Cerna M, Andel M: Association of HLA molecules with autoimmune diseases] Cas Lek Cesk. 2002 Dec 6;141(24):755-62. Review. Slovak

The published papers listed above give an overview of the diabetes gene associations in Czech population and introduce to the problems of expression studies for the T1D associated polymorphisms.

T1D is a polygenous disease and one of the predisposing factors is the genotype background, ascribed mainly to HLA genes, which was studied in our **Papers No. 1-4**, and **No. 10** and **11** (review in Slovak language). According to our published studies we can conclude that HLA-associations have shown strong age-dependence. Patients with autoimmune diabetes manifested after 35 years of age can be divided into two different groups having different HLA class II associations: type 1 diabtes mellitus (T1D) in adults with DQB1*0302 and DRB1*04 risk alleles and latent autoimmune diabetes in adults (LADA) with DRB1*03 risk allele (*HLA-DRB1*03* was associated with the group of LADA patients two times stronger than with the group of children diabetics).

*HLA-DRB1*04* gene frequency was significantly increased in T1D groups compared to controls and the power of association was comparable between the children and adult T1D groups. The occurance of HLA – DQB1 predisposition alleles in T1D adults has been less evident than in children (the relative risk values for *HLA-DQB1*0302* were almost three times higher in children with diabetes than in T1D adults, but whre its allele frequency was still more frequent than in patients with T2D and healthy controls). These data probably reflex increasing significance of environmental factors and decreasing significance of genetic factors in relation to age of disease onset.

We can confirm the difference in HLA allele frequencies between GADab positive/negative groups and CP negative/positive groups in autoimmune diabetes patients after 35 years of age. The *DRB1*04* and *DQB1*0302* alleles are associated

with decrease of CP levels while *DRB1*03* is a significant marker of autoantibody (GADab) development.

**Papers No. 1** and **No. 7** report the investigation of common variants within the genes coding for NFκB and IκB, *NFKB1* [4q24] and *NFKBIA* [14q13] respectively, for involvement in autoimmune diabetes and T2D complications. No T1DM association with any allele of the *NFKB1* microsatellite polymorphism could however be demonstrated in Danish T1D families as reported previously [172,173]. The differences in allele frequencies of *NFKB1* in Czech patients, compared with healthy controls, were observed only in the group of patients with adult onset of T1D. The frequency of A7 allele (size 132 bp, 20 repeats) was significantly increased in comparison with the control groups.

There is also evidence that the heterozygous genotype of *NFKBIA* is protective for diabetes onset in adulthood, according to the results in LADA patients (nonsignificantly) and T1D adults (significantly). In the mentioned groups the frequency of the heterozygotes is lowered and the frequency of the homozygotes is increased. A significant difference was observed for AA genotype in LADA group.

The results of the *HLA-DRB1*04* mRNA and *NFKB1* gene expression testing are also discussed in the **Paper No. 1,** partly in the **Paper No. 2.** The mRNA transcript levels of *HLA-DRB1*04* in circulating APC differ in diabetic patients with different onset. The mRNA of *HLA-DRB1*04* is significantly elevated in APC of diabetic patients with diabetes onset in adult comparing to the results in patients with onset in childhood. There was no difference in expression of *NFKB1* gene in APC in the above mentioned groups. We hypothesized that the increased HLA-DR expression is an age dependent factor probably due to accumulation of environmental stress. The newly published results have shown [174] that there could be an other

regulation factor as miRNA e.g. causing that the expression of the HLA-predisposing alleles is increased on the dendritic cells and B-cell lymphocytes and the expression of the HLA–protecting alleles is decreased. This disbalance could cause an autoimmune reaction in pancreatic $\beta$-cells. The prevention of T1D could be in that case based on an enhanced expression of HLA protective alleles on APCs.

**Paper No. 5** is discussing the impact of *IL-18* gene polymorphism on the T1D. The It has been reported by Nicoletti et al. that *IL-18* serum levels are increased during the subclinical stage of T1D [175]. The importance of *IL-18* is underscored by the observation that the murine *IL-18* maps to an interval on chromosome 9 close to the diabetes susceptibility gene IDDM2 of the NOD mouse [176]. Several polymorphims in the promoter region of human *IL-18* have been identified. [177]. Recently, Kretowski and co-workers have reported the role of *IL-18* gene polymorphisms at positions -607 and -137 in the predisposition to T1D in Polish population [178]. The conclusion of our genotype testing says that the *IL-18* gene polymorphisms at positions -607 and -137 are associated with genetic susceptibility to none of both types (T1D and LADA) of adult–onset diabetes mellitus in Czech population.

In **Paper No. 6** the *MIC-A* microsatellite polymorphism within the transmembrane region was tested. The *MIC*-A5.1 allele was found to be associated with genetic susceptibility to T1D in adults whereas no association has been proofed with LADA in Czech population.

**Paper No. 8** describes genetic analyses in the groups of patients with diabetes mellitus and its complications. We have found out, that the freqeuncy of Pro12Ala polymorphism in *PPARγ2* differes between controls and patients with T2D. The frequency of the Ala variant in the *PPARγ2* gene is higher in the control

group than in the group of T2D patients. This can be explained like a decreased risk of T2D for the Ala 12 cariers. Numerous clinical characteristics have been tested in connection to the Pro12Ala polymorphism, but no significant association to BMI and lipid levels was demonstrated in our patient samples so far.

Monogenetic studies have been done on glukokinase *(GCK)* gene in the group of MODY susceptible patients (see **Paper No. 9-10**). Within the directly sequence testing, six novel mutations in the *GCK* gene has been found in MODY patients and in 50% of children and adoslescents with asymptomatic hyperglycaemia.

# 4. Original papers

Paper 1: **Kolostova K**, Pinterova D, Novota P, Romzova M, Cejkova P, Pruhova S, Lebl J, Treslova L, Andel M, Cerna M: HLA, **NFKB1 and NFKBIA Gene Polymorphism Profile in Autoimmune Diabetes Mellitus Patients.** Exp Clin Endocrinol Diabetes 115:124-129, 2007

124 Article

# HLA, NFKB1 and NFKBIA Gene Polymorphism Profile in Autoimmune Diabetes Mellitus Patients

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Key words
- T1DM
- HLA
- NFKB1
- NFKBIA
- LADA

## Abstract

Type 1 diabetes mellitus (T1DM) is one of the long-time studied autoimmune disorders. The triggering of the autoimmune process has been ascribed to various genes active in the regulation of the cytokine gene transcription including the Rel/NF-κB gene family. In our study the gene polymorphism of HLA class II, NFKB1 (nuclear factor of kappa light polypeptide gene enhancer in B-cells 1) and NFKBIA (inhibitor of nuclear factor kappa B) was tested. Patients were divided into the subgroups in relation to the disease type: T1DM in children, T1DM in adults, and Latent Autoimmune Diabetes in Adults (LADA). HLA-DRB1*04 and HLA-DQB1*0302 have been detected as risk factors for T1DM in adults and particularly in children (P<0.0001, OR=22.9 and 46.5 respectively). HLA-DRB1*03 has been found as a single risk factor for LADA (P<0.0001, OR=4.9). We detected 15 alleles for the *NFKB1* gene polymorphism (CA-repeats) in the Czech population. The alleles were ranging in size from 114-142 bp corresponding to 10–25 CA repeats. Frequency of the A7 allele of NFKB1 gene has been significantly increased in T1DM adults (P<0.01). There was no difference in A and a G allele frequency of NFKBIA gene between the control group and patients, but the association of the AA genotype of NFKBIA gene has been found for LADA (P<0.05). Summarizing our results we concluded that there is a high probability of association of gene polymorphism from Rel/NF-κB family with an autoimmune diabetes course. Due to the results obtained in the epidemiological study we have been looking also for the function significance of the genetic predisposition. No significant changes have been observed by real time PCR testing of HLA-DRB1*04 gene and NFKB1 gene expression between T1DM diabetic group with different HLA, NFKB1, NFKBIA genetic background.

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## Abbreviations

| | |
|---|---|
| LADA | Latent autoimmune diabetes in adults |
| NFκB | Nuclear factor kappa B |
| NFKB1 | Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 gene |
| NFKBIA | Inhibitor of nuclear factor kappa B gene |
| T1DM | Type 1 diabetes mellitus |

## Introduction

Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disease characterized by a loss of tolerance towards own antigenic structures of beta-pancreatic cells. The destruction of beta cells subsequently leads to the loss of insulin production. T1DM is not a homogeneous disease, since several of its clinical features are different in children up to 6 years of age as compared to older patients (Csorba & Lyon, 2005). There are more factors, which trigger the autoimmune response in susceptible individuals; however, they are only partially known so far.

One of the predisposing factors is the genotype background, ascribed mainly to genes of the *HLA*. Out of extensive genetic and epidemiological studies, the Caucasoid population is known to have a significant association of insulin-depend-

**Table 1** Characteristics of the tested groups

| Features | Group 1 T1DM children | Group 2 T1DM adults | Group 3 LADA | Group 4 Control |
|---|---|---|---|---|
| 80 | n=179 | n=75 | n=31 | n=153 |
| Female | 60% | 52% | 44% | * |
| Age at disease onset (years) | 7.7 (1–16) | 22.1 (20–45) | 47.0 (25–64) | * |
| Duration of DM (years) | 4.9 (1–16) | 17.0 (1–50) | 15.0 (3–32) | * |
| Insulin therapy | yes | yes | yes | no |
| Fasting C peptide (pmol/L) | * | * | 478.0 (4.4–1522) | * |
| Anti-GAD (ng/ml) | * | * | 392.0 (5–2800) | * |

*data not available.

ent diabetes mellitus with the increased frequencies of haplotypes *HLA-DRB1*04-DQA1*0301-DQB1*0302* and *DRB1*0301-DQA1*0501-DQB1*0201*. The strength of associations is ethnically variable. The *HLA* genotype *DRB1*03-DQB1*0201/DRB1*04-DQB1*0302* confers a 25-fold increase in the risk of T1DM. (Jaini et al., 2002; Rewers et al., 2003; Shawkatova et al., 2000).

It is well known that T1DM results from the breakdown of peripheral tolerance. (Wheat et al., 2004) that is ended with cytokine-induced beta-cell death. Since the nuclear factor kappa-B (NFκB) plays an important role in cytokine-induced gene activation, it is a very attractive candidate for T1DM predisposition. NFκB is a transcription factor which interacts with kappa inhibitory proteins (IκB) to regulate gene expression (Curran et al., 2002) of a variety of processes including innate and adaptive immune responses, cell growth, apoptosis, tissue differentiation and inflammation. (Baldwin, 2001). The NFκB transcription factor complex has two alternative DNA binding subunits, NFKB1 and NFKB2. NFKB1 encodes two isoforms, the cytoplasmatic non-DNA-binding p105, and the 50 kDa DNA-binding p50 (Heron et al., 1995). To exert its effect, p50 binds to p65 (encoded by NFKB2) to form biologically active heterodimers, which activate transcription in promoter sequences of inflammatory genes (e.g. IL-12, TNF-α, IFN-γ), but alternatively p50 able to form homodimers that block gene transcription by binding to NFκB response sites in nuclues. Recent studies have investigated role of NFκB in the pathogenesis of various human diseases including immune deficiency, carcinogenesis and atherogenesis. Lately the possible link between NFκB and the development of insulin resistance, type 2 diabetes (Arkan et al., 2005; Cai et al., 2005; Chen, 2005; Evans et al., 2002) and in diabetic polyneuropathies (Haslbeck et al., 2005) has also been suggested.

This study investigated common variants within the genes coding for NFκB and IκBα, *NFKB1* [4q24] and *NFKBIA* [14q13] to test the probable genetic predisposition to autoimmune diabetes. The NFκB complex is inhibited by IκB proteins (NFKBIA or NFKBIB), which inactivate NFκB by trapping it in the cytoplasm. Phosphorylation of serine residues on the IκB proteins by kinases (IKBKA or IKBKB) marks them for destruction via the ubiquitination pathway, thereby allowing activation of the NFκB complex. NFKBIA is encoding for IkBα, which binds preferentially to p65. After degradation of IkBα, p65 tranlocates to the nucleus where it can form heterodimers with p50, released from p105 and following NFκB heterodimer complex binds to decameric DNA sequences and activates transcription of NFκB regulated target genes (Barnes & Karin, 1997; Bierhaus et al., 2001; Hayden & Ghosh, 2004).

A polymorphic dinucleotide CA microsatellite repeat, with 18 described alleles, has been identified close to the coding region of the human NFKB1 gene. *NFKBIA* genotypes (A/G in 3'UTR) have been determined in previous studies as wild-type 424 bp; variant 306 and 118 bp; and heterozygote 424, 306 and 118 bp after restriction digestion (Curran et al., 2002).

Following the results obtained in our epidemiological studies we have also focused on the probable functional significance of the tested gene polymorphisms. It has been demonstrated that variations of the density of *HLA class II* molecules on APCs influence the intensity and the nature of T cell response (Lechler et al., 1985). Due to the fact that NFkB participates even if indirectly on the regulation of HLA transcription we have finally decided to compare the mRNA level of the HLA-DRB1*04 allele and NFKB1 gene on APCs of peripheral blood leucocytes in diabetic patients in connection to the genotype background tested previously.

## Methods

### Subjects

The 267 individuals with a previous diabetes mellitus diagnosis, 159 ethnically matched controls for *NFKBIA* analysis and 58 controls for *NFKB1* genotyping were involved into the study. Criteria of the current WHO definitions for diagnosing diabetes were applied (World Health Organization Definition, 1999), considering patients' clinical features and laboratory data, including the presence of anti-islet autoantibodies (autoantibodies to glutamic acid decarboxylase - GAD65) and serum C-peptide (CP) level. All of the patients had insulin therapy. LADA was defined by a minimum 6 months long phase after diagnosis without insulin therapy. All subjects were informed and consented to participate before the study. The following parameters were recorded for each patient at the time of study: sex, age, disease duration, complications and family history (see **Table 1**). Both affected and control populations were recruited from individuals residing in the same geographical location in the Czech region and were from Caucasian descent.

### Genotyping

DNA was extracted from peripheral blood leukocytes using of DNA blood mini isolation method (Qiagen, Hilden, Gemany).

Genotyping. of the *NFKB1* dinucleotide repeat with use of fluorescently labeled primers was previously described by Ota et al. (Ota et al., 1999). Polymerase chain reaction (PCR) amplification was performed in a reaction mixture containing 25–50 ng genomic DNA, 0.5 mM each primer, 0.2 mM dNTPs, 1xPCR buffer, 2.5 mM $MgCl_2$ and DNA polymerase. PCR conditions were 4 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 52°C and 30 s at 72°C; followed by 5 min at 72°C. *NFKB1* alleles were then detected by

Table 2 The relative risk values and more additive statistics for T1DM associated HLA – alleles in the tested groups

| Groups | LADA | T1DM children | T1DM adults | Controls |
|---|---|---|---|---|
| Alelle frequencies | n=27% | n=188% | n=62% | n=99% |
| **HLA-DRB1*03** | **29.6** | **25.0** | **21.0** | **10.1** |
| P | 0.0001 | 0.0001 | n.s | |
| Relative risk | 3.25 (1.69–6.23) | 1.51 (1.25–1.81) | – | |
| Odds ratio | 4.94 (1.83–13.49) | 3.30 (1.78–6.17) | – | |
| **HLA-DRB1*04** | **18.5** | **43.8** | **34.6** | **6.5** |
| P | n.s. | 0.0001 | 0.0001 | |
| Relative risk | – | 3.12 (2.35–4.15) | 3.42 (2.32–5.04) | |
| Odds ratio | – | 22.87 (10.89–48.87) | 10.47 (4.53–24.65) | |
| **HLA-DQB1*0201** | **25.0** | **21.7** | **21.8** | **26.3** |
| P | n.s. | n.s. | n.s. | |
| Relative risk | – | – | – | |
| Odds ratio | – | – | – | |
| **HLA-DQB1*0302** | **14.3** | **30.8** | **29.0** | **5.1** |
| P | n.s. | 0.0001 | 0.0001 | |
| Relative risk | – | 8.98 (4.76–16.93) | 3.02 (2.12–4.30) | – |
| Odds ratio | – | 46.48 (16.15–140.23) | 9.49 (3.91–23.59) | – |

fragment analysis on the ALF express II detection system (Amersham Pharmacia Biotech, Uppsala, Sweden).

*NFKBIA* amplification was performed in a 20 $\mu$l final volume containing genomic DNA, 1xPCR buffer, 3.75 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 mM each primer and DNA polymerase. PCR conditions were initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 30 s, with a final extension of 2 min at 72°C. Following amplification, 10 $\mu$l of product was digested with HaeIII at 37°C overnight. Samples were then loaded into an ethidium bromide stained 2% agarose gel for genotype determination. Genotypes of 268 patients and 159 matched controls were determined as type (AA), 424 bp; variant (GG), 306 and 118 bp; and heterozygote (AG), 424, 306 and 118 bp (Curran et al., 2002).

*HLA class II* typing was performed according to the standard protocol of Genovision (SSP-PCR) (Genovision, Oslo, Norway). PCR conditions were initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 30 s, with a final extension of 10 min at 72°C.

### Gene expression testing

According to the genotyping study the independent sets of children and adults T1DM patients and sex- and age-matched healthy controls were chosen for the expression studies. 28 T1DM patients included in this study were adults, and 55 T1DM patients were children. The adult control set of patients was created in cooperation with blood transfusion stationary. The age of adults was 36,4±11,5 (mean±SD). The children - age matched control set was obtained from a phenylketonuria study. The age of children control set was 11, 4±8,2. (mean±SD). The control sets were matched by *HLA-DRB1*04* appearance.

### Real time RT-PCR analysis

Peripheral blood with EDTA was collected by venipuncture, and APCs were immediately separated by immunomagnetic separation by Dynabeads (Dynal HLA-class II, 210.04, Dynal, Oslo, Norway). Total RNA was extracted using the RNA blood mini kit (Qiagen, Hilden, Germany). RNA was reverse transcribed by Taqman® real time PCR reagents. The quantitative real time RT-PCR was performed in duplicates using the Taqman® PCR Kit in 96-well microtiter plates on the ABI PRISM 7000 Sequence Detector Systems, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The cDNA was amplified using specific primers for *HLA-DRB1*04* designed by Primer express® as well as of Taqman®MGB (**HLA-DRB1*04 gene forward primer** 5'ACACCCGACCACGTTTCTTG 3', **HLA-DRB1*04 gene forward primer** 5'TCCGTCCCGTTGAAGAAATG 3', **HLA-DRB1*04** Taqman®MGB probe 6-FAM-CACTCATGTTTAACCTGCT). Testing of *NFKB1* expression was done with assay on demand set of primers and probes. As an internal control the human beta actin was used (Applied Biosystems, Foster City, CA, USA). The $2^{-\Delta\Delta^{Ct}}$ [2-delta delta $C_t$] method was applied for relative quantification (Livak & Schmittgen, 2001).

### Statistical analysis

Allele and genotype frequencies, phenotype frequencies, and the frequency of an allele in the chromosomal pool of population were determined by direct counting for all groups of patients and controls. The genotype frequencies were tested for confirmation to Hardy-Weinberg equilibrium. For statistical establishment of significant differences genotype and allele distributions were compared between two populations using $\chi^2$ analysis, followed by Bonferroni correction for multiply comparisons. The strength of the observed associations was estimated by calculating odds ratios [relative risk (RR)] using the method by Woolf. The results from real time PCR were compared between the groups by one-way ANOVA testing and also by nonparametric Kruskall-Wallis statistics. The observed groups were also compared by Dunn's multiple comparison tests. P-value <0.05 was considered significant.

## Results

### The HLA association study

The *HLA* association study was performed to compare the risk values between the tested groups. *HLA*-associations have shown strong age-dependence (see **Table 2**). The relative risk values for *HLA-DQB1*0302* were almost three times higher in the group of children with diabetes than the relative risk values in T1DM of adults. On the other side *HLA-DRB1*03* was associated with the group of LADA patients two times stronger than with the group of children diabetics. *HLA-DRB1*04* was significantly increased in both T1DM groups compared to controls and the power of

Table 3 Frequencies of detected NFKB1 allele polymorphism in the tested groups

| NFKB1 | Lenght | Control Czech | Control Australia (Curran et al., 2002) | LADA | T1DM children | T1DM adults |
|---|---|---|---|---|---|---|
| gene | (bp) | n=58% | n=109% | n=34% | n=55% | n=67% |
| A01 | 114 | x | 2.9 | 1.5 | x | x |
| A02 | 116 | x | 2.9 | 1.5 | x | x |
| A03 | 118 | x | x | 2.9 | x | x |
| A1 | 120 | x | x | x | 0.9 | x |
| A2 | 122 | x | x | x | x | 1.5 |
| A3 | 124 | 23.2 | 23.5 | 20.6 | 19.1 | 23.1 |
| A4 | 126 | 10.3 | x | 2.9 | 10.0 | 6.0 |
| A5 | 128 | 4.3 | x | 2.9 | 1.9 | 2.2 |
| A6 | 130 | 7.8 | 14.7 | 14.7 | 4.5 | 11.2 |
| A7 | 132 | 1.7 | 2.9 | 2.9 | 0.9 | 13.4* |
| A8 | 134 | 8.6 | 14.7 | 14.7 | 10.9 | 12.7 |
| A9 | 136 | 35.3 | 2.9 | 27.9 | 38.4 | 24.6 |
| A10 | 138 | 2.6 | 2.9 | 4.4 | 4.5 | 2.5 |
| A11 | 140 | 2.6 | 5.9 | 4.4 | 8.1 | 0.7 |
| A12 | 142 | 1.7 | x | x | x | x |

*significant P value (P<0.05)

association was comparable between these two groups. The results of the study just confirm the previous results from the HLA typing studies, but were needed in the relation to the further NFKB1 and NFKBIA polymorphism testing.

### Distribution of NFKB1 polymorphism in Czech population

15 alleles for the *NFKB1* gene polymorphism (CA-repeats) in the Czech population were detected so far. The alleles were ranging in size from 114-142 bp corresponding to 10–25 CA repeats. The complete results can be seen in **Table 3**. The most frequent allele size in the patients and the control group was 136 bp (35.3%) corresponding to 22 CA repeats. The differences in allele frequencies, compared to healthy controls, were observed only in the group of T1DM adult patients. The frequency of A7 allele (size 132 bp, 20 repeats) was significantly increased in comparison with the control groups with a relative risk value of 1.88 (OR=10.69, P<0.01, CI=95%).

### Distribution of 3´ UTR NFKBIA polymorphism in Czech population

The genotype frequencies of the *NFKBIA* gene polymorphism in the control and patient groups are presented in **Table 4**. There was no difference in A and G allele frequency between the control group and patients, the difference was observed in distribution of genotypes between the patient groups. There is an evidence that the heterozygous genotype of *NFKBIA* is protective for diabetes onset in adulthood, according to the results in LADA group (nonsignificantly) and T1DM adults (RR=0.56, OR=0.44, P<0.01, CI=95%). In the mentioned groups the frequency of the heterozygotes is lowered and the frequency of the homozygous genotypes rises. A significant difference was observed for AA genotype in LADA group, with a relative risk value of 2.23 (OR=2.68, P<0.001, CI=95%) The AA and GG genotype frequencies in T1DM adults were increased, but with the border significance.

### Expression studies of HLA-DRB1*04 and NFKB1

The results obtained in mRNA quantification studies can be seen in **Table 5** for *HLA-DRB1*04* and in **Table 6** for *NFKB1* gene.

*HLA-DRB1*04*. gene expression was tested first among different groups of patients. The significant difference was found only in mRNA expression levels of *HLA-DRB1*04* between the group of T1DM children and the group of T1DM adults (P=0.034). The expression of *HLA-DRB1*04* was significantly higher in T1DM adult patients. The comparison was done with two specific control groups (positive for *HLA-DRB1*04*). Since there was no difference between the control groups with different age, we compared the control groups as one control set. No difference in the expression of *HLA-DRB1*04* has been observed after analysis among different HLA clas II, NFKBIA and NFKB1 genotypes.
No difference in the expression of NFKB1 gene has been observed among different types of diabetic patient groups and even after analysis based on the found HLA clas II, NFKBIA and NFKB1 genotypes.

## Discussion

Development of type 1 diabetes requires coordinated expression of genes responsible for initiation and progression of the disease, what is regulated by small number of transcription factors including the Rel/NF-kB family. In our study we have compared different groups of patients with autoimmune diabetes mellitus: 1) T1DM children 2) T1DM adults 3) LADA.
Based on the *HLA* - typing studies, we have concluded that *HLA – DR3* is significantly associated only with LADA (RR=3.25) and children diabetes (RR=1.51). *HLA – DQB1*0302* has three times stronger relative risk for diabetes in childhood (RR=8.98) than for diabetes in adults (RR=3.02). The results are in concordance with previous testing in Czech population (Cerna et al., 2003; Cinek et al., 2001; Novota et al., 2004). Surprisingly, there was only a border significance of *HLA-DQB1*0201* gene association in our T1DM children group. This is a difference from the results published in Czech population study by Cinek (Cinek et al., 2001). Generally, the found difference could be explained by different number of patients involved into the study what could change significance level.
Based on *NFKB1* and *NFKBIA* polymorphism studies, we have found out that A7 (132 bp) allele of *NFKB1* gene presents a risk

Table 4 Frequencies of NFKBIA genotypes in the tested groups

| NFKBIA genotype | Control Czech | LADA | T1DM children | T1DM adults |
|---|---|---|---|---|
| | n=159% | n=31% | n=173% | n=64% |
| GG (00) | 27.0 | 22.6 | 31.8 | 35.9 |
| AG (10) | 55.6 | 45.2 | 55.5 | 35.9* |
| AA (11) | 16.9 | 32.3* | 12.7 | 28.0 |

*significant P value (P<0.05)

Table 5 Statistical analysis of the data from relative mRNA quantification of the HLA-DRB1*04 gene

| Group | 1 | 2 | 3 |
|---|---|---|---|
| DRB1*04 | T1DM children | T1DM adults | Controls |
| *Number of values* | 55 | 28 | 10 |
| *Minimum* | 0,390 | 0,230 | 0,600 |
| *Maximum* | 2,510 | 4,390 | 1,410 |
| **Mean** | **1,132** | **1,845*** | **1,050** |
| *Std. Error* | 0,070 | 0,221 | 0,105 |

*significant P value (P=0.034)

Table 6 Statistical analysis of the data from relative mRNA quantification of the NFKB1 gene

| Group | 1 | 2 | 3 |
|---|---|---|---|
| NFKB1 | T1DM children | T1DM adults | Controls |
| *Number of values* | 24 | 20 | 20 |
| *Minimum* | 0,458 | 0,150 | 0,400 |
| *Maximum* | 2,460 | 3,370 | 3,000 |
| **Mean** | **1,120** | **0,991** | **1,335** |
| *Std. Error* | 0,127 | 0,274 | 0,155 |

for our group of T1DM adults (RR=1.88). In previous studies no association with *NFKB1* A7 allele was affirmed. Several reports on the association study about NFKB1 and NFKBIA with T1DM, T2DM, celiac disease, rheumatoid arthritis, systemic lupus erythematosus, breast cancer in a variety of ethnic groups (United Kingdom, Denmark, Spain, Australia) exist (Curran et al., 2002; Gylvin et al., 2002; Karban et al., 2004; Rueda et al., 2004; Smyth et al., 2006). No T1DM association with any allele of the *NFKB1* microsatellite marker could however be demonstrated in Danish T1DM families as reported previously (Gylvin et al., 2002; Karban et al., 2004). In contrast, the frequency of the A10 (138 bp) allele was significantly increased in patients with T1DM (0.17) compared with the normal controls (0.02) (Hegazy et al., 2001). However, the discrepancies in the frequencies of NFKB1 alleles in control sets of populations with different genetic origin show that it is not possible to compare the frequency of the risk alleles among various ethnics. The exact mechanism underlying the NFKB1 related disease susceptibility remains unknown. (Borm et al., 2005).

It is known that 3´UTR is a regulatory region essential for the appropriate gene expression of many genes. Any variation in 3´UTR of NFKBIA gene could alter the function of IκB. A long-lasting sustained activation of NFkB in the absence of decreased IkB in mononuclear cells from patients with type 1 diabetes has been reported (Bierhaus et al., 2001). The significant association in *NFKBIA* polymorphism testing was found only for the AA homozygous genotype in 3´UTR in the LADA group. Similarly the heterozygous *NFKBIA* conformation seems to have significant advantage for T1DM adults; otherwise it would be such frequent.

So far there is no evidence in literature about functional significance of our tested NFKB1 and NFKBIA polymorphisms. This was a reason why we decided to test *NFKB1* and *HLA-DRB1*04*-gene expression on the mRNA level. There was not, however, any difference in the *HLA-DRB1*04 and NFKB1* gene transcription between groups with different *NFKBIA* and *NFKB1* genotypes. The mRNA transcript levels of *HLA-DRB1*04* in circulating peripheral blood mononuclear cells differ significantly only in diabetic patients with different diabetes onset. We hypothesize that the increased *HLA-DRB1*04* mRNA expression could be a protecting factor in a group of T1DM adults.

Summarizing our results we conclude that there is a high probability of association Rel/NF-κB family genes with an autoimmune diabetes course, but the function of the genetic variations needs to be examined further.

## Acknowledgments

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# Autoimmune Diabetes Mellitus with Adult Onset and Type 1 Diabetes Mellitus in Children Have Different Genetic Predispositions

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ABSTRACT: **Type 1 diabetes with manifestation after 35 years of age is defined by CP <200 pmol/L and insulin therapy within 6 months after diagnosis, in comparison to latent autoimmune diabetes mellitus in adults (LADA) manifesting after 35 years of age, that is defined by minimum 6 months after diagnosis without insulin therapy, CP >200 pmol/L and antiGAD > 50 ng/mL. We aimed to find a possible genetic discrimination among different types of autoimmune diabetes. To accomplish this goal, we analyzed DNA samples from 31 LADA patients, 75 patients with adult onset of type 1 diabetes mellitus, 188 type 1 diabetic children, and 153 healthy adult individuals. We studied five genetic loci on chromosomes 6, 11, 4, and 14: HLA DRB1 and DQB1 alleles, major histocompatibility complex (MHC) class I-related gene-A (MIC-A) microsatellite polymorphism, interleukin (IL)-18 single nucleotide polymorphism, the microsatellite polymorphism of nuclear factor kappa B gene (NF-κB1) and the single nucleotide polymorphism of a gene for its inhibitor (NF-κBIA). HLA-DR3 was detected as the predisposition allele for LADA (OR = 4.94, $P < 0.0001$). Further we found a statistically significant increase of NF-κBIA AA genotype (OR = 2.68, $P < 0.01$).** Q2

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**On the other hand, DRB1˙04, which is linked with DQB1˙0302, was observed as a risk factor in patients withtype 1 diabetes mellitus (T1DM) onset after 35 years of age (OR = 10.47, $P < 0.0001$ and OR = 9.49, $P < 0.0001$, respectively). There was also an association with MIC-A5.1 (OR = 2.14, $P < 0.01$). Statistically significant difference was found inthe distribution of IL-18 promoter –607 (C/A) polymorphism between LADA and T1DM in adults ($P < 0.01$). We summarize that all subgroups of autoimmune diabetes have partly different immunogenetic predisposition.**

KEYWORDS: **T1DM; LADA; IDDM; HLA; autoantibodies**

## INTRODUCTION

Autoimmune diabetes mellitus, also called type 1 diabetes mellitus (T1DM), is usually caused by autoimmune destruction of islet β-cells in the pancreas.[1] The destruction of islet β-cells causes insulin deficiency and thereby a dysregulation in anabolism and catabolism. T1DM has features characteristic of most of the autoimmune diseases: (1) It is associated with specific human leukocyte antigens (HLA) class II haplotypes (particularly HLA DRB1*04–DQB1*0302, but also HLA DRB1*03–DQB1*0201). (2) Autoantibodies against autoantigens of β-cells circulating in the body are found (GAD65, IA-2, ICA, IAA). (3) Mononuclear infiltration of the pancreatic islet can be detected histologically.

There are indications that β-cell damage may be induced at any age and that the timing of the clinical presentation is highly variable, with the youngest patients diagnosed in infancy and the oldest at a later age.[2] Epidemiological data suggest that 30–50% of cases may develop clinical signs of T1DM after the age of 20 years. Such patients have many features of classic T1DM, but previous studies indicate that subjects presenting with the disease in adulthood are characterized by a longer symptomatic period before diagnosis, better preserved β-cell function, and a reduced frequency of insulin autoantibodies (IAA). Genetic predisposition to T1DM is less marked (decreased prevalence of HLA class II susceptibility alleles), and there is probably also a possible contribution of protective genes toward β-cell destruction.[3] Some studies have suggested that later disease onset is characterized by a higher frequency of DRB1*03.[4] Other studies found DRB1*04 associated with later onset of T1DM.[5] It was observed that the prevalence of the HLA- DQB1*0201/0302 (DR3/DR4) genotype reflects the age at onset of diabetes, and the 0302 genotype is associated with insulin dependency.

### *Aims of the Study*

The aims of this study are to analyze the genetic predisposition for autoimmune diabetes mellitus with onset after 35 years of age: (a) if it is a heterogeneous group of disease, in which we can further identify subtypes of

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diabetes, (b) or diabetes with adult onset, which has different immunogenetic predisposition compared to T1DM with childhood onset.

## PATIENTS

Two hundred eighty-one patients suffering from diabetes mellitus with manifestation after 35 years of age were divided into three groups:

1. T1DM CP $\leq$200 insulin <6 m. (N = 64)
2. Latent autoimmune diabetes in adults (LADA) CP $\geq$200 GADA $\geq$ 50 insulin >6 m. (N = 31)
3. T2DM CP $\geq$200 pmol/L GADA $\leq$ 50 ng/mL (N = 131)

LADA is considered to be a subtype of T1DM. Other tested groups were 188 children withT1DM and 159 healthy adult individuals.

## METHODS

We have analyzed five genetic loci localized on chromosomes 4, 6, 11, and 14 in patients with childhood- and adult-onset autoimmune diabetes, and compared to healthy control subjects:[6–11]

| Genes | Alleles |
|---|---|
| ■ HLA class II | DRB1 and DQB1 |
| ■ MIC-A (MHC class I-related gene-A) | A microsatellite polymorphism GCT in exon 5 |
| ■ NF-κB1 (nuclear factor-κ B) | CA repeating polymorphism in regulation region |
| ■ NF-κBIA (inhibitor I-κB) | A single nucleotide polymorphism A/G in 3′UTR |
| ■ IL-18 (interleukin-18) | A promoter polymorphism –137 G/C, –607 C/A |

## PREDISPOSITION GENES AND THEIR PRODUCTS

### *HLA Class II Alleles*

An association between HLA and autoimmune diabetes mellitus has been recognized for more than 20 years. Many studies have verified that *DQB1*0302* is a strong susceptibility gene and that the heterozygous combination of DQA1*0301-DQB1*0302 on the HLA-DR4 haplotype and DQA1*0501-DQB1*0201 on the HLA-DR3 haplotype results in a synergistically increased risk of T1DM. DQA1*0301-DQB1*0302 is the most prevalent risk conferring haplotype in Caucasians detected in 74% of T1DM patients followed by

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DQA1*0501-DQB1*0201 detected in 52% of Caucasian patients.[12] Also, there is agreement that *DQB1*0602* allele, linked with DR2, is a strong protective gene.[13] The protective effect of DQB1*0602 is dominant to the susceptibility effects of DQB1*0302-DQA1*0301 and DQB1*0201-DQA1*0501.[14] A single copy of this DQB1*0602 allele is adequate to confer significant negative association. HLA-DQ genes are of primary importance but HLA-DR genes modify the risk conferred by HLA-DQ.[15] The risk associated with an HLA genotype is defined by the particular combination of susceptible and protective alleles. The frequencies of predisposition alleles, DQB1*0302 and DQB1*0201, are usually increased, while frequency of protective DQB1*0602 allele is usually decreased.[16,17]

### *Major Histocompatibility Complex (MHC) Class I Chain-Related Gene A (MIC-A)*

A number of observations indicate that class II genes cannot explain all of the MHC associations with T1DM. A role for MHC complex genes other than class II genes has been suggested. Several studies have supported a role for both MHC HLA class III and class I genes in T1DM predisposition. The most studied class III genes are tumor necrosis factor and lymphotoxin (TNF-$\alpha$ and TNF-$\beta$). The strongest evidence for susceptibility genes in the class I region comes from recent systematic assessment of microsatellite markers spanning this region. Among them, one is MHC MIC-A. The MIC-A is located within the MHC class I region of the chromosome 6. It contains long open reading frames encoding three distinct extracellular domains ($\alpha$1, $\alpha$2, and $\alpha$3), a transmembrane segment, and a cytoplasmatic tail. It has been reported that MIC-A may be recognized by a subpopulation of intestinal $\gamma\delta$ T cells,[18] and may play a role in the activation of a subpopulation of NK cells that express the NKG2D receptor.[19]

Sequence analysis of the exon 5 encoding the transmembrane segment showed a trinucleotide repeat (GCT) microsatellite polymorphism within this region.[20] So far, six alleles of the exon 5 of the MIC-A gene, which differ in number of (GCT) repetitions at position 296, have been identified. These alleles contain 4, 5, 6, 9, 10 repetitions of GCT, and five repetitions of GCT with an additional nucleotide insertion (GGCT). The MIC-A alleles have been accordingly named A4, A5, A6, A9, A10, and A5.1, respectively.[21] Due to the proximity of the MIC-A locus to HLA-B and HLA-C, MIC-A polymorphisms have been investigated for associations with a variety of HLA class I-associated diseases. Some of these studies show a positive association between MIC-A and the disease in question, but this may be due to its linkage disequilibrium with HLA-B. However, most disease association studies have only concentrated on the MIC-A transmembrane region, focusing on the six variants

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so far identified. Recent studies of MIC-A transmembrane polymorphism focused on the investigation of its relationship to autoimmune diabetes mellitus showed an association of the A5 allele with the susceptibility to young-onset T1DM.[22,23] Conversely, this allele does not confer increased risk for adult-onset T1DM.[24]

### *NF-κ B and Its Inhibitor*

The gene encoding NF-κB, *NF-κB1*, on chromosome 4 with a microsatellite polymorphism (CA) inregulation region (18 alleles), and the gene encoding inhibitor of NF-κB, *NF-κBIA*, on chromosome 14 with single nucleotide polymorphism (A/G) in 3′UTR, are other candidates of genetic predisposition to diabetes mellitus for the reason of their relationship to inflammatory processes.[25,26] NF-κB is a transcription factor, which is implicated in the regulation of many genes that encode mediators of immune response, embryo and cell lineage development, cell apoptosis, inflammation, cell cycle progression, oncogenesis, viral replication, and various autoimmune diseases. The activation of NF-κB is thought to be part of a stress response as it is activated by a variety of stimuli, including reactive oxygen species (ROS) and AGEs, which Q3 are toxic products of nonenzymatic glycation due to long-term hyperglycemia and oxidative stress. At the cellular level NF-κB is activated through phosphorylation of inhibitor of NF-κB (IκB), what triggers subsequent translocation of NF-κB molecules into the nucleus, where they are bound with a consensus sequence (5′GGGACTTTCC-3′) of various genes, activating their transcription.

### *Interleukin (IL)-18*

On chromosome 11 is located the gene encoding IL-18, with two single nucleotide polymorphisms inpromoter region at position –607 and –137. The cytokine IL-18, the Th1 cytokine, and a member of the IL-1 superfamily, elicit several biological activities that initiate and promote host defense and inflammation following infection or injury.[27,28] It is a pleiotropic cytokine predominantly secreted by activated monocytes/macrophages involved in the regulation of innate and acquired immune response, playing a key role in autoimmune, inflammatory, and infectious diseases. IL-18 acts as a proinflammatory factor and, in synergy with IL-12, promotes development of Th1 lymphocyte response by induction of interferon-γ (IFN-γ) production, modulates activity of natural killer cells, increases TNF-α and IL-1 production by macrophages, upregulates the expression of adhesion molecules, and induces nitric oxide production in the area of chronic inflammation.[29] It was reported by Nicoletti *et al.*[30] that IL-18 serum levels are increased in the subclinical stage of T1DM in first-degree relatives of T1DM patients. The importance

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of IL-18 for the occurrence of T1DM is underscored by the observation that the murine IL-18 gene maps to an interval on chromosome 9 in the vicinity of the diabetes susceptibility gene from the nonobese diabetic (NOD) mouse, idd2.[31] Several polymorphisms in the promoter region of human IL-18 gene, located at chromosome 11q22.2–q22.3, have been identified.[32] Kretowski *et al.*[33] reported the role of IL-18 gene polymorphism at positions –607 and –137 in the predisposition to T1DM in Polish population.

## RESULTS

The most significant risk factor is on chromosome 6, human leukocyte antigens class II alleles, *HLA DRB1 and DQB1*. Odds ratio for single predisposition HLA alleles has been significantly different in different groups of patients. For DRB1*03 allele, it has been the highest in LADA patients (OR = 4.94 with CI 95% between 1.83 and 13.49), for DRB1*04 and DQB1*0302 alleles, it has been the highest in diabetic children (OR = 22.87 with CI 95% between 10.89 and 48.87, and OR = 46.48 with CI 95% between 16.15 and 140.23, respectively). In the case of T1DM with adult onset, predisposition alleles have been similar as in children, but odds ratio has been lower and proved stronger influence of DR alleles than DQ alleles (OR = 10.47 with CI 95% between 4.53 and 24.65, and OR = 9.49 with CI 95% between 3.91 and 23.59, respectively).

Analysis of gene expression of HLA-DRB1*04 in antigen-presenting cells of peripheral blood has described significantly increased quantity of mRNA of this allele in T1DM with adult onset in comparison to T1DM in children and to control groups of adults and children (FIG. 1). One HLA hypothesis points to a possible role of HLA-DR molecules as a protective factor, which in comparison to HLA-DQ molecules suppresses autoimmune reactions. Higher quantity of DR molecules in adult patients may suggest breaking autoimmune process.

On chromosome 6 is also located MHC *MIC-A*. Its exon 5, coding transmembrane region, has been tested for a microsatellite polymorphism (GCT) with six alleles. The results in this study suggest that MIC-A5.1 allele is associated with genetic susceptibility to adult-onset of T1DM (corrected $P$ = 0.009; OR = 2.14 with CI 95% 1.29–3.55), whereas it is increased, but not significantly in LADA. Different allele, MIC-A5, is associated with T1DM in children.

Since *NF-κB* is a transcription factor which is implicated in the regulation of many genes involving in inflammatory processes, a microsatellite polymorphism (CA) inregulation region of NF- κB1 gene and a single nucleotide polymorphism (A/G) in 3′UTR of NF-κBIA gene are other candidates of genetic predisposition to autoimmune diabetes mellitus. A statistically significant Q4 difference in allele frequencies of the NF-κB1 gene has been observed only in

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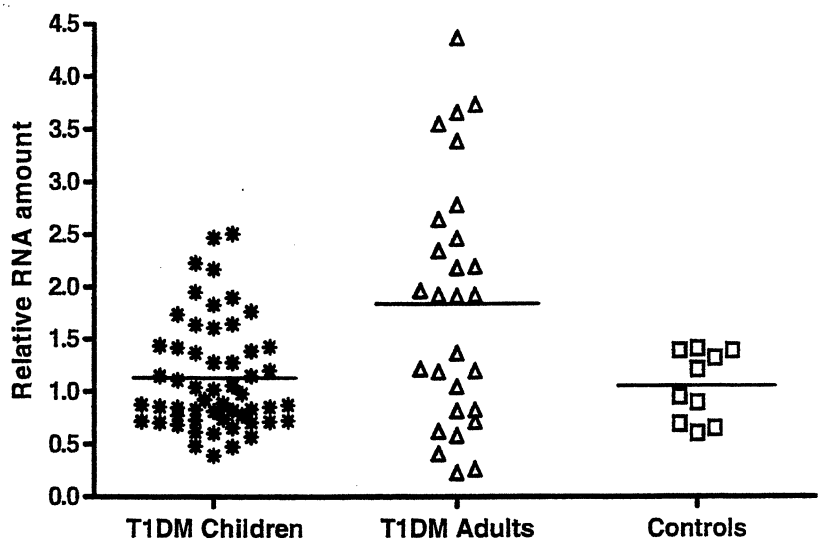


**FIGURE 1.** Relative quantification data from quantitative analysis of HLA-DRB1*04 mRNA levels in the tested groups of patients and controls. The expression of HLA-DRB1*04 was found significantly higher in T1DM adult patients. The comparison was done with two specific control groups (adult and children controls with HLA-DRB1*04 allele). The relative RNA amount for tested groups of type 1 diabetes (T1DM) with adult onset and in childhood was significantly different, *P*-value 0.034 (comparison by Kruskall–Wallis test, Dunn's multiple comparison test). The difference between patient groups and control groups was not significant by this type of testing.

T1DM with adult onset. The frequency of A7 allele (size 132 bp, 20 repeats) has been significantly increased in comparison to a control healthy group ($P < 0.01$; OR = 10.0 with CI 95% 1.25–215.6).

There was no difference in A and G allele frequencies of the NF-κBIA gene between the control group and patients, the difference has been observed in distribution of genotypes. There is an evidence that the heterozygous genotype, in comparison to a control healthy group, is protective for autoimmune diabetes with onset in adulthood: 55.6% in controls, 45.2% in LADA (nonsignificant decrease), and 35.9% in T1DM with adult onset ($P < 0.01$; OR = 0.4 with CI 95% 0.24–0.86). A statistically significant increase of the AA genotype frequency of NF-κBIA gene has been detected in autoimmune diabetes type LADA (32.3%, $P < 0.01$; OR = 2.7 with CI 95% 1.06–6.75) and in type 2 diabetes (42.3%, $P < 0.001$; OR = 2.6 with CI 95% 1.52–4.41) in comparison to a control healthy group (16.9%). In T1DM with adult onset, the genotype frequency has been also increased, but it has not reached a statistical significance. On the other hand, the values for diabetes in children are not different from control ones. The fact that polymorphism of NF-κB system is associated

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**TABLE 1. Overview of predisposition genes in autoimmune diabetes with different onset**

| Gene | LADA After 35 years of age | T1DM After 35 years of age | T1DM In children |
|---|---|---|---|
| HLA | DRB1*03 (OR = 5) | DRB1*04 DQB1*0302 (OR = 10) | DQB1*0302 (OR = 46) |
| MIC-A | Allele A5.1[n] | Allele A5.1 | Allele A5 |
| NF-κBIA | Homozygote AA | Homozygote AA [n] | – |
| NF-κB1 | – | allele A7 | – |
| IL-18 | Homozygote −607 CC ↑ (increased frequency) | Homozygote −607 CC ↓ (decreased frequency) | Allele −137 C |

[n]Observation was not statistically significant.

not only with autoimmunity, but also with metabolic disease, documents its central role in cell signaling.

Promoter polymorphism of *IL-18* gene is not associated with any group of diabetes, but a statistical significant difference has been found between autoimmune diabetes type LADA and adult-onset T1DM.

## CONCLUSIONS

We can summarize our observations in these findings:The main and unique HLA predisposition allele in autoimmune diabetes type LADA has been detected HLA-DR3 (OR = 5.0, $P < 0.0001$). Other HLA alleles have not been found having a significant association with the disease. Furthermore, the statistically significant increase of the AA genotype frequency of NF-κBIA gene (OR = 2.7, $P < 0.01$) has been present in comparison to a control healthy group. Contrary to this, the genetic risk factor in adult-onset T1DM has been detected DRB1*04 allele (OR = 10.0, $P < 0.0001$) that is in linkage disequilibrium with other predisposition allele DQB1*0302 (OR = 10.0, $P < 0.0001$). This group is also associated with microsatellite polymorphisms of MIC-A and NF-κB1 genes (MIC-A5.1 allele: OR = 2.14, $P < 0.01$; NF-κB1-A7 allele: OR = 10.0, $P < 0.01$).

The statistically significant difference has been observed inthe distribution of the promoter polymorphism at position –607 (C/A) of IL-18 gene between both groups of patients ($P < 0.01$), what supports our conclusions about partly different etiopathogenesis of these two groups of adult patients (TABLE 1).

Taken together, these observations suggest a general accepted conclusion that the presence of predisposing genes in autoimmune diabetes decreases with the age, probably due to increasing influence of environmental factors. Autoimmune diabetes with manifestation in adults (after 35 years ) may have partly different immunogenetic etiopathogenesis than autoimmune diabetes with man-

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ifestation in childhood. Allele polymorphism of NF-κB system presents a genetic risk factor only in autoimmune diabetes with adult onset, but not in diabetes of childhood. Q5

Furthermore, we can hypothesize that T1DM in children or adults might have different predisposition genes than LADA. Compared to fast-progressing adult-onset T1DM, slowly progressing adult-onset T1DM (LADA) can involve protective genes leading to a slow progressive β-cells destruction and insulin deficiency. The different mechanisms reflect different HLA association in these diseases. Allele polymorphism of HLA system is associated with autoimmune diabetes in both categories, but for each group of patients the specific association has been described. The most significant association is with DQB1 alleles in T1DM, but with DRB1 alleles in LADA.

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# HLA in Czech adult patients with autoimmune diabetes mellitus: comparison with Czech children with type 1 diabetes and patients with type 2 diabetes

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## Summary

Type 1 diabetes results from an autoimmune insulitis, associated with HLA class II alleles. The evidence about HLA allele association is not clear in patients diagnosed after 35 years of age. In this study we have analyzed HLA alleles of DQB1 and DRB1 genes by sequence specific primer (SSP)-PCR technique in adult patients with disease onset after 35 years of age. Two hundred and eighty-one patients were divided into three groups according to the insulin therapy, the level of C peptide (CP), and GAD antibodies (anti-GAD). Group 1 (type 1 diabetes in adults) was characterized by CP less than 200 pmol/L and anti-GAD more or less than 50 ng/mL ($n$ = 80). All of them had insulin therapy within 6 months after diagnosis. Group 2 latent autoimmune diabetes mellitus in adults (LADA) was defined by a minimum 6-month-long phase after diagnosis without insulin therapy, and was characterized by CP more than 200 pmol/L and anti-GAD more than 50 ng/mL ($n$ = 70). Group 3 (type 2 diabetes) was characterized by CP more than 200 pmol/L and anti-GAD less than 50 ng/mL ($n$ = 131). None ever had insulin therapy. In group 1, there was increased frequency of DRB1*04 (45.0% vs. controls 14.1%, OR = 5.0, $P < 0.0005$) and DQB1*0302 alleles (43.3% vs. controls 11.1%, OR = 6.1, $P < 0.00005$). There was increased frequency of DRB1*03 and DQB1*0201, and decreased frequency of DQB1*0602 (3.3% vs. controls 20.2%), but it was not significant. In group 2, there was a significantly increased frequency of DRB1*03 only (50.0% vs. controls 21.2%, OR = 3.7, $P < 0.05$). Compared with children with type 1 diabetes and adults with type 2 diabetes (group 3), we conclude that the presence of predisposing DQB1 alleles in adults with type 1 diabetes decreases with the age, probably due to environmental factors. Only the DRB1*03, but not the DQB1 gene, becomes the main predisposing allele in LADA patients. These findings suggest that the presence of HLA-DQB1*0302 identifies patients at high risk of requiring insulin treatment. Type 1 diabetes mellitus (DM) in children or adults may have partly different immunogenetic etiopathogenesis than LADA.

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## Introduction

Autoimmune diseases form a complex group of approximately 40 diseases affecting 5–7% of the general population. Type 1 diabetes mellitus (DM) is one of the most frequent and potentially severe with a prevalence of approximately 0.5% (Sinha *et al.*, 1990).

Type 1 diabetes mellitus develops as a result of selective autoimmune destruction of pancreatic β-cells, a process called insulitis. The presence of antibodies against islet-cells (ICA), insulin (IAA), glutamic acid decarboxylase (GAD-Ab) and tyrosine-phosphatase (IA2-Ab), and infiltration of pancreatic islands by B and T lymphocytes are all evidence of organ specific autoimmunity.

The exact cause of insulitis is not completely understood. The etiology is multifactorial and includes both genetic and environmental factors. Many papers have shown that HLA genes, the human main histocompatibility locus on the chromosome 6, are key genetic factors (Thomson *et al.*, 1988). The studies have proved that type 1 diabetes is associated with certain HLA class II alleles, namely DRB1*0301, DRB1*0401, DRB1*0405, DQB1*0201, DQB1*0302, DQA1*0301. Some alleles are also protective, namely DQB1*0602, DQB1*0603, DQB1*0301.

The HLA frequencies in Czech population are similar as those of the other Caucasian populations (Cerna *et al.*, 1992; Drabek *et al.*, 1998; Loudova *et al.*, 1999). Serological typing of the HLA system in young patients with type 1 diabetes in the Czech population has also proved HLA predisposition antigens similar to other Caucasians (Dvorakova & Majsky, 1979, Vondra *et al.*, 1996).

The association of HLA alleles with type 1 diabetes changes with age of onset. Generally, the younger the

patients the more significant is the HLA association (Tait *et al.*, 1995; Lohmann *et al.*, 1997; Graham *et al.*, 1999).

The evidence about HLA allele association is not clear, however. Some studies have suggested that later disease onset is characterized by a higher frequency of DRB1*03 (Lohmann *et al.*, 1997; Zevaco-Mattei *et al.*, 1999; Gambelunghe *et al.*, 2000). Other studies found DRB1*04 associated with later onset of type 1 diabetes mellitus (Tait *et al.*, 1995; Tuomi *et al.*, 1999). Analyses in non-diabetic offspring of probands with latent autoimmune diabetes mellitus in adults (LADA) concluded that LADA is a familial disease involving gene defects leading to a slow progressive β-cells destruction and insulin deficiency (Vauhkonen *et al.*, 2000).

The aims of our study were to analyze the frequency distribution of HLA DRB1 and DQB1 alleles in the adult onset diabetes in a Czech population and compare this data with those from controls, diabetic children and the healthy Czech population.

## Patients and methods

### Patients

We investigated 281 patients (172 women) with diagnosis of diabetes after 35 years of age. Their average age at disease onset was 53 years (range 35–81). The patients came consecutively for consultation to our diabetes centre and collaborating diabetes clinics. All patients had fasting C peptide, GAD and IA2 antibodies measured at the time of investigation. Body mass index (BMI) was calculated from weight and height. The therapy was recorded (Table 1).

We divided our patients into three groups: type 1 diabetes in adults, LADA, and type 2 diabetes. Patients with autoimmune diabetes (group 1 and 2) were negative for C peptide or positive for autoantibodies. All of them had insulin therapy. LADA (group 2) was defined by a minimum 6-month-long phase after diagnosis without insulin therapy. Patients with type 2 diabetes (group 3) were positive for C peptide and negative for autoantibodies. None ever had insulin therapy.

Group 1, type 1 diabetes in adults, comprised 80 patients (56 women) with an average BMI of 27 (SD = 4.6) and average duration of diabetes 16 years (SD = 14.2). All patients had fasting C peptide secretion less than 200 pmol/L. Anti-GAD more than 50 ng/mL was present in 50% of patients, anti-IA2 more than 0.9 U/mL was present in 15% of patients. Ten per cent of patients were positive for both antibodies, but 45% of patients were without these two antibodies.

Group 2, LADA, comprised 70 patients (40 women) with an average BMI of 32 (SD = 4.8) and average duration of diabetes 14 years (SD = 7.0). All patients had fasting C peptide secretion more than 200 pmol/L. Anti-GAD more than 50 ng/mL was present in 100% of patients and anti-IA2 more than 0.9 U/mL was present in 11% of patients.

Type 2 diabetic patients (group 3) comprised 131 persons (76 women) with an average BMI of 31 (SD = 5.5) and average duration of diabetes 13 years (SD = 6.6), with fasting C peptide secretion more than 200 pmol/L and anti-GAD less than 50 ng/mL. None ever had insulin therapy.

For comparison we used the results of HLA class II analysis of Czech diabetic children with type 1 diabetes mellitus (Cinek *et al.*, 2001). HLA typing performed in children was done by the same technique as in this study (SSP-PCR supplied by Genovision, Oslo, Norway).

We have compared the patient data with the data of the healthy Czech population, which was published earlier (Cerna *et al.*, 1992). The controls were genotyped in a previous study by oligonucleotide hybridization. We tested several control samples by the sequence specific primer (SSP)-PCR method used in this study to prove that we get the same results by both techniques. The study was approved by the ethical committee of Third Medical Faculty of Charles University at Prague. All patients gave

**Table 1.** Clinical features in 281 Czech adult patients with diabetes

| Features | Group 1 (*n* = 80) | Group 2 (*n* = 70) | Group 3 (*n* = 131) |
|---|---|---|---|
| Female | 61% | 57% | 58% |
| Age at disease onset (years)[a] | 43 (36–56) | 52 (35–71) | 53 (35–81) |
| Duration of DM (years)[a] | 16 (4–27) | 14 (4–29) | 13 (1–22) |
| BMI (kg/m$^2$)[a] | 27 (22–37) | 32 (27–46) | 31 (18–50) |
| Insulin therapy | Yes | Yes | No |
| (number) | 80/80 | 70/70 | 0/131 |
| Fasting C peptide[b] | – | + | + |
| (pmol/L)[a] | 63 (4–197) | 609 (51–2800) | 772 (1–50) |
| Anti-GAD[c] | +/– | + | – |
| (ng/mL)[a] | 193 (3–3000) | 379 (210–1753) | 8 (202–3370) |

[a] Average value with ranges in parentheses;
[b] + positive value ≥ 200 pmol/L, – negative value < 200 pmol/L;
[c] + positive value ≥ 50 ng/mL, – negative value < 50 ng/mL;
Group 1 (type 1 diabetes in adults): C peptide < 200 pmol/L, anti-GAD > or < 50 ng/mL;
Group 2 (LADA): C peptide > 200 pmol/L, anti-GAD > 50 ng/m, > 6 months without insulin:
Group 3 (type 2 diabetes): C peptide > 200 pmol/L, anti-GAD < 50 ng/mL.

informed consent to the use of their blood samples in this study.

#### Fasting C peptide

We used an immunoradiometric method (Immunotech, Prague, Czech Republic). The serum samples from 49 healthy individuals were analyzed and the results are as follows: average value 486 pmol/L, median 449 pmol/L, standard deviation 170 pmol/L, and minimal-maximal value 206–934 pmol/L. The level less than 200 pmol/L has been taken as decreased C peptide secretion.

#### GAD antibodies

The presence of IgG antibodies against GAD has been detected by the ELISA method (Roche Molecular Biochemicals, Mannheim, Germany). The test has 98% of specificity and 69% of sensitivity (Verge *et al.*, 1998). A level less than 32 ng/mL is considered as negative. To increase the precision, in our study, a level less than 50 ng/mL has been taken as normal. A level more than 50 ng/mL has been considered as a reliable sign of autoimmune insulitits.

#### IA2 antibodies

The presence of IgG antibodies against IA2 has been detected by the ELISA method (Roche Molecular Biochemicals). A level less than 0.9 U/mL is considered as negative.

#### Genomic DNA preparation

A salting out method (Miller *et al.*, 1988) was used to prepare genomic DNA from peripheral blood leukocytes. The QIAamp spin columns (QIAGEN GmbH, Hilden, Germany) were used for rapid purification.

#### Analysis of HLA DRB1 and DQB1 alleles

HLA DRB1 and DQB1 genes were typed using PCR with sequence specific primers (SSP-PCR) (Zetterquist & Olerup, 1992; Olerup *et al.*, 1993) supplied by Genovision.

#### Statistics

Allele, gene, and haplotype frequencies were calculated using standard methods. The significance of the differences in allele frequencies were evaluated by $\chi^2$ with Yates' correction and *P*-values were calculated. When a hypothesis that had not been tested was analyzed, the *P*-values were corrected by multiplying by the number of alleles tested. The strength of the observed associations was estimated by calculating odds ratios [relative risk (RR)] using the method by Woolf. When one of the RR values is 0 or close to 0, RR can be calculated by using Woolf's method with Haldane's correction.

## Results

#### Analysis of HLA DRB1 alleles

DRB1*04 was significantly increased in group 1 compared with controls (OR = 5.0, $P < 0.0005$) as was DRB1*03, but not significantly. DRB1*03 was significantly associated with group 2 compared with controls (OR = 3.7, $P < 0.05$). No differences in HLA DRB1 frequencies from the healthy population were observed in the patients with type 2 diabetes — group 3 (Table 2).

#### Analysis of HLA DQB1 alleles

DQB1*0302 was significantly increased in group 1 compared with controls (OR = 6.1, $P < 0.00005$), as was DQB1*0201, but not significantly. In the same group,

**Table 2.** Frequency (%) of DRB1 alleles in Czech patients with diabetes

| DRB1 alleles | Children ($n$ = 261) | Group 1 ($n$ = 80) | Group 2 ($n$ = 70) | Group 3 ($n$ = 131) | Control ($n$ = 99) |
|---|---|---|---|---|---|
| 01 | NT | 11.7 | 16.7 | 16.7 | 22.2 |
| 03 | NT | 40.0 | 50.0*(3.7) | 13.3 | 21.2 |
| 04 | NT | 45.0**(5.0) | 23.3 | 21.1 | 14.1 |
| 07 | NT | 16.7 | 13.3 | 26.7 | 36.4 |
| 08 | NT | 5.0 | 13.3 | 7.8 | 5.1 |
| 09 | NT | 0 | 3.3 | 3.3 | 1.0 |
| 10 | NT | 5.0 | 0 | 3.3 | 2.0 |
| 11 | NT | 11.7 | 30.0 | 25.6 | 23.2 |
| 12 | NT | 1.7 | 0 | 2.2 | 10.1 |
| 13 | NT | 11.7 | 13.3 | 15.6 | 22.2 |
| 14 | NT | 1.7 | 3.3 | 13.3 | 5.1 |
| 15 | NT | 11.7 | 20.0 | 20.0 | 23.2 |
| 16 | NT | 3.3 | 3.3 | 4.4 | 5.1 |

Data from children with diabetes obtained from Cinek *et al.* with permission for comparison.
Data from healthy control obtained from Cerna *et al.* are compared with patient groups.
Group 1 (type 1 diabetes in adults): C peptide < 200 pmol/L, anti-GAD > or < 50 ng/mL.
Group 2 (LADA): C peptide > 200 pmol/L, anti-GAD > 50 ng/mL, > 6 months without insulin.
Group 3 (type 2 diabetes): C peptide > 200 pmol/L, anti-GAD < 50 ng/mL.
NT, not tested, * $P < 0.05$, ** $P < 0.0005$, OR values are in brackets.

**Table 3.** Frequency (%) of DQB1 alleles in Czech patients with diabetes

| DQB1 alleles | Children ($n$ = 261) | Group 1 ($n$ = 80) | Group 2 ($n$ = 70) | Group 3 ($n$ = 131) | Control ($n$ = 99) |
|---|---|---|---|---|---|
| 0201 | 61.0*[10](3.4) | 41.7 | 42.9 | 24.4 | 30.3 |
| 0202 | NP | 6.7 | 14.3 | 19.1 | 19.2 |
| 0301 | 5.0*[15](0.06) | 18.3 | 28.6 | 31.3 | 38.4 |
| 0302 | 65.0*[15](9.0) | 43.3**(6.1) | 14.3 | 13.7 | 11.1 |
| 0303 | NP | 5.0 | 14.3 | 3.8 | 8.1 |
| 0304 | NP | 0 | 0 | 2.3 | 0 |
| 0401 | NP | 3.3 | 0 | 0 | 0 |
| 0402 | NP | 1.7 | 3.6 | 5.3 | 4.0 |
| 0501 | NP | 33.3 | 21.4 | 23.7 | 23.2 |
| 0502 | NP | 3.3 | 3.6 | 6.1 | 5.1 |
| 0503 | 1.0*(0.13) | 1.7 | 0 | 6.9 | 5.1 |
| 0504 | NP | 0 | 0 | 1.5 | 0 |
| 0601 | NP | 1.7 | 0 | 2.3 | 3.0 |
| 0602 | 1.0*[15](0.02) | 3.3 | 10.7 | 16.8 | 20.2 |
| 0603 | 4.0*[5](0.20) | 6.7 | 0 | 11.5 | 13.1 |
| 0604 | NP | 8.3 | 7.1 | 6.9 | 5.1 |

Data from children with diabetes used from Cinek *et al.* with permission for comparison.
Data from healthy control used from Cerna *et al.* are compared with patient groups.
Group 1 (type 1 diabetes in adults): C peptide < 200 pmol/L, anti-GAD > or < 50 ng/mL.
Group 2 (LADA): C peptide > 200 pmol/L, anti-GAD > 50 ng/mL, > 6 months without insulin.
Group 3 (type 2 diabetes): C peptide > 200 pmol/L, anti-GAD < 50 ng/mL.
NP, not published, * $P < 0.005$, ** $P < 0.00005$, *[5] $P < 10^{-5}$, *[10] $P < 10^{-10}$, *[15] $P < 10^{-15}$.
OR values are in brackets.

**Table 4.** Frequency (%) of DQB1 and DRB1 genotypes associated with type 1 diabetes

| | Children ($n$ = 261) | Group 1 ($n$ = 80) | Group 2 ($n$ = 70) | Group 3 ($n$ = 131) | Control ($n$ = 99) |
|---|---|---|---|---|---|
| DRB1 genotype | | | | | |
| 03/04 | NP | 17.0**(8.3) | 6.7 | 0 | 2.0 |
| DQB1 genotype | | | | | |
| 0201/0302 | 36.0*[15](22.0) | 15.0*(7.4) | 3.6 | 0.8 | 2.0 |
| 0201/0301 | 1.0**(0.1) | 0 | 3.6 | 6.1 | 8.1 |

Data from children with diabetes used from Cinek *et al.* with permission for comparison.
Data from healthy control used from Cerna *et al.* are compared with patient groups.
Group 1 (type 1 diabetes in adults): C peptide < 200 pmol/L, anti-GAD > or < 50 ng/mL.
Group 2 (LADA): C peptide > 200 pmol/L, anti-GAD > 50 ng/mL, > 6 months without insulin.
Group 3 (type 2 diabetes): C peptide > 200 pmol/L, anti-GAD < 50 ng/mL.
NP, not published, * $P < 0.05$, ** $P < 0.01$, *[15] $P < 10^{-15}$, OR values are in brackets.

DQB1*0602 was decreased compared with controls, but not significantly. In group 2, DQB1*0201 was increased compared with controls, but not significantly. DQB1*0602 was non-significantly decreased compared with controls up to 10.7 vs. 20.2%; all carriers had the second DQB1 allele 0201 (30% of all 0602) or 0302 (70% of all 0602) (Table 3).

### HLA DR4 subtyping

In patients with diabetes, we have distinguished three DRB1*04 alleles: DRB1*0401 (50% of all DR4), DRB1*0403 (25% of all DR4) and DRB1*0404 (25% of all DR4). For comparison, in controls, we distinguished five DRB1*04 alleles: DRB1*0401 (50% of all DR4), DRB1*0402 (7% of all DR4), DRB1*0404 (21% of all DR4), DRB1*0405 (14% of all DR4) and DRB1*0408 (7% of all DR4). Comparing the patients with the healthy population, we have not found any association of DRB1*04 subtypes with disease phenotypes.

### Heterozygosity for DRB1 or DQB1 genotypes

We have observed a statistically significant increased prevalence of high risk DRB1*03/DRB1*04 and DQB1*0201/DQB1*0302 heterozygotes among patients in group 1 compared with controls (OR = 8.3, $P < 0.01$ and OR = 7.4, $P < 0.05$). This risk became more significant when we divided this group into anti-GAD-positive and anti-GAD-negative patients, because predisposing heterozygotes were particularly associated with the anti-GAD-positive subgroup (25%, OR = 12.4, $P < 0.005$ both). The protective DQB1*0201/DQB1*0301 genotype has not been found in group 1 of patients with type 1 diabetes mellitus. Other DQB1 genotypes have not been significantly associated with the disease (Table 4).

| DQB1 genotype | Children | | | Group 1 | Control |
|---|---|---|---|---|---|
| | 0–4 years ($n$ = 82) | 5–9 years ($n$ = 98) | 10–14 years ($n$ = 81) | ($n$ = 80) | ($n$ = 99) |
| 0201/0302 | 45.0 | 36.0 | 27.0 | 15.0*(7.4) | 2.0 |

**Table 5.** Frequency (%) of DQB1* 0201/0302 heterozygotes in dependence on the age of type 1 diabetes manifestation

Data from diabetic children used from Cinek *et al.* with permission for comparison.
Data from healthy controls used from Cerna *et al.*
Group 1 (type 1 diabetes in adults): C peptide < 200 pmol/L, anti-GAD > or < 50 ng/mL.
Data from each group are compared with each other.
* $P < 0.05$, OR values are in brackets.

### The HLA class II association and age of onset

Comparing our patients with type 1 diabetes with children with type 1, diabetes (Cinek *et al.*, 2001), we found decreasing significance of predisposing alleles with increasing age of onset. The frequency of DQB1*0302 allele in children was 65%, in adult patients of group 1 43.3%, and in healthy controls 11.1% (Table 3). The frequency of DQB1*0201 allele in children was 61%, in adult patients of group 1 41.7%, and in healthy controls 30.3% (Table 3). A decreasing prevalence of genetic factors with increasing age of onset was not only found for predisposing single alleles, but also for DQB1* 0201/0302 heterozygotes (Table 5). While the prevalence of 0201/0302 heterozygotes among children diagnosed at the age of 0–4, 5–9, and 10–14 years was 45, 36 and 27%, respectively, the prevalence of 0201/0302 heterozygotes among adults diagnosed after the age of 35 years was 15% in the patients of group 1.

## Discussion

The classification of diabetes mellitus discriminate type 1, type 2, other specific types and gestational diabetes (Report, 2001). It has been reported that these types of diabetes involve separate genetic factors, suggesting that, in addition to the perceived differences in clinical manifestations and course, their etiologic mechanisms are distinct. The presence of islet cell autoantibodies in adult diabetic subjects who do not require insulin treatment for at least 6 months after the initial clinical diagnosis identifies the so-called latent autoimmune diabetes in the adult (LADA). Glutamic acid decarboxylase autoantibodies (GAD65Ab) are the best immune marker to identify LADA patients. The main goal of our study was to analyze HLA class II antigens in adult patients with autoimmune diabetes mellitus, to prove, if the basic data known from children are valid also in type 1 adult patients, and to compare these results with those from LADA patients. In view of the long and accurate follow-up in our study, we were able to further subdivide diabetic patients diagnosed after 35 years of age into three groups that differ in the presence of fasting C peptide and GAD antibodies, and insulin therapy, respectively.

The association of DQB1*0302 and DRB1*04 that was observed with the group of adult patients with type 1 diabetes with negative C peptide (group 1) suggests that these alleles represent a marker or risk factor for requiring insulin treatment, as was reported (Horton *et al.*, 1999). The highest risk in this group was for DQB1*0302. The odds ratio for DQB1*0302 was 6.1 (ratio 2.6–14.9). The odds ratio for DRB1*04 was lower, 5.0 (ratio 2.2–11.4). The frequencies of DQB1*0201 and DRB1*03 were increased, but not significant. The frequency of DQB1*0201/DQB1*0302 and DRB1*03/DRB1*04 genotypes in the C-peptide-negative group was statistically different from controls (OR = 7.4, ratio 1.7–33.2 and OR = 8.3, ratio 1.9–36.4), but lower than in children with diabetes. The pattern of predisposing alleles in adults with type 1 diabetes is similar with that in children with type 1 diabetes, but the prevalence seems to be age related. Whether these age-dependent differences reflect different environmental influences remains to be proven. Our observations correspond with results in Sweden, where the prevalence of the HLA-DQB1*0201/0302 (DR3/DR4) genotype reflects the age at onset of diabetes, and the 0302 genotype is associated with insulin dependency (Tuomi *et al.*, 1999).

The association of DRB1*03 only was found with the group of LADA patients with normal C peptide (group 2). The odds ratio for DRB1*03 was 3.7 (ratio 1.8–8.0). What was amazing, but very logical, was the exact match of the presence of C peptide with non-insulin-requirement. Previous studies proved that for early onset of type 1 diabetes, DQB1 alleles are a better marker of genetic predisposition than DRB1 alleles (Todd *et al.*, 1987). Our findings support the observations of some investigators that DRB1 alleles are the main marker for late onset (Lohmann *et al.*, 1997). Certain research groups have described DRB1*04 and DQB1*0302 as the main marker for LADA (Hosszufalusi *et al.*, 2003). Why different predisposing DR alleles are detected in different ethnic groups is not clear. The hypothesis been suggested that HLA-DRB1 does not predispose to autoimmune disease per se, but rather fails to provide efficient protection (Zanelli *et al.*, 2000). According to these facts we can hypothesize that type 1 diabetes mellitus in children or adults may have partly different immunogenetic etiopathogenesis than LADA. The different mechanism reflects different HLA association in these diseases. The most significant association is with DQB1 alleles in type 1 diabetes, but with DRB1 alleles in LADA.

Patients with type 2 diabetes without signs of autoimmune insulitis (fasting CP normal, GAD and IA2 antibodies negative, without insulin therapy) did not differ in HLA frequencies from healthy Czech controls.

The type of autoantibodies to islet cell antigens distinguish between acute-onset type 1 diabetes and LADA because GAD antibodies and ICA indicate slow disease progression, whereas the presence of IA2 antibodies is associated with an acute-onset clinical phenotype (Seissler *et al.*, 1998). That is probably why 50% of patients with type 1 diabetes in adults are negative for anti-GAD. These patients could be positive for other non-tested antibodies or their antibody production has burnt-out over the course of years. Additional testing for IA2 antibodies revealed that 15% of patients in group 1 (20% in anti-GAD+ and 10% in anti-GAD–) and 11% of patients in group 2 are positive for this autoantibody.

We conclude that Czech patients with autoimmune diabetes manifested after 35 years of age can be divided into two groups having different HLA class II associations: type 1 diabetes in adults with DQB1*0302 and DRB1*04 risk alleles, and LADA with DRB1*03 risk allele. The occurrence of HLA-DQB1*0302 (0201) and DRB1*04 (03) alleles in adults with type 1 diabetes was less frequent than in children, but more frequent than in patients with type 2 diabetes and healthy controls.

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# Diabetes mellitus in adults: association of HLA DRB1 and DQB1 diabetes risk alleles with GADab presence and C-peptide secretion

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**Abstract**

In our study, we investigated the relationship of HLA class II alleles to antibody production against glutamic acid decarboxylase (GADab) and to C-peptide secretion (CP) in diabetic patients. A group of 334 patients (190 women) diagnosed after 35 years of age and 99 control subjects were studied. Patients were divided into four groups according to concentrations of CP and GADab, respectively (CP high/low, GADab positive/negative). HLA DQB1 and DRB1 alleles were genotyped by SSP-PCR. The significance of DQB1 and DRB1 risk alleles was evaluated by examination of their odds ratios computed by testing 2 × 2 tables considering Bonferonis' corrected $P < 0.05$ as significant. We found strong association between the HLA DRB1*03 risk allele and presence of GADab, and close relationship of the HLA DRB1*04 and HLA DQB1*0302 risk alleles with decreased CP level. Taken together we conclude that the DRB1*04 and DQB1*0302 alleles are associated with progressive decrease of CP level, while DRB1*03 is a significant genetic marker of autoantibody (GADab) development.
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*Keywords:* HLA/DQ/DR; Glutamic acid decarboxylase (GAD); C-peptide (CP); Diabetes mellitus (DM)

## 1. Introduction

Diabetes mellitus with onset after the age of 35 years [1] is a heterogeneous group representing at least two major groups of diseases: insulin deficient (type 1 diabetes mellitus, T1DM) and insulin resistant diabetes mellitus (type 2 diabetes mellitus, T2DM) [2,3]. The etiology of the former is an autoimmune disorder and the etiology of the latter is a metabolic disorder. However, about 10% of patients initially diagnosed as patients with type 2 diabetes manifest autoimmune markers of β-cell destruction. These subjects have a slowly progressive form of autoimmune diabetes mellitus and are considered having latent autoimmune diabetes mellitus in adults (LADA) [4–7].

The most important gene loci defining risk to T1DM are located within the HLA gene region. HLA DQ molecules are of primary importance but HLA DR gene products modify the risk conferred by HLA DQ. The risk associated with an HLA genotype is defined by the particular combination of susceptible and protective alleles. The complicated analysis of HLA genotypes is simplified by strong linkage disequilibrium between HLA DRB1, DQA1 and DQB1 loci. Childhood T1DM is characterized by an abrupt onset and ketosis and is

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associated with HLA DRB1*04-DQA1*0301-DQB1*0302, and a high frequency of insulin and thyrosin-phosphatase autoantibodies [7,8].

The term latent autoimmune diabetes in adults was introduced to define adult diabetic patients initially non-insulin-requiring but with immune markers of type 1 diabetes that, in a number of cases, progress to insulin dependency [6]. It is characterized by the presence of glutamic acid decarboxylase 65 autoantibodies (GADab) and/or islet cell antibodies (ICA), and is associated with HLA DRB1*03-DQA1*0501-DQB1*0201 and/or with HLA DRB1*04-DQA1*0301-DQB1*0302 haplotype [5,9].

In patients diagnosed after 35 years of age, the relation of HLA risk alleles and GADab and/or C-peptide secretion (CP), generally used as a marker of insulin production, is not completely clear. In this study, we investigated the relationship between the presence of diabetes risk HLA alleles, and the presence of GADab and/or CP in diabetic patients diagnosed after 35 years of age.

## 2. Patients and methods

### 2.1. C-peptide level

Levels of CP were measured on an empty stomach (fasting C-peptide). For detection, we used an immunoradiometric method (Immunotech, Prague, Czech Republic). The serum samples from 99 healthy individuals provided control values: average value 486 pmol/l; median 449 pmol/l; standard deviation 170 pmol/l; and minimal–maximal value 206–934 pmol/l. According by values lower than 200 pmol/l were taken as decreased C-peptide secretion (marked as low CP, CP negative, or CP−).

### 2.2. GAD antibodies

The presence of IgG antibodies against GAD was detected by the ELISA method (Roche Molecular Biochemicals, Mannheim, Germany). The test had 98% of specificity and 69% of sensitivity. The cut-off point of the test was 32 ng/ml. Levels lower than this cut-off point were considered negative (marked as GADab negative, or GADab−), levels higher than 32 ng/ml were considered positive (marked as GADab positive, or GADab+) [10].

### 2.3. Genomic DNA preparation

The QIAamp spin columns (QIAGEN GmbH, Hilden, Germany) were used for extraction and purification of genomic DNA from peripheral blood leukocytes.

### 2.4. Analysis of HLA DRB1 and DQB1 alleles

HLA DRB1 and DQB1 alleles were genotyped using PCR with sequence specific primers (SSP-PCR) supplied by Genovision (Oslo, Norway) [11,12].

### 2.5. Statistics

Allele and gene frequencies were calculated using standard methods. The significance of the differences in allele frequencies were evaluated by $\chi^2$ and P values were calculated with Bonferoni correction. The strength of the observed associations was estimated by calculating odds ratios (OR) using the method by Woolf.

### 2.6. Group of patients

We investigated 334 patients (190 women) with diabetes mellitus with disease onset after 35 years of age. All of them were Caucasians and residents of the Middle region of Czech Republic. Diagnosis of diabetes was established according to the current WHO definitions and criteria for diagnosing diabetes [13]. The T1DM (49), LADA (48), and T2DM (237) patients were included. Patients with autoimmune diabetes (T1DM and LADA) were negative for C-peptide and/or positive for autoantibodies. All of them had insulin therapy. LADA was defined by a minimum 6 months long phase after diagnosis without insulin therapy. Patients with T2DM were positive for C-peptide. None ever had insulin therapy. The age at disease onset and duration of DM were recorded. For detailed characterization of patient groups see Table 1.

Table 1
Clinical characteristics of investigated patient groups

| | CP+ (≥200 pmol/l) | CP− (≤200 pmol/l) | GADab+ (≥32 ng/ml) | GADab− (≤32 ng/ml) |
|---|---|---|---|---|
| Number of patients | 285 | 49 | 97 | 237 |
| Number of female | 153 | 32 | 58 | 136 |
| Body mass index (BMI) | 31.3 (22.2; 50.1) | 28.0 (22.4; 36.7) | 31.1 (22.4; 45.5) | 31.1 (22.2; 50.1) |
| Age of onset of DM (years) | 53 (35; 81) | 44 (36; 56) | 52 (35; 71) | 53 (35; 81) |
| Duration of DM (years) | 12.35 (0; 31) | 14.46 (1; 29) | 13.27 (1; 31) | 12.48 (0; 31) |
| Diagnosis | | | | |
| T1DM, $N = 49$ | 0.0% (0/285) | 100.0% (49/49) | 50.5% (49/97) | 0.0% (0/237) |
| LADA, $N = 48$ | 16.8% (48/285) | 0.0% (0/49) | 49.5% (48/97) | 0.0% (0/237) |
| T2DM, $N = 237$ | 83.2% (237/285) | 0.0% (0/49) | 0.0% (0/97) | 100.0% (237/237) |

Values of BMI, age of onset of disease, and duration of DM are calculated as average. In brackets are minimal and maximal values.

Table 2
HLA DQB1 frequencies in investigated patient groups (GADab+, GADab−, CP+, and CP−)

| | GADab+ ($N = 45$) | GADab− ($N = 146$) | CP+ ($N = 153$) | CP− ($N = 38$) | Controls ($N = 99$) |
|---|---|---|---|---|---|
| DQB1*0201 | 0.467 | 0.267 | 0.288 | 0.447 | 0.303 |
| DQB1*0202 | 0.133 | 0.185 | 0.190 | 0.105 | 0.192 |
| DQB1*0301 | 0.267 | 0.315 | 0.333 | 0.184 | 0.384 |
| DQB1*0302 | 0.289 | 0.205 | 0.170 | 0.447 (6.48)* | 0.111 |
| DQB1*0303 | 0.111 | 0.048 | 0.065 | 0.053 | 0.081 |
| DQB1*0304 | 0.022 | 0.021 | 0.020 | 0.026 | 0.000 |
| DQB1*0402 | 0.067 | 0.048 | 0.059 | 0.026 | 0.040 |
| DQB1*0501 | 0.178 | 0.281 | 0.248 | 0.316 | 0.232 |
| DQB1*0502 | 0.022 | 0.068 | 0.059 | 0.053 | 0.051 |
| DQB1*0503 | 0.000 | 0.062 | 0.059 | 0.000 | 0.051 |
| DQB1*0504 | 0.000 | 0.014 | 0.013 | 0.000 | NT |
| DQB1*0601 | 0.022 | 0.021 | 0.020 | 0.026 | 0.030 |
| DQB1*0602 | 0.067 | 0.164 | 0.170 | 0.026 | 0.202 |
| DQB1*0603 | 0.022 | 0.110 | 0.098 | 0.053 | 0.131 |
| DQB1*0604 | 0.111 | 0.062 | 0.065 | 0.079 | 0.051 |

Statistically significant difference in frequency of allele DQB1*0302 in group CP− in comparison with healthy control. Data from healthy control used from Cerna et al. [14].
* $P_c < 0.001$; OR values in brackets.

## 3. Results

We investigated the distribution of HLA DRB1 and DQB1 alleles and the odds ratios of risk and protective alleles in the four patient groups (Table 1). Subsequently, we explored the relationship between HLA risk or protective alleles, and positivity (negativity) for GADab and values of CP, respectively.

In the GADab+ group, we found only one predisposing allele (DRB1*03, $P_c = 0.0002$). In the GADab− group, we did not find any association with HLA alleles. In the CP− group, we detected two risk alleles, DRB1*04 ($P_c = 0.0001$) and DQB1*0302 ($P_c = 0.0002$). In the CP+ group, we did not detected any HLA association. None protective alleles were ascertained in all these groups (see Tables 2 and 3). Odds ratios of the predisposing alleles were following: in the GADab+ group, OR = 4.90 (CI 95% = 2.21; 10.94) was calculated for allele DRB1*03; in the CP−group, OR = 5.80 (CI 95% = 2.36; 14.39) was calculated for allele DRB1*04; and OR = 6.48 (CI 95% = 2.43; 17.54) for allele DQB1*0302.

## 4. Discussion

We analyzed the frequencies of HLA class II alleles in patients with diabetes mellitus manifested after 35 years of age. We found two risk HLA alleles in the CP− group and one in the GADab+ group of patients. The frequencies of the DRB1*04 and DQB1*0302 alleles were significantly increased in the CP− group ($P_c < 0.001$ for both alleles). The odds ratio was higher for DQB1*0302 (OR = 6.48) than for DRB1*04 (OR = 5.80), what suggests DQB1*0302 as main risk allele in the CP−group. The frequency of DRB1*03 allele was significantly higher in the GADab+ group ($P_c <$ 0001). Its odds ratio was the lowest in the study (OR = 4.90). Our results are similar to data published by Caillat-Zucman et al. [7] or Krokowski et al. [8]. The only difference is in the frequency of the known risk allele DQB1*0201. The frequency of this allele was increased in our GADab+ group, but it did not reach statistically significant difference when compared with controls.

Table 3
HLA DRB1 frequencies in investigated patient groups (GADab+, GADab−, CP+, and CP−)

| | GADab+ ($N = 51$) | GADab− ($N = 111$) | CP+ ($N = 122$) | CP− ($N = 43$) | Controls ($N = 99$) |
|---|---|---|---|---|---|
| DRB1*01 | 0.157 | 0.198 | 0.164 | 0.233 | 0.222 |
| DRB1*03 | 0.569 (4.90)* | 0.162 | 0.221 | 0.465 | 0.212 |
| DRB1*04 | 0.314 | 0.270 | 0.230 | 0.488 (5.80)* | 0.141 |
| DRB1*07 | 0.118 | 0.252 | 0.230 | 0.163 | 0.363 |
| DRB1*08 | 0.098 | 0.081 | 0.090 | 0.070 | 0.050 |
| DRB1*09 | 0.020 | 0.027 | 0.033 | 0.000 | 0.010 |
| DRB1*10 | 0.000 | 0.045 | 0.033 | 0.047 | 0.020 |
| DRB1*11 | 0.255 | 0.225 | 0.262 | 0.140 | 0.232 |
| DRB1*12 | 0.000 | 0.018 | 0.016 | 0.000 | 0.101 |
| DRB1*13 | 0.137 | 0.135 | 0.156 | 0.093 | 0.222 |
| DRB1*14 | 0.020 | 0.108 | 0.107 | 0.000 | 0.050 |
| DRB1*15 | 0.176 | 0.171 | 0.197 | 0.093 | 0.232 |
| DRB1*16 | 0.020 | 0.054 | 0.041 | 0.047 | 0.050 |

Statistically significant difference in frequency of allele DRB1*03 in group GADab+ and allele DRB1*04 in group CP− in comparison with healthy control. Data from healthy control used from Cerna et al. [14].
* $Pc < 0.001$; OR values in brackets.

Our study showed that there is a difference in HLA class II allele frequencies between GADab+ group and CP−group. GADab positivity is strongly associated with the HLA DRB1*03 allele and CP negativity is linked to the DQB1*0302 and HLA DRB1*04 alleles. These results are supported by Gambelunghe et al. [9] who stated that GADab is closely associated with the presence of HLA DRB1*03-DQB1*02 haplotype. Finally, we conclude that the DRB1*04 and DQB1*0302 alleles are associated with decrease of CP levels while DRB1*03 is a significant genetic marker of autoantibody (GADab) development.

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# Interleukin IL-18 gene promoter polymorphisms in adult patients with type 1 diabetes mellitus and latent autoimmune diabetes in adults

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**Abstract**

Interleukin-18 (IL-18) gene promoter polymorphism is known as a genetic risk factor for child type 1 diabetes mellitus development. To test the role of IL-18 gene polymorphism in predisposition to adult type 1 diabetes (T1DM) and latent autoimmune diabetes in adults (LADA), we analysed SNPs at position −607 (C/A) and −137 (G/C) in the promoter region of IL-18 gene by sequence-specific PCR in 49 T1DM, 66 LADA patients and 139 healthy controls. We found differences in allele, genotype or haplotype distribution in tested patients when compared to frequencies found in control group but these differences did not reach statistical significance. However, there was a difference in −607 (C/A) allele and genotype distribution found in LADA and T1DM patients that reached statistical significance. These results suggest that the IL-18 gene promoter polymorphisms are not associated with adult type 1 diabetes or LADA susceptibility, and according to our findings genes involved in onset and progression of LADA and T1DM are probably different.
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*Keywords:* IL-18; Diabetes mellitus; Czech population

## 1. Introduction

Autoimmune diabetes in humans is believed to be a $T_H1$ lymphocyte-mediated disease, and both environmental and genetic factors play a role in its pathogenesis [1–3]. Type 1 diabetes mellitus (T1DM) is characterized by an abrupt onset and ketosis. It is associated with HLA DRB1*04-DQA1*0301-DQB1*0302, and a high frequency of insulin (IAA) and thyrosin-phosphatase (IA-2) autoantibodies [4,5]. The term latent autoimmune diabetes in adults (LADA) was introduced to define adult diabetic patients initially non-insulin-requiring but with immune markers of type 1 diabetes that, in most cases, progress to insulin dependency [6]. It is characterized by the presence of glutamic acid decarboxylase 65 autoantibodies (GADab) and/or islet cell antibodies (ICA), and is associated with HLA DRB1*03-DQA1*0501-DQB1*0201 and/or with HLA DRB1*04-DQA1*0301-DQB1*0302 haplotype [7,8].

It was recently reported by Nicoletti et al. [9] that interleukin (IL)-18 serum levels are increased in the sub clinical stage of type 1 diabetes in first-degree relatives of type 1 diabetic patients. IL-18, which is predominantly secreted by activated monocytes/macrophages, is a pleiotropic cytokine involved in the regulation of innate and acquired immune response, playing a key role in autoimmune, inflammatory, and infectious diseases [10]. IL-18 acts as a proinflammatory

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Table 1
Clinical characteristics of investigated patient groups

| | T1DM | LADA |
|---|---|---|
| Number of patients | 49 | 66 |
| Number of female | 22 | 39 |
| Body mass index (BMI) | 28.8 (22.4–45.5) | 31.5 (24.4–38.8) |
| Age at disease onset (years) | 30 (22–45) | 50 (35–71) |
| Duration of DM (years) | 18.7 (1–37) | 13.4 (1–31) |

Values of BMI, age of onset, and duration of diabetes are calculated as average. In brackets are minimal and maximal values.

factor and, in synergy with IL-12, promotes development of $T_H1$ lymphocyte response by induction of γ-interferon (IFN-γ) production, modulates activity of NK cells, increases tumour necrosis factor-α and IL-1 production by macrophages, up-regulates the expression of adhesion molecules, and induces nitric oxide production in the area of chronic inflammation [10,11].

Single nucleotide polymorphisms (SNPs) at positions −607 and −137 in IL-18 gene promoter are associated with child T1DM in some Caucasian populations [12]. The aim of our study was to evaluate the frequency of known polymorphisms in the IL-18 gene promoter in adult patients with T1DM and LADA in Czech population and to analyse the impact of these polymorphisms for disease onset and progression.

## 2. Patients and methods

### 2.1. Patients

A total of 115 adult Czech unrelated patients with autoimmune diabetes mellitus were studied after giving their informed consent. All of them were Caucasians and residents of the Middle region of Czech Republic. The diagnosis of type 1 diabetes mellitus and latent autoimmune diabetes in adults was based on criteria of the current WHO definitions for diagnosing diabetes [13], considering patients' clinical features and laboratory data, including the presence of anti-islet autoantibodies (autoantibodies to GAD65, IA-2 and insulin) and serum C-peptide level. All of the patients had insulin therapy. LADA was defined by a minimum 6 months long phase after diagnosis without insulin therapy. For detailed characteristics of investigated patients see Table 1.

### 2.2. IL-18 gene promoter polymorphisms

The single nucleotide polymorphisms at positions −607 (A/C) and −137 (C/G) in the promoter region of the human IL-18 gene, located at chromosome 11q22.2-q22.3, were analysed by sequence-specific PCR. Analysed genomic DNA was extracted from peripheral blood anticoagulated with EDTA using the QIAamp DNA Blood Kit (Qiagen GmbH, Hilden, Germany). For the position −607 two sequence-specific primers (5′-GTTGCAGAAAGTGTAAAAATTATTAC-3′ or 5′-GTTGCAGAAAGTGTAAAAATTATTAA-3′), a control primer (5′-CTTTGCTATCATTCCAGGAA-3′), and a common reverse primer (5′-TAACCTCATTCAGGACTTCC-3′) were used. For the position −137 two sequence-specific primers (5′-CCCCAACTTTTACGGAAGAAAAG-3′ or 5′-CCCCAACTTTTACGGAAGAAAAC-3′), a control primer (5′-CCAATAGGACTGATTATTCCGCA-3′), and a common reverse primer (5′-AGGAGGGCAAAATGCACTGG-3′) were used [14]. Control primers were used to amplify fragments covering the polymorphic sites as an internal positive amplification control. The PCR was performed in a 10 μl volume and reaction conditions were different from those used by Giedraitis et al [14]. The concentration of reagents and cycling conditions were optimized as follows: 1 μM of sequence-specific primer, 1 μM of control forward primer, 2 μM of common reverse primer, 2.5 mM $MgCl_2$, 200 μM of each dNTPs, 1.0 μl of 10× PCR buffer, 1 U of Taq polymerase (MBI Fermentas, Germany) and approximately 50 ng of genomic DNA. The reaction started with an initial denaturation at 95 °C for 4 min and followed in standard PCR protocol with 30 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 35 s, and extension at 72 °C for 50 s. A final extension at 72 °C for 5 min completed the reaction. All PCR products were separated by electrophoresis on 2% agarose gel and visualized, stained with ethidium bromide. We used different $Mg^{2+}$, dNTPs and primer concentration in our SSP—PCR protocol in comparison with original protocol used by Giedraitis et al [14], the total amount of Taq polymerase and cycling conditions were also slightly changed. These changes did not affect the sensitivity or specificity of our PCR test.

### 2.3. Statistical analysis

Allele, genotype or haplotype frequencies were calculated on patients and control subjects by direct counting. Statistical analysis of the differences between groups was determined by Chi-square test, or Fisher's exact probability test. The statistical difference of the allele, genotype or haplotype distribution of the IL-18 gene was analysed by Chi-square test for trend. Findings were considered statistically significant at a Bonferoni corrected $P$ value less than 0.05.

## 3. Results

We investigated the distribution of −607 (A/C) and −137 (C/G) SNP polymorphisms in IL-18 gene promoter in selected patient groups. We analysed allele, genotype and haplotype frequencies. Subsequently, observed frequencies were compared to those found in control group and $P$ values were calculated.

The allele frequencies of −607C and −137G were 43.9% and 65.3%, respectively, in 49 patients with type 1 diabetes,

Table 2
Frequency of alleles of IL-18 gene promoter polymorphisms in control subjects and adult patients with T1DM and LADA

| Loci | | Alleles | | | | |
|---|---|---|---|---|---|---|
| | | Control subjects ($N$=278) | T1DM ($N$=98) | $P$ | LADA ($N$=132) | $P$ |
| −137 | G | 198 (71.2%) | 64 (65.3%) | NS | 99 (75.0%) | NS |
| | C | 80 (28.8%) | 34 (34.7%) | | 33 (25.0%) | |
| −607 | C | 160 (57.6%) | 43 (43.9%) | NS | 85 (64.4%) | NS |
| | A | 118 (42.4%) | 55 (56.1%) | | 47 (35.6%) | |

Table 3
Frequency of genotypes of IL-18 gene promoter polymorphisms in control subjects and adult patients with T1DM and LADA

| Loci | | Genotypes | | | | |
|---|---|---|---|---|---|---|
| | | Control subjects ($N$=139) | T1DM ($N$=49) | $P$ | LADA ($N$=66) | $P$ |
| −137 | GG | 68 (48.9%) | 22 (44.9%) | NS | 36 (54.5%) | NS |
| | GC | 62 (44.6%) | 20 (40.8%) | | 27 (40.9%) | |
| | CC | 9 (6.5%) | 7 (14.3%) | | 3 (4.5%) | |
| −607 | CC | 43 (30.9%) | 9 (18.4%) | NS | 22 (33.3%) | NS |
| | CA | 74 (53.2%) | 25 (51.0%) | | 41 (62.1%) | |
| | AA | 22 (15.8%) | 15 (30.6%) | | 3 (4.5%) | |

Table 4
Frequency of haplotypes of IL-18 gene promoter polymorphisms in control subjects and adult patients with T1DM and LADA

| Haplotypes | Control subjects ($N$=139) | T1DM ($N$=49) | $P$ | LADA ($N$=66) | $P$ |
|---|---|---|---|---|---|
| −137CC/−607AC | 1 (0.7%) | 1 (2.0%) | NS | 1 (1.5%) | NS |
| −137CC/−607AA | 8 (5.8%) | 7 (14.3%) | NS | 2 (3.0%) | NS |
| −137CC/−607CC | 0 (0.0%) | 0 (0.0%) | NS | 0 (0.0%) | NS |
| −137CG/−607AC | 50 (36.0%) | 13 (26.5%) | NS | 26 (39.4%) | NS |
| −137CG/−607AA | 11 (7.9%) | 7 (14.3%) | NS | 1 (1.5%) | NS |
| −137CG/−607CC | 1 (0.7%) | 0 (0.0%) | NS | 0 (0.0%) | NS |
| −137GG/−607AC | 23 (16.5%) | 12(24.5%) | NS | 14 (21.2%) | NS |
| −137GG/−607AA | 3 (2.2%) | 0 (0.0%) | NS | 0 (0.0%) | NS |
| −137GG/−607CC | 42 (30.2%) | 9 (18.4%) | NS | 22 (33.3%) | NS |

or 64.4% and 75.0% in 66 patients with LADA. Allele frequencies found in LADA patients were similar to those observed in 139 healthy control subjects. Allele frequencies in T1DM patients and control group were different, but these differences were not significant (Table 2). The distribution of genotypes at positions −607 and −137 was not significantly different between patients with T1DM and healthy controls. We also did not find any statistical difference when genotypes at positions −607 and −137 were compared in LADA patients and healthy controls (Table 3). Genotype frequencies of these SNPs in healthy control subjects were statistically consistent with Hardy–Weinberg equilibrium. The haplotype frequencies in T1DM or LADA patients and control subjects were different, but these differences did not reach statistical significance (Table 4). We also found differences in allele and genotype frequencies between LADA and T1DM patients. The difference in distribution of −607 (A/C) alleles was near the statistical significance in T1DM and LADA patients ($P < 0.05$). Moreover, the distribution of −607 genotypes differed in T1DM patients when compared with LADA patients. This difference reached statistical significance with $P < 0.01$ (Table 5).

## 4. Discussion

In the present study we demonstrate that the susceptibility to adult type 1 diabetes mellitus as well as to latent autoimmune diabetes in adults is not associated with SNP polymorphisms at positions −607 and −137 in IL-18 gene promoter. Authors Kretowski et al [12], recently reported that SNP polymorphisms at positions −607 and −137 are associated with child type 1 diabetes mellitus in Polish population. The frequency of −137C allele was increased when compared to controls ($P = 0.002$) and the distribution of the −607 and −137 genotypes was different from controls ($P = 0.001$; $P = 0.0015$, respectively). Frequencies of haplotypes were also different in patients and controls [12]. Similar study of IL-18 gene promoter SNP polymorphisms was performed in Japanese population by Ide et al [15]. It showed that distribution of −607 genotypes in type 1 diabetic patients differed from controls ($P = 0.023$). These findings indicate that IL-18 promoter polymorphisms could play a part in the pathogenesis of T1DM. No studies investigating the connection between −137/−607 SNP polymorphisms in IL-18 gene promoter and LADA were performed up to date.

Table 5
Comparison of −607(C/A) alleles and genotypes distribution in adult patients with T1DM and LADA

| Locus | Allele/Genotype | T1DM | LADA | P |
|---|---|---|---|---|
| N | | 49 (98) | 66 (132) | |
| −607 | C | 43 (43.9%) | 85 (64.4%) | <0.05 |
| | A | 55 (56.1%) | 47 (35.6%) | * |
| −607 | CC | 9 (18.4%) | 22 (33.3%) | <0.01 |
| | CA | 25 (51.0%) | 41 (62.1%) | |
| | AA | 15 (30.6%) | 3 (4.5%) | |

The statistical significant differences were found between these two groups ($P < 0.05$ and $P < 0.01$, respectively).

The onset and progression of autoimmune diabetes mellitus is affected by both environmental and genetic factors. In our study we found differences in the allele, genotype or haplotype frequencies of the IL-18 gene polymorphisms between adult T1DM patients or LADA patients and healthy controls but these differences did not reach statistical significance. However, we found statistical significant difference in −607 allele and genotype distribution between LADA and T1DM patients. Effect of environmental or genetic factors on the onset of autoimmune diabetes mellitus seems to be different in child T1DM, adult T1DM and LADA. It is already known that different genetic risk factors can affect the onset and progression of LADA and T1DM. HLA class II alleles affect both diseases. However, the DRB1*03-(DQB1*0201) haplotype is associated with susceptibility to LADA and the DRB1*04-DQB1*0302 haplotype is associated with genetic susceptibility to adult T1DM [16]. The alleles of microsatellite polymorphism of MIC-A gene are also associated with the development of these diseases, the A5 allele with T1DM and the allele A5.1 with LADA [17,18]. Genes affecting autoimmune diabetes are also the CTLA-4 and INS-VNTR gene promoter. In this case the same alleles seem to be associated with the genetic susceptibility to T1DM or LADA [19,20].

In conclusion, the results in this study suggest that (a) IL-18 gene promoter polymorphisms at positions −607 and −137 are not associated with genetic susceptibility to adult type 1 diabetes mellitus or latent autoimmune diabetes mellitus in adults and (b) according to our findings we assume that differences in onset and progression of LADA and T1DM can be caused by different immunogenetic background of these two diseases.

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Paper 6: Novota P, **Kolostova K**, Pinterova D, Novak J, Weber P, Treslova L, Kovar J, Andel M, Cerna M: **Association of MHC class I chain related gene-A microsatellite polymorphism with the susceptibility to T1DM and LADA in Czech adult patients.** Int J Immunogenet 32:273-275, 2005

**Short Communication**

# Association of MHC class I chain related gene-A microsatellite polymorphism with the susceptibility to T1DM and LADA in Czech adult patients

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## Summary

The results in this study suggest that microsatellite polymorphism within the transmembrane region of MIC-A gene is associated with genetic susceptibility to adult-onset of type 1 diabetes mellitus (T1DM), MIC-A5.1 allele, corrected $P = 0.001$, whereas it is not associated with latent autoimmune diabetes in adults (LADA) in Czech population. According to our findings, we can hypothesize that adult-onset T1DM and LADA may have partly different immunogenetic aetiopathogenesis.

The human major histocompatibility complex class I chain-related gene A (MIC-A) is located within the MHC class I region of the chromosome 6 satellite polymorphism within this region (Fodil *et al.*, 1996; Ota *et al.*, 1997). So far, six alleles of the exon 5 of the MIC-A gene, which differ in number of (GCT) repetitions at position 296, have been identified (Ota *et al.*, 1997; Perez-Rodriguez *et al.*, 2000). Several recent studies of MIC-A transmembrane polymorphism were focused on the investigation of its relationship to autoimmune diabetes mellitus. It has been suggested that the A5 allele of exon 5 is associated with the susceptibility to young-onset type 1 diabetes mellitus (T1DM) (Gambelunghe *et al.*, 2000; Gupta *et al.*, 2003). Conversely, it does not confer increased risk for adult-onset T1DM (Gambelunghe *et al.*, 2001). In this paper we investigated the association of MIC-A exon 5 alleles with the adult-onset T1DM and latent autoimmune diabetes in adults (LADA) in Czech population based on case–control study.

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A total of 115 adult Czech unrelated patients suffering from autoimmune diabetes mellitus onset after 35 years of age were studied after giving their informed consent. All of them were Caucasians and residents of the middle region of the Czech Republic. The mean age was 50 years (35; 71) in LADA group and 46 years (35; 60) in T1DM group. The LADA group comprised of 54% of males and T1DM group 45% of males. The age and sex characteristics of control group were similar to investigated patient groups. The diagnoses of T1DM and LADA were based on criteria of the current WHO definitions for diagnosing diabetes (1999), considering patients' clinical features and laboratory data, including the presence of anti-islet autoantibodies, autoantibodies to GAD65, IA-2 and insulin, and serum C-peptide level. All of the patients had insulin therapy. LADA was defined by a minimum of 6-month-long phase after diagnosis without insulin therapy.

Analysed DNA samples were extracted from peripheral blood anticoagulated with EDTA using a QIAamp DNA Blood Kit (Qiagen GmbH, Hilden, Germany). PCR amplification of the polymorphic transmembrane region encoded by exon 5 was carried out with forward primer Cy5–5′-GCTGGTGCTTCAGAGTCATTGGC-3′ labelled at 5′ end with Cy-5 reagent and reverse primer 5′-GGAC-CCTCTGCAGCTGATGTTTTC-3′ (Generi Biotech, Hradec Králové, Czech Republic) (Novota *et al.*, 2004). Electrophoretic separation of PCR products was performed in the ALFexpress II DNA analysis system (Amersham Biosciences, Vienna, Austria).

Statistical analysis of the differences between groups was determined by Chi-square test, or Fisher's exact probability test. Findings were considered statistically significant at corrected *P*-value less than 0.05. The *P*-values were corrected for the number of alleles or genotypes observed among healthy controls. The strength of the observed associations was estimated by calculating odds ratios (OR) using the method by Woolf.

The most frequent allele in the adult-onset T1DM group was the A5.1 allele and the less frequent was the A5 allele with prevalence of 87.8% and 8.2%, respectively. Similarly, in the LADA group the most common allele was

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**Table 1.** Frequencies of alleles within the transmembrane region of MIC gene A in control subjects and adult patients with T1DM and LADA. Increased frequency of alleles A5.1 in T1DM with $P_c$ = 0.001 [OR = 491 CI(95%) = 1.94; 12.45]

| Allele | Control subjects *N* = 118 (236) | T1DM *N* = 49 (98) | $P_c$ | LADA *N* = 66 (132) | $P_c$ |
|---|---|---|---|---|---|
| A4 | 25.4% (30) | 20.4% (10) | NS | 15.2% (10) | NS |
| A5 | 24.6% (29) | 8.2% (4) | NS | 27.7% (15) | NS |
| A5.1 | 59.3% (70) | 87.8% (43) | 0.001 (OR = 4.91) | 63.6% (42) | NS |
| A6 | 32.2% (38) | 26.5% (13) | NS | 24.2% (16) | NS |
| A9 | 28.8% (34) | 24.5% (12) | NS | 42.4% (28) | NS |

NS, not significant

the A5.1 allele, but the rarest one was the A4 allele with frequencies of 63.6% and 15.2%, respectively. The frequency of the A5.1 allele was significantly increased in the adult-onset T1DM group when compared to controls. The corrected *P*-value was 0.001 and the odds ratio evaluated for this allele was OR = 4.91 with CI(95%) between 1.94 and 12.45 (Table 1).

The decreased incidence of the A5 allele was found among the adult-onset T1DM group when compared to controls, but this difference did not reach statistical significance. Increased frequencies of A5.1 and A9 alleles in the LADA group were found in comparison to controls. However, these differences did not reach statistical significance. Moreover, differences in allele frequencies among the T1DM and LADA groups were found, but these also did not reach statistical significance.

The frequencies of 15 MIC-A genotypes observed in controls and both disease groups were calculated and compared with each other. Increased frequencies of A4/A5.1, A5.1/A6 and A5.1/A5.1 genotypes and lower prevalence of A5/A5.1, A5/A9, A6/A9 and A4/A6 genotypes were found in the adult-onset T1DM group when compared to controls, but these differences did not reach statistical significance. However, increased frequency of A5.1/A9 genotype and decreased incidence of the A4/A6 allele was recorded in the LADA group when compared to controls, but these differences also did not reach statistical significance. When distributions of MIC-A genotypes between T1DM and LADA patients were compared, no statistical significant differences were found.

Our study shows that MIC-A microsatellite polymorphism is associated with the susceptibility to adult-onset T1DM. According to our results adult-onset T1DM development seems to be associated with the A5.1 allele. Conversely, we did not find association between microsatellite polymorphism in MIC-A gene and LADA.

Recent studies have confirmed that microsatellite alleles of MIC-A gene is associated with Behcet's disease (Mizuki *et al.*, 1999; Wallace *et al.*, 1999; Mok *et al.*, 2003), seronegative spondylarthropathies (Tsuchiya *et al.*, 1998) and inflammatory bowel disease (Orchard *et al.*, 2001; Ahmad *et al.*, 2002). Secondary association of the transmembrane MICA A5.1 allele with coeliac disease has been also suggested (Fernandez *et al.*, 2002; Lopez-Vazquez *et al.*, 2002a,b; Rueda *et al.*, 2003). Our finding that the transmembrane A5.1 allele is associated with the adult-onset T1DM is supported by several studies focused on the investigation of the relation between transmembrane MIC-A alleles and autoimmune diabetes mellitus. These studies showed that the microsatellite A5 allele is associated with the susceptibility to young-onset T1DM while the A5.1 allele is associated with adult-onset T1DM (Gambelunghe *et al.*, 2000, 2001; Gupta *et al.*, 2003). Findings that different alleles of MIC-A gene are associated with young-onset T1DM and adult-onset T1DM support the hypothesis that these two diseases could have different immunogenetic background. In contrast with several studies (Gambelunghe *et al.*, 2001; Berzina *et al.*, 2002), we have not proved that LADA development is associated with microsatellite polymorphism within the MIC-A gene.

Our study shows now that the microsatellite polymorphism of the MIC-A gene indicates the risk for autoimmune diabetes mellitus development. Until now, it has not been stated that MIC-A protein is normally expressed on pancreatic β-cells that's the way the association with autoimmune diabetes might be the result of linkage disequilibrium between the microsatellite polymorphism within the transmembrane region of the MIC-A gene and a still unidentified gene in the HLA region. Because the MIC-A gene is expressed only on intestinal epithelium, it could play a role in mucosal immunity and trigger autoimmune reactions.

In conclusion, the results in this study suggest that microsatellite polymorphism within the transmembrane region of MIC-A gene is associated with genetic susceptibility to adult-onset T1DM whereas we did not prove its association with LADA. According to our findings, we can hypothesize that adult-onset T1DM and LADA may have partly different immunogenetic aetiopathogenesis.

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Paper 7: Romzova M, Hohenadel D, **Kolostova K**, Pinterova D, Fojtikova M, Ruzickova S, Dostal C, Bosak V, Rychlik I, Cerna M: NFkappaB and its inhibitor IkappaB in relation to type 2 **diabetes and its microvascular and atherosclerotic complications.** Hum Immunol 67:706-713, 2006

# NFκB and Its Inhibitor IκB in Relation to Type 2 Diabetes and Its Microvascular and Atherosclerotic Complications

Marianna Romzova, Daniela Hohenadel, Katarina Kolostova, Daniela Pinterova, Marketa Fojtikova, Sarka Ruzickova, Ctibor Dostal, Vladimir Bosak, Ivan Rychlik, and Marie Cerna

ABSTRACT: Nuclear factor κ B (NFκB) is an important transcription factor that together with its inhibitor (IκB) participates in the activation of genes involved in immune responses. We examined the CA repeat polymorphism of the NFKB1 gene (encoding for NFκB) and A/G point variation in the 3′UTR region of the nuclear factor kappa B inhibitor alpha (NFKBIA) gene (encoding for IκB) in Czech and German patients with type 2 diabetes. The sample consisted of 211 patients, both with and without kidney complications, and 159 controls. Additionally, 152 patients with systemic lupus erythematosus (SLE) were genotyped for NFKBIA polymorphism. We observed a significant increase in the homozygous AA genotype of the NFKBIA gene when compared with the control group (the highest value was in diabetics without diabetic nephropathy [$p_c$* = 0.0015, odds ratio = 3.59]). No differences were seen between the SLE and control groups. With regard to the polymorphism of the NFKB1 gene, we did not observe any significant differences between the groups. Since the AA genotype of the NFKBIA gene presents a risk for type 2 diabetes development but not for diabetic nephropathy alone, we believe that the NFκB gene polymorphism can influence the pathogenesis of diabetes mellitus and affect its complications. Negative findings relative to other inflammatory autoimmune diseases, such as SLE, suggest a specific relationship between NFκB and type 2 diabetes mellitus. *Human Immunology 67, 706–713 (2006).* © American Society for Histocompatibility and Immunogenetics, 2006. Published by Elsevier Inc.

KEYWORDS: Nuclear factor κ B; inhibitor of nuclear factor κ B; diabetic nephropathy; type 2 diabetes; systemic lupus erythematosus

ABBREVIATIONS

| | |
|---|---|
| DN | diabetic nephropathy |
| DNTPs | deoxyribonucleotide triphosphates |
| HbAlc | hemoglobin Alc |
| IκB | inhibitor of nuclear factor κ B |
| IKK-β | IκB kinase β |
| INF-γ | interferon γ |
| NFκB | nuclear factor κ B |
| NDRD | nondiabetic renal diseases |
| PCR | polymerase chain reaction |
| SLE | systematic lupus erythematosus |

## INTRODUCTION

Diabetic nephropathy (DN) is the major cause of chronic renal failure in patients with diabetes mellitus. Nearly 30% of both type 1 and type 2 diabetic patients develop DN independently of glycemic control. The fact that DN manifests in only a subset of diabetics, together with racial/ethnic differences in the prevalence and family clustering, demonstrates its genetic independence from diabetes mellitus [1]. The risk factors that have been identified for the development of DN in longitudinal and cross-sectional studies include: race, genetic susceptibility, hypertension, hyperglycemia, hyperfiltration, smoking, advanced age, male sex, and a high-protein diet [2].

Nuclear factor κ B (NFκB) is a transcription factor that has been shown to be involved in the regulation of

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many genes that encode mediators of the immune response, embryo and cell lineage development, cell apoptosis, inflammation, cell cycle, oncogenesis, viral replication, and a variety of autoimmune diseases. Because it is activated by a variety of stimuli, the activation of NFκB is thought to be part of the stress response. These activating stimuli include reactive oxygen species and advanced glycation end products, which are toxic products of nonenzymatic glycation caused by long-term hyperglycemia and oxidative stress. At the cellular level, NFκB is activated through phosphorylation of an inhibitor of NFκB (IκB). Phosphorylated IκB is released from NFκB/IκB complex, allowing the translocation of NFκB molecules into the nucleus. Once in the nucleus, they bind to the consensus sequence (5′-GGGACTTTCC-3′) of various genes, thereby activating their transcription [3,4]. Recent studies have investigated the role of NFκB in the pathogenesis of various human diseases including neurologic disorders, immune deficiency, carcinogenesis, and atherogenesis. In addition, the possible link between NFκB and the development of insulin resistance and type 2 diabetes has also been suggested [3,5–8].

The NFκB transcription factor complex has two alternative DNA binding subunits, nuclear factor kappa B p 105 subunit (NFKB1) and NFKB2. The gene coding for NFKB1 is located on chromosome 4q23-q24 [9]. A polymorphic dinucleotide CA repeat, with 18 described alleles, has been identified close to the coding region of the human NFKB1 gene [10]. This polymorphism has recently been investigated for its role relative to increased susceptibility to type 1 diabetes mellitus (Kolostova et al., article in press) [11]. Encouraged by other studies that also suggest that an increased activation of NFκB is associated with the development of diabetic microvascular complications [12,13], we examined the CA repeat polymorphism of the NFKB1 gene in relation to diabetic nephropathy.

The gene coding for IκB (NFKBIA) has been mapped to chromosome 14q13, and A/G point variation in the 3′UTR region of NFKBIA has been detected. We also examined single nucleotide polymorphism of the IκB gene, looking for its involvement in the induction or progression of diabetic microvascular complications in the kidney.

In both analyses, we compared the entire group of diabetic patients (both those with and those without renal complications) with healthy controls drawn from Czech and German populations.

In addition, we also tested NFKBIA polymorphism in a second disease, systemic lupus erythematosus (SLE), to confirm or refute a specific association between NFκB and diabetic complications or diabetes itself.

## MATERIAL AND METHODS

### Subjects

The study of polymorphisms in the NFKBIA and NFKB1 genes involved 395 persons consisting of 246 diabetic patients and 159 control subjects. All subjects were of Caucasian descent and lived in either the Czech Republic or Germany.

The group of diabetic patients, most of whom were type 2 diabetics ($n$ = 211), were subdivided into three groups based on their renal status. The first group of patients ($n$ = 50) included persons with nondiabetic renal disease (NDRD). Diseases in this group included atherosclerotic renal disability, glomerulonephritis, focal segmental glomerulosclerosis, vascular nephrosclerosis, as well as inflammatory tubulointerstitial nephritis and chronic pyelonephritis. The second group of patients ($n$ = 118) consisted of persons with DN. The third group ($n$ = 78) consisted of patients who were excluded from groups 1 and 2 but were able to meet the following criteria: duration diabetes >15 years, normoalbuminuria (albumin <20mg/day), and were not using angiotensin-converting enzyme inhibitors, angiotensin II receptor antagonists, diuretics, or nonsteroidal anti-inflammatory drugs. All subjects were chosen on the basis of biochemical and clinical characterizations (Table 1).

For the genotyping of NFKBIA polymorphism in SLE patients, samples were collected from a group of affected persons ($n$ = 152) and a group of healthy controls ($n$ = 138). Both groups were chosen from the Czech and Slovak populations. The affected group was made up mostly of women (90%), with an average age of 47 years and an average SLE duration 17.5 years. The control group consisted of healthy persons with an average age of 40 years, with both sexes being almost equally represented.

Patients were recruited from the nephrology outpatient clinic of the 2nd Internal Medicine Department of the Faculty Hospital Kralovske Vinohrady in Prague, the private diabetology outpatient's clinic in Prague, the 5th Medical Department of the University Clinic in Mannheim, the Institute of Rheumatology in Prague, and the Institute of Rheumatology in Piestany. The control group came from blood donors recruited from the Blood Transfusion Department of the Faculty Hospital Kralovske Vinohrady in Prague. None of the healthy control subjects were taking any anti-inflammatory or immunosuppressive medication.

Written informed consents were obtained from all participants.

### Genotyping

DNA was extracted from collected samples using a modification of the Qiagen DNA blood maxi isolation method.

**TABLE 1** Clinical and biochemical characterizations of Czech and German patients in tested groups

| | Czech patients | | | German patients | |
|---|---|---|---|---|---|
| | NDRD | With DN | No DN | With DN | No DN |
| Women (%) | 40 | 40 | 45 | 46 | 53 |
| Average age (y) | 73 | 68 | 53 | 64 | 57 |
| Duration of DM (y) | 15.9 ± 8.65 | 18.5 ± 7.9 | 23 ± 8.1 | 13.7 ± 9.3 | 24.7 ± 8.4 |
| Hypertension (%) | 79 | 83 | 53 | 75 | 50 |
| Mean systolic BP | 144 ± 21.22 | 166 ± 26.0 | [a] | 142 ± 21.6 | 134.8 ± 21.6 |
| Mean diastolic BP | 83 ± 12.7 | 93 ± 11.6 | [a] | 79 ± 10.9 | 79 ± 12.0 |
| BP amplitude | 59 ± 15.74 | 73 ± 20.8 | [a] | 74 ± 11.11 | 68 ± 1.51 |
| History of MI (%) | 16 | 24 | [a] | 26 | 31 |
| History of stroke (%) | 13 | 7 | [a] | 12 | 6 |
| Diabetic retinopathy (%) | 18 | 49 | 7.5 | 91 | 44 |
| ACEi therapy (%) | 58 | 81 | 17.5 | 57 | 31 |
| Insulin therapy (%) | 45 | 46 | 20 | 55 | 53 |
| PAD (%) | 35 | 50 | 50 | 17 | 19 |
| HbA1 (g/L) | [a] | [a] | 5.6 ± 1.9 | 7.4 ± 1.55 | 7.4 ± 1.39 |
| Proteinuria (mg/L) | 0.39 ± 0.70 | 2.66 ± 0.82 | 8.75 ± 3.5 | 3.46 ± 2.2 | |
| Serum creatinin (μmol/L) | 169.5 ± 64 | 171 ± 89.4 | 103.9 ± 21.8 | [a] | [a] |

Abbreviations: DM = diabetes mellitus; BP = blood pressure; MI = myocardial infarction; ACEi = angiotensin-converting enzyme inhibitors; PAD = per oral antidiabetics; HbA1c = hemoglobin A1c.

[a] Unavailable data.

### NFKBIA

Genotyping of the NFKBIA point variation (A/G) polymorphism was performed using the restriction fragment length polymorphism (RFLP) method. Polymerase chain reaction (PCR) yielded 20 μl that contained: 50-100 ng genomic DNA, 1xPCR buffer, 2.5 mM MgCl2, 0.2 mM dNTPs, 0.5 mM of each primer, and 5U/μl Taq polymerase. Thermal conditions were: initial denaturation at 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 30 seconds, with a final extension of 72°C for 2 minutes. Following amplification, 10 μl of product was digested with HaeIII at 37°C. For genotype determination, samples were loaded into the wells of an ethidium bromide–stained 2% agarose gel.

We identified the following genotypes: the wild-type variant GG (nondigested) was characterized by fragment length 424 base pair (bp); the variant AA (completely digested) by 306bp and 118bp; and the heterozygote AG (partially digested) by 424bp, 306bp and 118bp fragments.

### NFKB1

Genotyping of CA repeat polymorphism in the NFKB1 gene involved the use of fluorescently labeled primers previously described by Ota *et al.* [10]. The PCR products were amplified under the following conditions: 20 μl of the reaction mixture contained 50-100 ng genomic DNA, 1xPCR buffer, 2.5 mM MgCl2, 0.2 mM dNTPs, 0.5 mM of each primer, and 5U/μl Taq polymerase. Thermal conditions were set at: 94°C for 4 minutes, followed by 30 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 30 seconds, with a final extension of 72°C for 5 minutes. We used the fragment analysis method, performed on ALFexpress fragment analyzer (Amersham Pharmacia Biotech, Uppsala, Sweden) with ALFwin software, for the detection of polymorphic alleles in the NFKB1 gene.

### Statistical Analysis

To determine significant differences in genotype and allele frequencies of the NFKBIA and NFKB1 genes, genotype and allele distributions were compared between affected and control populations using the $\chi^2$ test, followed by the Bonferroni correction for multiple comparisons ($p_c$). $p$ values $<0.05$ were considered significant.

## RESULTS

### Inhibitor of NFKBIA

Our study used PCR-based genotyping to investigate single-nucleotide polymorphism (A→G) in the 3′-UTR region of the NFKBIA gene in an attempt to access its possible role in the development or progression of DN in diabetic patients.

To determine whether this possible association is related to just nephropathy we compared diabetic patients with renal disease ($n = 117$) with those diabetic patients without renal disease ($n = 78$). We found no significant differences in allele or genotype frequencies ($\chi^2 = 2.75$; $p_c = 0.75$).

**TABLE 2** Frequencies of NFKBIA alleles and genotypes in tested groups with marked significant differences in comparison to the control group

| Studied groups | Frequencies (%) | | | | |
|---|---|---|---|---|---|
| | NFKBIA alleles | | NFKBIA genotypes | | |
| | f(A) | f(G) | f(AG) | f(AA) | f(GG) |
| Controls ($n$ = 159) | 45.0 | 55.0 | 56.0 | 17.0 | 27.0 |
| DM without DN ($n$ = 78) | 45.0 ($p$ = NS) | 55.0 ($p$ = NS) | 44.9 ($p$ = NS) | 42.3[a] $p_c$ = 0.0015[a] (OR = 3.59) | 12.8 ($p$ = NS) |
| DM with DN ($n$ = 111) | 57.0 ($p$ = NS) | 43.0 ($p$ = NS) | 53.2 ($p$ = NS) | 30.6[a] $p_c$ = 0.0381[a] (OR = 2.16) | 16.2 ($p$ = NS) |
| NDRD ($n$ = 40) | 52.5 ($p$ = NS) | 47.5 ($p$ = NS) | 20.0[a] $p_c$ = 0.0003[a] (OR = 0.20) | 42.5[a] $p_c$ = 0.0033[a] (OR = 3.61) | 37.5 ($p$ = NS) |
| T2DM ($n$ = 211) | 58.0 ($p$ = NS) | 42.0 ($p$ = NS) | 47.4 ($p$ = NS) | 34.6[a] $p_c$ = 0.0007[a] (OR = 2.59) | 18.06 ($p$ = NS) |

Abbreviations: NFKBIA = nuclear factor kappa B inhibitor alpha; DM = diabetes mellitus; DN = diabetic nephropathy; $p_c$ = $p$ value after Bonferroni's correction; NS = no significance; OR = odds ratio; T2DM = type 2 diabetes.

[a] Statistical significance.

With regard to allele frequencies we observed no differences between diabetic patients without DN and the control group. There was an increase in the frequency of the A allele in the diabetic NDRD group and the diabetic DN group as compared with the control group (Table 2), however, this increase was not statistically significant.

A statistically significant difference was observed in the frequencies of the NFKBIA genotypes between the diabetic group (Groups 1, 2, and 3) and the control group (Table 2). We observed a significant increase in the homozygous AA genotype in all tested groups; however, it was mainly seen in diabetic patients without DN (Groups 1 and 3) ($p_c$* = 0.0015, OR = 3.59). The expected decrease in frequency of the homozygous GG genotype did not prove to be significant when compared with the control group. An increased prevalence of the AA genotype ($p_c$* = 0.0033; OR = 3.61) was observed in the group of NDRD patients, but this was coupled with a significantly decreased prevalence of the AG genotype ($p_c$* = 0.0003; OR = 0.20).

Since our results suggested the involvement of NFKBIA polymorphism in the etiology of diabetes mellitus, we compared the allele frequencies of the controls with type 2 diabetic patients ($n$ = 211) collected in this study and observed that the AA genotype frequency was significantly increased ($p_c$* = 0.00075; OR = 2.59). There was also an increased frequency in allele A, but this was not statistically significant. To establish specificity of our findings, we also tested the NFKBIA polymorphism in SLE patients ($n$ = 152). We observed no differences in allele or genotype distribution between SLE patients and the control group.

## NFKB1

In addition to testing single-nucleotide polymorphism of the NFKBIA gene, we also sought an association between polymorphism in the NFKB1 gene and DN and type 2 diabetes. We tested 245 diabetic patients and 139 healthy controls for the polymorphism.

We identified 12 out of the previously described 18 alleles of the CA repeat in the regulatory region of the NFKB1 gene [11], ranging in size from 114 to 142bp, which corresponds to 12-26 CA repeats. The longest alleles (144-154bp), identified among United Kingdom subjects, were not found in our populations. The shortest ones (114-122bp) were found, but only in the control group. We did not find any statistical differences between the frequencies of NFKB1 alleles in diabetics with renal malfunction and those without. Compared with healthy controls, frequencies of observed alleles in type 2 diabetics were similar (Table 3). The most frequent alleles were A3 (23.2%) and A9 (35.3%).

We identified 43 genotypes in our samples of Czech and German populations, and we found no significant differences between the genotype frequencies of the diabetic groups compared with the control group. The most common genotypes were A3/A9 (124, 136) and A9/A9 (136, 136). Other frequent genotypes were: A6/A9, A4/A9, and A8/A9.

TABLE 3 Distribution and prevalence of the NFKB1 alleles among different populations (frequencies in %)

| | | Czech Republic | | Germany | Czech Republic | | | | United Kingdom | | Denmark | Spain | | | | Australia | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NFKB1 alleles | Length (bp) | C $n = 39$ | T2DM $n = 75$ | T2DM $n = 122$ | C $n = 37$ | T1DM $n = 67$ | LADA $n = 34$ | JDM $n = 55$ | C $n = 222$ | T1DM $n = 434$ | T1DM $n = 229$ | C $n = 200$ | RA $n = 197$ | SLE $n = 181$ | CD $n = 311$ | C $n = 109$ | C (BC) $n = 102$ | BC $n = 102$ |
| A01 | 114 | 0.4 | | | | | 1.47 | | | | | | | | | 2.94 | | |
| A02 | 116 | | | | | | 1.47 | | | | | | | | | 2.94 | 0.45 | |
| A03 | 118 | | | | | | 2.94 | | | | | | | | | | 0.98 | 1.96 |
| A1 | 120 | | | | | | | 0.9 | 0.45 | | | | | | | | | 0.98 |
| A2 | 122 | 0.7 | | | | 1.49 | | | 0.45 | 1.6 | 0.87 | | 0.5 | 0.4 | | | 24[b] | 25[b] |
| A3 | 124 | 22.7[b] | 21.9[b] | 18.1[b] | 23.7[b] | 23.13[b] | 20.58[b] | 19.1[b] | | 1.15 | 0.44 | 0.76 | 0.26 | 0.3 | | 23.5[b] | 11.8 | 6.37 |
| A4 | 126 | 5.8 | 3.4 | 8.2 | 10.5 | 5.97 | 2.94 | 10 | | 6.2 | 22.71[b] | 21.83[b] | 17.56[b] | 19.27[b] | 19.9[b] | | 0.49 | 1.98 |
| A5 | 128 | 3.6 | 0.7 | 2 | 4.4 | 2.23 | 2.94 | 1.9 | | 4.8 | 5.24 | 9.64 | 8.25 | 9.04 | 9.7 | | 4.4 | 8.3 |
| A6 | 130 | 8.6 | 10.3 | 13.1 | 7.9 | 11.19 | 14.7 | 4.5 | | 5.8 | 1.31 | 2.28 | 2.21 | 2.24 | 1.3 | 14.7 | 3.9 | 2.45 |
| A7 | 132 | 4.3 | 2.7 | 3.3 | 1.75 | 13.43[ab] | 2.94 | 0.9 | 0.45 | 5.8 | 9.17 | 4.57 | 7.25 | 6.35 | 6.8 | 2.94 | 8.8 | 13.7 |
| A8 | 134 | 9 | 7.5 | 6.6 | 8.8 | 12.68 | 14.7 | 10.9 | 19.8[b] | 6.2 | 4.59 | 6.09 | 8.75 | 7.34 | 9.7 | 14.7[b] | 37.8[b] | 34.8[b] |
| A9 | 136 | 3.7[b] | 43.2[b] | 43[b] | 36[b] | 24.6[b] | 27.9[b] | 38.4[b] | 9.9 | 14.9 | 10.26 | 11.68 | 10.2 | 10.8 | 12.1 | 2.94 | 3.4 | 1.96 |
| A10 | 138 | 3.6 | 5.5 | 3.3 | 2.6 | 2.47 | 4.41 | 4.5 | 2.7 | 17.5[b] | 34.93[b] | 32.99[b] | 36.28[b] | 34.67[b] | 28.6[b] | 2.94 | 3.9 | 2.45 |
| A11 | 140 | 2.9 | 2.7 | 2 | 2.6 | 0.74 | 4.41 | 8.1 | 9.9 | 10.6 | 3.093 | 5.08 | 3.5 | 4.27 | 5.8 | 5.88 | | |
| A12 | 142 | 1.4 | 2.1 | 0.4 | 1.75 | | | | 2.25 | 7.6 | 5.68 | 4.82 | 4.74 | 4.92 | 4.4 | | | |
| A13 | 144 | | | | | | | | 11.26 | 5.3 | 0.44 | 0.25 | 0.5 | 0.4 | 1.3 | | | |
| A14 | 146 | | | | | | | | 28.38[b] | 3.9 | 0.44 | | | | | | | |
| A15 | 148 | | | | | | | | 7.21 | 6.7 | | | | | | | | |
| A16 | 150 | | | | | | | | 5.41 | 1.6 | | | | | | | | |
| A17 | 152 | | | | | | | | 0.9 | 0.23 | | | | | | | | |
| A18 | 154 | | | | | | | | 0.9 | | | | | | | | | |

Abbreviations: NFKB1 = ; bp = ; C = controls; T2DM = type 2 diabetes mellitus patients; T1DM = type 1 diabetes mellitus patients; LADA = latent autoimmune diabetes in adult patients; JDM = juvenile diabetes mellitus patients; RA = rheumatoid arthritis; SLE = systematic lupus erythematosus patients; CD = celiac disease patients; C(BC) = controls for breast cancer patients; BC = breast cancer patients.

[a] Statistical significance found.

[b] Most frequent alleles in particular population.

## DISCUSSION

In this study we performed genetic analyses of two genes encoding NFKB1 and its inhibitor (NFKBIA) in patients with type 2 diabetes mellitus and SLE. The patients came from three central-European Caucasian populations. More than 200 type 2 diabetic patients, having had diabetes for at least 15 years, were tested. The diabetic patients were divided into a group of patients without complications, a group of patients with diabetic microvascular (DN) complications, and a group with macrovascular (NDRD) complications. Additionally, nearly 150 SLE patients were also tested. Because the Czech and German diabetic patients showed a similar distribution of NFKB1 and NFKBIA alleles, we put them together and compared them with the control group. There is evidence that Czech and German genetic backgrounds are similar, and other genetic studies have joined these two ethnic groups for increased validity [14,15]. Indeed, a study of NFKB1 gene polymorphism in Denmark used published data from the United Kingdom as their control group [16]. (Table 3). The Slovak and Czech patients, having originated from the same central-European Caucasian population, are considered to have a homogeneous genetic basis [17–19]. We also collected samples from a group of SLE patients and a control group and tested them for NFKBIA polymorphism. Since no divergences in the allele distribution between the two populations were observed, we included them in our study.

Although this study did not confirm any association between single-nucleotide polymorphism in the 3′UTR region of the NFKBIA gene or the CA repeat polymorphism of the NFKB1 gene and DN alone, we did detect an association between NFKBIA polymorphism and type 2 diabetes mellitus. In more than 200 type 2 diabetic patients we observed a significantly increased frequency of the AA genotype ($p_c$* = 0.00075; OR = 2.59). The value with the most statistical significance was observed for the AA risk genotype in diabetic patients without DN (Groups 1 and 3) ($p_c$* = 0.0033; OR = 3.61). We suspect that the AA genotype could represent a risk genotype for type 2 diabetes mellitus. Additional testing of 152 SLE patients proved our suspicion; we found no differences in allele or genotype frequencies, when comparing with the control group. The absence of an association between NFKBIA polymorphism and other diseases characterized by chronic inflammatory and autoimmune processes [20], where involvement of NFκB was presumed, indicates its specific relation to the pathogenesis of type 2 diabetes.

The AG genotype was significantly decreased in the NDRD group ($p_c$* = 0.0003; OR = 0.20), and probably renders protection against atherosclerosis. Our findings regarding the association between NFKBIA polymorphism and the NDRD group mirror the previous work of others. It suggests a possible role of NFκB in the degradation of the glomerular basement membrane and alteration of glomerular and tubular cell functions. The mechanism involves signaling pathways that trigger the transcription of genes, leading to hypertension, endothelial cell damage, and atherosclerotic changes under stress conditions. There has been an additional role hypothesized for NFκB in the etiopathogenesis of cardiovascular diseases [2,4,21].

It is known that 3′UTR is a regulatory region that is essential for the appropriate expression of many genes, specifically genes associated with the control of nuclear export, polyadenylation status, subcellular targeting, and rates of translation and degradation of mRNA [22]. These facts suggest a possible mechanism by which variation in the 3′UTR region of the NFKBIA gene could alter the function and structure of IκB. Aberrant IκB may not bind to NFκB effectively, allowing for sustained activity or preventing a reduction in activity. There is evidence that free fatty acids induce insulin activation of protein kinase C, which can cause insulin resistance in human skeletal muscle through the IKKβ/ IκBα/NFκB pathway [5,23,24]. NFκB-induced activation of several cytokines, such as interleukin 1-β and tumor necrosis factor α, leads to changes in the insulin receptor substrate, which contributes to the inhibition of glucose uptake by cells and thus causes insulin resistance [25,26]. This fact together with the proposed mechanism could explain why our findings point to the involvement of IκB in the pathogenesis of type 2 diabetes mellitus.

The IKKβ/ IκBα/NFκB pathway could also be involved in the initiation of the autoimmune process seen in type 1 diabetes mellitus [11]. The mechanism, however, is different from type 2 diabetes. Several studies have suggested a variety of factors, such as interleukin 1-β, interferon γ or double-stranded viral RNA as triggers of NFκB mediated β-cell destruction. This destruction is caused by the expression of a wide range of proapoptotic genes, such as, inducible nitric oxide synthase and tumor necrosis factor α [27]. These observations could explain the previous findings of Kolostova *et al.* (article in press) and the results of Hegazy *et al.* [11], which showed that CA repeat polymorphism of the NFKB1 gene is strongly associated with type 1 diabetes mellitus. The fact that this polymorphism is not a predisposing factor for type 2 diabetes mellitus in our study supports the idea that different signaling pathways involving IκB/NFκB are implicated in the pathogenesis of these two diseases.

Several reports on the association study about the CA repeat polymorphism of the NFKB1 gene exist. They were performed in a variety of ethnic groups (United

Kingdom, Denmark, Spain, and Australia) and involved a variety of diseases (type 1 and 2 diabetes mellitus, celiac disease, rheumatoid arthritis, SLE, and breast cancer). Surprisingly, different allele distributions (Table 3) were found in each ethnic control group. In Czechs, the most frequent alleles were A3 (124bp) and A9 (136bp) (Kolostova *et al.*, article in press). The most common alleles in the United Kingdom population were A8 (134bp) and A14 (146bp) [11]. In Spain, the most frequently reported alleles were A4 (126bp) and A10 (138bp) [28]. The most common alleles in the Australian population were A2 (122bp) and A8 (134bp) [29]. Beyond this, each study detected a different kind of genetic predisposition, specific only for a certain ethnic group. This divergence among populations may be explained by the genetic heterogeneity of the involved populations. Other genes, for instance MIC-A, also showed variations in allele distributions among control individuals [30,31]. An explanation for the different distribution of the allele frequencies among populations could be the difference in the methods used in detecting polymorphic variants of NFKB1 in these studies.

Although this study did not confirm an association between NFKBIA polymorphism and DN, we assume that NFκB is involved in the pathogenesis of both types of diabetes mellitus and its cardiovascular complications. This assumption is made on the basis of our current findings, which show an association between type 2 diabetes mellitus and our previous findings. Our previous findings showed NFKBIA polymorphism to be associated with autoimmune diabetes mellitus (Kolostova *et al.*, article in press). We suggest a dual mechanism for NFκB participation in their pathogenesis. Additional functional studies are necessary for further investigation.

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Paper 8 Pinterova D, Cerna M, **Kolostova K**, Novota P, Cimburova M, Romzova M, Kubena A, Andel M: **The frequency of alleles of the Pro12Ala polymorphism in PPARgamma2 is different between healthy controls and patients with type 2 diabetes.** Folia Biol (Praha) 50:153-156, 2004.

# Original Articles

# The Frequency of Alleles of the Pro12Ala Polymorphism in PPARγ2 Is Different between Healthy Controls and Patients with Type 2 Diabetes

( PPARγ2 / Pro12Ala polymorphism / type 2 diabetes / allele frequency / lipids )

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**Abstract. The aim of this initial case-control study was to determine the association between common Pro12Ala polymorphism in the PPARγ2 gene and type 2 diabetes in the Czech Republic. Furthermore, the effect of this polymorphism on phenotypic characteristics and on levels of lipids (total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides) was studied. One hundred thirty-three patients with type 2 diabetes and 97 control subjects were investigated. PCR and RFLP analysis were used for identification of individual genotypes. In the group of patients, three samples (2.26%) were identified as homozygous for the Ala/Ala genotype and 99 samples (74.44%) were homozygotes for the Pro/Pro genotype. Thirty-one samples (23.31%) were identified as Pro12Ala heterozygous. In the control group, six samples (6.19%) were homozygous for the Ala/Ala genotype and 61 samples (62.89%) were homozygotes for the Pro/Pro genotype. Thirty samples (30.93%) were identified as Pro12Ala heterozygous. The allele frequency for the Ala allele was lower in the type 2 diabetic group than in the control group (13.91% vs. 21.43%, P = 0.022). There was no difference (at P < 0.05) between the phenotypic characteristics (BMI, sex) studied in the group of patients according to the Pro12Ala genotype. There was no significant effect of the Pro12Ala polymorphism on lipid levels.**

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Abbreviations: PCR – polymerase chain reaction, PPAR – peroxisome proliferator-activated receptor, RFLP – restriction fragment length polymorphism.

Peroxisome proliferator-activated receptor (PPAR) γ is a transcription factor that has among others an important role in adipocyte differentiation and expression of the adipocyte-specific genes (Deeb et al., 1998; Zietz et al., 2002). PPARγ is activated by naturally occurring fatty acids and fatty acid derivates (Debril and Renaud, 2001). The biomolecular action of PPARγ is well documented. This protein heterodimerizes with another intracellular protein, the retinoid X receptor, and binds to specific DNA sequences noted as PPERs (Debril and Renaud, 2001). PPARγ activation is linked to an increased differentiation of preadipocytes to adipocytes. There are three already known forms of PPARγ: PPARγ1, PPARγ2 and PPARγ3. These are products of an alternative splicing (Šrámková et al., 2001). The Pro12Ala polymorphism resides inside exon 2, which is just in the form called PPARγ2 (Yen et al., 1997). The protective impact of the Ala genotype is probably based on less efficient stimulation of target genes and lower accumulation of adipose tissue and improved insulin sensitivity (Deeb et al., 1998; Hara et al., 2000).

Recently reported data are very inconsistent about the association of Pro12Ala polymorphisms in the PPARγ2 gene with type 2 diabetes. There are two large studies suggesting a decreased risk of type 2 diabetes for the Ala12 genotype in PPARγ2 (Deeb et al., 1998; Altshuler et al., 2000). Several subsequent publications failed to confirm the association (Mori et al., 1998; Mancini et al., 1999; Ringel et al., 1999; Clement et al., 2000), whereas others supported the data (Hara et al., 2000; Jacob et al., 2000; Mori et al., 2001).

## Material and Methods

### *Subjects*

DNA samples were obtained from 133 unrelated Czech patients with type 2 diabetes (characterization:

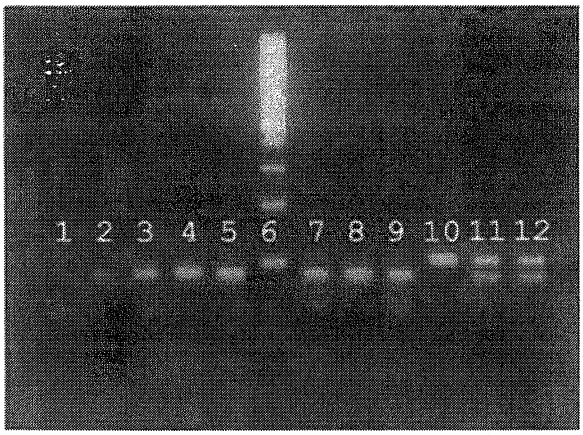


*Fig. 1.* Electrophoresis of PCR products after restriction on 2% agarose gel. Line 1: negative control. Lines 2,3,4,5,7,8,9: homozygotes for the Pro/Pro genotype. Line 6: DNA marker. Line 10: homozygotes for the Ala/Ala genotype. Lines 11,12: heterozygotes for the Pro/Ala genotype.

age > 35 years, C-peptide > 200 pmol/l, antiGAD < 50 ng/ml). The level of C-peptide was determined by an immunoradiometric method (Immunotech, Prague, Czech Republic). The presence of IgG antibodies against GAD was detected by ELISA (Roche Molecular Biochemicals, Mannheim, Germany). The levels of lipids were determined using automatic analyser KONELAB 60 (Labsystems CLD, Espoo, Finland) and commercially available kits (BioVendor, Brno, Czech Republic). Ninety-seven healthy subjects were used as a control. All of them were recruited from blood donors and no clinical details were available for this group. Informed consent was obtained from all subjects.

### *Genetic analysis*

Genomic DNA was isolated from peripheral blood using a commercially available kit (QIAamp Blood Kit, Qiagen, Hilden, Germany). The DNA samples were stored at -20°C.

The part of exon 2 containing codon 12 was amplified by using forward primer (Deeb et al., 1998) 26-mer 5'-GACAAAATATCAGTGTGAATTACAGC-3' and reverse primer 25-mer 5'-GTATCAGTGAAGGAACCGCTTTCTG-3'. The used PCR mix contained: 1x polymerase chain reaction (PCR) buffer for Taq polymerase, 200 µM dNTP (each), 1.5 mM $MgCl_2$, 0.4 µM primers, 2 U of Taq polymerase and 30–100 ng of the DNA sample. The PCR conditions were: denaturation for 2 min at 94°C, followed by 30 cycles of denaturation at 94°C for 25 s, annealing at 54°C for 30 s and extension at 72°C for 30 s, final extension at 72°C for 5 min. The result of the PCR reaction was a 106-bp fragment. This PCR product was visualized by electrophoresis on a 2% agarose gel in 1x TBE buffer.

The *Bse*LI restriction endonuclease was used for digestion. We changed the sequence of DNA with the reverse primer and we prepared the digest site for this inexpensive restriction enzyme. The digestion was done at 55°C for 1 h.

Then the final results were obtained from the second electrophoresis on an agarose gel (Fig. 1).

### *Statistical methods*

The statistical difference in allele frequencies between the group of patients and the control group was assessed by the binomial proportions test in the Statgraphics Plus software. To confirm the difference between groups of genotypes, the $\chi^2$ test was used in the EpiInfo 2000 software. The influence of the genotype on the clinical parameters was estimated by the ANOVA test. The P value < 0.05 was considered as significant.

## Results

### *Association of the Pro12Ala variant in the PPARγ2 with type 2 diabetes*

Genotype distribution in the group of patients was: 2.3% homozygous for the Ala/Ala allele, 23.3% Ala/Pro heterozygous, and 74.4% were Pro/Pro homozygous. In the control group, 6.2% were homozygous for the Ala/Ala allele, 30.9% Ala/Pro heterozygous, and 62.9% were Pro/Pro homozygous. There was no significant difference in the proportions of the different genotypes at codon 12 between the group of patients and the control group (see Table 1 below). The Ala-allele frequency was 21.4% in the control group and 13.9% in the patient group. The allele frequency for the Ala allele was significantly lower in the type 2 diabetic group than in the control group (13.91% vs. 21.43%, P = 0.022). These data suggest that the polymorphism Pro12Ala plays some role in type 2 diabetes in the Czech population.

*Table 1. Calculated P values for genotype frequencies*

| | No. of genotype | | | |
|---|---|---|---|---|
| | **Pro/Pro** | **Pro/Ala** | **Ala/Ala** | **Total** |
| **Patients** | 99 | 31 | 3 | 133 |
| **Controls** | 61 | 30 | 6 | 97 |
| **P value** | 0.06 | 0.19 | 0.13 | |

The odds ratios are not mentioned in the table because none of P values is significant.

### *Correlation between the PPARγ2 genotype and clinic parameters of the type 2 diabetic subjects with and without the Ala12 variant*

No relation between the polymorphism and BMI, sex or levels of total cholesterol, HDL- and LDL-cholesterol or triglycerides could be detected in the group of the patients.

*Table 2. Clinical characteristics of patients with the type 2 diabetes*

| Parameter | Total | Pro/Pro | Pro/Ala +Ala/Ala | P value |
|---|---|---|---|---|
| N (%) | 133 (100) | 99 (74.4) | 34 (25.6) | |
| Age [years] | 65.3 ± 9.6 | 64.6 ± 10.0 | 66.3 ± 9.0 | n.s. |
| BMI [kg/m] | 30.9 ± 6.3 | 31.2 ± 4.9 | 31.5 ± 5.8 | n.s. |
| Total cholesterol [mmol/l] | 6.3 ± 1.1 | 6.3 ± 1.1 | 6.4 ± 0.9 | n.s. |
| HDL [mmo/l] | 1.5 ± 0.3 | 1.5 ± 0.3 | 1.6 ± 0.3 | n.s. |
| LDL [mmol/l] | 3.9 ± 1.1 | 3.9 ± 1.1 | 4.0 ± 0.9 | n.s. |
| Triglycerides [mmol/l] | 2.7 ± 1.2 | 3.1 ± 1.7 | 2.4 ± 1.8 | n.s. |

n.s. – not significant

## Discussion

This study supports the hypothesis that the Pro12Ala polymorphism of the PPARγ2 gene plays a significant role in type 2 diabetes of the Czech population. Our results showed that the frequency of the Ala12 variant of the PPARγ2 gene is higher in the control group than in the group of patients. This can be explained by the fact that the proline to alanine substitution in the codon 12 in PPARγ2 is associated with a decreased risk of the type 2 diabetes. These data are consistent with several previous studies carried out on German (Jacob et al., 2000), Finnish (Deeb et al., 1998), Japanese (Hara et al., 2000; Mori et al., 2001) or Caucasian (Altshuler et al., 2000) populations and inconsistent with others (Mori et al., 1998; Mancini et al., 1999; Ringel et al., 1999).

Some authors compared numerous clinical characteristics and the Pro12Ala polymorphism between type 2 diabetic subjects and control subjects or type 2 diabetic subjects with and without the Ala12 variant. They found many various associations of the Pro12Ala polymorphism with BMI (Deeb et al., 1998), insulin sensitivity (Deeb et al., 1998; Koch et al., 1999; Hara et al., 2000; Jacob et al., 2000), changed concentrations of total cholesterol (Mori et al. 2001; Zietz et al. 2002) and LDL-cholesterol (Zietz et al., 2002). But it is obvious from our analysis of clinical characteristics that there is no significant diference in the BMI or lipid levels. It thus seems that for studying the role of the Pro12Ala polymorphism of the PPARγ2 gene in the genetic background of dyslipidaemia, much larger studies are needed.

In summary, we can conclude from our results that the Pro12Ala polymorphism of the PPARγ2 gene is associated with reduced risk of type 2 diabetes. This protective effect is evident among Ala12 variant carriers. We have further demonstrated that the polymorphism is not associated with BMI and changed lipid levels.

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Paper 9: Pinterova D, Ek J, **Kolostova K**, Pruhova S, Novota P, Romzova M, Feigerlova E, Cerna M, Lebl J, Pedersen O, Hansen T: **Six novel mutations in the GCK gene in MODY patients.** Clin Genet 71:95-96, 2007

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## Letter to the Editor

# Six novel mutations in the *GCK* gene in MODY patients

*To the Editor*:

Maturity-onset diabetes of the young (MODY; MIM# 606391) is a genetically and clinically heterogeneous form of diabetes mellitus, characterized by an autosomal dominant inheritance, early-onset non-insulin-dependent diabetes mellitus and by a primary defect in the pancreatic beta-cell function (1). Until now, six types of MODY diabetes have been identified, depending on the gene causing the disease (2). Screening for glucokinase (GCK) mutations in subjects with clinical characteristics of MODY allows distinguishing between patients with a benign metabolic condition (*GCK* mutation positive, clinical diagnosis MODY2) and those with a higher risk of progressive hyperglycemia associated with more prevalent and severe diabetic complications (*GCK* mutation negative). The first mutation in the *GCK* gene was reported in 1992 (3). Up to now, 195 mutations in *GCK* have been described, in 285 families (4). Diabetic complications are rare in GCK–MODY, thus GCK–MODY patients only need to be followed by annual HbA1c examination. Also, screening of *GCK* for heterozygous inactivating mutations allows to determine the subtype of MODY diabetes and to predict the lifelong prognosis.

All 12 exons (exons 1a, 1b, 1c and 2–10), the intron–exon boundaries and promotor region of *GCK* (GenBank accession number, AF041012-22) were screened; in 92 Czech probands fulfilling classical MODY criteria, using denaturing high-performance liquid chromatography as previously described (5). The nature of identified mutations was established by direct nucleotide sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) according to manufacture's instructions. Mutations were confirmed using a second, independent amplification of the affected part of *GCK* and re-sequenced the following day. The probands were recruited from pediatricians and endocrinologists from the entire Czech Republic. Fifteen different missense mutations were identified in 27 patients. Of these, six were novel missense mutations R250C (exon 7, c.748C>T), L315H (exon 8, c.944T>A), F316V (exon 8, c.946T>G), F419L (exon 10, c.1255T>C), I436N (exon 10, c.1307T>A) and A454E (exon 10, c.1361C>A). Some of the identified *GCK* missense mutations are located near putative functional domains: R250C was found in the close vicinity of a putative glucose binding site, while F419L was detected near a putative MgATP binding site and could thus affect binding kinetics (6). Five of these mutations co-segregated with hyperglycemia in the family, suggesting that the variants are new disease-causing mutations. For the novel R250C variant, family members were not available for co-segregation studies. All codons, which are changed by the six novel mutations, are conserved in the human, mouse, rat and chimpanzee genomes and we found none of these mutations in 50 unrelated healthy Czech Caucasian subjects. Therefore, we assume that the mutations are probably novel disease-causing mutations.

We also compared the clinical characteristics of patients with *GCK* mutations and those without mutation in *GCK* (data not shown in details). In short – the treatment of hyperglycemia with diet was more frequent ($p < 0.001$) in the group of probands with mutations in *GCK* and they had a significantly lower frequency of diabetic complications ($p = 0.02$). None of the patients with mutations in *GCK* was treated with insulin ($p < 0.001$). Moreover, *GCK* mutation carriers had a lower level of glycosylated hemoglobin ($p = 0.02$). The mean HbA1c (%) in GCK-positive probands *vs* negative was $5.7 \pm 0.2$ *vs* $6.5 \pm 0.2$.

In conclusion, we identified 29% of *GCK* mutation carriers among Czech MODY probands, confirming that mutations in *GCK* are a common cause of MODY in the Czech population. The present high relative prevalence of GCK–MODY, compared with some other European studies, might reflect not only a specific genetic background, but also the mode of recruitment, because most of the probands in the present investigation were recruited by

**Letter to the Editor**

pediatricians. The prevalence of known types of MODY differs in reports from various European populations. Mutations in *GCK* were described to be a common cause of MODY in France (7) and Italy (8) whereas mutations in *TCF1* (MODY3) predominated in the UK (9), Denmark (10) and Germany (11). Our findings again highlight the concept that molecular diagnostic methods in clinical practice may help to verify a diagnosis of MODY.

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Paper 10: Feigerlova E, Pruhova S, Dittertova L, Lebl J, Pinterova D, **Kolostova K**, Cerna M, Pedersen O, Hansen T: **Aetiological heterogeneity of asymptomatic hyperglycaemia in children and adolescents**. Eur J Pediatr 165:446-452, 2006

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ORIGINAL PAPER

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# Aetiological heterogeneity of asymptomatic hyperglycaemia in children and adolescents

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**Abstract** *Introduction:* Randomly estimated fasting hyperglycaemia in an asymptomatic individual may represent the first sign of pancreatic β-cell dysfunction. *Objective:* We aimed at specifying the genetic aetiology of asymptomatic hyperglycaemia in a cohort of children and adolescents. *Subjects and methods:* We analysed the aetiological diagnosis in 82 non-obese paediatric subjects (38 males) aged 0.2-18.5 years (median: 13.1) who were referred for elucidation of a randomly found blood glucose level above 5.5 mmol/l. In addition to fasting glycaemia and circulating levels of insulin and C-peptide, the subjects were tested by an oral glucose tolerance test and an intravenous glucose tolerance test and screened for mutations in the genes encoding glucokinase (*GCK*), HNF-1α (*TCF1*), Kir6.2 (*KCNJ11*) (if aged <2 years) and HNF-4α (*HNF4A*) (those with a positive family history of diabetes). *Results and discussion:* We identified 35 carriers of *GCK* mutations causing MODY2, two carriers of *TCF1* mutations causing MODY3, one carrier of a *HNF4A* mutation causing MODY1 and one carrier of a *KCNJ11* mutation causing permanent neonatal diabetes mellitus. Of the remaining patients, 11 progressed to type 1 diabetes mellitus (T1DM) and 9 had impaired glucose tolerance or diabetes mellitus of unknown origin. In 23 subjects, an impairment of blood glucose levels was not confirmed. We conclude that 39 of 82 paediatric patients (48%) with randomly found fasting hyperglycaemia suffered from single gene defect conditions, MODY2 being the most prevalent. An additional 11 patients (13%) progressed to overt T1DM. The aetiological diagnosis in asymptomatic hyperglycaemic children and adolescents is a clue to introducing an early and effective therapy or, in MODY2, to preventing any future extensive re-investigations.

**Keywords** Hyperglycaemia · Genetics · Children · MODY · Type 1 diabetes mellitus · Permanent neonatal diabetes mellitus

**Abbreviations** FPIR: First-phase insulin release · GCK: Glucokinase · *GCK*: Gene encoding glucokinase · $HbA_{1C}$: Glycosylated haemoglobin · HNF-1α: Hepatocyte nuclear factor-1α · *HNF4A*: Gene encoding HNF-4α · HNF-4α: Hepatocyte nuclear factor-4α · HNF-1: Hepatocyte nuclear factor-1 · IGT/DM: Impaired glucose tolerance/diabetes mellitus · IPF-1: insulin promotor factor · IVGTT: intravenous glucose tolerance test · *KCNJ11*: Gene encoding Kir6.2 · Kir6.2: Inwardly rectifying $K^+$ channel subunit · MODY: Maturity-onset diabetes of the young · NGT: Normal glucose tolerance · OGTT: Oral glucose tolerance test · PND: Permanent neonatal diabetes mellitus · SDS: Standard deviation score · T1DM: Type 1 diabetes mellitus · *TCF1*: Gene encoding HNF-1α

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## Introduction

For decades, the diagnosis of paediatric diabetes mellitus has being considered trivial. In the majority of patients, suggestive symptoms of recent polyuria, polydipsia and weight loss, in some cases associated with ketoacidosis, clearly indicate the need for blood glucose measurement to establish the diagnosis of type 1 diabetes mellitus (T1DM).

However, an unexpected finding of elevated blood glucose may arise from a random measurement in children without typical symptoms of diabetes, while elaborating various medical conditions; in others, a positive dipstick test for glycosuria may have led to a subsequent estimation of hyperglycaemia. As these children may suffer from pre-symptomatic progressive pancreatic β-cell dysfunction, a rapid and effective diagnostic action is required.

Maturity-onset diabetes of the young (MODY) is a family of monogenic forms of impaired β-cell function. The clinical diagnosis of MODY is based on (1) young age at onset (before 25 years of age), (2) familial occurrence with autosomal dominant inheritance and high penetrance and (3) no need for insulin treatment for at least 2 years following diagnosis [20, 22]. So far, six distinct MODY subtypes (MODY1–MODY6) have been defined according to the underlying genetic defect.

MODY2 is caused by mutations of the gene encoding glucokinase (GCK), an enzyme required for glucose phosphorylation in the pancreatic β-cells and in the liver cells (Fig. 1). The affected subjects exhibit mild hyperglycaemia from birth up to old age and are usually free of symptoms and severe organ damage. The age at diagnosis depends on the first blood glucose estimation.

The additional MODY subtypes (MODY1 and 3–6) result from defective β-cell transcriptional regulation (Fig. 1). The affected individuals usually manifest in late puberty or early adulthood and suffer from progressively impaired insulin secretion and impaired glucose regulation and a high risk of late diabetes-associated complications.

Neonatal diabetes mellitus (either transient or permanent) is characterised by hyperglycaemia revealed within the first months of life requiring insulin treatment [7, 12]. Transient neonatal diabetes mellitus resolves within a median of 3 months [17]. On the contrary, patients with permanent neonatal diabetes mellitus (PND) remain insulin dependent [17]. A defect of *KCNJ11* encoding the Kir6.2 subunit of the β-cell ATP-sensitive $K^+$ channel has recently been established as a cause for PND in a substantial proportion of affected children (Fig. 1) [7].

To make the spectrum of diabetic conditions among children and adolescents even more complex, type 2 diabetes mellitus is recently being reported among severely overweight young people from countries with an epidemic of obesity [14].

Here, we studied mutations and the phenotypic expression in the genes *TCF1*, *GCK*, *HNF4A* and *KCNJ11* in an unselected cohort of 82 children and adolescents, consecutively referred for investigation of asymptomatic fasting hyperglycaemia.

## Subjects and methods

### Subjects

Between January 1998 and December 2004, a total of 82 children and adolescents (38 males, 44 females; aged 2 months–18.5 years, median: 13.1 years) were referred by general paediatricians, paediatric endocrinologists or paediatric departments of local hospitals to the Department of Paediatrics of the 3rd Medical Faculty in Prague for elucidation of asymptomatic fasting hyperglycaemia. All patients were of Caucasian origin. Their body mass index (BMI) ranged between 13.7 and 25.0 kg/m$^2$ (median: 18.9). None of the subjects was severely overweight (BMI >97th percentile of the age- and gender-matched reference population) [3].

Non-symptomatic elevated fasting blood glucose was originally estimated either within the elaboration of an acute condition (tonsillitis, gastroenteritis/vomiting, bronchitis, influenza, pyelonephritis, otitis media, fatigue, abdominal pain, head injury, vertigo, dyspnoea, collapse or tachycardia) or following a positive glycosuria testing at a routine preventive examination or at urine examination for various other reasons. None of the patients suffered from polyuria/polydipsia/weight loss or was ketotic at initial evaluation.

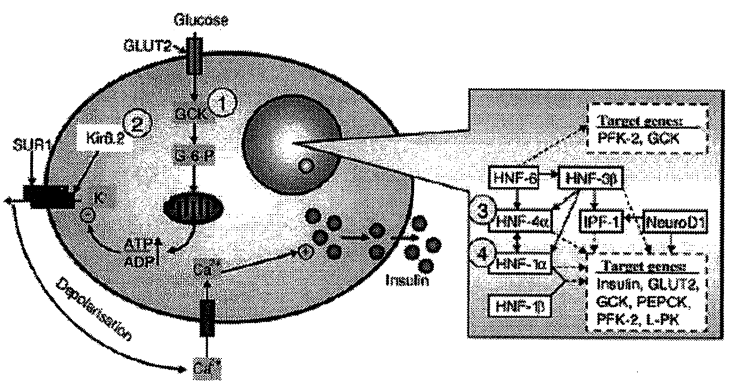


**Fig. 1** Main pathways regulating insulin secretion in β-cells (*left*) and the current concept of β-cell transcriptional regulation network (*right*). Glucose molecules enter β-cells via the GLUT2 membrane transporter. The cytoplasmic enzyme glucokinase (GCK) senses the glucose concentration and initiates subsequent steps leading to insulin release. Adenine nucleotides interact with the sulphonylurea-binding component (SUR1) of the inward rectifying potassium channel (Kir6.2). Potassium channel closure depolarises the cell membrane, opening voltage-gated calcium channels. Increased intracellular calcium concentration promotes exocytosis of insulin granules. Decreased GCK activity due to a heterozygous *GCK* gene mutation (1) is associated with persistent mild hyperglycaemia from birth up to old age (MODY2). Defects of Kir6.2 subunit of the potassium channel due to a *KCNJ11* mutation (2) lead to permanent neonatal diabetes (PND). Failure of transcriptional regulation results in gradual loss of insulin secretion. Affected individuals become hyperglycaemic in late childhood, adolescence or young adult age as seen in defects of HNF-4α (3) encoded by *HNF4A* (MODY1) or of HNF-1α (4) encoded by *TCF1* (MODY 3)

The age of patients at the first recognition of hyperglycaemia was 2 weeks–16.1 years (median: 12.0 years) and fasting plasma glucose level at the referring physician's office ranged from 5.6 mmol/l (101 mg/dl) to 20.9 mmol/l (376 mg/dl) (median: 7.1 mmol/l; 127 mg/dl). Those with fasting plasma glucose <5.6 mmol/l (101 mg/dl) at initial examination were excluded from the study cohort. At the first examination following referral, levels of fasting plasma glucose ranged from 4.0 mmol/l (71 mg/dl) to 22.6 mmol/l (407 mg/dl) (median: 5.9 mmol/l; 106 mg/dl).

## Study protocol

### *Baseline investigations*

The baseline evaluation included family history, fasting glycaemia, fasting serum insulin and C-peptide, and glycosylated haemoglobin ($HbA_{1C}$). Family history was considered positive if at least one parent and/or one sibling had diabetes mellitus.

All individuals were tested with an oral glucose tolerance test (OGTT) which was evaluated according to the American Diabetes Association (ADA) criteria [1]. Children with results within normal range (fasting plasma glucose <5.6 mmol/l and 2-h postload plasma glucose <7.8 mmol/l; <140 mg/dl) were considered to have normal glucose tolerance (NGT). Intravenous glucose tolerance test (IVGTT) to assess pancreatic β-cell function by estimating the first-phase serum insulin release (FPIR) was performed in all children above 2 years of age. All subjects were investigated for mutations in the genes encoding GCK and HNF-1α. The *KCNJ11* gene encoding the Kir6.2 subunit of the β-cell ATP-sensitive $K^+$ channel was analysed in all children younger than 2 years.

In addition patients with a positive family history of diabetes mellitus in first-degree relatives but with negative search for mutations in *GCK*, *TCF1* and *KCNJ11* genes were screened for mutations in the gene encoding HNF-4α.

Low-dose insulin therapy and home blood glucose monitoring were initiated in patients who had abnormal glucose tolerance (a pathological result during an OGTT) in association with decreased FPIR (lower than 1st percentile). Although that is still not a standard therapeutic option, we personally believe that it may prevent a rapid progression to overt T1DM.

### *Clinical follow up*

All study participants were followed prospectively. The median follow-up time was 3.9 years (range: 0.7–6.9 years). Venous blood was sampled for $HbA_{1C}$ and profiles of blood glucose levels over 24 h (three to five measurements before main meals and at bedtime) were performed every 6 months.

In those on insulin therapy, the treatment was aimed at maintaining near-normoglycaemia. The insulin requirements were recorded at regular outpatient visits every 3 months.

### *Ethics*

Informed written consent was obtained from all subjects and/or their parents before entering the study protocol. The study was approved by the Ethical Committee of the 3rd Faculty of Medicine, Charles University of Prague.

## Testing procedures

Both OGTT and IVGTT [2] were performed according to standard protocols. The subjects were on regular diet with unrestricted carbohydrate intake at least 3 days preceding the test. Excessive physical activity was not allowed 1 day before testing. The test was not provided in cases of acute illness and/or administration of drugs with potential effect on blood glucose levels (including inhaled corticosteroids). After an overnight fast for 10–12 h, testing was initiated between 8 and 9 a.m.

For IVGTT, two contralateral antebrachial veins were cannulated. Sampling was performed from one cannula to measure basal plasma glucose and serum insulin and C-peptide. Immediately thereafter, 0.5 g glucose per kg of body weight (maximum: 35 g) as a 40% aqueous solution was infused into the second cannula within 3 min±15 s. Serum insulin and C-peptide levels at time points 1 and 3 min were used for calculation of the first-phase insulin response (FPIR). The results were evaluated according to published standards [9, 18].

For OGTT, 1.75 g glucose per kg of body weight (maximum: 75 g) was given. Blood samples to measure plasma glucose and serum insulin and C-peptide were obtained at time points 0, 60 and 120 min. The results were evaluated according to ADA criteria [1].

## Routine laboratory assays

### *Plasma glucose*

Plasma glucose concentration was measured by the enzymatic hexokinase method using the automatic analyser Konelab 60 (Thermo Clinical Labsystem Oy, Espoo, Finland).

### *C-peptide and insulin*

C-peptide and insulin in serum were analysed by a chemiluminescent immunometric technique using the commercial sets Immulite 2000 C-peptide and Immulite 2000 Insulin (Diagnostic Products Corporation, Los Angeles, CA, USA).

*$HbA_{1C}$*

Estimation of $HbA_{1C}$ was performed with the DS5 Analyser (Drew Scientific Ltd., Barrow in Furness, Cumbria, UK) using cation exchange chromatography in conjunction with gradient elution. The assigned values of $HbA_{1C}$ were calibrated to the International Federation of Clinical Chemistry (IFCC) system (normal levels: 2.0–4.5%).

Genetic analyses

*Preparation of genomic DNA*

Genomic DNA was isolated from leukocytes in blood samples anticoagulated with ethylenediaminetetraacetate (EDTA).

*Analyses of genes encoding HNF-1α, GCK and HNF-4α*

Denatured high-performance liquid chromatography (dHPLC) and direct sequencing were used for analysis of all exons, the intron-exon boundaries and the promoter regions of the *TCF1* and *GCK* genes [4]. Analysis of the *HNF4A* gene and of its P1 promoter was performed by direct sequencing using ABI PRISM Dye Primer Cycle Sequencing Kit with AmpliTaq DNA polymerase FS.

*Analysis of the KCNJ11 gene*

The published primers and previously described protocol were used to carry out the polymerase chain reactions (PCR) in addition to fragment 6 for which the annealing temperature used was 70°C [8]. PCR were performed using AmpliTaq Gold and a Gene-Amp PCR system 9700 thermocycler (Perkin Elmer, Foster City, CA, USA). After PCR, the products were purified using an ExoSAP-IT treatment (USB Corporation, Cleveland, OH, USA), and all of them were sequenced in both directions using the BigDye Terminator v3.1Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The sequencing was performed on an ABI PRISM 3100-Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

Definition of diagnostic subgroups

Positive screening for mutations in the *HNF4A*, *GCK*, *TCF1* and *KCNJ11* genes was considered diagnostic for MODY1, MODY2, MODY3 and PND, respectively. Subjects with negative search for mutations in genes mentioned above, but with persistent increased fasting glycaemia and abnormally high plasma glucose response to an OGTT were considered diabetic. Of these, individuals with FPIR below the 1st percentile who required a daily insulin dose ≥0.5 IU/kg to maintain near-normoglycaemia within the follow-up period were considered to manifest an early (pre-manifest) phase of T1DM. Those with normal FPIR (and no insulin treatment) or those with low FPIR but daily insulin requirements <0.5 IU/kg were assigned as impaired glucose tolerance/diabetes mellitus (IGT/DM) of undetermined origin. β-Cell-specific autoantibodies were not included into the diagnostic work-up. Individuals negative for mutations in the analysed genes who had normal fasting plasma glucose and normal OGTT and physiological FPIR at re-evaluation following referral were considered as NGT.

Statistics

The values are expressed as mean±SEM if not given otherwise. The data on diagnostic subgroups were statistically evaluated if the entire subgroup included ≥9 individuals. The group differences in the clinical and metabolic variables were assessed by the one-way analysis of variance (ANOVA) and by Student's *t*-test where appropriate.

## Results

Monogenic forms of hyperglycaemia

The genetic findings are summarised in Table 1. One patient carried a heterozygous mutation in the *HNF4A* gene causing MODY1. In 35 subjects, we identified 19 different heterozygous mutations in the *GCK* gene (MODY2). In two individuals, we determined heterozygous mutations in the *TCF1* gene causing MODY3.

One patient carried a heterozygous mutation in the *KCNJ11* gene, an arginine-to-histidine substitution at position 201 (R201H), causing PND. He was originally investigated for prolonged cough at age 2.5 months. In spite of a blood glucose level of 22.6 mmol/l (407 mg/dl) at referral, he was free of diabetic symptoms. We have not previously reported this patient; however, the R201H mutation is known to be the most prevalent PND-causing variant within the entire *KCNJ11* gene [7].

Thus, in 39 of 82 patients (48%) the randomly found hyperglycaemia led to the disclosure of a single gene defect condition, MODY2 being the most prevalent. The clinical and biochemical phenotypes of affected individuals are summarised in Table 2.

Type 1 diabetes mellitus (pre-manifest phase)

Eleven children who tested negative for mutations in the screened genes (13% of the study group) had fasting hyperglycaemia (9.1±1.2 mmol/l; 164±22 mg/dl), abnormally high plasma glucose response to an oral glucose load and substantially decreased FPIR (13.7±3.0 mIU/l) (Table 2). None of them was ketotic at diagnosis or within

**Table 1** Genetic findings: 39 heterozygous mutations identified within the cohort of 82 unrelated children and adolescents with randomly found hyperglycaemia

| Gene | Identified mutations |
|---|---|
| *HNF4A* (1 subject) | Val121Ile |
| *GCK* (35 subjects) | Arg36Trp (1 subject) |
| | Glu40Lys (8 subjects) |
| | Gly44Asp (2 subjects) |
| | Phe150Leu (1 subject) |
| | Glu157Lys (1 subject) |
| | Ala188Thr (1 subject) |
| | Cys220Stop (4 subjects) |
| | Val226Met (2 subjects) |
| | Met251Val (1 subject) |
| | Glu268Stop (1 subject) |
| | Gly294Asp (1 subject) |
| | Leu315His (2 subjects) |
| | Phe316Val (1 subject) |
| | Gly318Arg (3 subjects) |
| | Ser383Leu (1 subject) |
| | Phe419Leu (1 subject) |
| | Cys434Tyr (2 subjects) |
| | Ile436Asn (1 subject) |
| | Ala454Glu (1 subject) |
| *TCF1* (2 subjects) | P379fsdelCT |
| | Arg229Stop |
| *KCNJ11* (1 subject) | Arg201His |

Identification of mutations in the *HNF4A*, *GCK* and *TCF1* genes in individual patients have been given in our previously published reports [15, Pinterova, submitted for publication]

the follow-up period of 3.8±0.4 years. The reason for the initial plasma glucose estimation was an intercurrent infection in five, fatigue in three and head injury in one subject. In two subjects, glycosuria was detected in a random urine sample at a preventive examination.

In all of the subjects, low-dose insulin therapy was initiated to prevent the development of overt clinical symptoms of diabetes and the daily insulin requirements to maintain near-normoglycaemia exceeded 0.5 IU/kg during the follow-up. Thus, these subjects were considered to suffer from T1DM that was randomly detected within the presymptomatic phase.

### Impaired glucose tolerance/diabetes mellitus (IGT/DM) of undetermined origin

Nine patients (11%) who tested negative at mutation screening had an abnormal plasma glucose response to an oral glucose load (OGTT) and a borderline fasting plasma glucose level (6.1±0.5 mmol/l; 110±9 mg/dl). Their FPIR ranged from low normal to moderately decreased values (54.4±19.5 mIU/l) (Table 2). The reason for the initial examination was glucosuria at a preventive examination (4), intercurrent infections (3) or fatigue (2). According to standard procedures, insulin therapy was initiated in those with FPIR below the 1st percentile. However, the daily insulin requirements to maintain near-normoglycaemia remained ≤0.3 IU/kg.

Members of this subgroup were not obese (BMI-SDS ranging from −0.61 to+0.63), making a diagnosis of type 2 diabetes mellitus improbable. However, eight of nine reported a history of diabetes in one parent (three cases

**Table 2** Clinical and metabolic characteristics of diagnostic subgroups of children with randomly found asymptomatic hyperglycaemia

| | T1DM | MODY1 | MODY2 | MODY3 | PND | IGT/DM | NGT |
|---|---|---|---|---|---|---|---|
| Number (%) | 11 (13) | 1 (1) | 35 (43) | 2 (2) | 1 (1) | 9 (11) | 23 (28) |
| Age at first detection of hyperglycaemia (years) | 9.1±1.2 | 14.0 | 11.2±0.7 | 15.9±0.1 | 0.2 | 11.2±1.5 | 9.6±1.0 |
| Sex (F/M) | 4/7 | 1/0 | 19/16 | 2/0 | 0/1 | 6/3 | 12/11 |
| BMI (SDS) | 0.25±0.30 | 0.92 | −0.15±0.13 | −0.06 ±1.01 | 0.18 | −0.04±0.25 | 0.12±0.20 |
| Fasting p-glucose (mmol/l) | 9.0±1.4* | 8.4 | 6.4±0.2* | 5.9[a] | 22.6 | 6.1±0.5* | 4.8±0.1* |
| Fasting s-insulin (mIU/l) | 7.3±1.6 | NA | 10.0±1.3 | 8.2[a] | NA | 6.8±1.1 | 6.7±0.7 |
| Fasting s-C-peptide (pmol/l) | 479±92 | 342 | 561±51 | 512±258 | NA | 645±83 | 508±69 |
| OGTT: 1-h postload p-glucose (mmol/l) | 12.6±1.1* | 10.6 | 10.7±0.6* | NA | NA | 11.6±1.2* | 6.0±0.5* |
| OGTT: 2-h postload p-glucose (mmol/l) | 10.4±0.6* | 10.1 | 9.3±0.5* | 11.5[a] | NA | 11.8±1.7* | 5.3±0.3* |
| FPIR (mIU/l) | 13.7±3.0*** | NA | 130.2 ±16.9** | 94.0[a] | NA | 54.4 ±19.5** | 113.8±20.1*** |
| $HbA_{1c}$ (%) | 6.6±0.7** | NA | 4.8±0.3** | 5.4±0.9 | 5.4 | 6.1±0.4** | 4.5±0.4** |
| Number of affected parents (2/1/0) | 0/3/8 | 0/1/0 | 2/28/5 | 0/2/0 | 0/0/1 | 0/8/1 | 0/5/18 |

Values are shown as mean ± SEM (single values for the one member groups). BMI was expressed as SDS according to recent local standards [3]. *T1DM* type one diabetes mellitus, *PND* permanent neonatal diabetes mellitus, *IGT/DM* impaired glucose tolerance/diabetes mellitus, *NGT* normal glucose tolerance, *SDS* standard deviation score, *FPIR* first-phase of insulin release during an ivGTT, *p* plasma, *s* serum, *NA* not available

*$p<0.0001$ (T1DM, MODY2, IGT/DM and NGT; ANOVA); **$p<0.005$ (T1DM, MODY2, IGT/DM and NGT; ANOVA); ***$p<0.01$ (T1DM vs. NGT; *t*-test) [a]In a single patient only

were classified as gestational diabetes and five cases as type 2 diabetes).

### Normal glucose tolerance

In 23 subjects (28% of the study cohort), we did not confirm the hyperglycaemia originally reported by the referring physician. These patients had normal fasting plasma glucose, OGTT and FPIR. The screening for mutations in the selected genes was negative. Furthermore, $HbA_{1C}$ and profiles of blood glucose levels remained normal during follow-up.

The initial examination of plasma glucose was provided when elaborating an intercurrent infection (11), fatigue (7) or abdominal pain (5).

### Summary of clinical laboratory data in diagnostic subgroups

The clinical and laboratory data on subgroups of patients with asymptomatic hyperglycaemia are summarised in Table 2. Individuals with the four most prevalent conditions (T1DM, MODY2, IGT/DM, NGT) were of similar age at first examination for hyperglycaemia, had similar age- and gender-matched body mass index (BMI) and similar fasting serum levels of insulin and C-peptide.

On the contrary, fasting plasma glucose, FPIR and $HbA_{1C}$ differed significantly among the subgroups, distinguishing children with pre-manifest T1DM by lower FPIR and higher fasting plasma glucose in association with increased $HbA_{1C}$.

## Discussion

Within the cohort of 82 children and adolescents with asymptomatic hyperglycaemia, we identified 35 mutation carriers in the glucokinase gene (MODY2) and 3 patients with mutations in genes encoding transcription factors: one case of MODY1, two cases of MODY3 and one infant with a *KCNJ11* mutation causing PND. Thus, in 39 patients (48% of the study cohort) the randomly found hyperglycaemia led to the disclosure of a single gene defect condition, MODY2 being the most common. Among the mutation-negative subjects, 11 (13%) developed T1DM, 9 (11%) had IGT/DM of unknown cause and 23 (28%) were glucose tolerant.

These findings have important clinical implications: 35 of 59 subjects who were confirmed to be hyperglycaemic by re-investigation suffered in fact from MODY2, a benign and non-progressive form of impaired glucose regulation. No diet or drug therapy is required in most of these patients [13] and an annual follow-up with $HbA_{1C}$ measurement would suffice. The risk of diabetes-associated complications is low [6]. However, affected women may require insulin therapy during pregnancy to prevent foetal macrosomia [5]. Also exact genetic diagnosis is important in order to prevent redundant periodic metabolic examinations of affected individuals.

The clinical diagnosis of MODY2 may be supported by a positive autosomal dominant family history of mild hyperglycaemia. If maternally transmitted, affected women may have a history of gestational diabetes mellitus in all pregnancies. If transmitted by the father, the diagnosis is not necessarily known. A simple estimation of parental fasting plasma glucose levels may be helpful. The affected grandparent may be known to have "mild type 2 diabetes mellitus" and being recommended to follow a "diabetic diet".

On the contrary, the additional MODY subtypes tend to manifest in later childhood, adolescence or young adulthood [21] and gradually develop to a symptomatic stage. These forms of diabetes are characterised by progressive decrease in β-cell function and high risk of microvascular complications. Therapy with insulin or oral hypoglycaemic drugs is required to maintain near-normoglycaemia [22]. A positive autosomal dominant family history of a clinically overt diabetes mellitus may help in establishing the clinical diagnosis of MODY.

Among infants, asymptomatic hyperglycaemia may be the first sign of PND. This condition is known to result from defects of the *KCNJ11* gene in a substantial proportion of cases [7]. Our patient carrying the R201H mutation within the *KCNJ11* gene was randomly detected to be hyperglycaemic at 2.5 months of age. His daily insulin requirements did not exceed 0.4 IU/kg and the metabolic regulation was excellent within the follow-up period of 3.3 years.

Currently, studies are ongoing to test the therapeutic potential of sulphonylurea derivates instead of insulin in children affected by mutations in the *KCNJ11* gene. Thus, genetic diagnosis may open new future treatment options in these children [16].

In 11 patients (13 %), random hyperglycaemia was apparently the first sign of T1DM. Early recognition of T1DM makes it possible to initiate insulin treatment before the clinical onset of the disease and to reduce the risk of unrecognised diabetic ketoacidosis upon manifestation of T1DM. The early stages of the disease process may be detected by decreased FPIR and by β-cell-specific autoantibodies. However, in young children the levels of autoantibodies may vary, introducing difficulties in the interpretation of the results [10, 11]. Temporary positive titres of an autoantibody against molecularly defined antigens (anti GAD65, anti-IA2 or anti-IAA) might also reflect the population variability [19]. Therefore, we performed the study irrespectively of autoimmune markers.

The IGT/DM group in which the molecular pathogenesis was not understood included nine (11%) of the examined subjects. They were all asymptomatic, had impaired glucose tolerance or diabetes mellitus according to diagnostic criteria and their FPIR was low normal or mildly decreased, making the diagnosis of T1DM unlikely. However, eight of them had a first-degree relative affected with diabetes mellitus indicating that undetected or unknown MODY gene variants may be involved in the

aetiology. Low-dose insulin therapy was in some of the subjects necessary to achieve near-normoglycaemia.

In the remaining subgroup of 23 subjects (28%) with NGT, a detailed clinical and metabolic examination did not reveal any metabolic disorder. The randomly found fasting hyperglycaemia might have been due to an isolated stress hyperglycaemia, postprandial blood sampling or errors in sample handling.

In conclusion, the underlying cause of a randomly found asymptomatic hyperglycaemia was fully elucidated in a considerable number of affected individuals in our study. For many of them, the final diagnosis bears a positive message on the benign nature of the condition; however, cases of pre-manifest type 1 diabetes mellitus and of MODY1 and MODY3 require a rapid and adequate treatment to prevent an unfavourable short-term and long-term outcome. In different populations, the results might differ largely. Cases of type 2 diabetes mellitus would probably be recognised among hyperglycaemic youngsters in countries with epidemic childhood obesity. However, the spectrum of newly established monogenic conditions is worthy of inclusion in the diagnostic work-up anyway.

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PŘEHLEDNÝ ČLÁNEK

# HYPOTÉZY ASOCIÁCIE HLA MOLEKÚL S OCHORENIAMI AUTOIMUNITNÉHO CHARAKTERU

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## SOUHRN

Príčinou autoimunitných ochorení je imunitná reakcia namierená proti rôznym autoantigénom. Medzi molekuly zahrnuté do procesu prezentácie antigénov imunitnému systému patria aj HLA molekuly, u ktorých sa dokázala asociácia s jednotlivými nozologickými jednotkami. Jednou z najsilnejších v literatúre popísaných asociácií je asociácia HLA-B27 s ankylozujúcou spondylitídou. Obdobne je tomu i pri asociáciách s HLA molekulami triedy II pri jednom z najčastejších autoimunitných ochorení – diabetes mellitus 1. typu. Diabetes 1. typu je orgánovo špecifické autoimunitné ochorenie charakterizované deštrukciou β-buniek Langerhansových ostrovčekov pankreasu. Príčina špecifického deštrukčného procesu prebiehajúceho v pankrease nie je známa, ale predpokladom iniciácie tohto procesu je existencia vlastného antigénneho peptidu, ktorý je opakovane prezentovaný imunitnému systému. Nasledujúci prehľadný článok poukazuje na najzaujímavejšie a najpravdepodobnejšie riešenia doposiaľ nezodpovedaných otázok o mechanizme HLA asociácie.

**Kľúčové slová:** HLA, autoimunita, HLA-asociácia, hypotéza, ankylozujúca spondylitída, HLA-B27, diabetes mellitus 1. typu, glutamátdekarboxyláza (hGAD65), enterovírusy (HERVs), regulácia expresie.

## SUMMARY

*Kološtová K., Černá M., Anděl M.: The HLA Molecules and their Association with Autoimmune Disease*

The etiology of autoimmunity diseases includes an immunological reaction against various autoantigens. The HLA molecules play an important role in the antigen presentation process to the immune system. The HLA genes were found as the most significant genetic predisposition factor. The strongest association described in the literature is the association of HLA-B27 with ankylosing spondylitis. Associations were since usually observed with HLA-DR and DQ genes. We are also focused on Diabetes mellitus 1. type. Diabetes is an organ specific autoimmune disease characterised by destruction of beta-cells of Langerhansen pancreatic islets. The cause of the destruction process is not known yet, but it seems to be triggered by repeated presentation of self-antigen hGAD65 to the immune system. This review presents the most interesting solutions and hypotheses of the unanswered question about HLA association mechanism.

**Key words:** HLA, association, autoimmunity, hypothesis, ankylosing spondylitis, HLA-B27, diabetes mellitus, glutamic acid decarboxylase (hGAD65), enteroviruses (HERVs), regulation of gene expression.

*Ko.*

*Čas. Lék. čes., 141, 2002, No. 24, p. 755–762.*

Asociácia autoimunitných ochorení s HLA molekulami triedy I a II bola potvrdená u ľudí aj u myší. Avšak jednotlivé autoimunitné ochorenia sú natoľko špecifické svojimi príznakmi, že sa doposiaľ nepodarilo vytvoriť všeobecne platný model zahŕňajúci vysvetlenie HLA asociácií, i keď známych je hneď niekoľko mechanizmov objasňujúcich autoimunitné procesy v spojení s HLA molekulami. Medzi najsilnejšie HLA asociované ochorenia patrí ankylozujúca spondylitída (AS) zo skupiny séronegatívnych spondylartritíd a diabetes mellitus 1. typu (DM1T). AS je asociovaná s génom triedy I – HLA-B27 a DM1T s HLA-DR/DQ génmi. Pre bližšie objasnenie HLA asociácií sme vybrali práve spomenuté ochorenia.

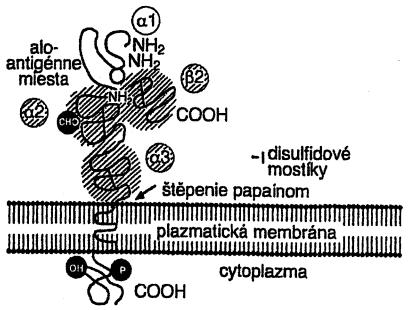
## HLAVNÝ HISTOKOMPATABILNÝ KOMPLEX

Genetický lokus zahrnutý do rejekčnej imunitnej odpovede tvorí oblasť MHC – hlavný histokompatibilný komplex (Major Histocompatibility Complex), u ľudí známy pod pojmom – humánny leukocytárny antigénový systém – HLA. HLA molekuly sú kódované v rámci MHC tvoriaceho komplex génov na krátkom ramene 6. chromozómu. Segment DNA je dlhý približne 3,6 Mb a predpokladá sa prítomnosť 224 génov. HLA antigény sú členom imunoglobulínovej superrodiny, pričom približne 39,8 % génov exprimovaných v rámci MHC lokusu je nepostrádateľných pre funkciu imunitného systému. HLA gény kódujú niekoľko izotypov, najdôležitejšie sú HLA molekuly triedy I (HLA-A, HLA-B, HLA-C) a HLA molekuly triedy II (HLA-DR, HLA-DQ, HLA-DP). Jedná sa o transmembránové antigény, ktoré viažu a prezentujú antigenické peptidické fragmenty, ktoré sú rozpoznávané imunitným systémom. Samotné HLA antigény sú antigénmi len v prípade, ak sú exponované cudziemu imunitnému systému, k čomu dochádza pri nehistokompatibilných transplantáciách a pravdepodobne autoimuntných poruchách. Okrem HLA génov triedy I a II sú týmto lókusom kódované molekulárne chaperóny (heat-shock proteíny Hsp 70, HLA-DM), cytokíny (tumour necrosis factor-α, lymfotoxín), zložky komplementu (C2, C4, faktor B), ktoré sú označované ako HLA molekuly triedy III.

HLA antigény triedy I sú exprimované na väčšine jadrových buniek organizmu s výnimkou neurónov a sú zahrnuté do procesu cytotoxickej odpovede proti vírusom infikovaným a rakovinovým bunkám. Ľudská HLA antigénová oblasť triedy I je kódovaná tromi lokusmi HLA-A, HLA-B, HLA-C. Ďalšia analýza HLA oblasti potvrdila i prítomnosť lókusov HLA-E, HLA-F a HLA-G. HLA-G antigény boli detekované na extravilóznom cytotrofoblaste, kde chránia bunky plodu pred NK-bunkami (natural killers). Molekula HLA antigénu triedy I

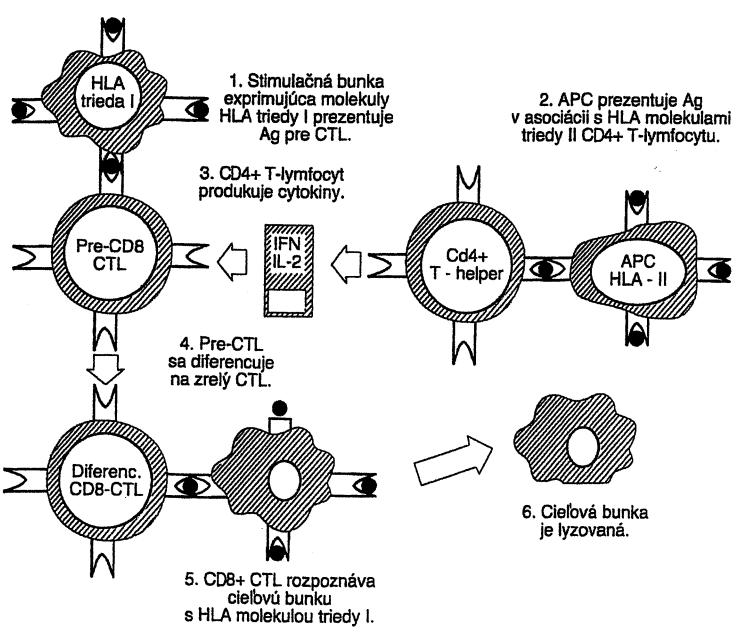
je heterodimérom, ktorý zahŕňa glykozylovaný ťažký peptidový reťazec (45kDa) – alfa (α) reťazec, nekovalentne asociovaný s β2-mikroglobulínom (12kDa) (obr. 1). Domény α1 a α2 tvoria platformu 8 antiparalelných β-listov a 2 α-helixov (vzdialené približne 10–18 A°), ktoré ohraničujú žliabok. Kryštalická štruktúra antigénov potvrdila prítomnosť difúznej hustoty práve v mieste žliabku, na čom sa zakladá tvrdenie o prítomnom väzbovom mieste pre procesované antigény. Toto tvrdenie je podmienené i pozorovaním najväčšieho počtu polymorfných miest práve v oblasti žliabku. Zámena aminokyselín v týchto polymorfných miestach vedie k dramatickej zmene tvaru celého žliabku a naväzujúceho sa peptidu. Žliabok nie je hladkou štruktúrou, pretože postranné reťazce aminokyselín navzájom interagujú a môžu tvoriť „vrecká" (pockets). Zámeny aminokyselín v týchto pozíciách tiež značne ovplyvňujú afinitu k procesovanému peptidu. HLA antigény triedy I prezentujú peptidy endogénneho pôvodu, vrátane virálnych produktov a tumorových antigénov. Krátko potom, čo sa prvý krát popísala funkcia T-lymfocytov sa zistilo, že T-lymfocyty lyzujú len tie cieľové bunky, ktoré zdieľajú ten istý povrchový HLA antigény triedy I. Táto vlastnosť bola pomenovaná MHC reštrikciou (1979) (1). Za objav MHC reštrikcie získali jej autori Zinkernagel a Doherty v roku 1996 Nobelovu cenu. Správne rozpoznávanie self a nonself-antigénov HLA molekulami je podmienené edukáciou lymfocytov v týmuse. Koreceptorom HLA molekúl triedy I je molekula CD8 prítomná na povrchu T-cytotoxických lymfocytov, ktorá väzbou s TCR komplexom a HLA molekulou triedy I indukuje imunitnú odpoveď (obr. 2).

HLA antigény triedy II sú exprimované na antigén-prezentujúcich bunkách (APC) – ako sú dendritické bunky, makrofágy, monocyty, B-lymfocyty, aktivované T-lymfocyty. Produkty HLA génov triedy II sú heterodiméry HLA-DR, HLA-DQ,

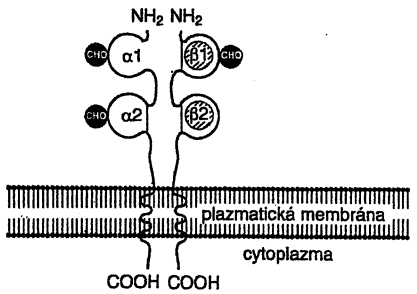



**Obr. 1.** Štruktúra HLA molekuly triedy I
Molekula HLA antigénu triedy I (HLA-A, B, C) je heterodimérom, ktorý zahŕňa glykozylovaný ťažký reťazec – α (45 kDa), nekovalentne asociovaný s β2-mikroglobulínom (12 kDa). Molekula sa delí na tri časti: cytoplazmatickú, transmembránovú a extracelulárnu. Domény α1 a α2 tvoria žliabok, v ktorom je lokalizovaná peptid-viažúca oblasť (PBR). HLA molekula triedy I viaže CD8-receptor na Tc-lymfocytoch.

HLA-DP pozostávajúce z ťažkého (α) a ľahkého (β) glykoproteínového reťazca (obr. 3). Molekulová hmotnosť α-reťazca je 30–34 kDa, β-reťazca je 26–29 kDa, čo sa mení s jednotlivými lokusmi. Početné dôkazy svedčia o podobnosti, respektívne zhode α a β-reťazcov po stránke štrukturálnej, rozdiel v molekulovej hmotnosti medzi α a β-reťazcom je dôsledkom rozdielnej glykozylácie. Koreceptorom HLA antigénov triedy II je molekula CD4. Štúdie zaoberajúce sa mutagenézou HLA-DR1 molekuly potvrdili, že sa táto viaže k CD4 β2-doménou. Následná asociácia CD4 s TCR komplexom sa považuje za iniciujúcu bunkovú aktiváciu T-helper buniek (väzbou dochádza k naviazaniu p56 k cytoplazmatickej časti CD4)



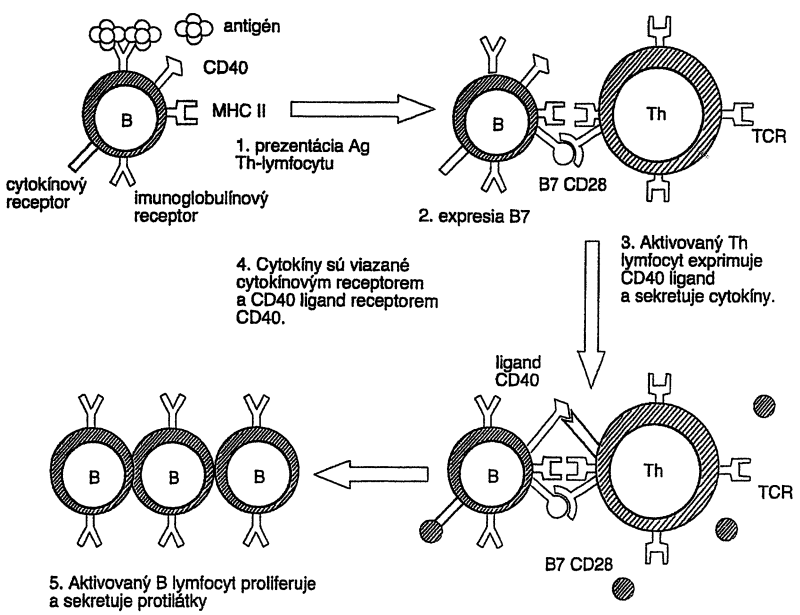

**Obr. 2.** Indukcia Tc-lymfocytov pri prezentácii antigénu HLA molekulami triedy I
Stimulačná bunka exprimujúca HLA molekulu triedy I prezentuje endogénny peptid ako antigén (Ag) pre-cytolymfotoxickému lymfocytu I (pre-CTL), ktorý vplyvom interleukínov a interferónu dozrieva na diferencovaný CD8+ lymfocyt. CTL-bunka následne lyzuje cieľovú bunku prezentujúcu stimulačná antigén. Interleukíny a interferón sú produkované Th-lymfocytmi po signále z APC-bunky (antigén prezentujúca bunka) exprimujúcej HLA molekuly triedy II v komplexe s Ag.



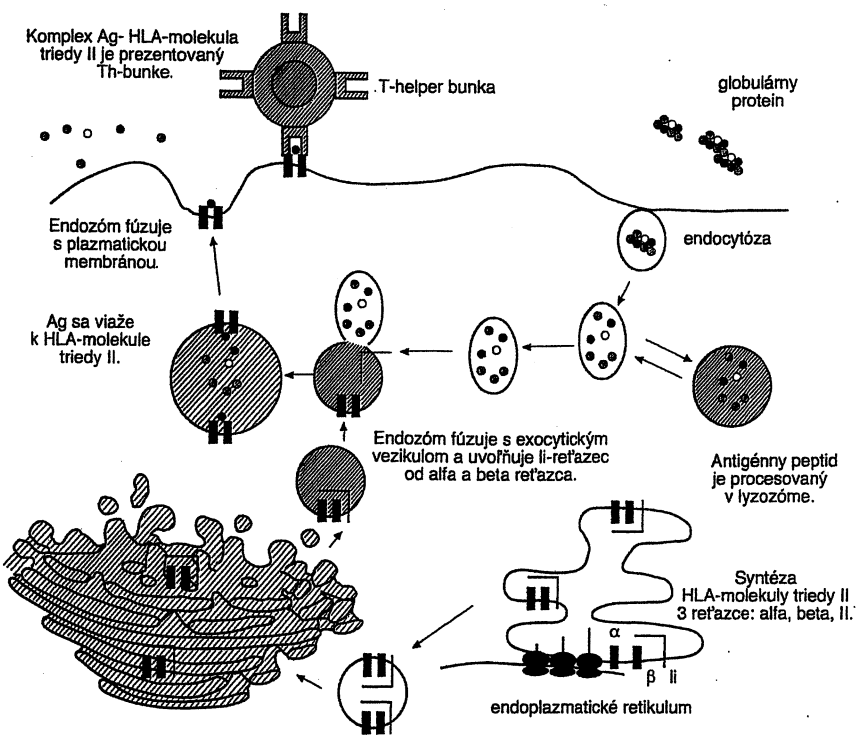

**Obr. 3.** Štruktúra HLA molekuly triedy II
Produkty HLA génov triedy II: (HLA-DR, DO, DP) sú heterodiméry pozostávajúce z ťažkého reťazca – α (30–34 kDa) a z ľahkého reťazca – β (26–29 kDa). Po stránke štrukturálnej sú oba reťazce zhodné, rozdiel je len na úrovni glykozylácie. Molekula sa skladá z troch častí: cytoplazmatická, transmembránová, extracelulárna. Peptid viažúcu oblasť tvoria domény α1 a β1. Doména β2 sa viaže k CD4 receptoru (koreceptor HLA-molekuly triedy II) na Th-lymfocytoch.

(obr. 4). Žliabok HLA antigénov triedy II je viac otvorený, čím umožňuje väzbu dlhším proteínom a tiež umožňuje väzbu postranných proteínových reťazcov vo vreckách.

Antigénové fragmenty prezentované HLA molekulami triedy I sú generované v cytosole a aktívne transportované pomocou chaperónov do endoplazmatického retikula. Vstup endogénnych peptidov do endoplazmatického retikula umožňujú membránové transportéry. K intracelulárnych chaperónom patria tapasín, calretikulín, calnexin, ERp57. Chaperóny sú zodpovedné aj za disulfidové výmeny na úrovni HLA molekúl. Peptidy (väčšinou nonaméry) sú v endoplazmatickom retikule asociované s nascentnými HLA molekulami triedy I, kde indukujú konformačné zmeny v ťažkom reťazci, čo posilní celkovú stabilitu komplexu. Ak sa peptid naviaže na HLA-molekulu, tapasín a calnexin disociujú (2). Po asociácii domény α3 s β2-mikroglobulínom je HLA molekula exprimovaná na povrchu, kde môže interagovať s receptormi špecifických T-lymfocytov. Obdobným procesom prechádzajú pri prezentácii antigénu exogénneho pôvodu aj HLA molekuly triedy II (obr. 5). Generácia samotných peptidových fragmentov pre prezentáciu HLA molekulami triedy I je závislá na aktivite veľkého multikatalytického proteázového komplexu v cytosole, ktorý je známy ako proteazóm (3). Tento enzýmový komplex je aktívny na proteínoch označených ubiquitínom. Po pripojení niekoľkých ubiquitínových reťazcov je proteín rozbalený a transportovaný do centra proteazómu, kde podlieha ATP-závislej katalytickej degradácii. Ľudské LMP2 a LMP7 gény, ktoré ležia v MHC oblasti triedy II (telomericky k HLA-DP), ako aj tretí non-HLA gén, kódujú neesenciálnu β-podjednotku 20S proteazómu, ktorá môže byť indukovaná interferónom-γ. Táto podjednotka môže nahradiť 3 z konštitutívnych podjednotiek, ktoré sú interferónom-γ suprimované (4). Toto môže meniť proteínové štiepenie v proteazóme. Gény kódujúce už spomínané membránové peptidové transportéry (transportéry asociované s processingom peptidov TAP1 a 2) sa nachádzajú v HLA oblasti II, sú členmi rodiny ATP-závislých membránových transportérov. Mutanti s deletovanými úsekmi TAP1 a 2 neexprimujú HLA molekuly, alebo ich exprimujú len v obmedzených množstvách. Táto porucha sa môže čiastočne vylepšiť pridaním sekundárnych peptidov k inkubovaným bunkám, ktoré sa viažu na ťažký reťazec HLA molekúl a stabilizujú ho. U potkanov polymorfizmus v TAP génoch ovplyvňuje charakter proteínov vchádzajúcich do ER (5). U ľudí je zatiaľ polymorfizmus TAP len minimálny a jeho úloha v ovplyvňovaní expresie HLA molekúl je tiež limitovaná (6). V Maroku sa identifikovala rodina s nefunkčným TAP2 génom, čoho následkom bola non-expresia HLA molekúl triedy I, ktorá spôsobuje rekurentné bakteriálne infekcie (7). Bunkové línie derivované z malo-




**Obr. 4.** Aktivácia B-lymfocytov
Antigén (Ag) transportovaný do B-lymfocytu je v komplexe s HLA molekulou prezentovaný Th-lymfocytu. Spojenie buniek je navodené kontaktom molekúl B7 a CD28. Aktivovaný Th-lymfocyt exprimuje CD40 ligand a sekretuje cytokíny, ktoré sú viazané B-lymfocytom a vedú k proliferácii B-lymfocytov a následne k produkcii protilátok.




**Obr. 5.** Processing antigénov
Globulárny antigénny proteín sa endocytózou dostáva do bunky, kde je lyzovaný v lyzozóme, alebo fúzuje v endozóme s exocytickým vezikulom Golgiho aparátu. V exocytickom vezikule Golgiho aparátu sa nachádza alfa a beta reťazec HLA molekuly viazaný invariabilným reťazcom (Ii). Po fúzii endozómu s vezikulom invariabilný reťazec (Ii) disociuje a uvoľňuje priestor pre naviazanie antigénneho peptidu na reťazec α a β. Endozóm fúzuje s plazmatickou membránou a komplex HLA-Ag je prezentovaný Th-lymfocytu.

bunkového pľúcneho karcinómu s defektným TAP1 génom neexprimujú HLA molekuly triedy I, čo by mohlo byť potenciálnym vysvetlením obchádzania imunologického dohľadu rakovinovými bunkami (8). Pri ankylozujúcej spondylitíde (AS) sa zdá byť polymorfizmus v LMP a TAP génoch irelevantný (9), hoci existujú tvrdenia, že iritída aj periférna artritída sú spojené práve s polymorfizmom LMP génu a TAP génu (10, 11).

## ANKYLOZUJÚCA SPONDYLITÍDA (AS) A HLA-B27

Asociácia AS a HLA-B27 je doposiaľ najsilnejšou popísanou v literatúre (OR = 87–92) (33, 35). Čo sa týka molekulárnej štruktúry HLA-B27 molekuly vieme veľmi veľa, jej úloha v patogenéze však ostáva predmetom diskusií. Všeobecne sa hovorí o piatich teóriách možnej HLA-B27 úlohy pri AS. Posledné dôkazy však potvrdzujú priamu úlohu HLA-B27 antigénu v patogenéze.

*Teória tio-reaktívnej hypotézy*

V ústí B-vrecka HLA-B27 molekuly sa na pozícii 67 nachádza cysteín oproti lyzínu, ktorý je na pozícii 70. Zmenená oxidácia reaktívnej tio-skupiny na cysteíne môže spôsobiť zmeny ako pri rozpoznávaní a prezentácii peptidu, tak i pri samotnom rozpoznaní vlastnej HLA-B27 molekuly (12). Robili sa pokusy i s bunkami ošetrenými homocysteínom (posilnenie homodimerizačnej úlohy cys 67), ktoré boli lyzované špecifickými CTL bunkami izolovanými z pacientov trpiacich spondylartropatiami, ale neboli lyzované špecifickými CTL bunkami izolovanými zo zdravých jedincov (14). Iné štúdie tvrdia, že B27 pozitívne bunky vystavené voľným radikálom naviazaným na monoklonálnej protilátke nevykazovali žiadne zmeny (13).

*Teória promiskuitného peptidu*

HLA-B27 fragmenty a fragmenty iných HLA molekúl triedy I sa našli i medzi peptidmi prezentovanými HLA molekulami triedy II, čo by mohlo byť impulzom k tvrdeniu, že HLA-B27 je prezentovaná imunitnému systému spúšťajúc autoimunitnú odpoveď (15). Tým by sa vysvetlil výskyt prevažne CD4-lymfocytov v mieste zápalu pri spondyloartropatiách. Nezistená silná asociácia s HLA molekulami triedy II len reflektuje na promiskuitné viazanie peptidov molekulami triedy II (16). Myšie a zvieracie modely spondylartropatií podporujú túto teóriu. U myší, transgénnych pre HLA-B27 ale s knockoutovaným β2m, sa B27 vyskytuje len vo veľmi malých množstvách na bunkovom povrchu (19). Tieto myši majú nízku hladinu CD8-lymfocytov, a B27 fragmenty sú prezentované HLA molekulami triedy II a spôsobujú artritídu. V transgénnych potkanoch je rozvoj artritídy závislý od počtu kópií HLA-B27 transgénu (17). U ľudí sa však HLA-B27 správa dominantne a nevyskytuje sa žiadny efekt dávky (18).

*Teória molekulového mimikry*

Molekulové mimikry medzi HLA-B27 a bakteriálnymi antigénmi boli objavené ako výsledok pozorovania humorálnych

krížových reakcií medzi HLA-B27 a baktériami (20). Druhou možnosťou je krížová reakcia medzi baktériami a B27-prezentovanými endogénnymi peptidmi. Existujú potenciálne ciele pre molekulové mimikry, dôkazom sú početné homológie medzi bakteriálnymi sekvenciami a HLA-B27 (21). Najprebádanejším je úsek z nitrogenázy *Klebsiella pneumoniae* – hexapeptid QTRDRE, ktorý je homologický s úsekom HLA-B*2705 práve v hypervariabilnej oblasti, kde je na mieste 77 kys. asparágová, ktorá sa u subtypov B*2702 a B*2704 nevyskytuje (tie nie sú s chorobou asociované). Nie je to však sekvencia rozhodujúca pre naviazanie peptidu, preto nemôže byť ani cieľom krížovej reakcie pri rozpoznaní T-lymfocytom. U pacientov s AS boli opakovane zistené zvýšené protilátky proti Klebsielle (22), ale nie je jasné, či sú naozaj príčinou AS. Na druhej strane existujú dôkazy potvrdzujúce, že s Klebsiellou krížovo-reagujúce T-bunky pacientov s AS sú odstraňované z periférneho krvného riečišťa a sú deponované v kĺbových štuktúrach (23). V centre pozornosti je i ďalší homologický úsek kódovaný plazmidom Shigella v jeho artritogénnej forme. Medzi HKA-B27 a HLA-B27 dochádza tiež ku krížovým reakciám, čo podporuje model molekulového mimikry pre AS. pretože aj alela HLA-B*2708 je serologicky typizovaná ako HLA-B7 (24).

***Teória viazaného génu***

Je akceptované, že sa HLA-B27 podieľa na patogenéze AS. Je však možné, že je len markerom génu, ktorý je s AS primárne asociovaný. Je možné, že takisto len niektoré HLA-B27 haplotypy môžu ovplyvňovať penetraciu alebo expresiu choroby, čo sa študuje pozorovaním TAP, LMP, a DR génov, 90 % pacientov s AS v kaukazoidných populáciách sú B27+ a ak zo súboru vylúčime pacientov s kontraindikáciu IBD (Intestinal Bowel Disease) je podiel pozitívnych 98 %, čo dokazuje prítomnosť silne asociovaného génu, ale pátranie po ňom bolo doposiaľ neúspešné i napriek použitým najmodernejším DNA-technikám (25). Podieľanie sa iných HLA i non-HLA génov na AS je pravdepodobné (26).

***Teória artritogénneho peptidu***

Táto hypotéza vychádza z toho, že krížové reakcie sú invokované artritogénnym peptidom, ktorý perzistuje v synoviu a kĺbových štruktúrach. Tieto reakcie sa odohrávajú na úrovni riadenej T-lymfocytmi a prezentáciou antigénu pomocou HLA-B27. Artritogénne peptidy môžu byť endogénneho alebo exogénneho pôvodu. Slabšie asociácie s AS boli nájdené i medzi génmi HLA triedy II: HLA-DR1, DR2, DR7 a DR8. Etnické rozdiely v haplotypovej väzbe s HLA-B27 však tieto málo preukazné nálezy vyvracajú.

Doposiaľ neuvádzanou teóriou je **TAPASÍN-BLOK** teória. Táto teória je sumarizáciou výsledkov, ktoré dokazujú priamu úlohu HLA-B27 génu pri rozvoji AS. Podkladom pre jej vznik boli závery z prác prezentovaných na druhom medzinárodnom kongrese Spondylartropatií v Gente (október 2000). Infekcia predchádzajúca vznik AS je vo väčšine prípadov spojená s baktériami čeľade *Enterobacteriacae* (rod *Salmonella typhi, Schigella* sp., *Campylobacter* sp. ap.). Baktérie tejto čeľade sú typické vnútrobunkovou parazitáciou a ich eliminácia je v bunkách HLA-B27+ znížená oproti bunkám s HLA-A2. Expresiou génov virulencie baktérií (u *Salmonella typhi*, sú to: groEL, sipB, spiC a mtgC – (27)) dochádza k zablokovaniu niektorých chaperonov (napr. tapasínu) v monocytických bunkách. Následkom tohto procesu je vznik homodimérov HLA-B27, ktoré sú nadexprimované najmä v bunkách tapasín-deficientných (28). Vznik HLA-B27 homodimérov potvrdili viaceré pracoviská. Primárnym predpokladom vzniku dimérov je nespárený aminokyselinový zvyšok cys 67 (29, 30). Homodiméry HLA-B27 sú zadržiavané nesprávne zbalené v endoplazmatickom retikule podstatne dlhšie než normálny komplex (5 hodín). Nesprávne zbaľovanie proteínu môže vyvolať stresový signál a následne intracelulárnu signalizáciu pre vznik choroby (31). U transgénnych zvierat sa potvrdil i podpôrný mechanizmus vzniku HLA-B27 homodimérov, ak sa vyskytuje blok pre β2 – mikroglobulín. Poukazuje sa i na fakt, že špecifické CD8+T-lymfocyty sú asociované s AS po reštrikcii cez HLA-B27 molekulu a na ňu naviazaný „artritogénny peptid" (32). Cytotoxicitu CD8 T-lyfmocytov ovplyvňuje aj špecifická expresia NK-receptorov na bunkovom povrchu CD8 intrasynoviálnych T-lymfocytov. Monocyt nesúci HLA-B27 homodimér s „artritogénnym peptidom" je schopný aktivovať CD4+ T-lymfocyty a vyvolať tak aktiváciu NK-buniek, makrofágov a granulocytov ako i nešpecifických Tc-lymfocytov.

## DIABETES MELLITUS 1. TYPU (DM1T) A HLA TRIEDA II

Diabetes mellitus 1. typu (DM1T) – je autoimunitné ochorenie, ktoré vykazuje orgánovú špecificitu prítomnosti jednotlivých autoprotilátok a je charakterizované imunitne sprostredkovanou deštrukciou β-buniek pankreatických Langerhansových ostrovčekov. Deštrukcia je iniciovaná interakciou medzi jednotlivými genetickými a enviromentálnych faktormi. Špecificky zameraný deštrukčný proces je výsledkom existencie vlastného antigénneho proteínu, derivovaného zo samotných β-buniek, ktorý je potom rozpoznávaný T-lymfocytmi.

Rozsiahle analýzy identifikovali v sérach pacientov niekoľko protilátok potenciálne namierených proti kandidátnym proteínom. Izoforma ľudskej glutamátdekarboxylázy hGAD65 je exprimovaná v pankreatických bunkách v pomerne vysokom množstve a protilátky proti tomuto enzýmu sú preukázateľné u 70 % novodiagnostikovaných prípadov diabetes 1. typu. Tieto protilátky sú prítomné v sérach pacientov niekoľko rokov pred rozvojom klinických prejavov samotného diabetes a predstavujú teda využiteľný predikčný marker pred nástupom ochorenia. Súčasné analýzy potvrdili, že supresiou expresie **GAD-proteínu** v prípade neobéznych NOD-myší sa zabráni rozvoju autoimunitného diabetes, čo by poukazovalo na priamu úlohu tohoto proteínu pri progresii ochorenia. Štúdie zaoberajúce sa reaktivitou T-lymfocytov s autoantigénmi u diabetikov potvrdili, že u pacientov dochádza k imunitnej odpovedi proti hGAD65 sprostredkovane CD4+ a CD8+ lymfocytmi, čo poukazuje na superantigénne vlastnosti spomenutého peptidu. Približne 70 % detských diabetikov kaukazoidnej populácie exprimuje alely HLA-DRB1*0401, *0402, *0405. Tieto alely sú vo väzbovej nerovnováhe s génom HLA-DQ*0302, ktorý je považovaný za priamy predikčný marker vysoko asociovaný s juvenilným diabetes 1. typu. Reakcie T-lymfocytov voči hGAD65 sú prítomné najmä u pacientov nesúcich v genóme práve uvedené haplotypy HLA. Tieto HLA-molekuly sa zúčastňujú procesu reštrikcie, ktorá je predpokladom imunitnej odpovede voči hGAD65. Nie je však vylúčené, že práve uvedené HLA alely sú schopné hGAD65-peptid alterovať do takej miery, že sa stane suprantigénom priamo aktivujúcim T-lymfocyty (36).

S diabetes sú asociované špecifické alely génov HLA-DR a HLA-DQ. Alely HLA-DQB1*0201 a HLA-DQB1*0302 sú asociované najsilnejšie a sú vo väzbovej nerovnováhe s alelami HLA-DR4 a HLA-DR3. Najväčšie riziko nesie jedinec

s haplotypom DR4/DR3. Transgénna myš s haplotypom HLA-DR3/HLA-DQB1*0302 spontánne stratila schopnosť tolerancie k peptidu GAD65. Hoci myši trpeli spontánnou inzulitídou, diabetes sa u nich nerozvinul. Myši exprimujúce HLA-DR3 alebo HLA-DQB1*0302 vykazovali špecifickú infiltráciu pankreatických ostrovčekov T-lymfocytami, ktorá sa vytratila, ak boli tieto antigény HLA koexprimované s HLA-DR2 a HLA-DQ6, ktoré sú považované za alely neutrálne k diabetes. Tento myší model dokazuje, že k rozvoju autoimunity je potrebná kombinácia HLA-DR a HLA-DQ alel (37). Predpokladá sa, že už samotná štruktúra HLA molekuly determinovaná príslušnou sekvenciou môže spôsobiť rozvoj diabetes. Je možné, že niektoré HLA-DQ alfa a beta reťazce vytvárajúce heterodiméry vykazujú zníženú stabilitu, čo naznačuje možnosť vzniku homodimérov HLAD-DQ, ktoré môžu iniciovať imunitnú reakciu (47).

Význam HLA molekúl v procese indukcie ochorenia je väčší u pacientov s diabetes diagnostikovaným pred 20. rokom veku pacienta. Tento fakt jasne dokumentuje, že s vekom rastie úloha vonkajších faktorov a klesá význam faktorov genetických. Taktiež však navodzuje otázku, či existuje **rozdiel v spektre HLA molekúl prezentujúcich antigény v detstve a v dospelom veku**. Ak sa expresia jednotlivých antigén-prezentujúcich HLA molekúl môže s vekom meniť, je pravdepodobné, že jednotlivé podľa veku rozdelené skupiny pacientov budú vykazovať asociácie s rozdielnymi HLA-DR/DQ antigénmi (38).

Pri skúmaní priameho prepojenia HLA molekúl s patologickým procesom pri diabetes 1. typu je potrebné zamerať sa na mechanizmus prezentácie jednotlivých antigénnych peptidov bunkám imunitného systému HLA molekulami. Správna funkcia HLA molekúl pri prezentácii jednotlivých antigénov je popísaná na obrázku 2 a 4. Antigénovo špecifické B-lymfocyty môžu procesovať antigén a exprimovať Ag-HLA komplex už hodinu po vystavení sa antigénu, ale až 3–4 dni si vyžaduje proces aktivácie a produkcie populácie Ag špecifických efektorov – CD4+ lymfocytov. Momentálne nie je jasné, ako B-lymfocyty predlžujú produkciu komplexu HLA-Ag, ktorý je lokalizovaný na ich bunkovom povrchu, až do doby, keď sa T-lymfocyty stanú aktívnymi. Skúmala sa teda funkcia **BCR-receptorov B-lymfocytov vo vzťahu k prezentácii antigénov** zisťujúc, či tieto bunky disponujú mechanizmom predlžujúcim expresiu komplexu Ag-HLA na ich bunkovom povrchu. Antigén naväzujúci sa na BCR-receptor B-lymfocytu prichádza do tzv. skorého endozómu, odkiaľ je presunutý do non-terminálneho neskorého endozómu. Ukázalo sa, že práve v neskorom endozóme sa komplex vyskytuje dlhšiu dobu, zatiaľčo antigény prichádzajúce do bunky „via fluid", sú lokalizované priamo do terminálneho lyzozómu a degradované. Ag-BCR komplexy v rámci výskytu v nonterminálnom endozóme preukazujú kolokalizáciu so chaperónom HLA molekúl triedy II – HLA-DM/H2. Táto kolokalizácia je vyššia než kolokalizácia spomenutého chaperónu s jeho regulátorom HLA-DO/H2. V konečnom dôsledku B-lymfocyty s prítomnými Ag-BCR komplexami môžu Ag-HLA komplex prezentovať na bunkovej membráne dlhšie. Ak teda existuje v rámci B-lymfocytov mechanizmus predlžujúci prezentáciu jednotlivých antigénnych komplexov, je možné, že práve tento mechanizmus je zodpovedný za opakované prezentácie antigénnych komplexov pri autoimunitných ochoreniach (39). Funkcia samotných HLA-DM a HLA-DO molekúl sa zistila len nedávno. HLA molekuly triedy II spolu s naviazaným peptidom sa pred samotnou prezentáciu na bunkovom povrchu lokalizujú do neskorých endozomálno-lyzozomálnych kompartmentov – **MIIC. HLA-DM a HLA-DO** sú tzv. modulačnými molekulami lokalizovanými tiež do MIIC. Zistilo sa, že HLA-DM/DO komplexy sa pravidelne recyklujú medzi plazmatickou membránu a MIIC. Obdobne ako pri HLAD-DM-beta reťazci a invariantnom reťazci HLA molekuly triedy II je aj cytoplazmatická doména HLA-DO-beta reťazca vybavená cieľovými sekvenciami pre lyzozomálne membrány MIIC organely. Tieto sekvencie nie sú esenciálne pre internalizáciu HLA-DM/DO komplexov do MIIC, ale ako sa ukázalo, sú podstatné pri lokalizácii komplexu HLA-DM/DO komplexov v rámci multivezikulárneho MIIC. HLA-DO určuje lokalizáciu HLA do internej (menej prípadov) alebo vonkajšej membrány MIIC. Správna distribúcia HLA molekúl v MIIC je esenciálna pre zvýšenie efektivity naviazania prezentovaného peptidu s HLA molekulou triedy II. HLA-DM/DO komplex predstavuje teda nový špecifický spôsob regulácie prezentácie antigénov (41).

Ďalším možným vysvetlením pre priame zahrnutie HLA molekúl do etiológie autoimunitných ochorení je fakt, že sa v rámci druhého intrónu génu HLA-DRB1*0401 našla **mikrosatelitová oblasť (GT)22(GA)15 – pomenovaná A9, viažúca regulačný proteín CTCF**. CTCF je transpkripčným faktorom pozostávajúcim z 11 zinkových prstov s viacerými špecifitami pri rozpoznávaní DNA sekvencií. CTCF reguluje expresiu viacerých génov, ako napr. ľudský onkogén c-myc alebo kurací lyzozým. CTCF viaže mikrosatelitovú oblasť v intróne HLA-DRB1*0401 silnejšie, než je tomu pri c-myc a lyzozýme, čo dokázala mnohonásobne znížená expresia sledovaného reportérového génu. Spomenutý prípad riadenia génovej expresie pomocou mikrosatelitovej oblasti je vôbec prvým známym, preto je snáď priskoro poukazovať na možnosti blokovania transkripcie exónu HLA-DRB1*0401, a teda následnú zmenu funkcie HLA molekuly pri prezentácii antigénov (42).

Molekulárno-genetické štúdie identifikovali v HLA oblasti na 6. chromozóme viac než 200 génov. V rámci tejto oblasti (jednej z najväčšou hustotou génov) boli identifikované i rozptýlené opakovania, ako sú SINEs a LINEs a takisto sekvencie ľudských endogénnych retrovírusov (HERVs). Prítomnosť retroelementov v HLA oblasti je priamym dôkazom plasticity genómu v rámci evolúcie ovplyvňovanej práve **retrosekvenciami**. Súčasne boli identifikované HERVs produkujúce superantigény, ktorých gény sa stali kandidátnymi génmi pre rizikové faktory pri diabetes 1. typu a sclerosis multiplex. Aktívne amplifikácie jednotlivých typov retroelementov môžu viesť k delécii jednotlivých oblastí v rámci -HLA génov. Regulácia génovej expresie závislá na HERV--LTR bola dokázaná už pri niekoľkých ľudských génoch. Sekvencia ERV9, ktorá je súčasťou LTR sekvencie identifikovanej v HLA-DR oblasti, je tiež sekvenciou viažucou niekoľko transkripčných faktorov a je považovaná za evolučne konzervovanú iniciačnú sekvenciu. Zaujímavosťou je, že aj interferón gama – responzívny element (IFN-γ-IRE) bol identifikovaný v rámci ERV9 sekvencie. Ako vieme, INF-γ je jediným známym induktorom HLA expresie. Preto sa poukazuje na možnosť, že ERV9 pôsobí ako enhancer génovej expresie pre HLA gény a je od neho závislá aj samotná promócia génovej expresie. Jeho prítomnosť však v rámci jednotlivých genotypov (napr. u HLA-DR1) dokázaná nebola. Práve jeho prítomnosť, resp. neprítomnosť by mohla byť rozhodujúcou pri riadení expresie HLA molekúl na bunkovom povrchu. Existujú štúdie zaoberajúce sa silou expresie HLA molekúl práve v súvislosti s dostatočnou intenzitou imunitnej odpovede závislej na transkripčných induktoroch. Endogénne retrovírusy boli lokalizované aj do druhého intrónu DRB7 pseudogénu v haplotype HLA-DR53. Tieto HERVs patria do rodiny HERV-K, ktorej

členom je aj gén HERV-K(C4) prítomný v HLA oblasti III. Ďalším členom tejto rodiny je novozistený IDDMK 22, ktorý bol izolovaný v skupine diabetikov. Ukazuje sa, že tento HERV-K vírus kóduje superantigén, ktorý aktivuje v komplexe s HLA molekulami triedy II T-lymfocyty, čo vedie ku klonálnej expanzii T-lymfocytov, a teda k potenciálnemu rozvoju autoimunitného ochorenia. Pre toto tvrdenie však neexistovali dôkazy. Avšak po identifikácii LTR sekvencii v HLA-DQ oblasti, ktoré vykazovali priame spojenie s HERV-K proteínmi typu **IDDMK 22**, sa o spomenutých HERV-K proteínoch uvažuje ako o faktoroch podporujúcich rozvoj autoimunitných ochorení svojou rozdielnou integráciou do jednotlivých HLA génov, najmä v oblasti HLA-DR. Takisto treba spomenúť i možné rekombinačné procesy v rámci HLA oblasti indikované homológiou retro- a Alu- sekvencií vedúce k štrukturálnym zmenám jednotlivých HLA molekúl (40). Podobné výsledky zaznamenala aj výskumná skupina Pascaula (43), ktorá skúmala na 5-koniec prvého exónu HLA-DQB1 alely u pacientov s reumatoidnou artritídou (RA). Asociácia s RA bola potvrdená v prípade prítomnosti LTR3, LTR5 – sekvencie. Pre prípadné potvrdenie asociácie LTR13 s diabetes 1. typu sú potrebné ďalšie výskumy. V rámci ľudského genómu bolo identifikovaných 6201 **alternatívnych splicingových produktov**. Takmer 42 % z nich sa vyskytuje v génoch, ktoré zabezpečujú funkciu imunitného a nervového systému. Aj malá zmena v rámci sekvencie, či už je spôsobená integráciou retroelementu alebo naviazaním sa vírusového proteínu, môže viesť k zmene splicingového miesta v HLA molekule, a tým meniť jej funkciu a vlastnosti pri rozpoznávaní self-antigénov (44).

V súvislosti s endogénnymi vírusmi a ich prepojením s autoimunitnými ochoreniami dokumentujú súčasné štúdie zvýšené **enterovírusové infekcie** u prediabetických detí indikujúc, že tieto infekcie môžu iniciovať a akcelerovať proces deštrukcie beta-buniek niekoľko rokov pred klinickou manifestáciou diabetes 1. typu. Pomocou RT-PCR sa sledovala prítomnosť RNA enterovírusov v súbore prediabetických detí a detí zdravých. Prítomnosť vírusovej RNA bola asociovaná so zvýšenou hladinou protilátok proti bunkám ostrovčekov (ICA) a proti GAD. Oproti prediabetickým deťom, v skupine novodiagnostifikovaných diabetikov prítomnosť vírusovej RNA zistená nebola. Enterovírusové infekcie môžeme teda radiť k rizikovým faktorom vzniku diabetes, prípadne iných autoimunitných ochorení. Ak uvážime, že protilátky proti GAD sú prítomné u diabetikov v rozdielnych množstvách v závislosti od ich HLA haplotypov, je možné predpokladať, že existujú homológie medzi proteínmi enterovírusov a bunkovými proteínmi. Primárna enterovírusová infekcia môže teda viesť k namiereniu imunitnej odpovede voči self-peptidom – autoantigénom (45). **Úloha enterovírusov** v etiológii diabetes 1. typu sa spomína tiež v **súvislosti s homológiou k GAD**. Známy je najmä neštrukturálny proteín 2C vírusu Coxackie 4 (CBV4), voči ktorému bola pozorovaná zvýšená odpoveď T-lymfocytov v skupine pacientov s novodiagnostifikovaným diabetes (46). Skupina pacientov s diabetes 1. typu s protilátkami proti bunkám Langerhansových ostrovčekov, ktoré sú u nich preukázateľné viac ako tri roky, vykazuje aj zvýšenú prítomnosť tyroido-gastrických autoprotilátok. To dokazuje, že **diabetes 1. typu nie je len orgánovo špecifickým ochorením, ale zahŕňa celý imunitný komplex**.

Pri rozlúštení tajomstva autoimunitných HLA asociovaných ochorení bude zo strany výskumných tímov preto potrebná ešte značná dávka fantázie.

**Skratky**

| | | |
|---|---|---|
| AS | – | ankylozujúca spondylitída |
| BCR | – | receptor B-lymfocytu |
| DM1T | – | diabetes mellitus 1. typu |
| CTCF | – | transkripčný faktor typu Zn-prstov |
| CTL | – | cytotoxický T-lymfocyt |
| ER | – | endoplazmatické retikulum |
| GA | – | Golgiho aparát |
| GAD | – | glutamátdekarboxyláza |
| HERVs | – | ľudské endogénne retrovírusy |
| hGAD65 | – | glutamátdekarboxyláza (Mr = 65, humánna) |
| HLA | – | ľudský leukocytárny antigén |
| ICA | – | protilátky proti bunkám pankreatických ostrovčekov |
| IDDM22 | – | superantigén rodiny HERV-K zapríčiňuje klonálnu expanziu T-lymfocytov pri autoimunitných ochoreniach |
| IFN-γ | – | interferón γ |
| IFN-γ-IRE | – | interferón-γ-responzívny element |
| LINEs | – | dlhé rozptýlené opakovania |
| LPM | – | gény kódujúce aparát proteazómu |
| LTR | – | dlhé terminálne opakovanie |
| MIIC | – | neskorý lyzozomálny kompartment |
| NK | – | natural killers-bunky |
| PCR | – | polymerázová reťazová reakcia |
| SINEs | – | krátke rozptýlené opakovania |
| TAP | – | transportéry asociované s processingom peptidov |

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## ALZHEIMEROVA CHOROBA

Na telefonní lince 844 1111 38 poradí vyškolení operátoři volajícím, kde hledat informace o demencích a Alzheimerově chorobě a kam se mohou obrátit o pomoc. Infolinka je v provozu od pondělí od pátku mezi 11.00 – 18.00 hodinou.

Na webových stránkách www.stari.cz se tazatel dozví informace o Alzheimerově nemoci, jaké jsou její příznaky, jak poznat, že se jedná o tuto nemoc a ne o důsledek přirozeného procesu stárnutí mozku, na koho se v případě podezření na onemocnění tohoto typu demence obrátit, jaké jsou možnosti léčby a jak se má rodina vyrovnat s tímto faktem.

Alzheimerova choroba je závažné onemocnění mozku, které postihuje nejvíce osoby starší 65 let. V současné době je v České republice diagnostikováno zhruba 70 000 osob s touto nemocí. Chorobu lze dnes léčit pomocí moderních léků nové generace, tzv. kognitiv. Pro úspěch léčby je však nutné včasné rozpoznání příznaků.

*/Z tiskové konference/*

# 5. Selected presentations

**Abstracts from conferences published in journals with Impact Factor**

Novota P, Cerna M, Kolostova K, Cejkova P, Zdarsky , Novakova D, Kucera P, Novak J, Andel M: Associations of HLA DQB1 and DRB1 alleles with the LADA in Czech population. European Journal of Immunogenetics, 2002: 29:147. Impact factor 1.355

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# 6. Conclusions

- HLA-BRB1*04 and HLA-DQB1*0302 have been detected as risk factors in T1D in adults and particularly in children ( P< 0.001 OR = 22.9 and 46.5 respectively)
- HLA-DRB1*03 has been found as a single risk factor for LADA ( P< 0.001 OR =4,9)
- The 15 alleles for NFKB1 gene polymorphism were detected in Czech population, the frequency of A7 allele (132bp, 20 repeats) was significantly increased in T1D adults
- There was no difference in A and G allele frequency of NFKBIA gene, but the association with AA genotype has been found for LADA patients (P<0.001 OR=2.68)
- Analysis of the HLA-DRB1*04 expression in APCs of peripheral blood has described singnificantly increased quantity of mRNA in T1D adults in comparison with T1D children, but there was not any difference in HLA-DRB1*04 and NFKB1 gene expression testing between different HLA, NFKB1, NFKBIA genetic background of T1D diabetic group
- MIC-A5.1 allele is associated with genetic susceptibility to T1D adult-onset (P<0.009 OR = 2.14)
- IL-18 SNPs at position -607(C/A) and -137 (C/G) are not associated with T1D or LADA susceptibility
- According to our findings we can conclude that the progression of the three types of autoimune diabtes (T1D in adults, T1D in children and LADA) is strongly influenced by different immunogenetic background and by impact of environmental factors modifying the ethiopathogenesis of diabetes

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