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Autoimmune Markers in Autoimmune Diabetes

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Stockholm 2003

To my family

Contents

ABSTRACT

Type 1 diabetes mellitus (T1DM) is an autoimmune disease, characterized by autoimmune mediated loss of insulin secreting ß-cells. The disease is associated with certain HLA class II haplotypes. HLA DR3-DQ2 and DR4-DQ8 are positively while DQ6 (DQA1*0102-DQB1*0602) is negatively associated with the disease in Caucasians. Taken together, DQ8 and/or DQ2 account for 89% of Swedish T1DM patients. Other genetic loci might be associated with T1DM. The hope is that by assessing multiple risk loci, pattern of alleles that substantially increase the sensitivity of genetic typing can be identified. Besides HLA-DQ and DR, polymorphism in another gene, MHC class I chain related gene-A (MICA), located in HLA class I region has been reported to influence the susceptibility to T1DM. Autoantibodies against β-cell antigens GAD65, IA-2 and insulin (IAA) are markers for T1DM and are rarely found in healthy population. Certain group of patients clinically diagnosed as type 2 diabetes mellitus (T2DM) does not respond to oral hypoglycemic treatment and require insulin therapy. Many of these T2DM patients are positive for T1DM associated autoantibodies and become insulin deficient because of autoimmune β-cell destruction. This form of diabetes is called as latent autoimmune diabetes in adults (LADA) or slow - onset T1DM. Viruses, in particular Coxsackie virus B (CBV), are one of the environmental factors proposed to be involved in disease pathogenesis.

Aim: To determine, in Swedish population, the association of MICA gene polymorphism with acute onset T1DM, with LADA, with antibodies against CBV among T1DM patients, with autoantibodies among T1DM patients and to determine the frequency of MICA alleles among newborn babies genetically at-risk to develop T1DM with respect to HLA-DQ.

Results and discussion: We found MICA5 to be positively associated among 0-35 year old T1DM independent of high-risk HLA. MICA5 with DR3-DQ2 gave a higher risk compared to risk with DR3-DQ2 alone. MICA6 was negatively associated with younger onset (0-20 years) T1DM. MICA5/5.1 was associated with T1DM and LADA. Association of DR3-DQ2/DR4-DQ8 and DR3-DQ2/DR3-DQ2 with LADA as reported earlier was confirmed. We found DR3-MICA5.1 to be more significantly associated with antibodies against CBV compared to DR3 alone or other high-risk HLA alone in the high incidence region of Linköping in southeast of Sweden. We still don't know how MICA polymorphism along with CBV infection might be important in T1DM pathogenesis. However, we cannot rule out that our observation could be a mere chance. Among autoantibodies, IAA are believed to appear first and are more prevalent in disease onset at younger age. IA-2 antibodies are associated with acute onset of the disease and are also more important in younger age. MICA alleles and genotypes, especially MICA5/5, showed association with IAA and IA-2 autoantibodies. Thus, the presence of MICA5/5 in addition to IAA or IA-2 autoantibodies could be a valuable marker for prediction strategies. Finally, we report the frequency of MICA alleles in Swedish newborn babies at genetically high-risk with respect to HLA-DQ; frequency of MICA5 was 38% in DQ8+, 35% in DQ2-DQ8+, 22.5% in DQ2+; frequency of MICA5.1 was 81% in DQ2+, 62% in DQ8+, 71% in DQ2-DQ8+; frequency of MICA6 was between 20-22% among the three groups. MICA5/5.1 was present in 19% of DQ2-DQ8+ and 12-13% of DQ2+ and DQ8+.

In conclusion: MICA appears to be an important in the etiology of T1DM. Inclusion of MICA typing in addition to HLA could be useful for screening of genetic markers associated with T1DM.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be reffered to in the text by their romal numerals.

I. **Gupta M**, Nikitina-Zake L, Zarghami M, Landin-Olsson M, Kockum I, Lernmark Å, Sanjeevi CB.
Association between the transmembrane region polymorphism of MHC class I chain related Gene-A and type 1 diabetes mellitus in Sweden.
Hum Immunol. 2003, 64(5):553-61

II. Törn C, **Gupta M**, Nikitina Zake L, Sanjeevi CB, Landin-Olsson M.
Heterozygosity for MICA5.0/MICA5.1 and HLA-DR3-DQ2/DR4-DQ8 are independent genetic risk factors for latent autoimmune diabetes in adults.
Hum Immunol. 2003, 64(9):902-9

III. **Gupta M**, Nikitina-Zake L, Landin-Olsson M, Kockum I, Sanjeevi CB.
Coxsackie virus B antibodies are increased in HLA DR3-MICA5.1 positive type 1 diabetes patients in the Linköping region of Sweden.
Hum Immunol. 2003, 64(9):874-9

IV. **Gupta M**, Graham J, McNeeny B, Nikitina-Zake L, Zarghami M, Landin-Olsson M, Kockum I, Hagopian WA, Palmer J, Lernmark Å, Sanjeevi CB
MHC class I chain related gene-A (MICA) is associated with IA-2 and IAA but not GAD in Swedish Type 1 diabetes mellitus (T1DM)
(manuscript)

V. **Gupta M**, Ludvigsson J, Sanjeevi CB.
The frequency of MICA in All Babies In southeast Sweden (ABIS) positive for high-risk HLA-DQ associated with Type 1 diabetes
(manuscript)

LIST OF ABBREVIATIONS

aa	Amino acids
ABIS	All Babies in southeast Sweden
ADA	American Diabetes Association
BMI	Body mass index
CBV	Coxsackie virus B
CMV	Cytomegalovirus
DAISY	Diabetes Autoimmunity Study in the Young
DiPiS	Diabetes Prediction in Skåne
DIPP	Diabetes Prediction and Prevention
DIS	Diabetes Incidence in Sweden
DPT	Diabetes Prevention Trial
ENDIT	European Nicotinamide Diabetes Intervention Trial
GAD	Glutamic acid decarboxylase
HBDI	Human Biological Data Interchange repository
HLA	Human leukocyte antigen
IA-2	Protein tyrosine phosphatase
IAA	Insulin autoantibody
IBD	Inflammatory bowel disease
ICA	Islet cell antibodies
IDDM	Insulin dependant diabetes mellitus
IFN	Interferon
IL	Interleukin
IVGTT	Intravenous glucose tolerance test
JDF-U	Juvenile Diabetes Foundation units
KIR	Killer-cell immunoglobulin-like receptor
LADA	Latent autoimmune diabetes of the adult
MHC	Major Histocompatibility Complex
MICA	MHC class I chain related gene-A
NIDDM	Non-insulin dependant diabetes mellitus

NK   Natural killer cell

NKG2D   An activating receptor present on NK-cells, γδ T-cells and αβ T-cells

NS   Not significant

OGTT   Oral glucose tolerance test

OR   Odds ratio

pc   Corrected P-value

P-value   Probability value

SCDS   Swedish Childhood Diabetes Study

sMICA   Soluble MICA

SSOP   Sequence specific oligonucleotide probe

T1DM   Type 1 diabetes mellitus

T2DM   Type 2 diabetes mellitus

TM   Transmembrane

TNF   Tumor necrosis factor

TRIGR   Trial to Reduce T1DM in the Genetically at Risk

WHO   World Health Organization

The tables in thesis have been numbered in Roman numerals. The tables reffered from papers have been numbered in Arabic numerals

INTRODUCTION

## Type 1 Diabetes Mellitus (T1DM)

Diabetes mellitus encompasses a family of disorders of carbohydrate metabolism that are characterized by hyperglycemia and the development of long-term complications. According to the World Health Organization (WHO), in 1985, diabetes was classified into insulin dependant diabetes mellitus (IDDM = type 1 diabetes) or non-insulin dependant diabetes mellitus (NIDDM = type 2 diabetes) based on clinical symptoms and the type of treatment used [1]. Later, in 1997, the American Diabetes Association (ADA) revised the classification of diabetes based on etiology of the disease [2].

Typical clinical symptoms of IDDM were weight loss, short duration of hyperglycemic symptoms, high blood glucose levels, ketonuria and ketoacedosis. Symptoms are often acute and need for insulin is obvious. The typical clinical signs of NIDDM were old age, long history of hyperglycemic symptoms such as thirst and polyuria, moderately elevated blood glucose levels, high body mass index (BMI) and absence of ketonuria [1].

Diabetes, according to ADA, is now classified as type 1 diabetes (T1DM), type 2 diabetes (T2DM), other specific types of diabetes (e.g. secondary diabetes), or gestational diabetes. T1DM is primarily insulinopenic that is subclassified as autoimmune T1DM (type 1a) or idiopathic T1DM (type 1b). Autoimmune type 1a diabetes is further divided into a rapid and slowly progressive form. The rapid form is the main diabetic form in children and adolescents and the slowly progressive is the adult form also known as Latent autoimmune diabetes of the adult (LADA). The most important factor differentiating type 1a from type 1b is the presence of islet autoantibodies. Type 1b has no known etiology.

Incidence: geography, age and gender

The incidence of T1DM varies greatly among countries. The incidence, from 1990-94 in children ≤14 years, has been reported to be highest in Sardinia (Italy), Finland (>36.5/100,000 per year) followed by Sweden (27.5/100,000 per year) and the lowest in China and Venezuela (0.1/100,000 per year) (fig. 1) [3]. Also, within Sweden, variation in incidence has been observed – the highest incidence being in Linköping region in the southeast of the country (fig. 2) [4]. The variation has been found to be large even within European countries - the incidence in Greece being just 9.1/100,000 per year [3].
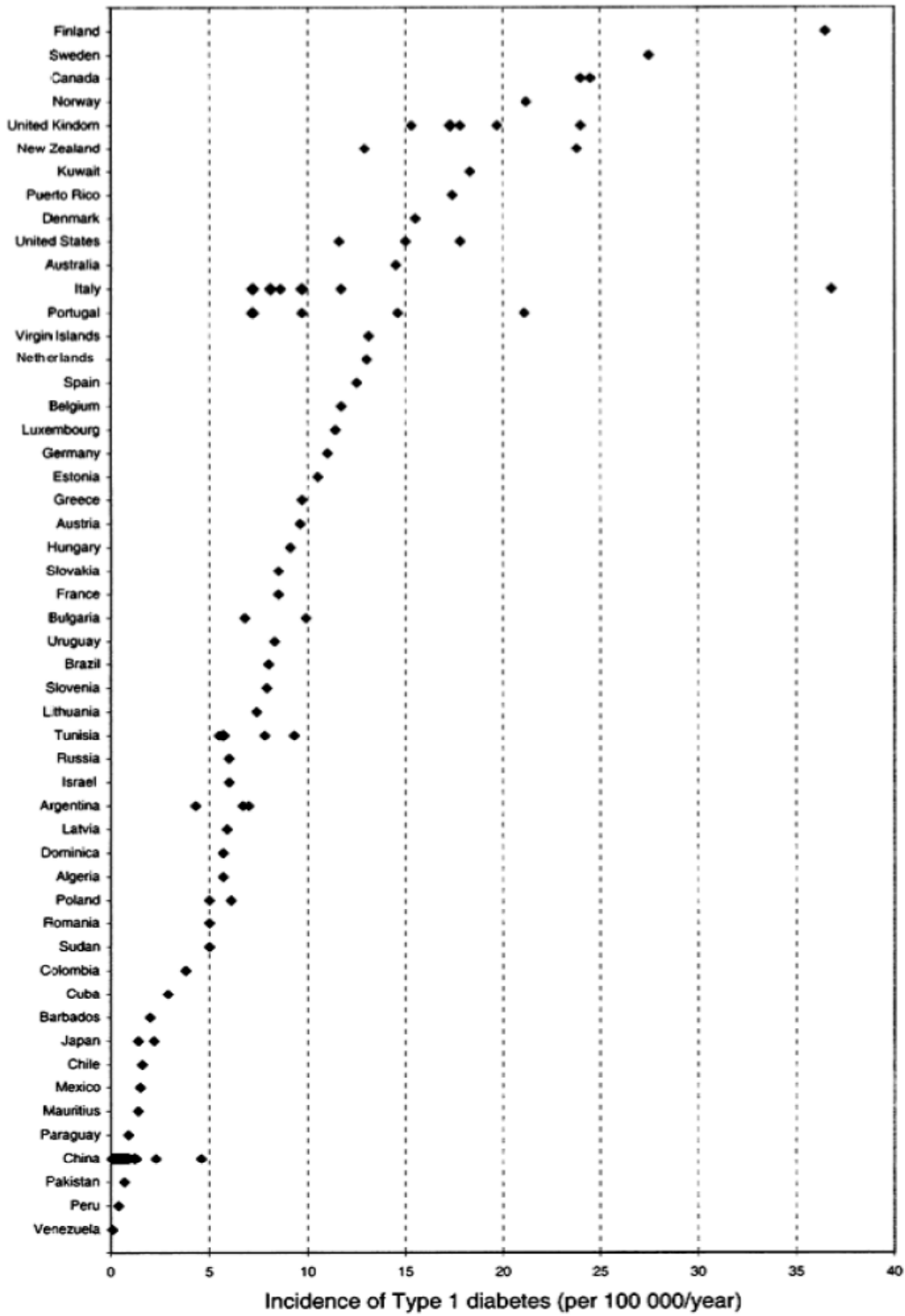
Figure 1: Age-standardized incidence (per 100,000 per year) of T1DM in children ≤14 years of age in 100 populations [3].

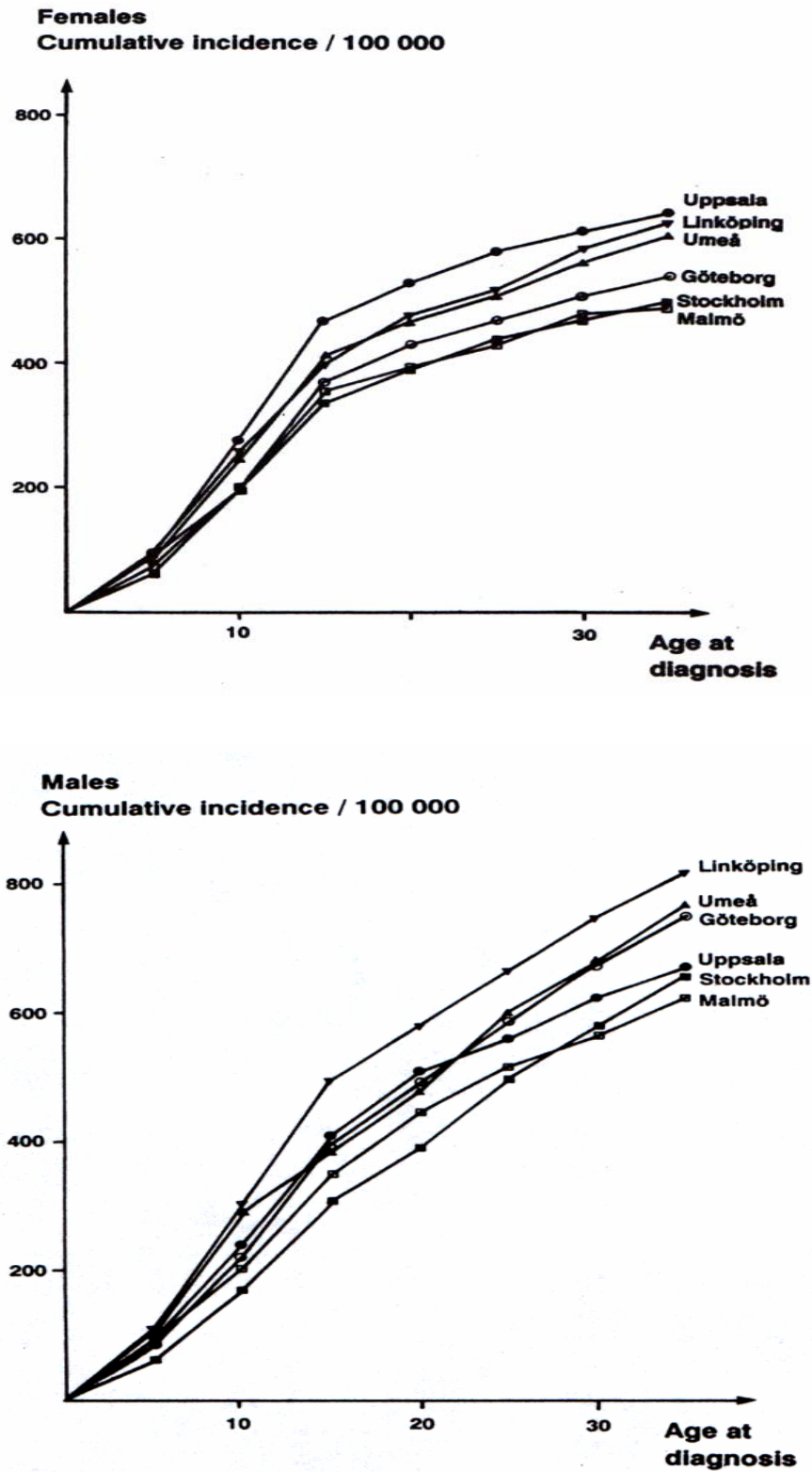**Figure 2:** Cumulative incidence per 100,000 of T1DM for females and males 0-34 years for six regions in Sweden 1983-1987 [4].

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T1DM is considered to be a disease of childhood. The incidence is highest among children 10-14 years of age [3, 5]. However, the disease may develop at any age. It has been estimated that 44% of cases develop T1DM after the age of 30 years with a rather stable incidence rate [6]. The peak incidence is observed around puberty after which the incidence falls [5]. Among the patients diagnosed from 1995 to 1998 in Sweden, the median age of diagnosis was 12.5 years for males and 10.4 for females [5].

In Sweden, the cumulative incidence in children ≤14 years does not differ between males and females (426 and 415 per 100,000 respectively). However, by the age of 35 years, it is much higher in males than females (748 and 598 per 100,000 respectively) [5]. The incidence peak occurs about two years earlier in females than males and remains lower than males afterwards.

Hypothetical stages in the development of autoimmune diabetes

Insulin is secreted by pancreatic β-cells located in the islet of Langerhans. It is believed that in T1DM, autoiimune destruction of β-cells results in loss of insulin secretion and hence hyperglycemia. The process of β-cell destruction can be viewed as roughly passing through five stages [7] (fig 3): 1) genetic predisposition, 2) autoantibody positivity, 3) abnormal insulin response during intravenous glucose tolerance test (IVGTT), 4) glucose intolerance during oral glucose tolerance test (OGTT), and 5) clinical diabetes.
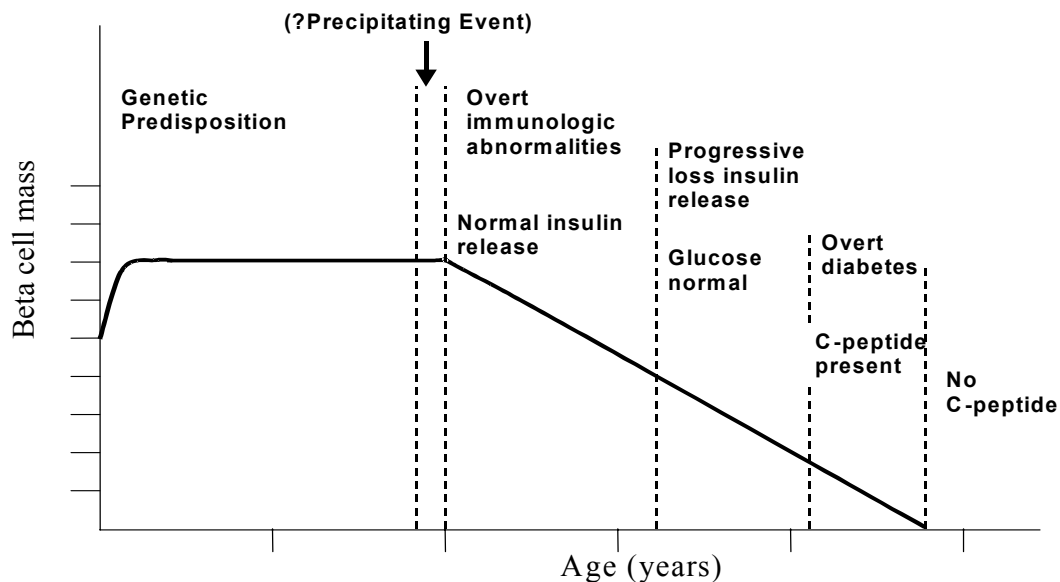
Figure 3. Hypothetical islet β-cell mass and stages in the development of autoimmune diabetes (from Eisenbarth, GS, New Engl J Med 1986; 314:1360-1368).

The genetically susceptible individual (stage 1) is exposed to an as yet unknown precipitating event that results in progressive β-cell destruction and development of autoantibodies towards β-cell antigens (stage 2). Cell-mediated autoimmunity is believed to mediate β-cell destruction. Autoimmune reaction results in a progressive loss of insulin release, with normal glucose homeostasis on a day-to-day basis but subnormal release when the first phase insulin response is measured during an IVGTT (stage 3). Once the β-cell mass is reduced to 20-30% of normal, overt diabetes develops (stage 4), although some residual endogenous insulin production remains. Eventually, the pancreas ceases all insulin production (stage 5), and the individual is entirely dependant on exogenous insulin. The prediabetes phase (stage 2 and 3) may last from months to years.

Genetic predisposition

T1DM is a polygenic disease, associated with several genes on different chromosomes. Although more than 20 different regions of the human genome have now been found to show some degree of linkage with the disease (Table I), most interest has focused on genes encoded in the Major Histocompatibility Complex (MHC) region.

Major Histocompatibility Complex (MHC) = IDDM1

The MHC complex, 3.6 megabase (Mb) long, is located on the short arm of chromosome 6 at position 6p21.3. A complete sequence and gene map of the complex has been reported [8]. The complex contains 224 identified genes (128 predicted to be expressed) and many of these are still of unknown function. Around 40% of the expressed genes are estimated to have immune system function [8]. The MHC complex has been divided into three regions: class I, II and III (fig 4). The proteins encoded by MHC genes in humans are called as Human Leukocyte Antigen (HLA).

Class I genes are located telomeric in the complex and code for a single polypeptide α-chain containing three domains: α1, α2, α3 associated with a β2 microglobulin. The classical class I molecules are HLA-A, -B and –C, expressed on most of the nucleated cells of the body and their function is to present antigens to CD8+ cytotoxic T-cells [9].

The class II genes are located at the centromeric end of the complex. The class II molecules (DP, DQ and DR etc.) are heterodimeric proteins of α and β chains consisting of four domains: α1, α2, β1 and β2. Among -DR and –DQ, DRA is non-polymorphic, while DRB1, DQA1 and DQB1 show a high degree of allelic variability. Class II molecules are expressed on the cells of immune system − monocytes, macrophages, B cells, dendritic cells and activated T-cells and their function is to present antigenic peptides to CD4+ T-cells [9]. In addition to their extreme polymorphism, strong linkage disequilibrium between the neighbouring genes within the HLA region is a hallmark [10].

Table I: Putative susceptibility loci for T1DM [11], [12]

Locus	Localization	Markers
IDDM 1	6p21.3	HLA-DRB1, DQA1
IDDM2	11p15	INSVNTR
IDDM3	15q26	D15S107
IDDM4	11q13	FGF3, D11S1337
IDDM5	6q25	ESR
IDDM6	18q21	JK, D18S487
IDDM7	2q31	HOXD8, D2S152
IDDM8	6q27	D6S264, D6S446
IDDM9	3q21-q25	D3S1576
IDDM10	10q11-q11	D10S193
IDDM11	14q24.3-q31	D14S67
IDDM12	2q33	CTLA4
IDDM13	2q35	D2S164
IDDM14	-	-
IDDM15	6q21	D6S283
IDDM16	14q32.3	D14S542, IGH
IDDM17	10q25	D10S554
IDDM18	5q33-q34	IL12B
Unnamed	1q42	D1S1617
Unnamed	16q22-q24	D16S3098
Unnamed	19p13	D19S247
Unnamed	19q13	D19S225
Unnamed	Xp13-p11	DXS1068
Unnamed	7p13	GCK
Unnamed	12q14-q15	IFNG
Unnamed	5p13-q13	D5S407

HLA in T1DM

A large number of studies have shown association between genes in the HLA region and T1DM. An association between HLA class I alleles and T1DM was first described in early 1970s [13, 14]. Subsequently, a closer association was found with HLA-DR3 and DR4 [15]. More recent observations have shown genes in the HLA-DQ region to be even more closely associated with T1DM than the DR genes [16]. Since then, several studies have confirmed the association of DQB1 and DRB1 genes with T1DM [17, 18].

Figure 4: Simplified map of the human MHC on the short arm of chromosome 6.
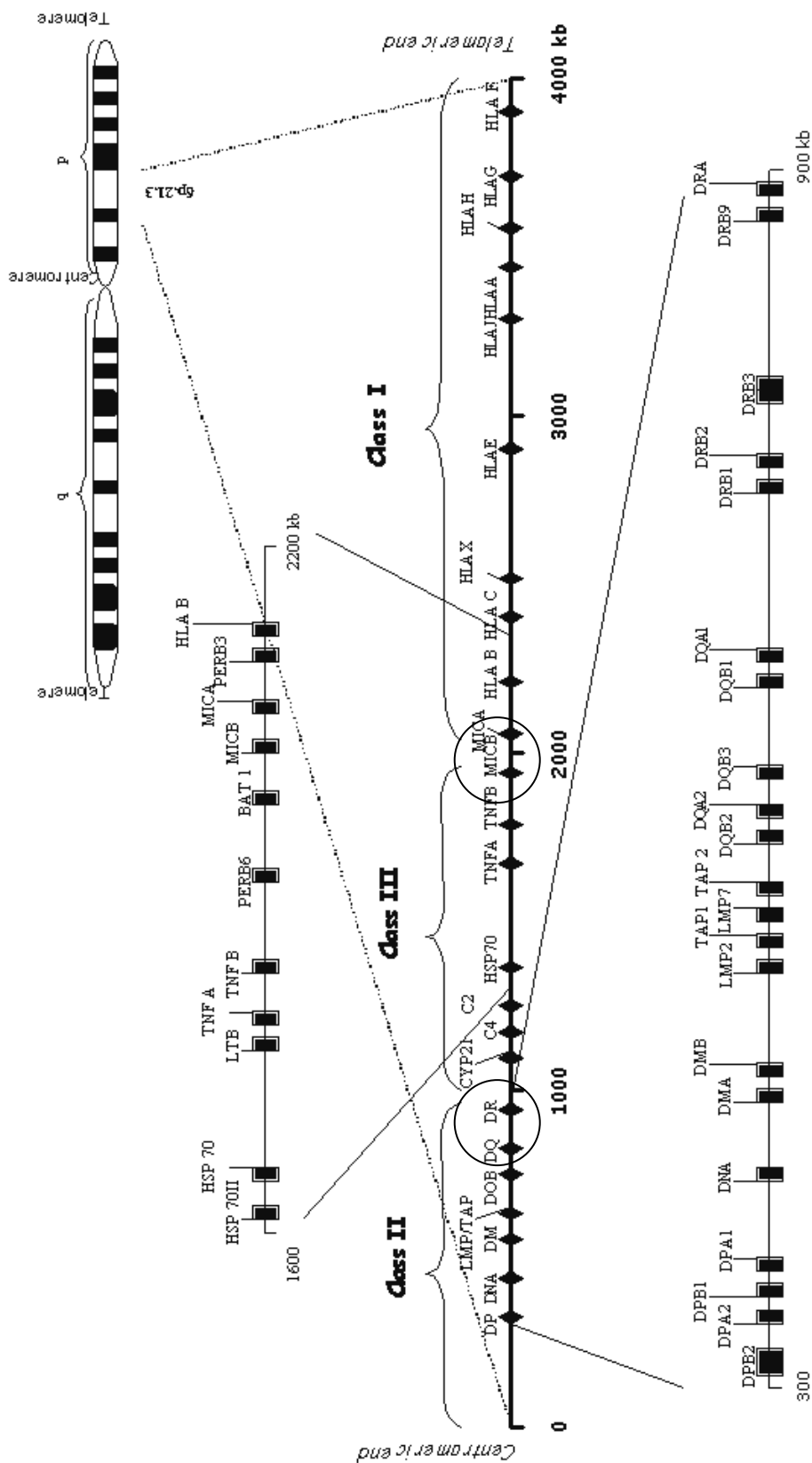
The human MHC contains more than 200 genes, more than 40 of which encode for HLA.

In Caucasians, two haplotypes DR4-DQA1*0301-DQB1*0302 (DR4-DQ8) and DR3-DQA1*0501-DQB1*0201 (DR3-DQ2), now referred to as high-risk haplotypes, are strongly associated with T1DM. The synergistic effect between these two haplotypes is marked [18-21]. The strongest association is found in DQ2-DQ8 heterozygotes. On the other hand, DRB1*15- DQA1*0102-DQB1*0602 (DR15-DQ6) is negatively associated with the disease [18, 21, 22]. In Sweden, high-risk haplotypes account for as many as 89% of the T1DM patients but not all patients [19]. However, considering our current understanding of the genetics of T1DM, genetically most susceptible individuals defined by HLA typing have a low absolute risk for developing T1DM [19, 23]. The hope is that by assessing multiple risk loci, patterns of alleles that substantially increase the sensitivity of genetic typing can be identified. Further investigations are therefore underway to determine other genes associated with the disease.

Reports suggest that there is another gene in the HLA region besides HLA-DQ and DR that influences susceptibility to T1DM [24]. MHC class I chain related gene-A (MICA) gene lies in this suggested region [25].

The MIC genes: MICA

The major histocompatibility complex class I chain-related (MIC) gene family was first discovered by Bahram *et al* [25]. The family consists of seven genes, MICA to MICG, located in the human HLA region centromeric to the class I genes between the Tumor Necrosis Factor (TNF) loci and HLA-B genes (fig 4; only MICA and MICB are shown). Among the MIC genes, MICA and MICB code for cell surface proteins while the other members of the family are pseudogenes. MICA and MICB genes are located 46.4 kb and 141.2 kb centromeric to HLA-B respectively and length of the genes is 11.7 kb and 12.9 kb respectively [26].

Structure

MICA and MICB chains fold similar to typical class I chains with three extracellular domains (α1, 2 and 3), a transmembrane (TM) segment and a cytoplasmic tail, each encoded by a separate exon (exon 2 to 6) but are not associated with a β2-microglobulin [25]. MICA gene encodes a 383 amino-acid (aa) polypeptide with a relative molecular mass of 43 kDa. Unlike class I molecules, MICA molecules are not involved in peptide binding and antigen presentation [25-27].

Expression

MICA molecules are not expressed constitutively. It has been shown that the expression of MICA is stress induced e.g. after heat shock [28], viral infection [29], bacterial infection [30] or oxidative stress [31]. MICA was first identified to be expressed in the cells of intestinal epithelium where it is recognized by Vδ1 bearing $\gamma\delta$ T-cells [32]. MICA is also

expressed on a wide variety of tumors, particularly of epithelial origin including renal, breast, colon and lung [33]. It has been suggested that stress-induced expression of MICA (and MICB) may serve as an immune surveillance mechanism for the detection of damaged, infected or transformed epithelial cells. Expression has also been reported on fibroblasts, keratinocytes, epithelial cells, freshly isolated monocytes [34].

Function

MICA is a ligand for an activating receptor NKG2D present on γδT-cells, CD8+CD28-αβT-cells, natural killer (NK) cells [35] and CD4+CD28- αβT-cells [36]. Engagement of NKG2D by MICA activates cytolytic responses from γδT-cells and NK-cells against transfectants and epithelial tumors expressing MICA [35]. MICA also co-stimulates CD4+CD28- αβT-cells in synovial tissue of rheumatoid arthritis patients [36]. According to Groh *et al* [29] Cytomegalovirus (CMV) induced the expression of MICA and concurrent downregulation of HLA class I molecules on fibroblasts and endothelial cells. MICA was also expressed on lung sections of patients with CMV interstitial pneumonitis. MICA thus induced after viral infection could bind to NKG2D on CMV specific CD8+CD28- αβT-cells and deliver a co-stimulatory signal and augment T-cell antigen receptor dependent cytolytic and cytokine responses [29].

NKG2D can also be induced on CD8+CD28- αβ T-cells by interleukin (IL)-15 [37] and on CD4+CD28-T-cells by TNFα and IL-15 [36].

Normally, epithelial cells inhibit NK-cell mediated cell lysis via inhibitory HLA class-I-specific killer cell immunoglobulin-like receptors (KIR) expressed on NK-cells (fig. 5). The inhibitory KIR receptors on NK-cells recognize HLA class I molecules expressed on epithelial cells and this prevents cytolytic action of NK-cells. However, during stress, cellular transformation leads to induction of MICA on the surface of epithelial cells and recognition of MICA thus induced by NK-cell receptor NKG2D leads to NK-cell activation and release of perforin and granzymes. Inhibitory recognition of HLA class I molecules can moderate or tune the activating signal. Maximal NK-cell activation occurs when the target-cells down-regulate HLA class I molecules (fig 5) [38].

It has been shown that human tumor cells spontaneously release a soluble form of MICA (sMICA) encompassing the three extracellular domains, which is present at high levels in sera of patients with gastrointestinal malignancies [39]. Groh *et al* [40] showed that sMICA induced endocytosis and degradation of NKG2D on a large number of tumor infiltrating T-cells from individuals with cancer. This mode of T cell silencing may promote tumor immune evasion and by inference, compromise host resistance to infections. Thus, in case of tumors, it is suggested that expression of MICA by nascent tumors might, to some extent, be effective in mobilizing responses from effector T-cells and NK-cells, its shedding at progressive stages of tumor growth probably promotes immune evasion [40].
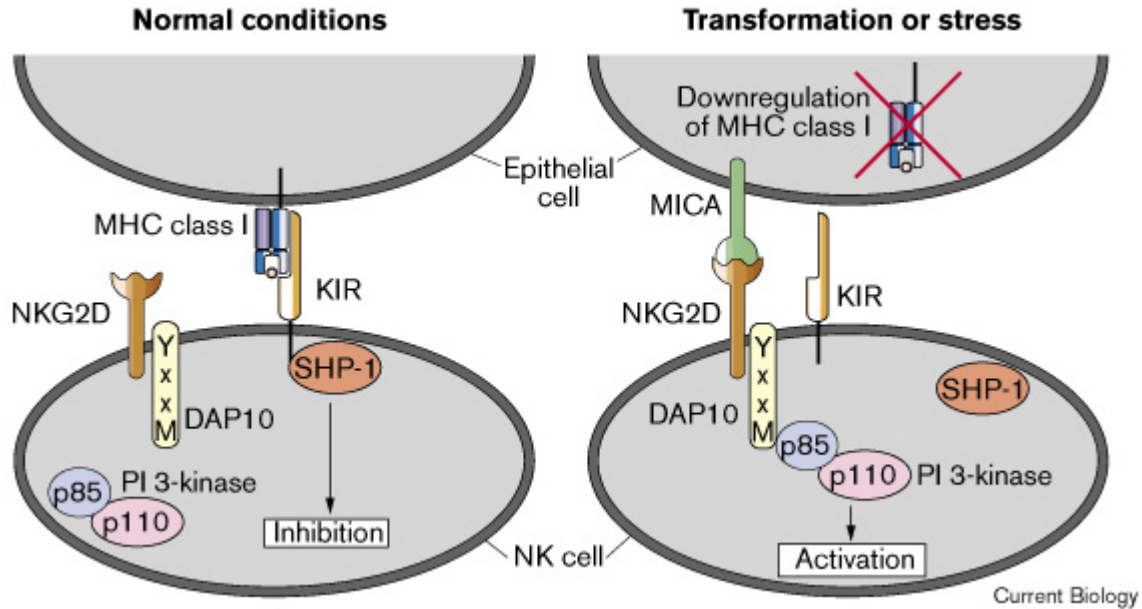
Figure 5: MICA, NKG2D and NK-cell recognition. Normal epithelial cells inhibit NK-cells via inhibitory MHC class-I-specific receptors (KIR), which recruit and activate the protein tyrosine phosphatase SHP-1. Dephosphorylation of activating signal molecules by SHP-1 prevents NK-cell activation. Cellular transformation leads to induction of stress proteins including MICA ('stress out'). Recognition of MICA by NKG2D–DAP10 'turns on' the NK-cell. Engaging the NKG2D receptor complex induces binding of the p85 subunit of PI 3-kinase to a YxxM motif in the DAP10 cytoplasmic domain, leading to NK-cell activation. Inhibitory recognition of MHC class I molecules could moderate or 'tune' the activating signal. Maximal NK-cell activation would occur when the target cell downregulates MHC class I molecules as shown on the right [41].

MICA polymorphism

The exon 5 of MICA gene which codes for the TM region of the protein contains a microsatellite polymorphism with short tandem repeats of GCT [(GCT)$_n$ →polyalanine)] [42]. Depending on the number of repeats, the alleles have been named as MICA-4, -5, -6 or -9. Another allele, -5.1 has an additional insertion of G (GCT→GGCT) with 5 GCT repeats. MICA-7 and -10 have also been reported by Rueda B *et al* and Perez-Rodriguez M *et al* respectively in specific populations [43, 44]. There are, however, not many studies describing functional consequences of the TM polymorphism of MICA. It has been reported that dihydrophobic Leu-Val tandem sequence at position 344-345 (numbering according to that in MICA5 allele) in the cytoplasmic tail of MICA is responsible for targeting the protein to the basolateral plasma membrane of the gut epithelial cells, the prime site of contact with effector NK and intraepithelial T-cells. In MICA5.1, an additional insertion of G creates a frame shift mutation resulting in a premature termination codon within the transmembrane region, as a result of which the MICA5.1 molecule is not translated till Leu-Val sequence at position 344-345. This denies MICA5.1 molecule of the Leu-Val sorting signal. This results in localization of MICA5.1 molecules to the apical plasma membrane region instead of basolateral plasma membrane [45].

The MICA gene also displays an unusual distribution of a number of variant amino acids in its extracellular domain α1, α2, and α3 [46]. Comparison of allelic variants of MICA has revealed a large difference in NKG2D binding that is associated with a single amino acid substitution at position 129 in the α2 domain. Varying affinities of MICA alleles for NKG2D may affect thresholds of NK-cells triggering and T-cell modulation [47].

Komatsu-Wakui *et al* [48] identified a MICA-MICB null haplotype, which is associated with HLAB*4801 variant. In this haplotype, large-scale deletion (of approximately 100 kb), including the entire MICA gene, and a MICB gene that possessed a stop codon was found. However, homozygous individuals are healthy, with no deficiency being immediately apparent.

MICA and disease associations

The TM region polymorphism of MICA gene has been shown to be associated with several autoimmune disorders including psoriasis, ankylosing spondylitis [46], autoimmune

Table II: Association of MICA alleles with T1DM in different populations

MICA allele	Population studied	Association	Reference
5 and 5.1	HBDI T1DM families	Positive	[56]
5	Latvians Asian Indians Italians	Positive	[54, 55, 57]
4	Japanese Koreans Spanish Basques	Positive	[52, 53, 58]
9	Chinese	Positive	[51]
6	Japanese Koreans	Negative	[52, 53]
Genotype: 4/5.1 Genotype: 9/9	Brazilian	Positive	[59]

Addison's disease [49] and juvenile rheumatoid arthritis [50]. Previous studies in different ethnic populations have reported different MICA allele associations with T1DM (Table II). In Chinese population, MICA9 is positively associated with T1DM [51], whereas in Japanese [52] and Koreans [53] MICA4 is positively and MICA6 is negatively associated while in Latvians [54] and Asian Indians [55] MICA5 is positively associated.

Environmental trigger: Coxsackie virus B (CBV)

Despite a growing knowledge of T1DM, the triggers of β-cell autoimmunity remain elusive. Both, genetic and environmental factors are involved in disease pathogenesis. It is clear that concordance amongst identical twins is not 100% and the incidence of T1DM is increasing world wide suggesting the importance of environmental factors [7]. Among environmental factors, diet (N-nitroso compounds, cow's milk, duration of breast feed), stress and viral infections have been proposed [60, 61]. Among viruses, the role of enteroviruses, Coxsackie virus B (CBV) in particular, in the pathogenesis of T1DM has been studied widely [62, 63]. Within enteroviral genus, there are over 60 different serotypes of human enteroviruses including 3 polioviruses, 23 coxsackie A serotypes, 6 coxsackie B serotypes (CBV 1 to 6), 28 echovirus serotypes and four numbered serotypes [64]. The association of CBV with T1DM is based on the isolation of CBV from pancreas of fatal cases of T1DM [65] and based on reports of increased prevalence of antibodies to CBV in patients at onset of T1DM compared to matched control subjects [66, 67].

Studies have shown that the prototypes strains of CBV-3, CBV-4 and CBV-5 can infect insulin-producing β-cells in primary adult human pancreas and functionally impair β-cell function or lead to β-cell death. CBV-3 and CBV-5 induced most severe signals of β-cell dysfunction [68]. Another study from the same group showed that even when prototype strains were not destructive, highly destructive strains could be found among field isolates of the serotype [69].

The mechanism of CBV induced β-cell death leading to T1DM has not been fully elucidated. However, there are hypothesis about their role in pathogenesis of the disease. One is a mechanism called "molecular mimicry" which is based on a sequence homology between a foreign antigen e.g. a viral protein, and a host protein. It is postulated that immune reactivity against the virus can lead to a cross-reactive response to the homologous sequence of the host protein [63, 70-72]. Other mechanism can be direct lytic infection or indirect through bystander activation of autoreactive T-cells [71].

"Molecular mimicry" between the viral protein P2C of CBV and human GAD65 (an enzyme that converts glutamic acid to gamma-amino butyric acid) can be one of the possible mechanisms to initiate the autoimmune response. It is known that amino-acid residues 250-273 of human GAD65 and residues 28-50 of CBV4-P2C (and also other serotypes of CBV [63]) carry residues PEVKEK. The PEVKEK motif found in both CBV and GAD65 bind to T1DM associated DR3 but not to DR1 or DR4 [63]. Immunization of mice with CBV4-P2C could induce T-cell immune responses that cross-reacted with GAD65 peptides corresponding to the region of sequence similarity [63, 70-72]. It has also been reported that β-cells critically depend on antiviral interferons (IFNs) to lower their permissiveness to CBV4 infection and that in mice with pancreatic β-cells that had defective IFN responses, CBV4 manifested an acute form of diabetes that resembled the T1DM that develop in humans after severe enteroviral infection [73].

Autoantibodies

In T1DM, cell-mediated immune attack targeted against self-antigens is believed to result in formation of autoantibodies against islet antigens. Thus, antibodies do not appear to play an etiological role in β-cell destruction, but they serve as important markers of β-cell autoimmunity. The first autoantibody that was discovered to be directed against β-cells in diabetic patients was islet cell antibodies (ICA) [74] followed by insulin autoantibodies (IAA) [75]. Later, autoantibodies against Glutamic acid decarboxylase-65 (GAD65) and Protein tyrosine phosphatase (IA-2) were discovered and found to be associated with T1DM (Table III). GAD is an enzyme that catalyzes the production of GABA (gamma-amino butyric acid) from glutamic acid. GAD exists in two isoforms: GAD67 (594 aa) and GAD65 (585 aa). GAD65 is one of the antigens in β-cells [76]. IA-2 is a transmembrane protein (979 aa, 106 kDa) and a member of protein tyrosine phosphatase family expressed in the islets of Langerhans and in the central nervous system [76]. Many studies have reported association of IA-2 with T1DM (Table III). IA-2 mRNA shows different splicing in different tissues. A version lacking exon 13 is expressed in thymus and spleen and this could result in loss of tolerance of the full-length version expressed in pancreatic islets [83]. ICAs are polyclonal antibodies that react with proteins from all cells of the islet (e.g. α, β, γ, δ and PP cells) [84].

Since the autoimmune reactions resulting in formation of autoantibodies begin months to years before the clinical diagnosis of T1DM (fig. 3), these can be detected before the clinical diagnosis of T1DM and hence are used as markers for the impending disease [20, 84].

It is believed that different HLA types predispose to the development of different autoantibodies. DQ8 is positively associated with ICA, IAA and IA-2 autoantibody formation and DQ2 is positively associated with GAD65 antibodies. DQ2 is negatively associated with IA-2 autoantibodies [77, 80, 85-87].

In a study on eight European populations, despite wide variation in the background incidence of childhood diabetes, ICA of 20 JDF-U (Juvenile Diabetes Foundation Units) or more or multiple antibodies were not associated with nationality. Thus, it is possible that the rate of progression to diabetes varies between autoantibody positive first-degree relatives in high and low-risk environments [88].

Latent autoimmune diabetes in adults (LADA)

At the time of diagnosis some adult patients appear to have T2DM based on clinical findings but are positive for T1DM associated autoantibodies. Compared to T1DM, these patients have higher age, higher BMI, higher C-peptide levels and no immediate need for insulin [89]. However, such patients differ from non-autoimmune T2DM since they are

Table III: Frequencies of autoantibodies in different ages and populations

Ref	Population studied	No. of subjects studied	Age-at-onset (yrs)	IA-2	GAD65	ICA	IAA
[77]	Holland	200 T1DM	0-5	75	25	87.5	-
			6-10	68	77	86	-
			11-15	77	69	85	-
			16-20	70	71	80.5	-
			21-25	46	65.5	62.5	-
			26-30	36	59	68	-
			31-40	23	61	77	-
[78]	Germany	23 T1DM	<40 (12-38)*	39	74	83	-
		24 T1DM	>40	0	29	46	-
[79]	Belgium	312 T1DM	0-9	-	64	86	78
			10-19	-	80	84	43
			20-39	-	78	60	29
[80]	Sweden	491 T1DM	0-15		70	83	56
		415 controls			4.1	4.1	2.8
[77]	Holland	785 T2DM	40-96	0.1	2.8	7.6	
[81]	Finland	1122 T2DM			9.3		
		383 controls			4.4		
[82]	Caucasians	3672 T2DM	25-34		34	21	
			35-44		14	9	
			45-54		9	6	
			55-65		7	4	

younger, have lower BMI and have lower C-peptide [89]. Different studies have shown between 3 to 34% of clinically diagnosed T2DM patients to be positive for ICA or GAD65 antibodies (Table III) [77, 81, 82]. Studies have also shown that most of these patients will become insulin dependent within the next few years after diagnosis [81, 82]. These antibody positive patients not classified as T1DM on clinical grounds are often referred to as LADA patients. This form of diabetes has also been called slowly progressive form of T1DM.

Genetics of LADA

LADA, like classical T1DM, is associated with HLA class II genes. Ludvigsson *et al* [90] demonstrated in 1986 that T1DM is a genetically heterogeneous disease and that DR3 is associated with a more slowly progressive form of the disease. Tuomi *et al* [81] showed that 0201/0302 and 0302/x genotypes are increased in frequency in GAD65 antibody positive T2DM (41%) compared to GAD65 antibody negative T2DM (21%) and controls subjects (24%). However, compared to classic T1DM, frequency of 0201/0302 was decreased among LADA patients (34% vs. 13%) while frequency of 0302/x did not differ. Frequency of *0602(3) was similar among GAD65 antibody positive T2DM, GAD65 antibody negative T2DM and healthy controls. Other studies have reported that among LADA patients, DR3-DQ2 is significantly increased [91, 92].

In an Italian study [93], MICA5 was associated with younger onset T1DM (<25 years; odds ratio [OR]=12.5) independent of high risk DR3-DQ2/DR4-DQ8. The odds ratio of simultaneous presence of MICA5 and DR3-DQ2/DR4-DQ8 was 388. Also, MICA5.1 was associated with adult-onset T1DM (>25 years; OR=3.4) and LADA (OR =7.0) independent of DR3-DQ2/DR4-DQ8 and a combination of MICA5.1 and DR3-DQ2/DR4-DQ8 conferred an increased risk for adult-onset T1DM (OR=18.2) and LADA (OR=34.4).

Screening

In genetically at-risk individuals, sometime after the exposure to one of the proposed environmental trigger(s), β-cell autoimmunity can be detected by analyzing autoantibodies againt islet antigens. For the assessment of the risk for future progression to T1DM, the long subclinical period offers an opportunity to identify these individuals before clinical manifestation of the disease or even before autoimmune process has started.

To determine these factors, a large cohort of genetically susceptible children needs to be followed prospectively with frequent measurements of candidate exposures from birth up to early childhood – the time when prediabetic autoimmunity often develops. Early detection of autoimmune process against islet-antigens is also a pre-requisite for the identification and recruitment of pre-diabetic subjects into trials aiming to halt or slow β-cell death [94]. To find individuals at high-risk, it is essential to perform screening studies.

Many studies for risk assessment or prevention of T1DM have been implemented in the first degree relatives of T1DM patients [20, 94, 95]. However only around 10% of childhood cases at onset of the diseases have a first degree relative with the disease i.e. 90% of potential cases occur in the general population [96]. Thus, studies for risk assessment should be based on general population.

Screening studies are being done using mainly two approaches. One is to detect autoantibodies in all newborn babies or newborn babies who have a first-degree relative with T1DM [97]. The other approach is to define at birth the individuals at increased genetic risk by analyzing the risk genes or risk alleles and subsequent follow-up of those at risk for autoantibody formation [98]. Until recently, non-HLA genetic markers of T1DM risk have not been sufficiently characterized to be included in a screening program [99]. The benefit of adding non-HLA markers to the screening program remains to be determined.

There are several screening studies going at different places in the world. Diabetes Autoimmunity Study in the Young (DAISY) study in the US [98] and Diabetes Prediction in Skåne (DiPiS) study in Sweden (Ivarsson, S http://www5.medfak.lu.se/forskning/medfak/projects_details.php?Proj=375) identify individuals from general population at genetically high-risk for the disease and follow these individuals for immune markers. The BABY-DIAB study in Germany observes the development of autoimmunity in children of parents with T1DM and of mothers with gestational diabetes [97].

Other studies trying intervention using different approaches include European Nicotinamide Diabetes Intervention Trial (ENDIT) in 18 European countries, Canada and the USA [94], Trial to Reduce T1DM in the Genetically at Risk (TRIGR) in Finland (TRIGR: http://www.trigr.org/about.html) and Diabetes Prevention Trial-Type 1 (DPT-1) in the USA and Canada [95].

ENDIT tests preventive therapy using nicotinamide in high-risk first-degree relatives of patients with T1DM [94]. TRIGR is testing whether delayed exposure to intact food proteins will reduce the chances of developing T1DM later in life (TRIGR: http://www.trigr.org/about.html). The DPT-1 in US and Canada consisted of two clinical trials that sought to delay or prevent T1DM. One DPT-1 trial tested low-dose insulin injections and the other tried oral insulin (DPT: http://www.niddk.nih.gov/patient/dpt_1/dpt_1.htm).

Diabetes Prediction and Prevention (DIPP) study in Finland aims at prediction as well as trial for prevention [99]. This study identifies newborns at genetic high-risk for T1DM. Those at genetically high-risk are considered for immunological follow-up (ICA and IAA, GAD65 and IA-2 antibodies if positive for ICA). At-risk and antibody-positive children are offered the possibility to participate in a prevention trial, which evaluates the efficacy of nasally administered insulin to delay or prevent the onset of clinical T1DM.

The aim of the present thesis was to determine the association of MICA gene polymorphism with acute onset T1DM and LADA in Swedish population. Since, the expression of MICA can be induced by viruses, we also aimed to determine, if, among T1DM patients, the polymorphism of MICA is related to antibodies against Coxsackie virus B (CBV), a virus speculated to have role in the pathogenesis of autoimmune T1DM. We also looked if MICA polymorphism is associated with autoantibodies, which are markers for impending T1DM. We also determined the frequency of MICA alleles among newborn babies genetically at-risk to develop T1DM with respect to HLA-DQ.

SPECIFIC AIMS

1. To determine the association of MICA gene polymorphism with T1DM, age-at-onset of T1DM and gender in Swedish population (paper I).

2. To determine the association of MICA gene polymorphism with LADA in Swedish population (paper II).

3. To determine if there is a relation between antibodies against Coxsackie virus B (CBV) and MICA gene polymorphism among T1DM patients from Sweden (paper III).

4. To determine if MICA gene polymorphism is associated with autoantibodies in T1DM (paper IV).

5. To determine the frequency of MICA alleles in Swedish newborn babies positive for high-risk HLA-DQ associated with T1DM (paper V).

MATERIALS AND METHODS

Subjects

The Swedish Childhood Diabetes Study (SCDS): Papers I, III and IV

Since 1977, incident cases of children ≤14 years developing T1DM are registered in the Swedish Childhood Diabetes registry [100]. Cases are registered in all the 44 pediatric clinics in 24 counties in Sweden that participate. All patients who were registered in the registry between September 1, 1986 and December 31, 1987 were asked to participate in SCDS. During the period, 515 patients were registered [101] and 497 (94%) of these patients donated blood sample.

For patients above 7 years (n=359) two age, sex and geographically matched individuals were selected as controls (n=718) from the Swedish population registry. Of these 718, 371 individuals volunteered to donate a blood sample. Ethical reasons precluded contacts to controls less than 7 years of age and instead another child treated at hospital for reasons other than diabetes was selected at the hospital of the index case. Fifty-two controls aged below 7 years donated a blood sample. Thus, a total of 423 controls donated a blood sample, which corresponds to 52% of those asked to participate. These subjects registered in SCDS have been studied in paper I, III and IV.

The Diabetes Incidence in Sweden (DIS) study: Papers I and IV

Since 1983, incident cases of diabetes between the age of 15 to 34 years are registered in the Diabetes Incidence in Sweden registry [102]. During 1987 and 1988, blood samples were obtained from 474 patients classified as T1DM. The type of diabetes was classified by the treating physician according to WHO criteria, 1985. Two controls were selected from the Swedish population registry for each patient. The controls were matched for age, sex and geography with the patient. Blood samples were obtained from 279 controls. These patients and controls have been studied in paper I and IV.

These two studies administered over an overlapping period were combined for a total of 971 T1DM patients and 702 controls. Of these, DNA samples from 670 patients (age: 0–20 years = 503, 21–35 years = 167; males = 397; females = 273) and 534 healthy controls (age: 0–20 years = 409, 21–35 years = 125; males = 274; females = 260) were available for MICA genotyping. MICA genotyping for the exon 5 microsatellite polymorphism was successfully determined for 635 of 670 diabetic patients and 503 of 534 healthy controls. The number of individuals for whom both MICA and HLA-DR-DQ typings were successful was 591 patients and 462 controls. Informed consent was obtained from all the participants in the study. This study was approved by the Ethical Committee at Karolinska Institute.

LADA study - Paper II

This study (paper II) was focused on T1DM patients with onset in adulthood. From October 1, 1995 until December 1, 1998, 100 patients newly diagnosed with diabetes were clinically classified as T1DM in a defined area in the southern part of Sweden. However, sporadic cases were reported from the Children's Department at Lund University Hospital and these patients were also included. The clinical classification was done by the treating physician based on clinical observations such as weight loss and ketoacidosis.

From October 1, 1995 until November 3, 1999, 1557 newly diagnosed diabetic patients from the same geographical area were clinically classified as T2DM or considered to have a type of diabetes that was unclassifiable. A total of 119 healthy blood donors resident in the same area as patients were invited as control. All patients and controls donated a blood sample for analysis of autoantibodies and genotyping. A total of 60 patients clinically classified as T2DM or unclassifiable diabetes out of the 1557 reported patients were positive for at least one of ICA, GAD65 autoantibodies or IA-2 autoantibodies and were considered as LADA patients.

Since the age spans for T1DM patients (median age 35; 9-89 yrs), LADA patients (median age 48; 19-79 yrs) and controls (median age 35; 19-65 yrs) were similar, the same set of controls was used for both T1DM and LADA patients.

DNA extraction and complete genotyping for HLA and MICA was successful in 94% (262/279) of all the subjects, 98% (98/100) of T1DM patients, 85% (51/60) of LADA patients and in 95% (113/119) of controls. Only patients and controls with complete genotyping were taken into account in paper II.

Informed consent was obtained from all the participants in the study. This study was approved by the Ethical Committee at Lund University (LU 44-95 and LU 526-00)

ABIS study- Paper V

It is known that the incidence of T1DM is highest in Linköping region in southeast of Sweden (fig 2) [4]. All Babies In Southeast Sweden (ABIS) study in five counties in southeast Sweden covers a population of 1.1 million. This study is conducted from Linköping through 11 obstetric clinics, 6 pediatric clinics, 6 neonatal wards, 55 health care centers, 70 maternal health care centers, and 250 well baby clinics in the ABIS region.

The main purpose of the ABIS project is to assess the risk of future progression to T1DM in the general child population and to prospectively study the importance of environmental factors for the development of T1DM, together with some other so-called autoimmune diseases such as celiac disease and inflammatory bowel disease (IBD) [103]. Another purpose is to try to identify high-risk individuals from the material collected; the aim is to

develop methods for identifying those children who can be diagnosed as having a higher risk of developing childhood diabetes [103]. The study has been approved by the Research Ethics Committees at the Faculty of Health Sciences, Linköping University and the Medical Faculty, Lund University, Sweden.

Mothers-to-be of 21,700 children born in five counties in southeast Sweden between October 1, 1997 and October 1, 1999 were asked to participate in the study. Of these 17,055 (78.6%) mothers gave consent for the participation. The population represents very well the general population of Sweden.

At the time of birth, umbilical cord blood from the newborns was collected in EDTA and stored at -20°C for the typing of genetic markers HLA and MICA. Serum samples were also collected at the time of birth, and then at 12, 30 and 60 months of age when the child visits the well baby clinic. Autoantibodies against GAD65 and IA-2 are being tested in these serum samples at the University of Linköping.

In paper V for this thesis, so far, samples from 2821 newborns have been tested for the high-risk HLA markers DQ2 (DQA1*0501-DQB1*0201), DQ8 (DQA1*0301-DQB1*0302) and the protective DQ6 (DQB1*0602). Of these 2821 samples, those positive for DQ2 or DQ8 (n=1013) are being typed for MICA TM polymorphism. So far, 499 of 1013 DQ2 or DQ8 positive samples have been typed and have been reported in paper V.

Methods

MICA genotyping

MICA alleles were determined using a fluorescence-based automated fragment size analysis. The TM region of the MICA gene (exon 5) was amplified by polymerase chain reaction (PCR) using 5'-CCTTTTTTTCAGGGAAAGTGC-3' as the forward primer and 5'-CCTTACCATCTCCAGAAACTGC-3' as the reverse primer as described [104]. The reverse primer was labeled at the 5' end with the fluorescent reagent HEX, TET or 6-FAM (Pharmacia Biotech, Sweden).

The PCR reaction was carried out using enzyme activation and initial DNA denaturation for 10 minutes at 96°C followed by 34 cycles of denaturation at 96°C for 30 sec, annealing at 55°C for 30 sec and polymerization at 72°C for 30sec with a final extension cycle at 72°C for 7 minutes. Following amplification, the numbers of GCT trinucleotide repeat units were determined using Perkin-Elmer ABI 373 DNA sequencer (Perkin-Elmer, Norwalk, CT, USA) and output file was analyzed using Genescan and Genotyper softwares (Perkin-Elmer). The TAMRA 500 was used as an internal standard for correct determination of fragment sizes. The different alleles of MICA correspond to fragment sizes as follows: MICA4: 179 bp, MICA5: 182 bp, MICA5.1: 183 bp, MICA6: 185 bp, MICA9: 194 bp.

HLA-DR and -DQ genotyping

For paper II and V, typing for DQA1, DQB1, and DRB1 genes was done by PCR - sequence specific oligonucleotides probes (SSOP). The polymorphic regions from exon 2 of DQA1, DQB1 and DRB1 genes were amplified by PCR using specific primers [105]. The amplified products were dotted onto nylon membranes under denaturing conditions. The membranes were hybridized with SSO, which were 3'end labeled with α^{32}P-dCTP and washed under specific stringency conditions for the respective probe before exposure to x-ray film [105]. For paper under SCDS and DIS study, HLA typing of DQA1 and DQB1 was carried out as above while DR typing was done by restriction fragment length polymorphism [106].

CBV antibody assay

Immunoglobulin M (IgM) antibodies against procapsid of CBV serotypes 3 and 5 were assayed by μ-antibody capture radioimmunoassay-technique using ^{35}S-labeled CBV as previously described in detail [107, 108]. Data for CBV antibodies was provided by Prof. G. Dahlquist, Umeå University.

For paper III on subjects ≤14 years, data for IgM antibodies against procapsid of CBV serotypes 3 and 5 was available for 298 patients and 246 controls (46 patients and 39 controls from Linköping). HLA and CBV data was available in 289 patients (45 from Linköping). MICA and CBV data was available in 253 patients (41 from Linköping). HLA, MICA, and CBV data was available in 248 patients (40 from Linköping).

IAA

IAA were measured by radiobinding assay using acid-charcoal extraction and cold insulin displacement as described elsewhere [75, 109]. The results were expressed as percent binding. For paper IV, the IAA results were available for 591 of 670 patients.

GAD65 autoantibodies

For ≤14 years old, antibodies to radiolabeled human GAD Mr 65,000 were quantified by immunoprecipitation assay using fluorographic densitometry as described earlier [80, 110]. For 15 to 35 years old, GAD65 autoantibodies were analyzed by radioligand binding assay as described [111]. Positive and negative control sera were included in each assay and the antibody levels were expressed as an index defined as (cpm in unknown sample-negative control) / (positive control-negative control). For paper IV, GAD65 autoantibody index measurements were available on 651 of 670 patients.

IA-2 autoantibodies

Autoantibodies to IA-2 (also called ICA512) were measured by radioligand binding assay [112]. The 3' portion of the ICA512cDNA: residues 602–979 (corresponding to the cytoplasmic portion of the protein) was amplified by RT-PCR from human HTB-14 glioblastoma cells [113]. In vitro translation with ^{35}S-methionine yielded a polypeptide of 46-kDa highly precipitable by diabetic sera. Radiobinding assays used scintillation counting of protein A-Sepharose pellets. The levels of IA-2 autoantibodies were expressed as an index calculated with the same formula as used for GAD65 autoantibody radioassay. For paper IV, the results for IA-2 autoantibody were available for 647 of 670 patients.

ICA

For paper II, ICAs were analyzed using an immunofluoresence assay described previously [114]. The lower limit of positivity for the pancreas used in this study was 9 (Juvenile Diabetes Foundation-units) JDF-U.

Statistical analysis

For papers I to IV, differences in allele, haplotype or genotype frequencies between the groups were tested by the Chi-square method. Yates' correction or the Fisher's exact tests were used when necessary. The odds ratio (OR) was calculated as described previously [115, 116]. For paper I and IV, the p-values were corrected (pc) for the number of comparisons, according to the number of alleles or genotypes observed among the subjects studied: 5 for MICA alleles, 15 for MICA genotypes (paper I and IV) and 52 for MICA-HLA haplotypes (paper IV). A pc<0.05 was considered significant. For paper II, 95% confidence intervals excluding unity were considered as significant. For paper III, the p-values less than 0.05 were considered significant.

The strongest HLA association in paper I was tested using the method of Svejgaard and Ryder [117]. In this analysis, MICA gene polymorphism (factor A) was compared with HLA-DR3-DQ2 and with HLA-DR4-DQ8 (factor B) for association with T1DM. Also, the association of MICA5/5 (factor A) and DR3-DQ2/DR4-DQ8 (heterozygous; factor B) with T1DM was tested. Association of MICA alleles with age-at-onset and with gender in T1DM was tested by logistic regression analysis using the SAS system for Windows, version 8.01 (SAS Institute Inc., Cary, NC, USA).

In Paper II, for univariate analysis, the different genotypes of MICA were grouped as MICA5.0/5.0, MICA5.0/5.1, MICA5.1/5.1, MICA5.0/x, MICA5.1/x, and MICAx/x, where x denotes alleles other than MICA5.0 or MICA5.1. To estimate the importance of HLA genotypes by univariate analysis, the different genotypes of HLA were grouped as DR3-DQ2/DR3-DQ2, DR3-DQ2/DR4-DQ8, DR3-DQ2/x, DR4-DQ8/DR4-DQ8, DR4-DQ8/x, DR15-DQ6/x, x/x where x denotes other haplotypes than DR3-DQ2 or DR4-DQ8 (DR15-

DQ6/x included both homozygotes and heterozygotes due to its low prevalence). In the multiple logistic regression analysis diabetes or not diabetes was the dependent variable, and the independent factors compared were MICA5/5.1, MICA5.1/x (including homozygotes), MICA5/x (including homozygotes), DR3-DQ2/DR4-DQ8, DR3-DQ2/x (including homozygotes), DR4-DQ8/x (including homozygotes), and DR15-DQ6/x (including homozygotes). In the multiple logistic regression analysis homozygotes were included into the respective groups with single risk haplotypes, due to relatively low number of homozygous individuals. Confidence intervals below or above 1.0 were considered as significant. The statistical calculations were done with the Statistical Package for Social Sciences (SPSS), version 6.1 for Macintosh (SPSS, Inc, Chicago, IL, USA).

In paper 5, the frequency of risk alleles or genotypes of HLA and MICA were expressed as percent.

RESULTS AND DISCUSSION

MICA is associated with T1DM

Our study showed that MICA5 is associated with T1DM. The univariate analysis showed allele 5 of MICA was significantly increased in Swedish T1DM patients between the age of 0-35 years (Table 2, paper I). MICA genotype -5/5.1 was significantly increased in patients compared with controls (Table 2, paper I). Logistic regression analysis showed that MICA5 is associated with T1DM independent of high-risk HLA DR3-DQ2 and DR4-DQ8.

Our finding of MICA5 to be positively associated with the disease is in agreement with the previous reports in other populations (Table II in introduction). However, association of MICA5 with T1DM in our study was weaker than that in Italians [57] (OR=1.81 vs. 6.1 respectively). This could be because of higher frequency of MICA5 in Swedish general population (24.5%) compared to Italians (15%) or because of environmental factors being different in two countries.
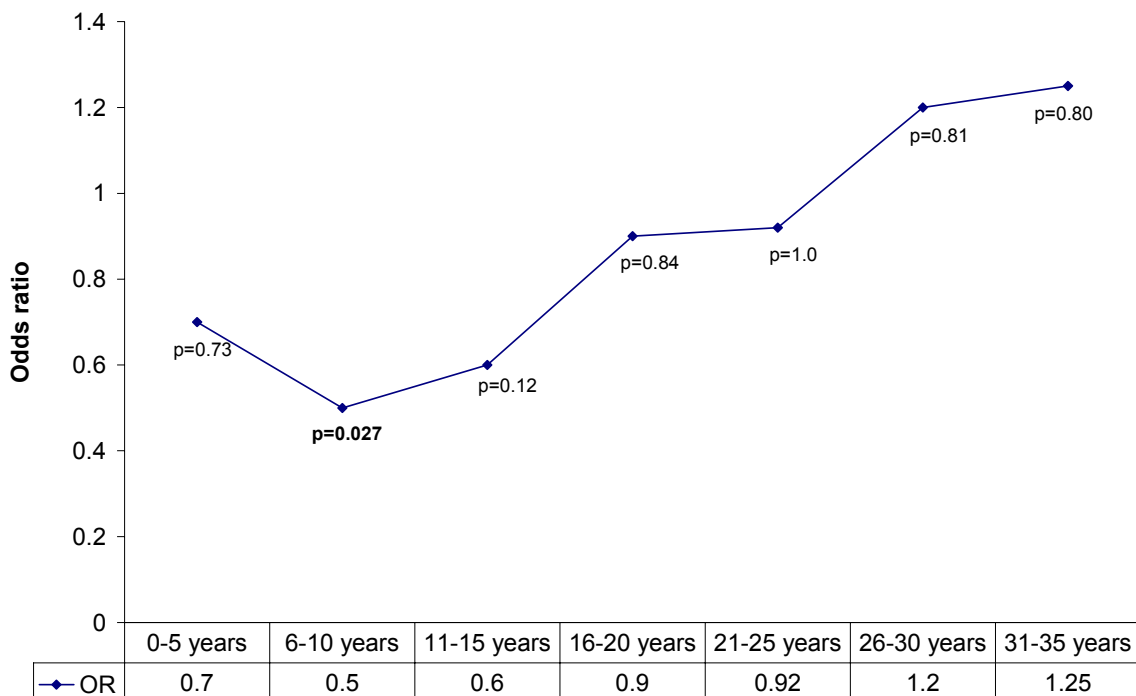
Test of strongest association

Svejgaard and Ryder analysis (Table 3 and 4, paper I) showed that MICA5 increased the risk for disease in combination with DR3-DQ2 compared to the risk with MICA5 alone or DR3-DQ2 alone (comparison 8 vs. comparison 1 and 2, Table 4, paper I). However, the combined risk with the simultaneous presence of MICA5/5 and DR3-DQ2/DR4-DQ8 (OR = 13.91) was not higher than the risk with DR3-DQ2/DR4-DQ8 alone (OR = 18.97; Table 4, paper I). MICA5 was independently associated with the disease in subjects stratified for the absence of DR3-DQ2 haplotype (comparison 4). MICA5 was in linkage disequilibrium with DR4-DQ8 in both patients and controls (comparison 9 and 10) and only in patients with DR3-DQ2 (comparison 9).

Thus, DR3-DQ2/DR4-DQ8 heterozygous combination was the strongest genetic marker for the disease. MICA5 was associated with the disease and increased the risk when in combination with DR3-DQ2. Though MICA5 was in linkage disequilibrium with DR4-DQ8, it also contributed to the risk for disease independent of high-risk HLA as shown by logistic regression. The finding that MICA5 and DR3-DQ2 together are observed more often than expected in patients might indicate that both are involved in disease susceptibility (Table 3 and Table 4, paper I). In Italian T1DM, the risk with simultaneous presence of both MICA5 and DR3-DQ2/DR4-DQ8 was 172 fold that associated with the absence of both markers [57].

As shown in Table II of introduction, different MICA alleles have been reported to be associated with T1DM in different ethnic populations. We can not rule out that different observations of MICA association with T1DM in different populations could also be due to different linkage disequilibrium of MICA alleles in the HLA region.

Effect of MICA on age-at-onset of T1DM:

It is known that the risk for T1DM with positively associated DQ2-DQ8 and negatively associated DQB1*0602 is attenuated with increasing age [18, 78]. We divided our patients and controls into two age groups; those aged 20 years or less (younger onset group) and those aged above 20 years (older onset group) to see if MICA polymorphism determines the age-at-onset. The risk associated with MICA5, analyzed by univariate analysis, was higher in younger onset group compared to the older onset group (Table 5, paper I) but the 95% confidence interval for the two overlapped. MICA6 was negatively associated with the disease in the younger onset group (Table 5, paper I). This negative association was not observed in overall (0-35 years) group or in older onset group. Logistic regression analysis did not confirm MICA5 to be associated with the age-at-onset, but confirmed the negative association of MICA6 to be associated with age-at-onset. We believe that the lack of association of MICA5 with univariate analysis in the older onset group was because of smaller number of subjects in older onset

	0-5 years	6-10 years	11-15 years	16-20 years	21-25 years	26-30 years	31-35 years
OR	0.7	0.5	0.6	0.9	0.92	1.2	1.25

Figure 6: Odds ratio with MICA6 in different age groups (of 5 years) in 0-35 year old population

group (patients=165, controls=125) compared to younger onset group (patients=470, controls=378). Thus, the association of MICA5 is not related with the age-at-onset of T1DM. However, negative association of MICA6 is related to age-at-onset and is stronger in younger age. Figure 6 shows the increasing odds ratio (decreasing negative association) with increasing age-at-onset of the disease. MICA6 has previously been shown to be negatively associated with the disease in Japanese and Koreans (Table II in introduction).

Among MICA genotypes, the risk conferred by MICA5/5.1 was higher in younger onset group than the risk in older onset group (OR=4.8, pc=0.02 vs. OR=2.33, pc=NS) as tested by univariate analysis. However, as for MICA5, the lack of association with MICA5/5.1 in the older onset group may very well be because of smaller number of subjects in older onset group compared to younger onset group. Also, the 95% confidence interval for -5/5.1 between the two age groups overlapped.

Unlike the Italian population [93], where MICA5.1 was associated with older onset (25 to 42 years old) of the disease, we did not find MICA5.1 to be associated with the disease in our cohort of older onset patients (21 to 35 years old). This could be because: 1) we have studied patients only up to the age of 35 years, and not above, 2) the number of subjects in older group in our study, although large, was not sufficient to detect a statistical significance, or, 3) Italians are a different population. Itlains have a lower frequency of MICA5.1 in general population (33%) compared to Swedes (62.6%).

Thus, in Swedish T1DM, only MICA6 is negatively associated with the age-at-onset of the disease and association is stronger in younger age.

Effect of gender

The incidence of T1DM among 0-35 year old in Sweden is higher in males compared to females (21.4 vs. 17.1) [5]. Our aim was to determine if association of MICA5 is related with gender in T1DM. In our study, according to univariate analysis, MICA allele 5 and genotype –5/5.1 were significantly increased in males but not in females (Table IV). The male to female ratio for presence of MICA5 among patients was

Table IV: Frequency and Odds ratio with MICA5 and –5/5.1 for T1DM in males and females in the age group of 0-35 years

	Patients	Controls	OR	95%CI	P	pc
MICA5 (females)	92/259 (35.52)	64/247 (25.91)	1.57	1.07-2.3	0.025	NS
MICA5 (males)	**143/376 (38.03)**	**59/256 (23.05)**	**2.05**	**1.43-2.93**	**0.0001**	**0.0005**
MICA 5-5.1 (females)	15/259 (5.79)	12/247 (4.86)	1.2	0.55-2.62	0.7	NS
MICA 5-5.1 (males)	**33/376 (8.78)**	**5/256 (1.95)**	**4.8**	**1.85-12.54**	**0.0003**	**0.0045**

1.07 (38.03% vs. 35.52%). However, logistic regression analysis did not demonstrate MICA5 association with the disease to differ between males and females. Moreover, the 95% confidence interval of the risk with MICA5 and MICA 5/5.1 for males and females overlapped (Table IV). We believe that the significance observed for MICA5 by univariate analysis but not by logistic regression in males and not in females is because of the number of female subjects studied being smaller (patients=259, controls=247) than the number of male subjects (patients=376, controls=256). Thus, the associations observed in males but not in females could merely be because of the difference in number of subjects in each group. Hence, we believe that MICA is not related with the gender of patients. None of the earlier reports on T1DM has shown MICA association to differ between males and females. However, none of these previous reports had such large number of subjects.

MICA is associated with LADA

It is known that the strength of association of HLA haplotypes/genotypes vary between T1DM and LADA. In paper II, MICA5/5.1 was associated with LADA (Table 3 and 6, paper II). However, none of the MICA alleles or genotypes was associated with T1DM, neither in the complete group including all patients (Table 3 and 6, paper II) nor in the group including patients up to 25 years of age (Table 4, and 7, paper II). This is in contrast with our observation of the association of MICA5/5.1 with T1DM (0-35 years) in paper I. The fact that no association of MICA gene polymorphism with T1DM was seen in paper II could be because of the smaller number of T1DM subjects which was not enough to detect weak association of –5/5.1 with statistical significance.

This raises a question whether MICA5/5.1 can distinguish younger onset T1DM and LADA? Our data indicates that MICA5/5.1 does not differentiate between T1DM and LADA qualitatively but does quantitatively i.e. strength of association vary between the two. In paper II, MICA5/5.1 gave an odds ratio of 8.5 in 51 LADA patients studied while in paper I, the odds ratio was 2.33 in 635 T1DM patients studied. This shows that association of MICA5/5.1 is much stronger with LADA than with T1DM. As shown by Gambelunghe *et al* [93] that MICA5 is associated with younger onset of the disease (<25 years at onset) and MICA5.1 is associated with older onset of the disease (>25 years at onset) and with LADA, we could not show different associations of MICA polymorphism with T1DM and LADA as clearly.

In paper II, we did not find MICA allele 5 to be associated with T1DM which was associated with T1DM in paper I. This could be because the number of T1DM subjects in paper II was much smaller than in paper I. This is supported by the fact that when we divided subjects in paper I with respect to six regions to which they belonged (each region having between 68 to 125 patients and controls), MICA5 did not show significant association with T1DM in any of the regions (see Table V). However, it should also be noted that the in paper II, patients were mainly from the adult clinic with age range much wider (9-89 years) than in paper I (0-35 years)

Table V: Odds ratio with MICA5 and -5.1 in different regions in Sweden

	Göteborg (n_p=123, n_c=98)			Linköping (n_p=90, n_c=82)			Lund (n_p=80, n_c=77)			Stockholm (n_p=125, n_c=74)			Umeå (n_p=92, n_c=68)			Uppsala (n_p=102, n_c=124)		
	OR	CI	P	OR	CI	P	OR	CI	P	OR	CI	P	OR	CI	P	OR	CI	P
MICA5	1.6	0,91 - 2,81	0.12	1.96	0,97- 3,9	0.08	1.57	0,77 - 3,19	0.21	2	1,05- 3,8	0.043	1.58	0,8- 3,11	0.23	2.07	1,14- 3,76	0.023
MICA5.1	0.95	0,55- 1,62	0.9	1.05	0,57- 1,95	0.87	0.76	0,39 - 1,46	0.5	0.76	0,42- 1,36	0.38	0.91	0,47- 1,76	0.86	0.77	0,44- 1,33	0.43

n_p=number of patients, n_c=number of controls

DR3-DQ2/DR4-DQ8 was associated with T1DM and with LADA (Table 5 and 6; paper II). DR4-DQ8/x (including homozygotes) was associated with T1DM (Table 6, paper II). DR3-DQ2/DR3-DQ2 was associated with LADA by univariate analysis (Table 5, paper II). DR15-DQ6 was a protective factor for T1DM (OR = 0.062) and also to some extent for LADA (OR = 0.66; Table 5 and 6, paper II). DR3-DQ2/DR4-DQ8 is a well established risk factor for T1DM, especially at a younger age [18] and LADA [118]. DR4-DQ8 has previously been reported to be of higher importance for T1DM [78]. DR3-DQ2 has been reported to be related to a more slowly progressive form of type I diabetes [78, 90] and this is supported by our findings of DR4-DQ8 alone as a significant risk factor for T1DM and homozygosity for DR3-DQ2 as a risk factor for LADA.

Association of DR3-DQ2/DR4-DQ8 with T1DM in those <25 years old was stronger than in overall patients (OR= 22 vs. 14). This is in agreement with the fact that frequency of DR3-DQ2/DR4-DQ8 is inversely related to the age-at-onset of the disease [18].

Viral etiology of T1DM: is MICA involved?

Polymorphism of MICA and HLA and infection with CBV has been associated with T1DM. Also it is known that in Sweden, Linköping is the region with highest incidence (fig. 2 in introduction). Our aim in this study was to see whether there is an increased frequency of antibodies against CBV in those carrying high-risk HLA-DR3, -DR4, MICA5, and MICA5.1 among patients from Linköping and patients from whole of Sweden.

In paper III, we found that antibodies against procapsids of CBV 3 or 5 were neither increased in all Swedish T1DM patients compared to all Swedish controls (25.83% vs. 21.13%) nor in Linköping patients compared to Linköping controls (26.08% vs. 23.07%).

In Linköping region, antibodies to CBV 3 or 5 were increased in DR3 positive patients versus DR3 negative patients (36.6% vs. 6.66%; p < 0.04) and DR3-DR4 positive patients versus DR3-DR4 negative patients (43.5% vs. 9.09%; p < 0.02) (Table 1, paper III). Also, frequency of CBV antibodies was increased in DR3-MICA5.1 positive patients versus DR3-MICA5.1 negative patients and in DR3-DR4-MICA5.1 positive patients versus DR3-DR4-MICA5.1 negative patients in Linköping (p < 0.02 for both; Table 3, paper III). The increase in the frequency of CBV antibodies in the later analysis was higher than in DR3 positive patients or in DR3-DR4 positive patients (Table 3 vs. Table 1, paper III). This suggests that MICA5.1 has an influence on immune response against CBV 3 or 5 infection in patients from Linköping. The increased susceptibility in DR3-DR4 positive individuals may solely be due to the presence of DR3 in these individuals.

When the patients from the whole of Sweden were divided based on the presence or absence of DR3, DR4, or DR3-DR4 (heterozygous); MICA5, -5.1 or -6; and DR3-

MICA5.1, DR4-MICA5.1 or DR3-DR4-MICA5.1, no difference in the frequency of CBV antibodies was observed in any of the three groups.

It is difficult to say how association of CBV-IgM to MICA5.1 could be relevant in pathogenesis of T1DM. There is evidence that early innate immune response (via NK-cells) against CBV4 may directly be responsible for the development of diabetes in mice [73]. MICA is one of the activating factors for NK-cells [35]. Induction of MICA expression after viral infection (CMV) has been shown [29]. It needs to be tested if MICA expression can be induced by CBV infection, particularly in the β-cells. Induced expression of MICA after CBV infection might be important in the pathogenesis of T1DM through NKG2D bearing NK-cells and also αβ T-cells.

We cannot rule out the possibility that significance seen in Linköping region, which is a high incidence area in Sweden [96], and not in the whole of Sweden could be due to chance. However, it is also possible that the environmental factors in the Linköping region, which are important for interaction with susceptibility genes, are different than in other areas in Sweden.

It has been shown that even when the prototype strain of a serotype is not destructive, highly destructive sub-strains can be found among field isolates of the serotypes [69]. It is possible that a different sub-strain of CBV 3 or 5 [63] might be prevalent at the time of sample collection in Linköping. It is also possible that in Linköping, CBV and T1DM are independently associated with DR3 i.e. DR3 positives are more prone to infection and also DR3 positive subjects are at risk to develop T1DM. This can be addressed by looking at larger group of CBV infected non-T1DM subjects for DR3 or DR3 positive and negative subjects for CBV. We could not study this in our material because of smaller number of CBV infected healthy subjects.

MICA is associated with autoantibodies in T1DM

Association of different high-risk HLA haplotypes with T1DM associated autoantibodies is known. In this study, we determined if MICA gene polymorphism is associated with autoantibodies.

MICA and single antibody: In this analysis, we compared antibody positive patients with patients negative for the corresponding antibody. *For IAA*: Frequency of MICA-5/5 and -5.1/5.1 were significantly increased in IAA positives (Table 1, paper IV). Among alleles, frequency of MICA5.1 and MICA9 and among genotypes frequency of MICA5/5.1 and -5.1/9 were significantly decreased in IAA positives (Table 1, paper IV). *For IA-2 autoantibodies*: Frequency of MICA5/5 was significantly increased and MICA5.1 was decreased in IA-2 autoantibody positives (Table 1, paper IV). *For GAD65 autoantibodies*: None of the alleles or genotypes of MICA were significantly associated with the presence or absence of GAD65 autoantibodies.

MICA and two antibodies: *IAA and/or IA-2 autoantibody positive vs. those negative for both*: frequency of MICA-5/5 was increased while that of MICA -5.1 and -9 as well as -5.1/9 was decreased in those with antibody positivity (Table 2a, paper IV). *IAA and IA-2 autoantibody positive vs. those negative for either or both the antibodies*: frequency of MICA5/5 was increased while that of MICA5.1 and -5/5.1 was decreased in those with antibody positivity (Table 2b, paper IV).

MICA and three antibodies: All patients with any of the three antibodies were positive for MICA5/5 and none of the patients with zero antibodies was positive for MICA5/5. MICA6 was decreased in the group carrying any of the three antibodies or in group with all three antibodies when compared to the group with no antibodies (data not shown).

In our study, we showed that in T1DM, MICA gene polymorphism is associated with autoantibody against insulin and IA-2 but not against GAD65. This is the first report to show association between MICA gene polymorphism and autoantibodies in T1DM. MICA5/5 was positively associated with both IAA and IA-2 autoantibodies considered either individually or together. MICA5.1/5.1 was positively associated with IAA alone. MICA5.1 was negatively associated with IAA and IA-2 autoantibodies considered individually or in combination. MICA-9 and -5.1/9 were negatively associated with IAA and with IAA and/or IA-2 antibody formation while MICA-5/5.1 was negatively associated with IAA formation and with formation of both IAA and IA-2 antibodies together.

MICA is important in early events of autoimmunity and events before acute onset of the disease

Among T1DM associated autoantibodies, IAA is believed to appear first [97, 119] and is more prevalent in disease onset at younger age [79, 97]. IA-2 antibodies are associated with acute onset of the disease and are also more important in younger age [77, 120]. GAD65 antibodies are more important in older onset of the disease and also LADA (Table III in introduction) [77, 120].

MICA molecules are a part of innate immune system that interacts with NK-cells, $\alpha\beta$ T-cells and also $\gamma\delta$ T-cells [35]. The role of polymorphic MICA molecules in T1DM pathogenesis is not known, but role of NK-cells and CBV infection in T1DM pathogenesis has been shown [73, 121]. We speculate, that one of the ways in which MICA might be involved in disease pathogenesis could be that after an environmental trigger e.g. viral infection, MICA molecules are upregulated [29] on β-cells. These MICA molecules, upregulated soon after the environmental insult, can activate NK-cells (and probably also co-stimulate CD4+ and also CD8+ $\alpha\beta$ T-cells) via interaction with NKG2D receptor [29, 35, 36] and these activated cells are important in early β-cell destruction. In this regard, it is very interesting observation that MICA gene polymorphism especially genotype 5/5 is associated with the formation IAA and IA-2 antibodies. Also, all subjects

carrying MICA5/5 developed at least one of the three antibodies tested. Thus, the presence of MICA5/5 in addition to IAA or IA-2 could be a valuable marker for prediction strategies.

However, since not all individuals who develop autoantibodies develop T1DM and also not all T1DM patients are necessarily positive for autoantibodies at the time of diagnosis, it suggests that immune reaction can take different / multiple paths for disease development and development of autoantibodies in different individuals. Other factors such as immune regulatory mechanisms and other genes might also affect cytokine levels important in T1DM pathogenesis.

MICA in newborn screening study

At present, voluntary genetic screening for risk alleles is an irreplaceable tool in research looking for susceptible individuals for intervention trials. ABIS is one of such studies but it does not include intervention. In the ABIS newborn screening study, we have so far typed 2821 of 17,055 newborn babies for high-risk HLA. Out of 2821, 563 subjects were positive for DQ2, 583 subjects were positive for DQ8, 133 subjects were positive for DQ2-DQ8 (heterozygous) and 1013 subjects were positive for either DQ2 or DQ8 (Table 1, paper V). Out of 1013 babies positive for either DQ2 or DQ8 we have so far typed 499 babies for MICA alleles the frequencies for which are shown in Table 2 of paper V; these include 293 of 563 DQ2 positives, 275 of 583 DQ8 positives and 69 of 133 DQ2-DQ8 (heterozygous) positives (Table 3, paper V).

Frequency of MICA5 was 38% among DQ8+, 35% among DQ2-DQ8 (heterozygous) positives and 22.5% among DQ2+. Frequency of MICA5.1 was 81% among DQ2+, 62% among DQ8+ and 71% among DQ2-DQ8 (heterozygous) positives. Frequency of MICA6 was between 20-22% among the three groups. Frequency of MICA5/5.1 was 19% among DQ2-DQ8 (heterozygous) positives while it was between 12-13% among those positive for DQ2, DQ8, DQ2 or DQ8.

It has been shown that in Sweden, 89% of T1DM carry either DQ2 or DQ8 [18]. MICA5 has been shown previously to be positively associated with the disease in Swedish population and increase the risk for disease especially in combination with DR3-DQ2 (Table 4, paper I). In this study, we anticipate that a higher proportion of those positive for MICA5-DQ2 will develop T1DM than the proportion carrying DQ2 alone.

The genetic markers typed in newborns in ABIS study would be re-evaluated in conjunction with autoantibody markers which are being analyzed at University of Linköping. The results from this study would be useful to develop an approach for identifying children who are at high-risk to develop T1DM. Also, since all subjects in this study are being followed for environmental exposures, the results from genetic typings, autoantibody analysis and environmental exposures would be useful in retrospect after

many children would have developed T1DM, the peak incidence for which is between 10-14 years of age.

SUMMARY OF RESULTS

1. In Swedish population up to the age of 35 years, MICA5 and MICA5/5.1 are positively associated with T1DM independent of high-risk HLA. MICA5 in combination with DR3-DQ2 increases the risk for the disease.

2. MICA6 is negatively associated with T1DM and association is stronger in younger age. MICA5 and MICA5/5.1 did not appear to be associated with age-at-onset of the disease.

3. MICA gene polymorphism is not associated with gender in T1DM.

4. MICA5/5.1 is also associated with LADA and association is stronger as compared to acute onset T1DM.

5. DR3-MICA5.1 in relation to CBV-3 and CBV-5 infection seems to be important in disease pathogenesis in the high-incidence region of Linköping. It is difficult to say what role MICA5.1 might play in disease pathogenesis. It is also possible that our findings are due to chance. Such an association should be confirmed in other studies.

6. MICA gene polymorphism is associated with IAA and IA2 autoantibodies and is important in early events of autoimmunity and events before the acute onset of the disease.

7. Inclusion of MICA typing in addition to HLA could be useful for screening of genetic markers associated with T1DM.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all the people who helped me in so many ways to make this thesis become a reality. The work in this thesis would have not been possible without support and encouragement from all:

My supervisor, **Dr. Carani B. Sanjeevi**, for introducing me to the world of HLA and immunogenetics, for his continuous supervision, support, constructive criticism and encouragement throughout my PhD studies.

Prof. Kerstin Brismar, my co-supervisor and Chairman of the Department of Molecular Medicine, thank you for your support, encouragement, long scientific discussions and for seeing the positive side of human nature.

Words fall short to thank **Dr. Ingrid Kockum** for the warmth and friendliness throughout my association with her. Discussions about statistics and your scientific point of view have taught me a lot about data interpretation. For your support throughout, answering my endless questions and constant help while thesis writing… I cannot thank you enough.

This journey through years was not possible without support from **Emma Tham**. Thanks for trust in me, understanding me and making me move forward

Liene Nikitina-Zake, not only my colleague and co-author but also great and understanding friend, who made me laugh during tough times. Thanks for teaching me everything about PCR and ABI prism. I learnt a lot about geography from you but you still owe me a PhD in geography. The pains you took to teach me swimming enabled me to swim at-least 25 meters.

The past members of my group: **Mehran** for allowing me to use the map of MHC in this thesis and for all the unscientific discussions, telling me about his exciting experience in paragliding, parachuting, trying unsuccessfully to teach me how to drown when in water, **Annika Andersson** for friendship, help in translating Swedish correspondence to English, and assisting me in experiments, **Giovanni Gambelunghe** for discussions and humor, **Aija** for discussions about HLA and diabetes and **Late Laura** for teaching me DNA extractions and dot blotting, **Ken** 'the self promoted computer professional' for solving computer problems and help in formatting this thesis, **Vali**, **Ershad** and **Lisen** to create a nice environment to work.

Maria Malec for being a nice and understanding friend and giving advices during difficult times. **Fabio Sanchez** for sharing our deep knowledge in genetics and advice even at mid-night.

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