Exploring the Grey Zone between Type 1 and Type 2 Diabetes

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Exploring the Grey Zone between Type 1 and Type 2 Diabetes

ACADEMIC DISSERTATION

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With the permission of the Medical Faculty of Lund, to be presented for public examination in the Grand Hall at the Medical Research Center, Entrance 59, Malmö University Hospital, on February 20, 2009 at 9.00 a.m.

Faculty Opponent

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Exploring the Grey Zone between Type 1 and Type 2 Diabetes

Abstract
T1D diabetes in children and young adults and is characterized by autoimmune destruction of pancreatic beta-cells. In adults the same situation is often referred to as Latent Autoimmune Diabetes in Adults (LADA). T2D is characterized by impaired insulin secretion and/or insulin resistance. Maturity-onset diabetes of the young (MODY) is an autosomal dominant diabetes with primarily defect in beta-cell function. The goal of this thesis was to genetically dissect autoimmune and non-autoimmune diabetes in young (15-34 years old) and middle-aged (40-59 years old) Swedish diabetic patients. In Study I, fPc-peptide concentrations after diagnosis were markedly lower in patients with Ab+ than in Ab-. Irrespective of clinical classification Ab+ patients showed increased frequencies of at least one risk HLA-DQB1 genotypes than Ab- patients. Ab-patients with risk HLA-DQB1 genotypes had significantly lower fPc peptide concentrations than those without risk genotypes. In Study II, the T2D-associated genotypes of TCF7L2 CT/TT were significantly more common in young GADA+ than in GADA- patients. No such difference was seen in middle-aged patients, in whom the frequency of the CT/TT genotypes of TCF7L2 was similarly increased in GADA+ and GADA- groups, suggesting that young GADA+ patients have T2D, middle-aged GADA+ patients (LADA) are different from their young GADA+ (T1D) counterparts and share genetic features with T2D. In Study III, young GADA- patients had increased frequency of common variants in T2D genes. Middle-aged GADA+ diabetic patients with more than 12 risk alleles had decreased fPc-peptide concentrations than patients with less than 9 risk alleles. Also, GADA+ patients with more than risk alleles had an earlier age at onset than GADA- patients with less risk alleles. Distribution of T2D-associated risk alleles was quite similar in middle-aged patients regardless of presence of GADA. In Study IV, no significant difference in frequency of common variants in MODY genes was seen between Ab+ and Ab- individuals. In Ab+ diabetic patients carriers of the T2D-associated T allele of HNF-1α gene had higher age at onset of diabetes, but more severe symptoms of diabetes than G allele carriers. In Ab+ diabetic patients carriers of the T2D-associated G allele of HNF-1α gene had less severe symptoms of diabetes at diagnosis than A allele carriers. One patient had a frameshift mutation in exon 4 designated "Pro291Hisfsx174" in the HNF-1α gene. Our studies clearly show that genetic markers markedly improve the classification of diabetes and together with islet antibodies they might be help for diagnosis and treatment of different diabetic subgroups.

Key words: T1D, T2D, LADA, MODY, islet antibodies (ICA, GADA, IA-2A), plasma C-peptide, HLA-DQB1, INS VNTR, PTPN22, TCF7L2, GWAS, BMI.
Exploring the Grey Zone between Type 1 and Type 2 Diabetes

ACADEMIC DISSERTATION

Ekaterine Bakhtadze

Lund University
Department of Clinical Sciences
Diabetes and Endocrinology
Malmö University Hospital
To my beloved mother and father, family and friends

All our dreams can come true, if we have the courage to pursue them.

Walt Disney

What you get by achieving your goals is not as important as what you become by achieving your goals.

Zig Ziglar
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### Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Ab</td>
<td>Antibodies</td>
</tr>
<tr>
<td>ADA</td>
<td>American Diabetes Association</td>
</tr>
<tr>
<td>ADAMTS9</td>
<td>A Disintegrin-like and Metalloproteinase with Thrombospondin Type 1 Motif</td>
</tr>
<tr>
<td>ARG</td>
<td>Arginine</td>
</tr>
<tr>
<td>BACH2</td>
<td>BTB and CNC homology 1, basic leucine zipper transcription factor 2</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CAMK1D</td>
<td>Calcium/Calmodulin-dependent Protein Kinase 1-delta</td>
</tr>
<tr>
<td>CAPN10</td>
<td>Calpain 10</td>
</tr>
<tr>
<td>CDKAL1</td>
<td>CDK5 regulatory subunit associated protein</td>
</tr>
<tr>
<td>CDKN2A/2B</td>
<td>Cyclin-dependent kinase inhibitor 2A2B</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CLEC16A</td>
<td>C-type lectin domain family 16, member A</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte-associated antigen-4</td>
</tr>
<tr>
<td>CYP27B1</td>
<td>Cytochrome P450, family 27, subfamily B, polypeptide 1</td>
</tr>
<tr>
<td>ICA</td>
<td>Islet cell antibodies</td>
</tr>
<tr>
<td>IFG</td>
<td>Impaired fasting glucose</td>
</tr>
<tr>
<td>IGT</td>
<td>Impaired glucose tolerance</td>
</tr>
<tr>
<td>IFIH1</td>
<td>Interferon-induced with helicase C domain 1</td>
</tr>
<tr>
<td>IGF2BP2</td>
<td>Insulin-like growth factor 2 mRNA binding protein 2</td>
</tr>
<tr>
<td>DISS</td>
<td>Diabetes Incidence Study in Sweden</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyriboonucleic acid</td>
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<tr>
<td>FpC-peptide</td>
<td>Fasting plasma C-peptide</td>
</tr>
<tr>
<td>FTO</td>
<td>Fatso-fused toe (fat mass and obesity associates gene)</td>
</tr>
<tr>
<td>GADA</td>
<td>Glutamic acid decarboxylase antibody</td>
</tr>
<tr>
<td>GCK</td>
<td>Glucokinase gene</td>
</tr>
<tr>
<td>GIP</td>
<td>Gastric inhibitory polypeptide</td>
</tr>
<tr>
<td>GLP-1</td>
<td>glucagon-like peptide 1</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association study</td>
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<tr>
<td>HbA1c</td>
<td>Glycosylated hemoglobin</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HHEX</td>
<td>Hematopoietically expressed homeobox</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HNF</td>
<td>Hepatocyte nuclear factor</td>
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<tr>
<td>IL2RA</td>
<td>Interleukin 2 receptor alpha</td>
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INS VNTR  Alleles of variable-number tandem repeat of insulin gene
JAZF1  Juxtaposed with another zinc finger
JDF  Juvenile Diabetes Foundation
KCNJ11  Potassium inwardly-rectifying channel Subfamily J, member 11
kDa  kilodalton
LADA  Latent autoimmune diabetes in adults
LD  Linkage Disequilibrium
LDL  Low density lipoprotein
LOD  Logarithm of the odds
MDC  Malmö Diet and Cancer Study
MODY  Maturity onset diabetes of the young
mRNA  Messenger ribonucleic acid
NOTCH2  Notch Homolog 2
OR  Odds ratio
PCR  Polymerase chain reaction
PRKCG  Protein kinase C, theta
PPARG  Peroxisome proliferator-activated receptor gamma
PTPN2  Protein tyrosine phosphatase, non-receptor type 2
PTPN22  Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)
RIA  Radioimmuno assay
RU  Relative units
SBE  Single base extension
SLC30A8  Solute carrier family 30 (z-transp) member 8, ZnT-8
SNP  Single nucleotide polymorphism
T1D  Type 1 diabetes mellitus
T2D  Type 2 diabetes mellitus
TCF7L2  Transcription factor-7-like 2
THADA  Thyroid Adenoma Associated
TRP  Tryptophan
TSPAN8  Tetraspanin 8
UBASH3A  Ubiquitin associated and SH3 domain containing, A
UKPDS  United Kingdom Prospective Diabetes Study
WFS1  Wolfram syndrome 1
WHO  World Health Organisation
ZnT8  Zinc transporter antibodies
Abstract

T1D is most common in children and young adults and is characterized by autoimmune destruction of insulin producing pancreatic beta cells, presence of certain risk genotypes such as HLA-DQB1, INS VNTR, PTPN22 and need of insulin for survival. In adults the same situation is often referred to as Latent Autoimmune Diabetes in Adults (LADA), with age at onset after 35 years and non-insulin requiring for at least 6 month after diagnosis. On the other hand, T2D is characterized by impaired insulin secretion and/or insulin resistance, which coexists with excessive hepatic glucose production and abnormal fat metabolism. Environmental factors causing insulin resistance are puberty, pregnancy, weight gain (central obesity “apple type”) and sedentary lifestyle. Usually T2D is diagnosed after 45 years of age and in some cases is diagnosed when patients develop vascular and neuropathic complications. TCF7L2 is by far the strongest T2D-associated gene. Maturity-onset diabetes of the young (MODY) is a monogenic form of diabetes inherited in an autosomal dominant fashion (individual has one copy of a mutant gene and one normal gene on a pair of autosomal chromosomes) characterized by nonketotic diabetes, age at onset before 25 years and primarily defect in beta-cell function. Until now, mutations in six genes have been identified as the cause of different forms of MODY, i.e. HNF-4α (MODY 1), glucokinase (GCK) (MODY 2), HNF-1α (MODY 3), IPF1 (MODY 4), HNF-1β, formerly TCF2 (MODY 5) and NeuroD1 (MODY 6).

The goal of this thesis was to genetically dissect autoimmune (T1D and LADA) and non-autoimmune (T2D and MODY) diabetes in young (15-34 years old) and middle-aged (40-59 years old) Swedish diabetic patients for proper diagnosis and treatment of the disease in the future.

To fulfill our goals we have selected 1642 young (15-34 years old) adult diabetic patients from Diabetes Incidence Study in Sweden (DISS) and 1619 middle-aged (40-59 years old) diabetic patients from Diabetes Registry in Southern Sweden. We determined genetic markers: HLA-DQB1 (study I and II), PTPN22, Ins VNTR, TCF7L2 (study II), PPARG, KCNJ11, IGF2BP2, WFS1, CDKAL1, JAZF1, CDKN2A/2B, IHEX, SLC30A8 and FTO (study III) and MODY genes- HNF-4α, GCK, HNF-1α and HNF-1β, formerly TCF2 (study IV), measured islet antibodies (ICA, IA-2A and GADA) and C-peptide (marker of beta-cell function instead of insulin).

In Study I we evaluated whether HLA-DQB1 genotypes facilitates the classification of diabetes as compared with islet antibodies among young (15-34 years) adult diabetic patients. Islet antibodies were found among 83% clinically considered to have T1D, 23% with T2D and 45% with unclassifiable diabetes.
fpC-peptide concentrations after diagnosis were markedly lower in patients with than in those without islet antibodies. Irrespective of clinical classification, patients with islet antibodies showed increased frequencies of at least one risk HLA-DQB1 genotypes compared with patients without. Antibody negative patients with risk HLA-DQB1 genotypes had significantly lower fasting fpC-peptide concentrations than those without risk genotypes. We concluded that Assessment of islet antibodies is necessary for an etiological classification of diabetic patients. HLA-DQB1 genotyping does not improve the classification in patients with islet antibodies. However, in patients without islet antibodies, HLA-DQB1 genotyping together with fpC-peptide measurement may be of value in the differentiation between idiopathic T1D versus T2D.

In Study II we evaluated whether genetic markers associated with T1D (HLA-DQB1, INS VNTR and PTPN22) and T2D (TCF7L2) could help to discriminate between autoimmune and non-autoimmune diabetes in young (15-34 years) and middle-aged (40-59 years) diabetic patients. Frequency of risk genotypes HLA-DQB1, PTPN22 CT/TT, INS VNTR class I/I and INS VNTR class IIA/IIA was increased in young and middle-aged GADA+ compared with GADA- patients. T2D-associated genotypes of TCF7L2 CT/TT of rs7903146 were significantly more common in young GADA- than in GADA+ patients. No such difference was seen in middle-aged patients, in whom the frequency of the CT/TT genotypes of TCF7L2 was similarly increased in GADA- and GADA+ groups. We concluded that common variants in the TCF7L2 gene help to differentiate young but not middle aged GADA+ and GADA- diabetic patients, suggesting that young GADA- patients have T2D and that middle-aged GADA+ patients (LADA) are different from their young GADA-positive (T1D) counterparts and share genetic features with T2D.

In Study III we genotyped a panel of 10 novel T2D-associated risk genotypes in young (15-34 years) and middle-aged (40-59 years) GADA+ and GADA-diabetic patients and evaluated how they would modify the clinical phenotype. Young GADA- patients had increased frequency of risk variants in the PPARG, IGF2BP2, WFS1, JAZF1 and CDKN2A/2B genes compared with an elderly non-diabetic control group. Also risk variants in JAZF1 (AA) and CDKN2A/2B (TT) were more common in GADA- than in GADA + young diabetic patients. As expected middle-aged GADA- patients had increased prevalence of risk variants in the PPARG, IGF2BP2, WFS1, CDKAL1, JAZF1, SLC30A8, CDKN2A/2B, KCNJ11 and FTO genes compared with non-diabetic controls with no significant difference compared with GADA+ patients. Middle-aged GADA-diabetic patients with more risk alleles (≥12) had decreased C-peptide concentrations than patients with less risk alleles (≤9). Also, GADA+ patients with more risk alleles had an earlier age at onset than GADA+ patients with less risk alleles. Distribution of T2D-associated risk alleles was quite similar in
middle-aged patients regardless of presence of GADA. T2D-associated risk genotypes modify the disease phenotype (age at onset and C-peptide) in middle-aged but not in young diabetic patients.

In Study IV we evaluated whether common variants in MODY genes can discriminate between autoimmune and non-autoimmune diabetes in young adult diabetic patients and screened antibody negative diabetic patients with ≥ 3 members with diabetes in the family for HNF-4α, GCK and HNF-1α mutations. No significant difference in frequency of common variants in MODY genes was seen between Ab+ and Ab- individuals. In Ab+ diabetic patients carriers of the T2D-associated T allele of the HNF-1α gene had higher age at onset of diabetes, but severe symptoms of diabetes (weight reduction and/or polyuria) than G allele carriers. Finally, in Ab- diabetic patients carriers of the T2D-associated G allele of HNF-1β gene had less frequent weight reduction and/or polyuria and ketonuria at diagnosis than A allele carriers. One patient had frameshift mutation in exon 4 designated “Pro291fsinsC” in the HNF-1α gene. Common variants in MODY genes do not discriminate between young patients with autoimmune and non-autoimmune diabetes but they do influence onset and presentation of the disease.

Our studies show that genetic markers clearly improve the classification of diabetes and together with islet antibodies they might be of help for diagnosis and treatment of different diabetic subgroups.
1. Introduction to diabetes mellitus (DM)

Diabetes is a disease recognised from the ancient times. The first description comes from the 3rd dynasty Egyptian papyrus by the physician Hesy-Ra in 1550 BC, who mentioned polyuria (frequent urination) as a symptom. The term diabetes (passing through) was introduced by the Greek physician Aretaeus in 150 A.D, who described it as “the melting down of flesh and limbs into urine.” In 164 A.D, another Greek physician, Galen of Pergamum mistakenly diagnosed diabetes as an ailment of the kidneys. The sweet taste of diabetic urine, described in ancient Indian texts and noted by Avicenna (980-1037) and Morgagni (1635-1683), was attributed to the passage of absorbed water and nutrients unchanged into the urine. In 1000s diabetes was commonly diagnosed by “water tasters”, who tasted urine from the people suspected to have diabetes and since it was sweet-tasting, the Latin word for honey – mellitus – was incorporated into the condition's name and “diabetes mellitus” was coined. In 1774 Mathew Dobson confirmed that the sweet taste of urine and blood was due to high content of glucose, which suggested that diabetes was not just a kidney problem, as it was previously been believed (1). In 1866, the British physician George Harley first made the distinction between types of diabetes, one “gaining weight and strength on diet, so called excessive formation of sugar diabetes and another, in the same regimen of diet loosing both flesh and energy, so called defective assimilation of sugars (mal-nutrition) diabetes.” This was followed by French physician Etienne Lancereaux in 1880, who made a distinction between lean and obese diabetes: “Le diabète maigre et le diabète gras.”

Insulin was discovered in 1921 (3) and insulin treatment had been initiated in increasing number of diabetic patients. By that time physicians were classifying the patients using clinical characteristics, such as age at onset, body weight and insulin requirement. The first test used to distinguish between the two main forms of diabetes was response to insulin. Austrian investigators Falta and Boller drew attention to the existence of insulin-sensitive and -resistant forms of diabetes (4). Insulin-sensitive patients readily suppressed urinary excretion of glucose and developed hypoglycemia in response to a few units of insulin, whereas withdrawal of insulin rapidly resulted in glycosuria and ketosis. These features were lacking in insulin-insensitive patients. A clear distinction between insulin-sensitive and -resistant forms of diabetes was made by the British scientist Harold Himsworth in 1936 (5). He developed a challenge test in which glucose was given by mouth while insulin was injected intravenously. Based upon careful anthropometrical measurements of diabetic patients, two different groups, pancreatic diabetes and diabetes of pituitary origin, were described by Draper, in 1940. These findings were adopted by Lister in 1951, who noted “two broad groups of diabetics- group I - the young, thin, non-arteriosclerotic group with normal blood pressure and usually an acute onset of the disease, and group
II - the older, obese, arteriosclerotic group with hypertension and usually an insidious onset (6)’’.

2. Diabetes mellitus- diagnosis and classification, pathogenesis and epidemiology

Diabetes mellitus is a multifactorial metabolic disorder which results from the complex interaction of genetic and environmental factors. Diabetes comprises a group of disorders with distinct pathophysiological mechanisms, but sharing the common phenotype of hyperglycaemia (increased blood sugar levels). The end point of the disease process is insulin deficiency, which can be absolute or relative in the coexistence of insulin resistance (response to insulin by the target tissues, such as muscle, liver and adipose tissue). The result is chronic hyperglycaemia, caused by reduced insulin secretion, decreased insulin utilisation and increased liver glucose production, which in the long run lead to diabetic complications. Diabetes is a leading cause of end-stage renal disease (ESRD), non-traumatic lower extremity amputations, adult blindness and cardiovascular complications.

The first guideline for the diagnosis and classification of diabetes was published by WHO in 1965 (7) and was entirely based on age at onset of diabetes (juvenile and maturity-onset). In 1976 the names type 1 and type 2 diabetes were reintroduced (8). According to the classification of diabetes by US National Diabetes Data Group in 1979 (9), the terms juvenile and maturity onset diabetes were replaced by insulin-dependent diabetes mellitus (IDDM) or type 1 and non–insulin-dependent diabetes (NIDDM) mellitus or type 2 diabetes. Patients with any form of diabetes may require insulin therapy; for this reason, the new classification based on disease etiology emerged. In 1997 ADA (10) and in 1998 WHO (11) introduced new diagnostic criteria for diabetes classification where the terms type 1 and type 2 diabetes were retained and a plasma glucose level of 7.0 mmol/l (126 mg/dL) instead of 7.8 mmol/L (140 mg/dL) was recommended as a new threshold for diabetes. The American Diabetes Association (ADA) in 2003 (12) reviewed its diagnostic criteria and recommended a new threshold for impaired fasting glucose (IFG) 5.6 mmol/l (100 mg/dL) instead of 6.1 mmol/L (110 mg/dL). The new diagnostic criteria are based on the following premises: 1) symptoms of diabetes (polyuria, polydipsia and unexplained weight loss) together with random plasma glucose concentration above 11.1 mmol/L (200mg/dL) or 2) fasting plasma glucose concentration above 7.0 mmol/l (126mg/dL) on two different days or 3) two-hour 75g oral glucose tolerance test (OGTT) value above 11.1 mmol/l (200mg/dL) (Table 1). In the latest updates by WHO in 2006 (13) and ADA in 2009 (14) no changes to the diabetes classification were made.
Table 1. Comparison of 1999 WHO and 2003 ADA diagnostic criteria

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<th>ADA 2003</th>
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<tr>
<td>Diabetes</td>
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<tr>
<td>Fasting glucose</td>
<td>≥7.0 mmol/l</td>
<td>≥7.0 mmol/l</td>
</tr>
<tr>
<td>2-hour glucose*</td>
<td>or 11.1 mmol/l</td>
<td>or 11.1 mmol/l</td>
</tr>
<tr>
<td>IGT</td>
<td>Fasting glucose</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;7.0 mmol/l (if measured)</td>
<td>Not required</td>
</tr>
<tr>
<td></td>
<td>or</td>
<td></td>
</tr>
<tr>
<td>2-hour glucose</td>
<td>≥7.8 and &lt;11.1 mmol/l</td>
<td>≥7.8 and &lt;11.1 mmol/l</td>
</tr>
<tr>
<td>IFG</td>
<td>Fasting glucose</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.1 to 6.9 mmol/l (measurement recommended)</td>
<td>5.6 to 6.9 mmol/l (measurement not recommended)</td>
</tr>
<tr>
<td></td>
<td>(if measured should be &lt;7.8 mmol/l)</td>
<td>(if measured should be &lt;11.1 mmol/l)</td>
</tr>
</tbody>
</table>

*Venous plasma glucose 2-hours after 75g oral glucose load


Etiologically diabetes is divided into four general subclasses: 1) type 1, primarily caused by autoimmune destruction of pancreatic beta-cell and characterized by absolute insulin deficiency 2) type 2, characterized by insulin resistance and relative insulin deficiency or primarily insulin secretory defects with or without insulin resistance 3) “other” specific types of diabetes (associated with identifiable clinical conditions or syndromes) and 4) gestational diabetes mellitus (Figure 1).

In addition to these clinical categories, two forms of pre-diabetes 1) impaired fasting glucose (IFG) and 2) impaired glucose tolerance (IGT) have been defined to describe intermediate metabolic states between normal glucose homeostasis and overt diabetes. Both impaired fasting glucose and impaired glucose tolerance are strong risk factors for future diabetes as well as cardiovascular disease (15, 16). In most types of diabetes, the individual traverses from normal glucose tolerance to abnormal glucose tolerance and to overt diabetes (Figure 1).
Figure 1. Disorders of glycemias: etiological types and clinical stages (13, 14).
*Even after presenting with 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.

The prevalence of diabetes is increasing dramatically worldwide, being 30 million in 1985, 177 million in 2000 and it is estimated to reach 366 million by the year 2030 (17). The increase in the prevalence of type 2 diabetes is much more rapid, due to population growth, aging, urbanization, and increasing prevalence of obesity and physical inactivity (18-20).

2.1 Type 1 diabetes (T1D)

Type 1 diabetes is a multifactorial disease, which is primarily caused by autoimmune destruction of pancreatic beta-cells leading to absolute insulin deficiency (Figure 2) and need of insulin for survival. Antibodies against islet cell (ICA), insulin (IAA), glutamic acid decarboxylase (GADA), protein tyrosine phosphatase (IA-2A) and, recently discovered zinc transporter Slc30A8 are autoimmune markers rather than the cause of type 1A diabetes (21). Severe insulin deficient diabetes without islet antibodies, so called type 1B diabetes, has also been reported (22). It has been recognized that destruction of pancreatic beta-cell is mediated by cytokines or by direct CD8 cytotoxic T-lymphocyte activity (23-26). At T1D onset, islet antibodies are present in about 98% of children (27, 28) and 85% of young adults (29, 30). Features of diabetes do not become evident until the majority of beta cells are destroyed (~80%).
The onset of T1D is acute and presents as polyuria (frequent urination), polydipsia (thirst), weight loss, fatigue and blurred vision. Infection is reported to precipitate the disease onset, although only congenital rubella and parotitis infections have a clear association with the disease (31). Enterovirus, coxsackievirus and retrovirus infections have also shown an association with type 1A diabetes. Other environmental risk factors seem to include early introduction of bovine milk, fast weight gain during early life, toxins, psychological stress, sterile environment and vitamin D deficiency (32-39).

There are clear geographical variations in the occurrence of the disease. T1D is more prevalent in Finland, Scandinavia, and Sardinia; less prevalent in most of Southern Europe and the Middle East; and uncommon in Asian nations (40-42). It accounts for 10-15% of diabetes among children and young adults of Caucasian origin. In addition, T1D incidence rates appearing to be increasing (42), at least in children.

Figure 2. Hypothetical model of development autoimmune type 1 (type 1A) diabetes. Individuals with a genetic predisposition are exposed to an immunological trigger that initiated an autoimmune process, resulting in a gradual decline in beta cell mass (Modified from Eisenbarth GS. Type 1 diabetes mellitus: a chronic autoimmune disease. N Engl J Med 1986; 314: 360-368 (43))

2.2 Type 2 diabetes (T2D)
Type 2 diabetes is a multifactorial disease characterised by impaired insulin secretion and insulin resistance, which coexists with excessive hepatic glucose production and abnormal fat metabolism. Environmental factors causing insulin
resistance are puberty, pregnancy, weight gain (central obesity “apple type”) and
sedentary lifestyle. In the prediabetic state, with increased insulin resistance,
pancreatic beta-cells are capable to produce enough insulin to maintain glucose
tolerance within normal range. When beta-cells no longer are able to
compensate for the insulin resistance diabetes becomes manifested (44). Increasing adipocyte mass accompanying obesity in T2D results in increasing
levels of non-esterified free fatty acids (NEFFA) which further impairs glucose
utilization in skeletal muscle, promotes glucose production by the liver, and
impairs beta cell function (45) (Figure 3).

![Pathophysiology of hyperglycaemia in T2D](image)

**Figure 3. Pathophysiology of hyperglycaemia in T2D (45).**

Usually T2D is diagnosed after 40 years of age. Classical symptoms of the
disease are mild and may exist several years before patients seek medical
attention. Moreover, in some cases, patients are diagnosed when they develop
vascular and neuropathic complications. Ketosis rarely develops, but can appear
as a result of fasting and increased oxidation of fat. T2D patients are not
dependent on insulin for survival, but they may require it for proper glycaemic
control.

T2D accounts for >95% of all diagnosed cases of diabetes globally. The
prevalence of T2D is highest in the Pima/Papago Indians (50%), and certain
Pacific islands (e.g. Nauru) (42%), high in Middle East (17%-20%),
intermediate in countries such as India and the United States (8-11%), and
2.3 Latent Autoimmune Diabetes in Adults (LADA)

In clinical practice main diagnostic criteria of diabetes are related to phenotypic presentation, e.g. age at onset, BMI and insulin requirement. However, it is not that uncommon that disease presentation does not exactly follow the classical frame. For example approximately 25% of patients who develop T1D are diagnosed after the 35 years of age (49). In 1970’s Irvine and colleagues reported that the presence of ICA was observed in 11-14% of adult onset diabetic patients who did not require insulin for at least 3 month after diagnosis (50, 51). First in 1986 (52) and then in 1988, Groop and colleagues showed that insulin requiring T2D patients had high prevalence of ICA as well as the T1D risk HLA genotypes. The name Latent Autoimmune Diabetes in Adults (LADA) was subsequently introduced to classify these patients (53, 54). Thereafter, several other names, including slowly progressive forms of T1D (55) and 1.5 diabetes (56) have also been proposed to define this form of diabetes, but LADA is the most commonly used eponym. It was later demonstrated that GADA are the most prevalent islet autoantibodies present in LADA patients, whereas IAA and IA-2A are less prevalent (57).

Clinically LADA is characterized by lower BMI, less features of metabolic syndrome, more severe reduction in insulin secretion and rapid progression to insulin treatment as compared to the common forms of T2D (58-60). Definition of age for LADA has varied from 25 (UKPDS) to 40 years (61, 62). LADA accounts for approximately 10% of the patients initially diagnosed as having T2D (63).

Presently, the diagnosis of LADA is based on: adult onset diabetes (>35 years), presence of GADA and insulin independence at diagnosis (59).

2.4 Maturity Onset Diabetes of the Young (MODY)

First described by Fajans and colleagues in 60’s, maturity-onset diabetes of the young (MODY) is a monogenic form of diabetes inherited in an autosomal dominant fashion (individual has one copy of a mutant gene and one normal gene on a pair of autosomal chromosomes) characterized by nonketotic diabetes, age at onset before 25 years and primarily defect in beta-cell function (64). In monogenic forms of diabetes, environmental factors play a minor role in determining whether or not a genetically predisposed person develops clinical diabetes (65).
Until now, mutations in six genes have been identified as the cause of different forms of MODY, i.e. \textit{HNF-4\alpha} (MODY 1) (66), glucokinase (MODY 2) (67, 68), \textit{HNF-1\alpha} (MODY 3) (69), \textit{IPF1} (MODY 4) (70), \textit{HNF-1\beta}, formerly \textit{TCF2} (MODY 5) (71) and \textit{NeuroD1} (MODY 6) (72). Of them all except \textit{GCK}, represent transcription factors involved in beta-cell development. Although the exact prevalence of MODY is not known, it is suggested that it accounts for 1\% to 5\% of all diabetes cases (64). In addition, there is increasing evidence that common variants in MODY genes also predispose to T2D (73-82).

3. Metabolic basis of diabetes

Diabetes develops due to inadequate production of insulin by pancreatic beta-cells and/or resistance to actions of insulin (stimulation of glucose uptake, inhibition of hepatic glucose production and lypolysis). Glucose is a major regulator of insulin secretion. A glucose level >3.9 mmol/l enhances protein translation and processing and thereby stimulates insulin synthesis. Amino acids, ketones, gastrointestinal peptides and neurotransmitters also influence insulin secretion. Glucose enters the beta-cells via the glucose transporter GLUT2. It is phosphorylated by glucokinase (the rate-limiting step of glucose metabolism) to form glucose-6-phosphate. Further processing generates ATP, which inhibits the ATP-sensitive potassium channel inducing beta-cell membrane depolarization, opens the voltage dependent calcium channel which result in calcium influx into the cell and insulin release (83) (Figure 4). Insulin secretion has a pulsatile pattern, with small secretory bursts occurring about every 10 minutes.

Insulin secretion is higher when glucose is given orally than intravenously, because of the simultaneous release of gut peptides known as incretins (e.g., glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP)). Insulin acts through the insulin receptor, which is a heterodimer containing two \(\alpha\) and \(\beta\) chains linked by disulfide bridges. Target tissues for insulin action are muscle, fat and liver whereas glucose uptake in the brain is insulin independent. A number of other hormones, such as glucagon, growth hormone, cortisol and catecholamines counteract the effect of insulin. Glucagon is secreted by pancreatic alpha cells when blood glucose or insulin levels are low and stimulates glycogenolysis and gluconeogenesis by the liver and renal medulla. Glucagon release is normally inhibited by hyperglycaemia, but this effect can be impaired in people with impaired glucose tolerance and diabetes (84).
Figure 4. Insulin release from pancreatic beta-cells.

4. Measures of beta-cell function (C-peptide)

The precursor of insulin, proinsulin and C-peptide (connecting peptide) were described by Steiner in 1967. Measuring C-peptide to assess beta-cell function was introduced in 1972 (85-87). Insulin is produced by beta-cells in the pancreatic islets of Langerhans. It is initially synthesized as 86 amino-acid single chain precursor polypeptide -preproinsulin, which after proteolytic processing produces proinsulin. Proinsulin is structurally related to insulin-like growth factors I and II, which bind weakly to the insulin receptor. Proinsulin is then cleaved to produce equimolar amounts of insulin A (21 amino acids) and B (30 amino acids), which are connected by disulfide bounds and C-peptide (Figure 5). The mature insulin molecule and C-peptide are stored together and co-secreted from the secretory granules directly into the portal vein in response to raised glucose (88). Although secreted in equimolar amounts insulin is less useful than C-peptide for the assessment of beta-cell function. The reason for this is that about 50% of insulin is degraded in the liver whereas metabolism of C-peptide mainly occurs in the kidney. Also, in insulin-treated patients insulin measurements represent the sum of endogenous and exogenous insulin. It
though has a longer half-life than insulin which makes it less suitable for assessment of early phase insulin secretion than insulin (89). C-peptide measurements are also useful in the evaluation of hypoglycaemia. It is still an open question whether C-peptide has any biological function (90). Although it has been ascribed some effect in diabetic complication, predominantly neuropathy, these findings have been difficult to confirm (91-93). Plasma or serum C-peptide can be measured in the fasting state, non-fasting or after oral or intravenous glucose stimulation. Since fasting C-peptide measurements are more standardized than post-prandial test, in this thesis fasting C-peptide measurements were used (94).

![Molecular structure of proinsulin.](image)

Figure 5. Molecular structure of proinsulin.

5. Autoantibodies in T1D and LADA

5.1 Islet cell antibodies (ICA)

Antibodies against the pancreatic islets of Langerhans, islet cell autoantibodies (ICA) are directed against cytoplasmic antigens in all islet cells (95). Islet cell autoantibodies were first described by Bottazzo in 1974 (96). ICA is present in about 80% of patients classified as having type 1 diabetes (97, 98), in 5-10% of patients classified as having type 2 diabetes (62, 99, 100) and less than 3% of the healthy population (97, 101). The number of patients having ICA decreases with time after the diagnosis of diabetes (102). The disadvantage of ICA measurement assay is that antibody is not antigen(s) specific and results are investigator-dependent as the assay requires microscopic assessments of
immunofluorescence. Also it requires pancreas from persons with blood group O. Therefore, there was a continuous search for other more specific antigens.

5.2 Glutamic acid decarboxylase antibodies (GADA)

In 1982 the 64-kDa protein was found to be immunoprecipitated with circulating auto-antibodies in serum from T1D patient’s (103). In 1990, the 64-kDa protein was identified as glutamic acid decarboxylase (GAD), a target antigen for auto-antibodies in patients with T1D and latent autoimmune diabetes in adults (LADA) (104). Also, very high levels of GAD antibodies (GADA) are found in the rare progressive neurological disease Stiff-Man Syndrome. GAD is the rate-limiting enzyme in the conversion of glutamic acid to the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). GAD exists in two isoforms GAD 65 (65kDa) and GAD 67 (67kDa). Only GAD 65 is present in the pancreas, and most of the GADA in T1D patients reacts with GAD 65 (105). GAD 65 is a membrane-attached protein of 585 amino acids located in synaptic-like microvesicles, which are present in nerve cells, pancreatic islets and testis. In the pancreatic islets GABA is thought to be involved in paracrine signalling (106). In contrast to the situation in Stiff-Man Syndrome, in T1D GADA reacts with different structures (epitopes) on the GAD 65 molecule (107), starting within the middle portion and rapidly spreading to the other regions of the molecule (108).

5.3 Protein tyrosine phosphatase antibodies (IA-2A)

In 1990, immunoprecipitation of islet cell extracts with serum of T1D patients demonstrated reactivity against a non-GAD 64kDa protein (109). Further treatment of this protein with trypsin resulted in 40kDa and 37kDa fragments, later identified as two transmembrane protein tyrosine phosphatase-like proteins called IA-2 (ICA512) and IA-2ß (Phogrin) (110, 111). Antibodies against IA-2 are more common than against IA-2ß in T1D patients. IA-2ß has 74% identity to the intracellular and 26% identity to the extracellular domains of IA-2. IA-2 is a 979 amino acid protein, which consists of a signal peptide, extracellular, transmembrane and intracellular domain. Autoantibodies to IA-2 are largely conformational in nature and are directed exclusively to epitopes in the intracellular domain (112). IA-2 is expressed in neuroendocrine cells throughout the body, including pancreatic alpha and beta cells. The protein is an integral component of dense core vesicles (DCV). Disruption of IA-2 has been associated with impaired insulin release and glucose intolerance (113, 114), whereas over-expression of IA-2 promoted beta-cell apoptosis (115). These findings though await replication in independent studies.
5.4 Insulin autoantibodies (IAA)
Although insulin antibodies have been detected in serum from patients treated with bovine and pig insulin since early 70’s, it was realized in 1983 that insulin antibodies could be detected also in patients not treated with insulin, i.e. autoantibodies against insulin (IAA). These antibodies though have value only in patients not treated with insulin (116, 117). IAA have been reported to be more common in young children with diabetes than in adolescents and adult diabetic patients (118-120).

5.5 ZnT8 (Slc30A8) antibodies (ZnTA)
The Zinc transporter (Slc30A8) protein has recently (2007) been identified as a novel and major autoantigen in T1D (121). This molecule is a multspanning transmembrane protein that resides in the insulin secretory granules. The use of an antigen construct spanning the C-terminal amino acids 268–369 resulted in an autoantibody assay with disease sensitivity of 50% a specificity of 98% and a positive predictive value of 37% for T1D. Autoantibodies to Slc30A8 do not usually appear before the age of 3 years.

It is important to keep in mind the possibility that the primary autoantigen has not yet been identified for T1D. Due to the low prevalence of IAA in young adults and the very recent identification of ZnTA, these antibodies have not been studied in this thesis.

6. Genetics
6.1 Genetic variation
Genetic variation consists of both chromosomal aberrations and difference in DNA sequence between individuals. Such variations can consist of tandem repeats represented by satellites (alphoid DNA), minisatellites (variable number of tandem repeats, VNTR and telomere), microsatellites (short tandem repeats, STR) and telomeric sequencies, insertions or deletions of one or more bases and single base substitution so called single nucleotide polymorphism (SNP) (122). SNPs are commonly biallelic, but rarely can have three or more alleles. The number of SNPs is estimated to be around 10 million, i.e. on average one SNP every 300 base pairs (bp) (123, 124).
Closely linked alleles on the same chromosome which are inherited together are called haplotypes. Haplotype may also refer to two or more loci on the chromosome.
6.2 Identifying the genes for polygenic diseases

The first observed link between genes and disease came at the beginning of the 20th century, by the description of alcaptonuria by Archibald Garrod as the first inborn errors of metabolism. Garrod described the more of inheritance and the strong penetrance of the disease suggesting a Mendelian mode of inheritance. This initial discovery paved way for the identification of thousands of rare Mendelian diseases, such as cystic fibrosis, Huntington disease and etc, all of which show early onset and strong genetic component. In contrast, common forms of human diseases, such as diabetes, show complex pattern of inheritance and late onset. Unlike to the identification of the genetic causes of Mendelian diseases, the identification of genetic causes of complex diseases is more difficult, because there is no clear inheritance pattern, many common genetic variants contribute to the disease phenotype, they show low penetrance and complex interaction with other genes and the environment (125).

Several approaches are being used in the search for susceptibility loci predisposing to polygenic diseases: linkage studies, association studies and genome-wide association studies (GWAS).

**Linkage studies**

Linkage is the phenomenon describing a departure from the independent assortment of two loci, i.e. the tendency for alleles at loci that are close together on the same chromosome to be transmitted together, as an intact unit, through meiosis. Linkage analysis is a tool to map genes in families to determine whether two genes show linkage (are linked) when passed on from one generation to the next. Linkage studies analyze whether there is linkage between a chromosomal region and disease assuming no knowledge of the underlying defects and searching for loci based upon their location in the genome. This is usually performed by using 400 to 500 highly polymorphic microsatellite markers, ca 10 cM apart, spread over entire genome. Linkage is reported as a logarithm of odds (LOD) score (126). A LOD score greater than 3.0 is considered evidence for linkage, whereas a LOD score less than –2.0 excludes the linkage.

**Association studies**

If there is a prior strong candidate gene for the disease, the best approach is to search for association between SNPs in the gene and the disease. This can either be a case-control or nested cohort study. In a case-control study the inclusion criteria for the cases are predefined and thereafter matched individual controls are searched for (or selected), representing the same ethnic group as the cases. In a cohort study affected and unaffected groups- not individuals- are matched. Ideally cohorts are population-based but often they represent consecutive patients from an outpatient clinic. It is preferable that controls are older than cases to exclude the possibility that they still will develop the disease. The
question of matching is crucial for the results, matching for a parameter influenced by the genetic variant (e.g. BMI) might influence its effect on a disease like T2D. If cases and controls are not drawn from the same ethnic group, a spurious association can be detected due to ethnic stratification.

One way to circumvent this problem is to perform a family-based association study. Distorted transmission of alleles from parents to affected offspring would indicate that the allele showing excess transmission is associated with the disease. The untransmitted alleles serve as control. This transmission disequilibrium test (TDT) represents the most unbiased association study approach but suffers from the drawback of low power as only transmissions from heterozygous parents are informative. The prerequisite of DNA from parents usually enrich for individuals with an earlier onset of the disease. It is still debated whether common or rare variants are the cause of common complex diseases. The haplotype approach would work for common but not for rare variants. The common variant-common disease hypothesis assumes that relatively ancient common variants increase susceptibility to common diseases like diabetes. These variants would be enriched in the population, as they have been associated with survival advantage during the evolution, so called thrifty genes (127). Storage of surplus energy during periods of famine may have been beneficial for survival, while in the Westernized society we rather need genetic variants which would waste energy.

**Genome-wide association studies (GWAS)**

GWAS have recently been applied to identify the loci increasing susceptibility to complex common diseases. With GWAS it became possible to examine SNPs across the whole genome, without prior knowledge of disease mechanisms, and potentially identify totally novel susceptibility factors. GWAS involve scanning thousands of samples, either as case-control cohorts or in family trios, utilizing hundreds of thousands of SNP markers. Power is important for GWAS and is mandatory to have sufficient number of rigorously characterized cases and controls. Also crucial for the success is a comprehensive map of >500,000 carefully selected SNPs, accurate genotyping facilities, and proper processing of the enormous data sets and replication of identified associations in an independent populations.

The number of tests is a major factor in determining the statistical power of GWAS. Although genetic markers are not strictly independent because of LD, the current convention is to apply a Bonferroni correction (which assumes independence and is thus overly conservative) by dividing the conventional P value of 0.05 by the number of tests performed. This requires P values = 5x10^{-7} to 5 x10^{-8} range to define a stringent level of significance in GWAS (128).
6.3 Hardy-Weinberg equilibrium

“There is not the slightest foundation for the idea that a dominant trait should show a tendency to spread over a whole population, or that a recessive trait should die out”, stated G.H Hardy (129). Gene frequencies can be high or low regardless of how the allele is expressed and can change, depending on the conditions that exist. Changes in gene frequencies over time results in evolution. The Hardy-Weinberg (HW) principle describes a hypothetical situation in which there is no change in the gene pool (frequencies of alleles) over time, hence no evolution. Consider a population with the alleles A and a, p the frequency of the dominant allele A and q the frequency of the recessive allele a, i.e. p + q = 1. The Hardy-Weinberg equilibrium assumes random distribution of alleles, which can be described as $p^2 + 2pq + q^2$. If the frequencies of A and a remain unchanged from generation to generation the Hardy-Weinberg equilibrium (HWE) is met. This though requires that 1) the population is large enough to minimize random sampling errors, 2) random mating, 3) no mutation, 4) no migration.

6.4 Power of genetic studies

Power of a statistical test is the probability that the test will reject the null hypothesis. Power depends on sample size, frequencies of the predisposing alleles, genotypes, or haplotypes and effect size. The most important factor influencing the power is the sample size (130). Power calculations in this thesis were performed using the Genetic Power Calculator (www.psycho.uni-duesseldorf.de/aap/projects/gpower/)

7. Genetics of diabetes

7.1 T1D

Inherited genetic factors influence both susceptibility and resistance to T1D. There is significant familial clustering with an average prevalence of 6% in siblings compared to 0.4% in general population. The sibling relative risk ($\lambda_S$) can be calculated as the ratio of the risk to siblings over the disease prevalence in the general population, and thus $\lambda_S = 6/0.4 = 15$ (131, 132). In addition, the overall rate of concordance of T1D in monozygotic twins is 50% (133) and in dizygotic twins 11% (134).

**HLA**

Association between human leukocyte antigen (HLA) and T1D was described almost 35 years ago (135). HLA is so far the strongest genetic loci associated with T1D and explains about 40% of familiar aggregation of the disease (Figure
The major determinants of diabetes susceptibility are DR and DQ molecules of HLA class II genes (135-142). They are membrane glycoproteins, expressed on antigen presenting cells and involved in antigenic peptide presentation to T-helper lymphocytes. The ability of class II MHC molecules to present antigen is dependent on the amino acid composition of their antigen-binding sites. Amino acid substitutions may influence the specificity of the immune response by altering the binding affinity of different antigens for class II molecules. Because of close proximity, usually HLA alleles are inherited together creating the T1D risk haplotypes: DQA1*0501-DQB1*0201 also called DQ2 and DQA1*0301-DQB1*0302 called DQ8 (138). The haplotype DQA1*0102-DQB1*0602 provides dominant protection from T1DM and is called DQ6 (143). Assuming that DQA1 and DQB1 alleles are in linkage disequilibrium (LD) (alleles of different genes are non-randomly associated with each other on a haplotype) we genotyped only the HLA-DQB1 gene in thesis to explain risk associated with T1D.

![Class II, Class III, Class I](image.png)

**Figure 6. The human leukocyte antigen (HLA) complex on chromosome 6.**

**Insulin gene**

The insulin gene association with T1D was first described in 1984 (144). The insulin gene is located on chromosome 11p15 and is the second strongest genetic risk factor for T1D. A polymorphism in the insulin minisatellite or variable number of tandem repeats (INS VNTR) of 14-15bp oligonucleotides is
located 596bp upstream of the insulin gene translation initiation site (144). There are three main types of INS VNTR defined by their size: class I (26–63 repeats), class II (approximately 80 repeats) and class III (140–200 repeats). Class I INS VNTRs are associated with T1D and are thought to influence insulin mRNA expression within the thymus (145-148). Some studies have shown association between the INS VNTR class III and T2D (149, 150).

**PTPN22 gene**
Protein tyrosine phosphatase (PTPN22) gene is located on chromosome 1p13 and the third confirmed gene influencing T1D risk. The gene is encoding a lymphoid-specific phosphatase that acts as a negative regulator of T-cell activation by dephosphorylating T-cell receptor activation-dependent kinases (151, 152). It contains a gain of function mutation resulting in increased susceptibility to autoimmunity. The amino acid change (TRP to ARG) disrupts binding of PTPN22 to an intracellular kinase, Csk (153).

**Other genes associated with T1D**
The cytotoxic T-lymphocyte-associated antigen-4 gene (CTLA4) encodes a costimulatory molecule that is expressed on the surface of activated T cells. Protein product of variants in the CTLA4 locus transmits inhibitory signals of T-cell activation (154, 155). The association of CTLA4 with T1D is predominantly seen in patients with co-existing autoimmune thyroid disease (AITD) (154). Also a noncoding SNP in the interleukin 2 receptor alpha (IL2RA) gene are associated with T1D. IL2RA is also known as CD25 and expressed on regulatory T cells (156-158).

**Genome-Wide Association Studies for T1D**
During the last years GWAS have identified a number of novel T1D susceptibility loci. Most consistent associations with T1D have been shown for the interferon-induced helicase (IFIH1) (159-161) and C-type lectin domain family 16 gene A, formerly KIAA0350, (CLEC16A) (162-164). The role of other genes remain to be validated CYP27B1, PTPN2, UBASH3A, BACH2 and PRKCQ (162, 165-173).

### 7.2 T2D
T2D has a strong genetic component. This is supported by a clear familial aggregation of the disease and from twin studies showing higher concordance in monozygotic twins (70%) than in dizygotic twins (20-30%) (174-177). If both parents have T2D, the risk approaches 70% (178). The sibling relative risk (rs) for T2D is 3.5 (35% vs 10%) (179). In the Botnia prospective study we have
demonstrated that first-degree family history is associated with 2-fold increased risk of future T2D (180).

A challenge has been to identify genetic variants that explain the excess risk associated with a family history of diabetes. From a long list of candidate genes, variants in only three have been consistently associated with T2D: TCF7L2, KCNJ11, and PPARG (181-186). However, in 2007, a number of novel genetic variants (CDKL1, IGF2BP2, the locus on chromosome 9 close to CDKN2A/2B, FTO, HHEX, SLC30A8, and WFS1) were shown to increase susceptibility to T2D in several studies. Furthermore, a recent meta-analysis identified six novel variants (JAZF1, CDC123/CAMK1D, TSPAN8/LGR5, THADA, ADAMTS9, and NOTCH2) that are associated with T2D (187-193).

**TCF7L2**

The transcription factor 7-like 2 gene (TCF7L2) encodes for a transcription factor involved in the Wnt signaling pathway. TCF7L2 is by far the strongest T2D associated gene. TCF7L2 is one of the earliest examples of a gene discovered to be associated with T2D based upon its position in the genome rather than function. TCF7L2 was identified in a follow-up analysis of a region on chromosome 10q identified through linkage analysis. The mechanisms by which it increases the risk of T2D seem to include impaired beta cell function, possibly through an impaired incretin (GLP-1 and GIP) effect on insulin secretion (194). Notably, the risk of diabetes associated with the TCF7L2 gene is abolished by treatment with metformin or lifestyle intervention (195).

**PPARG**

The peroxisome proliferator-activated receptor gamma (PPARG) gene encodes for a nuclear receptor regulating adipogenesis. The gene is also expressed in the pancreatic beta cells (196, 197).

In the 5’ untranslated end of the gene is an extra exon B that contains a SNP changing a proline in position 12 of the protein to alanine. The rare Ala allele is seen in about 15% of Europeans and was shown to be associated with increased transcriptional activity, increased insulin sensitivity and protection against T2D in an initial study (198). This study was followed by a number of studies, which could not replicate the initial finding. However, an analysis of parent–offspring trios showed excess transmission of the Pro allele to affected offspring and a meta-analysis combining the results from all published studies showed a highly significant association of the Pro12Ala with type 2 diabetes (183). The Pro12Ala polymorphism is now one of the best-replicated genes for T2D.

Mutation in PPARG gene can also cause familial lipodystrophy type 3 with early-onset diabetes and insulin resistance (199). The PPARG receptor agonists thiazolidinediones represent a novel group of anti-diabetic drugs aimed to reduce insulin resistance (200).
**KCNJ11**
The beta cell ATP-sensitive K+ channel (KATP) is composed of two subunits: a high-affinity sulfonylurea receptor (SUR1) and an inward rectifier (Kir6.2) (201). As described above, after glucose transport into the pancreatic beta-cells, the increase in ATP closes the K+ channels resulting in calcium influx, membrane depolarization and insulin release (83). A Glu23Lys polymorphism (E23K) in *KCNJ11* has been associated with T2D and a modest impairment of insulin secretion (184, 202). In addition, activating mutations in the *KCNJ11* gene leave the channel open causing permanent neonatal diabetes (203, 204); this effect is markedly stronger than that seen for the E23L polymorphism (205, 206). *KCNQ1* encodes another potassium channel that has recently been implicated in type 2 diabetes in two Japanese GWAS (207, 208). The ATP dependent K+ channels are targets for the anti-diabetic drugs sulfonylureas.

**WFS1**
In a study of 1536 SNPs in 84 candidate genes for T2D (191) only one of these genes, *WFS1* was associated with T2D. *WFS1* encodes wolframin, a protein that is defective in individuals with the Wolfram syndrome. This syndrome is characterized by diabetes insipidus, juvenile diabetes, optic nerve atrophy and deafness. Thereby *WFS1* can be considered a functional candidate gene for T2D.

**Genome-Wide Association Studies for T2D**
The year 2007 brought a real breakthrough for disease genetics, not at least for genetics of diabetes.
The reason was that several GWAS using DNA chips with more than 500,000 SNPs in a large number of patients with type 2 diabetes and controls were published (187-190, 192). The transcription factor gene *TCF7L2* was on top of the list of each WGAS with a joint $p$-value in three scans of $10^{-50}$. In addition to *TCF7L2*, *PPARG*, *KCNJ11* and *WFS1* the first GWAS identified at least six novel genes/loci for T2D: *CDKN2A/2B*, *IGF2BP2*, *CDKAL1*, *SLC30A8* and *HHEX*.

In 2008 a meta-analysis of three large GWAS revealed additional six loci associated with T2D (*JAZF1*, *CDC123/CAMK1D*, *TSPAN8/LGR5*, *THADA*, *ADAMTS9* and *NOTCH2*) (193). Most of the newly identified T2D genes are affecting insulin secretion rather than insulin resistance (44, 209-214). The *CDKN2A/2B*, *CDKAL1*, *JAZF1*, and *CDC123/CAMK1D* genes encode for cell cycle proteins, *HHEX* encodes for a transcription factor, and *SLC30A8* for the above-described Zn transporter, ZnT8.

Of the new T2D genes, the fat mass and obesity associated (*FTO*) gene increases risk of T2D by increased adiposity (215, 216). Interestingly, the same variants in the *FTO* gene are also associated with increased physical activity in children (217).
In the first successful linkage study of a complex disease like T2D, Graeme Bell and co-workers reported in 1996 significant linkage between T2D in Mexican American sib pairs and a locus on chromosome 2q37 (also referred to NIDDM1) (218). Three intronic SNPs in the gene coding for calpain 10 (CAPN10) could explain most of the linkage (219). Calpains are Ca\(^{2+}\)-dependent cysteine proteases. It has been shown that in diabetes, calpains play a role in regulating insulin secretion and insulin action (220-223). The CAPN10 association with T2D has been controversial (224, 225) since it has not been replicated in all studies, nor in the GWAS.

Calpains are also associated with other diseases, such as limb girdle muscular dystrophy type 2A, cataracts, Alzheimer's disease and also contribute to ischemic tissue damage following stroke, myocardial infarction and traumatic brain and spinal cord injuries (226).

**CAPN10**

Figure 7. Chromosomal locations of some genes studied in this thesis.
7.3 LADA
Although there are limited number of studies which have tried to dissect the genetics of LADA, LADA has been shown to share HLA predisposition with T1D (227-230). However, there have not been any reports showing association between LADA and T2D genes until this question was addressed in the current thesis and an accompanying paper by Cervin et al (231, 232).

7.4 MODY genes

**GCK (glucokinase) (MODY 2):** Glucokinase is highly expressed in the pancreatic beta cell and the liver. It catalyzes the transfer of phosphate from adenosine triphosphate (ATP) to glucose to generate glucose-6-phosphate. Glucokinase functions as the glucose sensor in the beta cell by controlling the rate of glucose entry into the glycolytic pathway (glucose phosphorylation). In the liver glucokinase promotes storage of glucose as glycogen in the postprandial state. Heterozygous mutations leading to partial deficiency of glucokinase cause MODY2, whereas homozygous mutations resulting in complete deficiency of the enzyme lead to permanent neonatal diabetes mellitus (233). Mutation in the GCK gene was the first described genetic cause of MODY (67, 68).

**Hepatocyte nuclear factors (HNFs)**

HNF-1α, HNF-4α and HNF-1β constitute an interacting network of transcription factors that function together to control gene expression during embryonic development and in adult tissues where they are co-expressed. In the pancreatic beta cell, these transcription factors regulate insulin gene expression as well as expression of proteins involved in glucose transport and metabolism and mitochondrial and lipoprotein metabolism (234-236). HNF-1α, HNF-4α and HNF-1β are the members of the nuclear receptor family. HNF-1α and HNF-4α are expressed in the liver, gut, kidney and pancreas (237-239). The expression of HNF-1α is regulated at least in part by HNF-4α (240).

**The HNF-1α gene (MODY3):** Heterozygous mutations in the gene encoding HNF-1α are the most common cause of MODY diabetes (241). It has been shown that common variants in the HNF-1α gene (I27L, A98V) are associated with an increased risk of type 2 diabetes (73, 78, 242, 243).

**The HNF-4α gene (MODY1):** The expression of HNF-4α isoforms is regulated by a proximal promoter (P1), which is liver specific and an alternative promoter (P2) found 46 kb upstream of P1, mainly regulating transcription in beta-cells (244, 245). It has been shown that common variants in both P1 and P2
promoter of the HNF-4α gene are associated with an increased risk of type 2 diabetes (77, 246-248).

**The HNF-1β gene (MODY5)** is also referred to as TCF2. Mutations in the gene cause various phenotypes including abnormalities in kidney, pancreas, and genital tract formation (249-251). The risk variants of HNF-1β gene could confer the susceptibility for type 2 diabetes development (73, 78, 80, 252).

**The IPF-1 (Insulin promoter factor-1) gene (MODY 4),** also referred to as PDX1, is homeodomain-containing transcription factor that was originally isolated as a transcriptional regulator of the insulin and somatostatin genes (253, 254). It also plays a central role in the development of the pancreas as well as in regulating expression of a variety of pancreatic islet genes (255). Homozygous mutation in the IPF-1 gene causes pancreatic agenesis whereas heterozygous IPF-1 mutations have been associated with T2D (218).

**The NeuroD1 (Neurogenic differentiation factor-1) gene (MODY 6),** also referred to as Beta2, a basic helix-loop-helix transcription factor is required for normal pancreatic islet development (256). Mutations in the NeuroD1 gene represent rare causes of MODY.
8. Aims of the presented study
The goal of this project was to study genetic factors associated with autoimmune and non-autoimmune diabetes in young (15-34 years) and middle-aged (40-59 years) Swedish diabetic patients

The specific aims were:

1) To evaluate the role of HLA-DQB1 genotypes and islet antibodies in the classification of diabetes in young adult diabetic patients

2) To study whether genetic markers associated with type 1 (HLA-DQB1, INS VNTR and PTPN22) and type 2 diabetes (TCF7L2) could help to discriminate between autoimmune and non-autoimmune diabetes in young and middle-aged diabetic patients

3) To study associations between 10 novel type 2 diabetes-associated risk genotypes with autoimmune and non-autoimmune diabetes in young and middle-aged diabetic patients

4) To study whether common variants in MODY genes can discriminate between autoimmune and non-autoimmune diabetes in young adult diabetic patients and to screen for MODY mutations in antibody negative diabetic patients with a strong family history of diabetes (≥ 3 members in the family)
9. Diabetic patients and healthy controls

9.1 Diabetes Incidence Study in Sweden (DISS)

The Diabetes Incidence Study in Sweden was initiated in 1983 (257). The study is a nationwide registry of incident cases of diabetes aged 15 to 34 years. Diabetes was classified at registration by the reporting physician as T1D, T2D, gestational or unclassifiable. Registration form includes age, gender, height, body weight, diagnosis date, family history of diabetes, diabetic symptoms (polyuria, weight loss, fatigue), coma, duration of symptoms (weeks), initial treatment (diet, oral hypoglycemic agents or insulin), degree of ketonuria, presence of ketoacidosis (bicarbonate <15 mmol/l and/or pH <7.3). Fasting or random diagnostic blood glucose values were also reported. From 1998, blood samples were taken for DNA, islet antibody analysis (ICA, GADA and IA-2A), fasting plasma C-peptide was measured 3-6 month after diagnosis. The objectives of the DISS study were to follow the trends in incidence of diabetes in young adult population and find the clues to the putative aetiology and pathogenesis of diabetes and its complications. During a five-year period (Jan 1, 1998 to December 31, 2002), 2077 young adult (15-34 years of age) diabetic patients were reported to the Diabetes Incidence Study in Sweden (DISS).

9.2 Scania Diabetes Registry

A diabetes registry in Southern Sweden (Diabetes 2000) was initiated in 1996 and hitherto 7,461 patients have been registered. The majority of the patients (4,981) have been registered at the department of Endocrinology, University Hospital Malmö, whereas the remaining were registered at the Trelleborg hospital or health care centers in the Malmö and Trelleborg regions. The registry includes information about onset of diabetes, and mode of treatment. At registration and at least once a year thereafter the following measurements were performed: body weight, height, blood pressure, fasting concentrations of plasma glucose, HbA1c, serum total cholesterol, HDL-cholesterol and triglycerides in addition to the urinary albumin excretion rate (AER) and P-creatinine. Plasma glucose, C-peptide and GAD antibodies (GADA) are measured at the registry inclusion. At annual follow-ups, signs of retinopathy, nephropathy, neuropathy and macrovascular disease are recorded. All patients gave their informed consent and the registry was approved by the Swedish Data Inspection Board and the Ethics Committee of Lund University.

9.3 Skaraborg Population Registry

216 subjects without diabetes from the county of Skaraborg (280, 000 inhabitants), Sweden were used as controls for HLA-DQB1 genotyping (258).
9.4 The Malmö Preventive Project (MPP)
Malmö Preventive Project was initiated in Malmö, Sweden in 1974 as a population-based health screening. The main aim was to identify individuals at risk of development of diabetes and cardiovascular diseases (259, 260). Today DNA and follow up data are available for about 17,000 individual (44) out of 33,000 participants. As controls in this study, we included 11,923 non-diabetic Swedish individuals (age >40 years) without family history of diabetes or antihypertensive treatment from the Malmö Preventive Project (MPP).

10. Methods

10.1 Islet autoantibodies
ICA were determined by a prolonged two-colour immunofluorescence assay by incubating patient plasma with pancreas cryosections from blood group 0 subjects as antigen (261). The detection limit for ICA was 4 Juvenile Diabetes Foundation (JDF) units for the first pancreas used in samples tested until April 1999 and 5 JDF units for the second pancreas used in samples tested from April 1999 and onwards. In the last ICA Proficiency Test (13th) our ICA assay performed with 100% sensitivity and 100% specificity (ICA is not included in the Diabetes Autoantibody Standardization Program (DASP)).

GADA in young patients were measured by a radioligand binding assay, based on human $^{35}$S-labeled recombinant GAD 65 (262). Overnight incubations with the $^{35}$S-GAD 65 were made at +4°C for each patient plasma sample duplicate. Then two aliquots from each incubation were incubated with protein A Sepharose for 45 minutes on a 96-well filtration plate for collection of immunocomplexes. After filtering and washing, the bottom of each well was punched into a scintillation bottle, and the radioactivity was counted in a liquid scintillation counter. Pooled serum from three blood donors served as a negative control and plasma from a patient with high levels of GADA, diluted in negative control serum, served as a positive control. The results are presented as GADA index=$100 \times (cpm \text{ of mean activity of all four measurements for sample}-cpm \text{ of the negative control})/(cpm \text{ of the positive control}-cpm \text{ of the negative control})$. A GADA index >4.6 was considered as positive (97.5 percentile of 165 non-diabetic controls aged 7-34 years). In the first DASP (2000) the GADA assay showed a sensitivity of 80% and a specificity of 96%, in the second (2002), a sensitivity of 88% and specificity of 87% and in the third DASP (2003), a sensitivity of 82% and a specificity of 93%.

GADA in the middle-aged patients were measured by a radioligand binding assay using $^{35}$S-labelled in-vitro translated recombinant GAD 65 prepared using human GAD 65 DNA from plasmid pGAD65cDNAII (263). Human plasma was
incubated with $^{35}$S-labelled GAD 65 during overnight at +4°C on a plate shaker. Then duplicates of plasma $^{35}$S-GAD 65 were incubated with 50% protein A Sepharose on precoated microfilter plates for 50 minutes at +4°C. Since protein A interacts with the Fc region of IgG subclasses, they precipitate on Sepharose. After dissolving the precipitate in scintillation liquid activity was determined on a beta scincillation counter (264). The results are presented as relative units (RU): \( \text{RU} = 100 \times \frac{\text{cpm of sample mean-cpm of three negative controls}}{\text{cpm of a positive internal reference serum-mean cpm of three negative controls}} \). The cut-off limit for positivity was 5 RU, which is comparable to 32 IU/ml according to the new WHO standard (265) and represents mean+3 SD of 296 healthy control participants. In the first DASP (2000) the GADA assay showed a sensitivity of 76% and a specificity of 94%, in the second (2002) a sensitivity of 88% and a specificity of 96%, and in the third DASP (2003) a sensitivity of 88% and a specificity of 94% (266). From the beginning of the year 2000 (in 41% of registry patients), the results were given as IU/ml (267).

**Comparison of two assay methods of GADA measurement.** To evaluate the correlations between two assay methods of GAD antibody measurements used in young (DISS study) and middle-aged patients (Scania Diabetes Registry), we selected \( n=276 \) young diabetic patients and re-ran GAD antibodies by the method used in the middle-aged patients. In total 62% of them were GADA positive by the method applied in the young subjects and 61% of them were GADA positive with the method used in the Registry. Eight of the GAD-positive patients with the first assay were negative with the second assay (7%). The correlation coefficient between two methods was \( r=0.93 \) with \( p<0.0001 \) (Table 2).

<table>
<thead>
<tr>
<th>GADA measurements in young diabetic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reruns (Method used in Diabetes Registry)</td>
</tr>
<tr>
<td>Positive</td>
</tr>
<tr>
<td>161 (95%)</td>
</tr>
<tr>
<td>Negative</td>
</tr>
</tbody>
</table>

**IA-2A assay principle is the same as for the $^{35}$S GADA assay:** Human $^{35}$S labelled recombinant IA-2 was synthesized in the same way as $^{35}$S GAD$_{65}$ described above, in the young diabetic patients, using circular pSP64 poly (A) vector with an IA-2ic insert (268). IA-2A index $>1.0$ was considered as positive (97.5 percentile of 165 non-diabetic controls aged 7-34 years). In the first DASP
(2000), our IA-2A assay showed a sensitivity of 58% and a specificity of 100%, in the second (2002), a sensitivity of 62% and specificity of 100%, and in the third DASP (2003), a sensitivity of 64% and a specificity of 100%.

10.2 C-peptide
Plasma C-peptide was determined by radioimmunoassay (RIA) (Peninsula Laboratories, Inc, Belmont, Ca, USA) in both in young (age 15–34 years) (142) and middle-aged (age 40–59 years) diabetic patients (267). The reference range for healthy participants after 12 h fasting was 0.25 to 0.75 nmol/l. The detection limit was 0.10 nmol/l. A C-peptide concentration 0.1-0.25 nmol/l was considered as low and <0.10 nmol/l as unmeasurable (beta-cell failure) (269). The reference range for healthy subjects after 12-h fasting was 0.25 to 0.75 nmol/l.

10.3 Genotyping
**HLA-DQB1 genotyping - Paper I and Paper II**
HLA-DQB1 typing was performed by time-resolved fluorometry based triple-label hybridization assay. Using a primer pair with biotinylated 3’ primers, the 158 bp second exon of HLA-DQB1 gene was amplified by PCR. The amplification product was bound to streptavidin-coated microtitration plates and denatured with NaOH. After washing, bound DNA was assessed using two different hybridisation mixtures with lanthanide (III) chelate-labelled DNA probes specific for the HLA-DQB1 alleles. One mixture contained europium (Eu)-labelled and samarium (Sm)-labelled internal reporter probes for DQB1*0602 and *0603 alleles (*0602-*0603) and DQB1*0603 and *0604 (*0603-*0604) alleles respectively; a terbium (Tb)-labelled consensus sequence-specific probe (Tb-DQB1 control) was used as control of PCR amplification. The other mixture contained Tb-, Sm- and Eu-labelled probes specific for DQB1*0201, DQB1*0301 and DQB1*0302 alleles, respectively. To measure probe hybridisation, microtitration plates were evaluated by time-resolved fluorescence (Delfia Research Fluorometer; Wallac OY, Turku, Finland) (Figure 8). Different emission wavelengths and delay times were used to distinguish the signals of each lanthanide label (270). From 1564 genotyped DISS patients, genotyping success rate was 1537 (98%) and from 1365 diabetes registry patients 1350 (99%). The risk HLA-DQB1 genotypes include *02/*0302, *0302/X, *0302/*0604 (270, 271) and in the second paper we refer to risk HLA-DQB1 without any further risk genotype specification. In control subjects, HLA-DQB1 locus was amplified by PCR, followed by dot-blotting onto nitrocellulose filters, and hybridization using the radioactively end labelled sequence-specific oligonucleotide probes, then autoradiography was done (258).
Figure 8. *HLA-DQB1* assay, time-resolved fluorometry output. Code row shows different *HLA-DQB1* alleles (brown outline). PCR row shows PCR in sterile water. Kons column shows DNA amplification for each sample (outlined in blue). Black circles outline fluorescent signal for *HLA-DQB1* alleles for each sample. Note column (outlined in black) shows HLA-DQB1 genotypes for each sample.

**TaqMan allelic discrimination method - Paper II, Paper III and Paper IV**

The ABI Prism 7900 Sequence Detection System (7900HT system; Applied Biosystems, Foster City, CA, USA) uses fluorescent probes to discriminate between the two different alleles of the SNP. It starts with a PCR reaction to amplify the desired DNA fragment and allele specific probes are used to detect the alleles. The probes are designed to make either a perfect match or a mismatch. The fluorescent signal is sent out when the polymerase cleave attached probes so that the dye is separated from the quencher. As PCR cycles proceed, the fluorescent signal will increase exponentially and will thereby be detectable (Figure 9).
Figure 9. TaqMan allelic discrimination assay. The output signals are plotted in a scatterplot with one fluorescent colour signal intensity on the y-axis (FAM) and the other on the x-axis (VIC). Three clusters that represent the three genotypes (wt/wt, wt/pm and pm/pm) could be determined. Signals that fall outside of these clusters are “undetermined”

**Insulin VNTR (rs689 and rs3842755), PTPN22 (rs2476601) and TCF7L2 (rs7903146 and rs10885406)**

Two SNPs associated with INS VNTR were genotyped, i.e. rs689 (-23HphI variant, a surrogate for the subdivision of VNTR into class I [A] and III [T] alleles) and rs3842755, which also allows subdivision of class III into IIIA (C) and IIIB (A) alleles (145, 272, 273). The SNP rs2476601 (also denoted 1858C/T) was genotyped in the PTPN22 gene (274, 275). From young diabetic patients 1,564 DNA samples were available and genotyping success rate was 98% for PTPN22, 98% for INS VNTR rs689 and 98% for INS VNTR rs3842755. In genotyped 1312 middle-aged diabetic patients genotyping success rate was 99% for PTPN22, 98% for INS VNTR rs689 and 96% for INS VNTR rs3842755. Random samples (3.2%) for each SNP were re-genotyped with reproducibility of 99.9%.

SNP rs7903146 in the TCF7L2 gene, previously shown to be associated with T2D, and a more recently described microsatellite marker, captured by SNP rs10885406, were genotyped (182, 276). In young diabetic patients 1,564 DNA
samples were available and genotyping success rate was 98% for TCF7L2 rs7903146 and 98% for TCF7L2 rs10885406. In middle-aged diabetic patients 1614 DNA samples were available and genotyping success rate was 96% for TCF7L2 rs7903146 and 99% for TCF7L2 rs10885406. Random samples (3.2%) for each SNP were re-genotyped for quality control. The T allele of TCF7L2 rs7903146 was in strong linkage disequilibrium with the G allele of TCF7L2 rs10885406 (D'=0.99; r²=1.0).

PPARG (rs1801282), KCNJ11 (rs5219), IGF2BP2 (rs4402960), WFS1 (rs10010131), CDKAL1 (rs7754840), JAZF1 (rs864745), CDKN2A/2B (rs10811661), HHEX (rs1111875) and FTO (rs9939609)

Genotyping in all young patients (n=1,564) and in the LADA and adult T1D patients (n=348) was performed by an allelic discrimination method on the Applied Biosystems 7900HT platform. Genotyping for control subjects (n=11,923) was performed using allelic discrimination method for IGF2BP2 (rs4402960), FTO (rs9939609), WFS1 (rs10010131), HHEX (rs1111875), and JAZF1 (rs864745).

HNF-4α (rs4810424, rs2144908 and 3212198), GCK (rs1799884), HNF-1α (rs1169288) and HNF-1β (rs757210 and rs4430796)

Genotyping in all young patients (n=1,564) and in a selection of controls (n=5,795) was performed by an allelic discrimination method on the Applied Biosystems 7900HT platform.

KASPar® SNP genotyping method - Paper III. PCR SNP genotyping system, The KASPar® assay system, Kbioscience, UK (http://www.kbioscience.co.uk/) relies on the discrimination power of a novel form of competitive allele specific PCR to determine the alleles at a specific locus within genomic DNA for SNP typing, i.e. on the ability of the Taq DNA polymerase to discriminate the polymorphism and not the hybridisation of probes. Similar to Taqman, KASPar PCR plates are read on ABI Prism 7900 Sequence Detection System (Applied Biosystems) and output plot looks similar to TaqMan plot with three clusters of genotypes.

SLC30A8 (rs13266634) in patients and controls was genotyped by KASPar genotyping method.

Sequenom MassARRAY iPLEX platform (San Diego, CA, USA) – Paper III and Paper IV

Sequenom MassARRAY iPLEX platform is the leading technology for SNP Genotyping for sub-whole genome study applications. The MassARRAY® System is widely used for fine mapping and validation studies and for routine applications that employ fixed SNP panels. iPLEX assay is based on single-base primer extension (SBE) biochemistry with the sensitivity and accuracy of
Matrix-assisted laser desorption/ionization (MALDI)- time-of-flight (TOF) mass spectrometry (MS) detection (Sequenom, San Diego, CA, USA). Assays can be multiplexed up to 40 SNPs in one individual reaction allowing for throughput levels of up to 150,000 genotypes per instrument and day (277). Genotyping for the novel T2D genes in middle-aged GAD negative \( (n = 1,270) \) diabetic patients was performed using Sequenom MassARRAY platform (277), except for the \( FTO \) \((rs9939609)\) gene which was genotyped by an allelic discrimination method on the Applied Biosystems 7900HT platform. Genotyping for \( PPAR\gamma \) \((rs1801282)\), \( KCNJ11 \) \((rs5219)\), \( CDKAL1 \) \((rs7754840)\) and \( CDKN2A/2B \) \((rs10811661)\) in the control subjects \((n = 11,923)\) was performed using the Sequenom MassARRAY platform. Genotyping for the MODY genes - \( HNF-4\alpha \) \((rs4810424, \hspace{0.5cm} rs2144908 \hspace{0.5cm} \text{and} \hspace{0.5cm} rs3212198)\), \( GCK \) \((rs1799884)\), \( HNF-1\alpha \) \((rs1169288)\) and \( HNF-1\beta \) \((rs757210 \hspace{0.5cm} \text{and} \hspace{0.5cm} rs4430796)\) in the rest of control subjects \((n = 6,128)\) was performed using the Sequenom MassARRAY platform. The \( rs2144908 \) failed genotyping by this platform and therefore we only had possibility to analyse data \((n = 5,795)\) obtained by the TaqMan allelic discrimination assay.

Average genotyping success rate was 98% among young, 96% among middle-aged diabetic patients and >95% for type 2 genes and >98% for MODY genes in control subjects. 3.2% of random samples for each SNP’s were re-genotyped, with reproducibility of 99.9%. All SNPs were in Hardy–Weinberg equilibrium \((p > 0.05)\), apart from tree-allelic SNP \( rs757210 \) of the \( HNF-1\beta \) gene.

10.4 Sequencing

Sequencing of \( HNF-4\alpha \), \( GCK \), \( HNF-1\alpha \) constructs were performed using Thermo Sequenase II dye terminator cycle sequencing premix kit with four fluorescence-labeled didoxynucleotides on an ABI PRISM 3730 Sequencer (Applied Biosystems, Foster City, CA, USA)

10.5 Statistical analysis

Continuous data are presented as median and 1\(^{st}\) and 3\(^{rd}\) quartile. Categorical variables were compared by two-tailed Fisher’s exact test or chi-square \((\chi^2)\) test, with Bonferroni adjustment for multiple comparisons (the normal \( p \) value of 0.05 divided by the number of tests being performed). Odds ratios (OR) and 95% confidence intervals (CI) were calculated by use of the formula \( \frac{a*d}{b*c} \), where \( a \) is the number of diabetic patients with one of the genotypes; \( b \) is the number of control subjects with the same genotype; \( c \) is the number of patients without this genotype; and \( d \) is the number of control subjects without the
corresponding genotype. The significance of differences in continuous variables between groups was assessed by nonparametric Mann-Whitney U test. Genotype association with plasma C-peptide was assessed by linear regression analysis, using log transformed C-peptide as a dependent variable. Genetic models were derived from http://pngu.mgh.harvard.edu/~purcell/. All statistical tests were performed by SPSS version 13.0 (SPSS, Chicago, IL, USA), JMP version 5 for MAC OS X (SAS Institute, Cary, NC, USA) and whole genome association analysis toolset PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/). Linkage disequilibrium (LD) between two TCF7L2 SNPs rs in paper II was analyzed using Haplovie 4.0 and D’ values were calculated (278). In papers (II-IV) Hardy-Weinberg equilibrium was calculated in Microsoft excel sheet provided by Tufts University (Boston, USA) (www.tufts.edu/~mcourt01/Documents/Court%20lab%20%20HW%20calculator.xls). Power of the studies (II-IV) was calculated by using G•Power statistical power calculator (Version 2.1.2 for Macintosh) (http://www.psycho.uni-duesseldorf.de/aap/projects/gpower/how_to_use_gpower.html) (130).
11. Results

11.1. Paper I: HLA-DQB1 genotypes, islet antibodies and beta-cell function in young adult (15-34 years) diabetic patients

The aim of this study was to evaluate whether HLA-DQB1 genotypes facilitate the classification of diabetes as compared with assessment of islet antibodies by investigating young adult diabetic patients.

There were 2,077 young adult patients (aged 15–34 years old) included over a 5-year period in the nationwide Diabetes Incidence Study in Sweden. Islet antibodies were measured at diagnosis in 1,869 patients, fasting plasma C-peptide after diagnosis in 1,522, while HLA-DQB1 genotypes were determined in 1,743. Of the 2,077 incident diabetic patients, 2,018 were classified by the reporting physicians: 1,395 (69%) were given a diagnosis of clinical T1D, 366 (18%) of clinical type 2 diabetes and 257 (13%) could not be classified (unclassifiable diabetes) (Figure 10).

![Flow chart of DISS patients recruited during the 5-year study period (1998–2002 inclusive).](image)

There was a clear male preponderance among clinical T1D patients (ratio of
men:women=1.9) (Table 3). Patients with clinical T1D were younger (24 vs 30 and 28 years, p<0.0001) and had a lower BMI (22 vs 32 and 25, p<0.0001) than patients with clinical T2D or unclassifiable diabetes. A family history of diabetes was more frequent in clinical T2D (53%) than in clinical T1D (22%, p<0.0001) or unclassifiable (38%, p=0.004) diabetes (Table 3). Among all patients, 1,250 (67%) were positive for islet antibodies (Ab+). Islet antibodies were found in 83% of patients clinically considered to have T1D 23% with T2D and 45% with unclassifiable diabetes. After diagnosis, median fpC-peptide concentrations were markedly lower in patients with islet antibodies than in those without (0.24 vs 0.69 nmol/l, p<0.0001).


<table>
<thead>
<tr>
<th></th>
<th>Incident cases (n= 2,077)</th>
<th>Type of diabetes</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type 1 (n= 1,395)</td>
<td>Type 2 (n= 366)</td>
<td>Unclassifiable (n= 257)</td>
</tr>
<tr>
<td>Male/Female ratio</td>
<td>1.6</td>
<td>1.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Age at onset (year)</td>
<td>26 (20, 30)</td>
<td>24 (19, 29)</td>
<td>30 (26, 32)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23 (21, 27)</td>
<td>22 (20, 24)</td>
<td>32 (27, 37)</td>
</tr>
<tr>
<td>With family history of diabetes</td>
<td>407 (30)</td>
<td>211 (22)</td>
<td>130 (53)</td>
</tr>
<tr>
<td>Insulin treatment:</td>
<td>1694 (83)</td>
<td>1339 (97)</td>
<td>142 (39)</td>
</tr>
<tr>
<td>Patients with antibodies (Ab+)</td>
<td>1250 (67)</td>
<td>1056 (83)</td>
<td>79 (23)</td>
</tr>
<tr>
<td>Follow-up P-C-peptide</td>
<td>0.31 (0.17; 0.59)</td>
<td>0.24 (0.14; 0.40)</td>
<td>0.74 (0.48; 1.14)</td>
</tr>
</tbody>
</table>

Data are presented as absolute numbers (%) or median, 25th and 75th percentile

Irrespective of clinical classification, patients with islet antibodies showed increased frequencies of at least one of the risk-associated *HLA-DQB1* genotypes compared with patients without (Table 4).
Table 4. Frequencies of HLA-DQB1 genotypes among type 1, type 2, and unclassifiable Ab+ versus Ab- diabetic patients.

<table>
<thead>
<tr>
<th>HLA-DQB1 genotypes</th>
<th>Type of diabetes</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Ab+ (n)</td>
<td>Ab- (n)</td>
<td>p-value</td>
<td>Ab+ (n)</td>
<td>Ab- (n)</td>
<td>p-value</td>
</tr>
<tr>
<td>*02/*0302</td>
<td>271</td>
<td>20</td>
<td>&lt;0.0001</td>
<td>16</td>
<td>15</td>
<td>0.0005</td>
</tr>
<tr>
<td></td>
<td>(27)</td>
<td>(10)</td>
<td></td>
<td>(22)</td>
<td>(6.3)</td>
<td></td>
</tr>
<tr>
<td>*0302/X</td>
<td>257</td>
<td>30</td>
<td>0.0018</td>
<td>19</td>
<td>29</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>(26)</td>
<td>(15)</td>
<td></td>
<td>(26)</td>
<td>(12)</td>
<td></td>
</tr>
<tr>
<td>*0302/*0604</td>
<td>74</td>
<td>4</td>
<td>0.0039</td>
<td>3</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>(7.4)</td>
<td>(2.0)</td>
<td></td>
<td>(4.1)</td>
<td>(1.3)</td>
<td></td>
</tr>
<tr>
<td>Non-risk:</td>
<td>398</td>
<td>142</td>
<td>&lt;0.0001</td>
<td>36</td>
<td>190</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>(40)</td>
<td>(72)</td>
<td></td>
<td>(49)</td>
<td>(80)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
<td>196</td>
<td>74</td>
<td>237</td>
<td>107</td>
<td>122</td>
</tr>
</tbody>
</table>

Data are presented as absolute numbers (%).

Antibody-negative patients with risk-associated HLA-DQB1 genotypes had significantly lower median fpC-peptide concentrations than those without risk-associated genotypes (0.51 vs 0.74 nmol/l, p=0.0003) (Figure 11).
Figure 11. Levels of fpC-peptide measured at follow-up in relation to islet antibodies and HLA-DQB1 genotypes.

Conclusions:
1) 67% of patients with newly diagnosed diabetes at the age of 15-34 years have T1D
2) Islet antibodies strongly contribute to the etiological classification of diabetes.
3) Islet antibodies are more closely associated with beta-cell impairment than risk HLA-DQB1 genotypes.
4) Risk HLA-DQB1 genotypes are associated with islet antibodies and do not add much to the classification of diabetes in Ab+ individuals.
5) Risk HLA-DQB1 genotypes are, however, associated with low fpC-peptide concentrations in the absence of islet antibodies presumably identifying non-autoimmune T1D
6) Absence of islet antibodies and high fpC-peptide concentrations predict a T2D phenotype.
11.2. Paper II: *HLA-DQB1, INS VNTR, PTPN22* and *TCF7L2* in young (15-34 years) and middle-aged (40-59 years) diabetic patients

In this study we tested whether genetic markers associated with T1D or T2D could help to discriminate between autoimmune and non-autoimmune diabetes in young (15-34 years) and middle-aged (40-59 years) diabetic patients. In 1,642 young and 1,619 middle-aged patients we determined *HLA-DQB1* genotypes, *PTPN22* and *INS* VNTR polymorphisms, polymorphisms in the *TCF7L2* gene, GAD and IA-2 antibodies and fasting plasma C-peptide.

As expected, young GADA-positive diabetic patients had a higher frequency of risk *HLA-DQB1* (60 vs 25%; \( p=9.4\times10^{-34} \)), *PTPN22* CT/TT (34 vs 26%; \( p=0.0023 \)), *INS* VNTR (class I/I) AA (69 vs 53%; \( p=1.3\times10^{-6} \)) and *INS* VNTR (class IIIA/IIIA) CC (75 vs 63%; \( p=4.3\times10^{-6} \)) genotypes than young GADA-negative patients. The frequency of T2D-risk genotypes CT/TT of *TCF7L2* (rs7903146) was significantly increased in young GADA-negative (53 vs 43%; \( p=0.0004 \)) compared with GADA-positive diabetic patients (Table 5).

Middle-aged GADA-positive patients also had a higher frequency of risk genotypes *HLA-DQB1* (45 vs 18%; \( p=1.4\times10^{-16} \)), *PTPN22* CT/TT (31 vs 23%; \( p=0.034 \)), *INS* VNTR (class I/I) AA (69 vs 51%; \( p=8.5\times10^{-5} \)) and *INS* VNTR (class IIIA/IIIA) CC (73 vs 60%; \( p=0.008 \)) genotypes than middle-aged GADA-negative patients. There was no difference in T2D-associated CT/TT genotypes of the *TCF7L2* gene between middle-aged GADA-positive and GADA-negative patients (Table 5), both of whom showed a significantly higher frequency than controls (55 vs 43%; \( p=1.5\times10^{-9} \) and 24 vs 20%; \( p=0.0007 \)).
Table 5. Prevalence of HLA-DQB1, PTPN22, INS VNTR and TCF7L2 in young (15–34 years) and middle-aged (40–59 years) GADA-positive and GADA-negative diabetic patients.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Young (15–34 years)</th>
<th>Middle-aged (40–59 years)</th>
<th>p value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GADA+</td>
<td>GADA-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GADA+</td>
<td>GADA-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-DQB1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Risk (*02/*0302, *0302/X, *0302/*604)</td>
<td>572 (60) 115 (25) 5.7x10^{-33} 78 (45) 208 (18) 8.3x10^{-16}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-risk</td>
<td>387 (40) 341 (75) 94 (55) 965 (82)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTPN22 (rs2476601)</td>
<td><strong>CC</strong> 631 (66) 344 (74) 0.014 86 (69) 908 (77) 0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT+TT</td>
<td>324 (34) 120 (26) 39 (31) 267 (23)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INS VNTR (rs689)</td>
<td><strong>AA (class I/I)</strong> 634 (69) 248 (53) 8x10^{-8} 87 (69) 588 (51) 0.0005</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT+TT (class I/III+III/III)</td>
<td>295 (31) 216 (47) 39 (31) 573 (49)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INS VNTR (rs3842755)</td>
<td><strong>CC (class IIIA/IIIA)</strong> 707 (75) 290 (63) 2.6x10^{-5} 71 (73) 691 (60) 0.048</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC+AA (class IIIA/IIIB+ IIIB/IIIB)</td>
<td>242 (25) 173 (37) 26 (27) 469 (40)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCF7L2 (rs7903146)</td>
<td><strong>CC</strong> 538 (57) 217 (47) 0.0024 88 (44) 610 (45) NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT+TT</td>
<td>407 (43) 246 (53) 109 (56) 733 (55)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as absolute numbers (%). Risk genotypes are highlighted.

Conclusions:
1) TCF7L2 genotypes help to distinguish between autoimmune and non-autoimmune diabetes in young but not in middle-aged diabetic patients.
2) Genetically young and middle-aged Ab+ diabetic patients are similar with respect to increased prevalence of T1D risk genotypes.
3) Addition of TCF7L2 to HLA genotyping in young adults improved the prediction of diabetic subtype.
4) It is likely that young GADA- diabetic patients do have true T2D.
5) If the age at onset for definition of LADA is lowered below 35 years, the LADA group will include an increasing number of patients with classical T1D.

11.3. Paper III: Novel T2D genes in young (15-34 years) and middle-aged (40-59 years) diabetic patients

In this study we evaluated whether common variants in novel T2D diabetes genes can discriminate between autoimmune and non-autoimmune diabetes in
young (15-34 years) and middle-aged (40-59 years) subjects. We genotyped following SNPs: PPARG, KCNJ11, IGF2BP2, WFS1, CDKAL1, JAZF1, CDKN2A/2B, HHEX, SLC30A8 and FTO, measured GAD autoantibodies and C-peptide.

Altogether, 62% of the young and 13% of the middle-aged patients had GAD autoantibodies. The frequency of common variants in the PPARG (OR=1.3, \( P=0.02 \)), IGF2BP2 (OR=1.1, \( P=0.02 \)), WFS1 (OR=1.1, \( P=0.04 \)), JAZF1 (OR=1.2, \( P=0.018 \)) and CDKN2A/2B (OR=1.3, \( P=0.013 \)) genes was increased in young GADA- patients compared with an elderly non-diabetic control group. Also young GADA-negative diabetic patients had higher frequency of the JAZF1 AA (32% vs 23%; \( P=0.02 \)) and CDKN2A/2B TT (74% vs 67%; \( P=0.03 \)) genotypes than young GADA+ patients.

GADA- middle-aged showed the expected increased frequency of T2D diabetes associated risk alleles in the PPARG (OR=1.2, \( P=0.008 \)), IGF2BP2 (OR=1.2, \( P=8.7 \times 10^{-7} \)), WFS1 (OR=1.2, \( P=0.0003 \)), CDKAL1 (OR=1.3, \( P=2.6 \times 10^{-10} \)), JAZF1 (OR=1.2, \( P=2.4 \times 10^{-9} \)), SLC30A8 (OR=1.2, \( P=3.5 \times 10^{-6} \)), CDKN2A/2B (OR=1.3, \( P=6.5 \times 10^{-6} \)), KCNJ11 (OR=1.1, \( P=0.0008 \)), and FTO (OR=1.3, \( P=3.7 \times 10^{-8} \)) genes compared with non-diabetic control subjects. The risk score indicating a high genetic risk of T2D (\( \geq 12 \) risk alleles) was associated with decreased fasting C-peptide concentrations (0.84 vs 0.97 nmol/l; \( P=0.009 \)) in GADA- and an earlier age at onset (47 vs 51 years; \( P=0.039 \)) in GADA+ (Figure 12) middle-aged patients.

Of individual genotypes, carriers of the T2D-associated risk C allele of CDKAL1 (CC: 0.73 and CG: 0.87 vs GG: 0.97 nmol/l; \( P=0.0002 \)) had lower C-peptide concentrations than G allele carriers.
In this study the frequency distribution of T2D diabetes associated risk alleles was quite similar to that of LADA, but different from non-diabetic controls and T1D patients, (Figure 13). These findings lend further support to the view that LADA patients share several genetic features with T2D. The study was, however, underpowered to detect a significant difference between middle-aged GADA+ patients and non-diabetic controls, e.g. the power to detect a difference in frequency for CDKAL1 was only 37%.

Figure 12. Fasting plasma C-peptide concentrations in GADA- (A) and age at onset of diabetes in GADA+ (B) middle-aged (40-59 years) GADA- diabetic patients.
Figure 13. Frequency distributions of type 2 diabetes risk alleles in T1D, LADA, T2D patients and healthy controls.

Conclusions:
1) An increase of T2D risk variants in young GADA- diabetic patients suggests a resemblance with T2D
2) Middle-aged GADA- diabetic patients with a high genetic risk score (≥ 12 risk alleles) had reduced fasting C-peptide concentrations.
3) The risk variants in the CDKAL1 gene resulted in a step-wise decrease in fasting C-peptide concentrations in middle-aged GADA- patients and to some extent in the GADA+ patients.
4) Middle-aged GADA+ diabetic patients with a high genetic risk score (≥ 12 risk alleles) had earlier age at onset than those with a low risk score.
5) LADA patients share several genetic features with T2D.

11.4. Paper IV: Common variants and rare mutations in MODY genes in young (15-34 years) diabetic patients
In this study we evaluated whether common variants in MODY genes can discriminate between autoimmune and non-autoimmune diabetes and screened for MODY mutations in young adult (15-34 years) diabetic patients. We
genotyped the following SNPs in MODY genes: HNF-4α, GCK, HNF-1α and HNF-1β in 1,642 young adult diabetic patients (15-34 years) with and without islet auto-antibodies. Eight patients with ≥ 3 family members with diabetes were sequenced for possible mutations in the HNF-4α, GCK and HNF-1α genes. Out of 1,642 young diabetic patients, 1,138 (69%) had islet antibodies and 31% were antibody negative. No significant difference in frequency of common variants in HNF-4α, GCK, HNF-1α and HNF-1β genes was seen between Ab+ and Ab- individuals nor between diabetic patients and non-diabetic controls (Table 6).

Table 6. Frequency of common variants in MODY genes in young (15-34 years at onset) Ab+ vs. Ab- diabetic patients.

<table>
<thead>
<tr>
<th>Chr</th>
<th>Gene SNP</th>
<th>Genotypes</th>
<th>N Ab+/Ab-</th>
<th>Controls</th>
<th>Ab+</th>
<th>Ab-</th>
<th>p-valuea Ab+ vs. Ab-</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>HNF4-α (MODY1) rs4810424</td>
<td>CC</td>
<td>1070/466</td>
<td>338 (3.0) 3135 (28) 7702 (69)</td>
<td>40 (3.7) 284 (27) 746 (70)</td>
<td>12 (2.6) 134 (29) 320 (70)</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CG</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>GG</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>1051/464</td>
<td>138 (3.7) 1104 (29) 2514 (67)</td>
<td>43 (4.0) 280 (27) 728 (69)</td>
<td>14 (3.0) 136 (29) 314 (68)</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CC</td>
<td>1062/460</td>
<td>2014 (17) 5662 (48) 4075 (35)</td>
<td>180 (17) 519 (49) 363 (34)</td>
<td>87 (19) 225 (49) 148 (32)</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT</td>
<td></td>
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</tr>
<tr>
<td>7</td>
<td>GCK (MODY2) rs1799884</td>
<td>AA</td>
<td>1032/458</td>
<td>279 (2.5) 2872 (26) 8013 (72)</td>
<td>20 (1.9) 261 (25) 751 (73)</td>
<td>6 (1.3) 125 (27) 327 (71)</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>HNF-1α (MODY3) rs1169288</td>
<td>GG</td>
<td>1072/464</td>
<td>5183 (46) 4882 (44) 1109 (9.9)</td>
<td>493 (46) 459 (43) 120 (11.0)</td>
<td>190 (41) 223 (48) 51 (11.0)</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>HNF-1β (MODY5) rs757210</td>
<td>CC+G</td>
<td>1040/451</td>
<td>4549 (42) 4604 (43) 1651 (15)</td>
<td>446 (43) 450 (43) 144 (14)</td>
<td>186 (41) 181 (40) 84 (19)</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT+G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>1063/460</td>
<td>3426 (30) 5601 (49) 2307 (20)</td>
<td>349 (33) 525 (49) 189 (18)</td>
<td>137 (30) 214 (46) 109 (24)</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
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<td>AG</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>GG</td>
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</tbody>
</table>

Data are presented as absolute numbers (%). Risk genotypes are highlighted.

SNP rs1169288 in the MODY 3 gene refers to the common variant I27L which previously has been associated with increased risk of T2D (78, 279). Among Ab+ patients, carriers of T2D-associated T (L in I27L) allele of SNP rs1169288 in the HNF1-α gene had higher age at onset of diabetes (26 vs 24 years; p=0.0006, recessive model of inheritance) and more severe symptoms at diabetes onset such as weight reduction and/or polyuria (97% vs 92; p=0.0008, dominant model of inheritance) than G allele carriers (Figure 14).
Figure 14. Age at onset (years) (A) and symptoms at onset (weight loss and/or polyuria) (B) in different genotype carries of the HNF-1α (rs1169288) gene in young adult Ab+ diabetic patients.

Among Ab- diabetic patients carriers of the T2D-associated G allele of the HNF-1β (rs4430796) gene (TCF2) had less severe diabetic symptoms (76% vs 86%; p=0.015, dominant model of inheritance) and less ketonuria (29% vs 46%; p=0.003, recessive model of inheritance) at diagnosis than A allele carriers (Figure 15).
Out of 355 diabetic patients without islet auto-antibodies 57% had no family history of diabetes, 34% had 1 family member, 6% had 2 family members and 3% had 3 or more family members with diabetes.

Out of 8 patients from pedigrees with at least 3 affected members, only one Ab-patient who was 26 years at diagnosis and treated with oral anti-diabetic drugs had a common frameshift mutation in exon 4 designated “Pro291fsinsC” in the HNF-1α (MODY 3) gene (Figure 16). It is the most common MODY 3 mutation described in a number of Swedish patients (65, 280, 281).
Figure 16. Pedigree figure with MODY 3 diabetic patient (marked by star). Squares represent males, circles females and rhombus an unknown gender.
12. Discussion
Although the ADA and WHO definitions provide criteria for the diagnosis of diabetes they provide little information on how to subclassify patients. In the clinic, most diabetic patients are classified as having either T1D or T2D but these classifications often more reflect our need to classify than follow hard criteria. Therefore a relatively large group of patients belong to the grey zone where clinical classification is more subjective than real. The age group between 15-34 years is becoming increasingly important with the ever younger onset of T2D. DISS is a unique study which can help to subclassify diabetic patients in this grey zone of diabetes. We have used a combination of markers (auto-antibodies, C-peptide, genetic markers) to classify these patients into subgroups. Including middle-aged patients also allowed us to distinguish between autoimmune diabetes in young and middle-aged (LADA) patients. The papers included in the thesis thus provide a first comprehensive attempt to classify diabetic patients across the age groups of 15-59 years into subgroups.

Strengths: The major strength of DISS is that we recruited a large cohort of incident diabetic patients aged 15-34 years from the whole country. Studies were large enough to detect effects of genotypes on clinical phenotypes with a power > 90%.

Weaknesses: 1) all statistical analyses were not corrected for multiple comparison (78) 2) Although we used different GADA assays in young and middle-aged diabetic patients, assay reruns showed correlation coefficient of 0.93 with \( p < 0.0001 \) between two methods suggesting that this would not significantly have influenced the results, 3) theoretically, if some middle-aged patients with low GADA titres were falsely labelled as GADA+ this could give an impression of similarity between middle-aged GADA+ and GADA- patients. This is an unlikely explanation, as by subdividing the middle-aged GADA+ patients by the median of the GADA titres, the results were virtually unchanged.

12.1 Study I
This study showed that 67% of young adult diabetic patients had autoimmune T1D if islet antibodies were used as an objective diagnosis of autoimmune T1D. If immeasurable or low fpC-peptide after diagnosis was included as a further objective criterion for clinical T1D, only another 70 patients without islet antibodies were added to the clinical T1D group. Hence, including patients with low or immeasurable fpC-peptide, the proportion of T1D would increase with only 4% leading to 71% with objective T1D among all incident young adults between 15 and 34 years of age. Among those classified as having T1D, 83% had objective T1D base on islet antibodies. Antibodies also are seen among 23% classified having T2D and 45% having unclassifiable diabetes, as previously
reported (282, 283). This emphasizes that classification should be based upon clear criteria as presence of islet antibodies rather than symptoms and clinical impressions. We observed that in the presence of islet antibodies, risk-associated \textit{HLA-DQB1} genotypes do not contribute to the classification of T1D per se, as seen also previously (284-286). Indeed, in line with the Belgian Diabetes Registry (141), the only suggestion that risk \textit{HLA-DQB1} genotypes may contribute to an etiological classification of diabetes was among Ab- diabetic patients. The explanation for this finding could be that islet antibodies not detected by the current assays may be present in Ab- diabetic patients with risk-associated \textit{HLA-DQB1} genotypes and low fpC-peptide. Later development of antibodies or disappearance of previous antibodies should also be considered. It can be argued that we did not determine \textit{HLA-DQA1} genotypes in our patients. However, the linkage disequilibrium between alpha and beta chain alleles is very strong. Additional information obtained by typing for \textit{HLA-DQA1} would thus be of limited added value (270). Furthermore, our association study of \textit{HLA-DQB1} loci may be considered as a cost-effective way of assessing the role of HLA in the classification of diabetes in individuals aged 15-34 years.

\section*{12.2 Study II}

In this study, we provide a comprehensive genetic and clinical characterisation of antibody-positive and -negative diabetes across the age groups 15 to 59 years. The key finding of our study was that \textit{TCF7L2} CT/TT genotypes helped distinguish between young GADA-positive and GADA-negative patients (age 15-34 years) but not between middle-aged (40-59 years) GADA-positive and GADA-negative diabetic patients. These findings may have important implications for diagnosis of diabetic subgroups. Consistent with previous reports (142, 228, 230), genetically, young and middle-aged antibody-positive patients were similar with respect to increased prevalence of risk genotypes \textit{HDLQ1}, \textit{PTPN22} CT+TT, \textit{INS} VNTR class I/I and \textit{INS} VNTR class IIIA/IIIA. Although \textit{HLA} genotyping together with measurements of islet antibodies has been used to identify individuals at risk of T1D, no genetic markers for T2D have been available until the identification of the strong association between common variants in the \textit{TCF7L2} gene with T2D. Therefore, addition of \textit{TCF7L2} to \textit{HLA} genotyping in young adults could clearly help to improve the prediction of diabetic subtype. When we subdivided the young adult patients into two age groups (15-25 and 25-34 years), we observed a stronger discriminatory effect of variants in the \textit{TCF7L2} gene in the older than in the younger group. Our failure to find a difference in the frequency of the T2D-associated T allele of rs7903416 in the \textit{TCF7L2} gene between middle-aged GADA-positive and GADA-negative patients, while finding a significantly higher frequency than in young GADA-positive patients, indicates that autoimmune diabetes in the
middle-aged is different from autoimmune diabetes in young adults. These findings complement and add to our previous paper (231) showing that frequency of the *TCF7L2* variant markedly differs between young and middle-aged autoimmune diabetes, further supporting the view that LADA represents an admixture of T1D and T2D. It also shows that if age at onset for definition of LADA is lowered below 35 years, the LADA group will include an increasing number of patients with classical T1D.

12.3 Study III
The novel findings of this study was 1) an increase of T2D risk variants (*JAZF1* and *CDKN2A/2B*) in young GADA- diabetic patients suggesting resemblance with T2D and 2) a modifying effect of T2D- associated genotypes on C-peptide in GADA- and age-at-onset in GADA+ middle-aged patients. An “accelerator” hypothesis has been put forward by which a genetic background of T2D would result in earlier onset of autoimmune diabetes by imposing an earlier stress on beta-cells by the insulin resistance characteristic of T2D (287). Our data indeed support the view that T2D associated genes influence the phenotype in patients with autoimmune diabetes but that the acceleration of diabetes onset and beta-cell deterioration is only seen in middle-aged but not in young patients. Also, the data rather suggest that further impairment in beta-cell function rather than in insulin action is the accelerator culprit. Although the mechanism by which the new T2D associated risk genotypes impair beta-cell function is not fully understood, one common hypothesis is that most of them would impair proliferation of beta-cells. Also, risk variants in the *CDKAL1* gene resulted in a step-wise decrease in fasting C-peptide concentrations in middle-aged GADA- patients and to some extent in the GADA+ patients.

12.4 Study IV
Novel findings in the present study were that 1) Ab+ patients, T2D-associated T allele carriers in the *HNF-1α* (rs1169288) presented with more severe diabetic symptoms than G allele carriers 2) Ab+ patients, the same T allele was associated with a delayed onset of the disease 3) Ab- patients, T2D-associated G allele carriers in the *HNF-1β* (TCF2: rs4430796) gene presented with less severe diabetic symptoms and ketonuria than A allele carriers.

Mutations in the *HNF-1α* gene cause decreased renal threshold for glucose reabsorption and correspondingly glucosuria (288, 289), increased level of liver enzymes (290), reduced pancreatic volume (291) and could modify the age at onset of diabetes (292). It has been shown previously that functional variant in the *HNF-1α* gene (I27L) was associated with an increased risk of T2D
independent of sex, age at onset and BMI, with an effect size similar to the novel T2D genes, influence transcriptional activity and insulin secretion in vivo (78, 279).

We do not know whether polyuria in the Ab+ patients with the T allele of rs1169288 reflects a reduced renal threshold or simply is a reflection of a more severe disease. It has been shown that the HNF-1α knockout mouse has a severe renal Fanconi syndrome with polyuria and glucosuria, caused by renal proximal tubule dysfunction. This defects in glucose reabsorption is caused by significant reduction in expression of high capacity/low affinity sodium-glucose transporter-2 (SGLT-2) (293, 294).

In patients with MODY 3, age at onset differs with location of the mutation in the gene. Patients with mutations that affected exons 8-10 in the HNF-1α gene were diagnosed later than patients with mutations in exons 1–6 (295). The SNP I27L (rs1169288) is located in the exon 1 region of the HNF-1α gene. It has previously been shown that a family history of T2D influences the phenotype of patients with T1D and vice versa (296-298). With the notion that this polymorphism (I27L) in the HNF-1α gene is associated with increased risk of T2D, the effect on age at onset in the Ab+ patients could reflect co-inheritance of T2D genes or a shared family history of T2D.

It has been shown that MODY 5 patients carrying mutations in the HNF-1β gene can show similar clinical symptoms and signs as patients with T1D and insulin dependency (299, 300). Our finding of less severe diabetic symptoms in carriers of the T2D-associated G allele of HNF-1β gene in Ab- patients might indicate that they share more of a T2D genetic background and phenotype.

Taken together, the data from these studies suggest that diabetes represents a continuum where T1D and T2D make up the extremes (Figure 17). In the grey zone between these two forms of diabetes we find LADA which shares genetic features with both T1D and T2D and should be considered as an admixture of the two rather than as a subgroup of T1D. The data also convincingly show that co-sharing other genes between subgroups can influence the clinical phenotype. This is though only the beginning, with more refined methods we can hope that classification of diabetes can be more precise and possibly one day be used for predicting course of the disease and response to treatment, i.e. a step towards personalized medicine.
Figure 17. Diabetes is a continuum where the relative contribution of impaired beta-cell function and insulin sensitivity differs between different subgroups.
Conclusions

**Study I:** Presence of islet auto-antibodies is more important than C-peptide or T1D risk-associated *HLA-DQB1* genotypes in the classification of diabetes among young adults (15-34 years old)

**Study II:** Genetically, Latent Autoimmune Diabetes in Adults (LADA) is an admixture of both type 1 and type 2 diabetes, sharing *HLA-DQB1* risk genotypes with type 1 and *TCF7L2* risk variants with type 2 diabetes.

**Study III:** Type 2 diabetes associated risk genotypes modify the disease phenotype (age at onset and C-peptide) in middle-aged but not in young diabetic patients.

**Study IV:** Common variants in MODY genes do not discriminate between young patients with autoimmune and non-autoimmune diabetes but they do influence onset and presentation of the disease.
Populärvetenskaplig sammanfattning

Diabetes kännetecknas av förhöjda halter av glukos i blodet och flera vanliga former förekommer. Typ 1 (T1D) diabetes är vanligast förekommande hos barn och ungdomar och karakteriseras av att de insulin producerande cellerna (s.k. betacellerna) förstörs av kroppens eget immunförsvaret, en autoimmun reaktion. Patienter med T1D diabetes kräver insulin för överlevnad och har ofta riskgenotyper så som HLA-DQB1, INS VNTR, PTPN22. Hos vuxna beskrivs samma situation som Latent Autoimmune Diabetes in Adults (LADA) med en diagnos efter 35-års ålder och utan insulinbehandling de första sex månaderna.

Typ 2 diabetes (T2D) karakteriseras av minskad insulinsekretion och nedsatt insulinverkan tillsammans med ökad produktion av glukos från levern och onormal fettmetabolism. Miljöfaktorer som ökar insulinresistens är pubertet, graviditet, viktuppgång (framför allt central fetma, s.k. ”äpple form”) och fysisk inaktivitet. T2D diagnosticeras vanligtvis efter 40-års ålder, i vissa fall efter att patienten utvecklat kärl och neurala komplikationer. TCF7L2 genen associerar starkt med typ 2 diabetes.

Maturity-onset diabetes of the young (MODY) är en monogen form av diabetes som nedärvs dominant (dvs. patienten har en muterad och en normal variant av på de två kromosomerna) och karakteriseras av diabetes utan förekomst av ketoner, sjukdomsdebut innan 25-års ålder och orsakas av en störning i betacellsfunktionen. Hittills har mutationer i sex gener visat sig orsaka MODY, HNF-4α (MODY 1), glucokinase (GCK) (MODY 2), HNF-1α (MODY 3), IPF1 (MODY 4), HNF-1β (MODY 5) och NeuroD1 (MODY 6).

Målet med denna avhandling var att undersöka genetiska skillnader mellan autoimmun (typ 1 diabetes och LADA) och icke-autoimmun (typ 2 diabetes och MODY) diabetes i unga (15-34 år) och medelålders (40-59 år) diabetiker från Sverige för att förbättra framtidens diagnoser och behandling.

I första studien undersökte vi huruvida HLA-DQB1 genotyper kunde förbättra klassificering av diabetes jämfört med antikoppar mot insulinproducerande celler i unga (15-34 år) diabetespånsanter. Antikoppar (ICA, IA-2A eller GADA) mot insulinproducerande celler upptäcktes hos 83% av patienter som kliniskt karakteriserades som T1D, hos 23% av patienter med T2D och hos 45% av patienter vars diabetes inte kunde klassificeras enligt kliniska symptom. Hos patienter med antikoppar var C-peptidnivåerna, ett mått av betacellsfunktion, lägre vid diagnosen än hos patienter utan antikoppar. Oberoende av klinisk klassificering hade patienter med antikoppar högre frekvens av HLA-DQB1 risk genotyper än patienter utan antikoppar. Patienter utan antikoppar och bärare av HLA-DQB1 risk genotyper hade lägre C-peptidnivåer än de som inte hade risk gennomen av HLA-DQB1. Från dessa resultat drog vi slutsatsen att antikroppsmätningar är nödvändiga för att klassificera patienter med diabetes. Genotypning av HLA-DQB1 bidrar inte till bättre klassificering hos patienter.
med antikroppar. I patienter utan autoantikroppar kan genotypning av HLA-DQβ1 tillsammans med mätning av C-peptidnivåer bidra till bättre urskiljning mellan T1D och T2D.

I studie II undersökte vi om genetiska markörer associerade med T1D (HLA-DQB1, INS VNTR och PTPN22) och T2D (TCF7L2) kunde hjälpa till att skilja mellan autoimmun och icke-autoimmun diabetes hos unga (15-34 år) och medelålders (40-59 år) diabetiker. Frekvensen av riskgenotyper av HLA-DQB1, PTPN22 CT/TT, INS VNTR klass I/I och INS VNTR klass IIIA/IIIA var högre hos både unga och medelålders individer med GADA antikroppar jämfört med individer utan GADA antikroppar. T2D riskgenotypen (CT/TT) i TCF7L2 var vanligare hos unga individer utan GADA antikroppar jämfört med individer med GADA antikroppar. Hos medelålders individer var frekvensen av CT/TT genotyper förhöjt hos både individer med och utan GADA antikroppar. Av dessa resultatt kunde vi dra slutsatsen att variationen i TCF7L2 kunde skilja mellan unga men inte medelålders patienter med och utan GADA antikroppar. Detta tyder på att unga patienter utan GADA antikroppar har T2D och att medelålders patienter med GADA antikroppar (LADA) skiljer sig från unga patienter med GADA antikroppar (T1D) och delvis genetisk bakgrund med T2D.


I studie IV undersökte vi om vanliga varianter i MODY gener kunde urskilja mellan autoimmun och icke-autoimmun diabetes hos unga diabetiker. Vi letade efter mutationer i de vanligast förekommande MODY gener (HNF-4α, GCK och HNF-1α) hos individer utan autoantikroppar med tre eller fler familjemedlemmar med diabetes. Ingen skillnad i frekvens mellan individer med
eller utan antikroppar observerades bland de undersökta MODY generna. Individer med autoantikroppar som bar på den T2D associerade T-allelen i $HNF-1\alpha$ genen hade senare sjukdomsdebut och en svårare sjukdomsbild med viktnedgång och polyuri än de individer som saknade allelen. Hos individer utan antikroppar hade bärare av den T2D associerade G-allelen i $HNF-1\beta$ genen mindre ofta problem med viktnedgång, polyuri och ketonuri än individer utan allelen. Bland patienter med flera familjemedlemmar med diabetes kunde vi identifiera en individ med en MODY mutation i exon fyra i $HNF-1\alpha$ genen. Våra resultat tyder på att vanliga varianter i MODY gener inte kan urskilja mellan autoimmun och icke-autoimmun diabetes hos unga individer, däremot kan de påverka åldern vid insjuknande och sjukdomsbilden.

Våra studier visar att genetiska markörer tydligt förbättrar klassificeringen av diabetes och tillsammans med antikroppar mot insulinproducerande celler kan hjälpa vid diagnosen och behandlingen av olika diabetesgrupper.
Georgian Summary (ქართულ სიტყვები წარწერა)

გავიანება I შეიძლება ბრძოლა ჰქონოდა საზოგადოებაზე და ახალგაზრდაფეხბურთელმა. იმ ხარისხებით, რომ საფრთხემდე ახალგაზრდის ხარისხებთან შედარებით შეიძლება მომეწოდების გამო ამოღებული გულის ტრაუმა. აქ, რომ გამოვიყენებთ მოსაზრები (HLA-DQB1, INS VNTR, PTPN22) ანსამბლს, და იმ ზომანთა საჭიროებას სოციალურ სენსიტიზაციის. სიმჭიდროვე ადამიანებმა ინტენსიური ბადირბატის ხელში ჰყავს ვიზომული ალგორითმით დანერგება ბიოჰერეგულობი (LADA). ფაქტობრივად, 35 წლის შემდეგ და იმ მონაწილე დანერგების შემდეგ ჰყავთ ყველაზე პოპულარული საკუთართვის პერიოდულმა დანერგება დიაპაზონი (LADA). ცოცხალმა სურს უპატივი და იმ საჭიროებას ინდივიდუალური ხელში ჰყავს 2013 ი.წ. დასამუშავება.

გავიანება II შეიძლება ბრძოლა ჰქონოდა ამათაკავშირში საზოგადოებაზე და ახალგაზრდაფეხბურთელმა. იმ ხარისხებით, რომ საფრთხემდე ახალგაზრდის ხარისხებთან შედარებით შეიძლება მომეწოდების გამო ამოღებული გულის ტრაუმა. აქ, რომ გამოვიყენებთ მოსაზრები (HLA-DQB1, INS VNTR, PTPN22) ანსამბლს, და იმ ზომანთა საჭიროებას სოციალურ სენსიტიზაციის. სიმჭიდროვე ადამიანებმა ინტენსიური ბადირბატის ხელში ჰყავს ალგორითმით დანერგება დიაპაზონი (LADA). ცოცხალმა სურს უპატივი და იმ საჭიროებას ინდივიდუალური ხელში ჰყავს 2013 ი.წ. დასამუშავება.
არსებულ ანტიკომბუტი ალგორითმი იძგა პლატფორმად დათვალიერებული ნიშა 1 დათვალიერი შემდეგ 83%-ში, ნიშა 2 დათვალიერი შემდეგ 23%-ში და არსებულ პლატფორმად დათვალიერი შემდეგ 45%-ში, ფართოდ განსხვავებით C-პროპენის კოლუნგრუტა დათვალიერების შემდეგ შესაძლო ოფ. არ არსებულია შესაძლო არკამბოლში. თუმცა არქამბოლში შიდა გარემანგვან ლანგვანგი პლატფორმა შექმნა. არსებული ალგორითმი მოქვეყნა, არსებულ ალგორითმ ფსევდო გარემანგვანი HLA-DQB1 ეროვნული მდგომარეობი შეიძლება ცოდნივი სოფელში, არსებულ ალგორითმი არგუნტი, HLA-DQB1 ეროვნული შექმნილ არქამბოლში შექმნა არკამბოლ შიდა დათვალიერა პლატფორმა კოლუნგრუტა. ვიდრე შორის, თუმცა არ გარემანგვან ლანგვანგი გარემანგვან უფრო გარემანგვან ფსევდო გარემანგვან პლატფორმა. ვიდრე შორის, არსებულ პლატფორმა გამოყენება HLA-DQB1 ეროვნული მდგომარეობი არქამბოლში არსებულ ალგორითმ შექმნილ არქამბოლში. თუმცა, არსებულ პლატფორმა შექმნილ არქამბოლში HLA-DQB1 ეროვნული მდგომარეობა C-პროპენის დათვალიერი შექმნა არქამბოლში. კოდა, არსებული პლატფორმა HLA-DQB1 ეროვნული მდგომარეობი არქამბოლში შიდა დათვალიერ დათვალიერ 1 დათვალიერი ფორმის ნიშა 2 დათვალიერი დათვალიერები შორის.
გულისხმოვანი აბსხვავებით GADA+ პაციენტის პატიოსნობი ჰაერდული C-ეპიფანი (12) გვერდი ქმნის ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიფანი (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეპიჭა (29) გვერდი ჰაერდულ C-ეpig.
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