CHARLES UNIVERSITY IN PRAGUE

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Molecular aspects of genetic predisposition to type 2 diabetes mellitus and its monogenic forms

PhD thesis

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Content

Abbreviations

ADA	American Diabetes Association
ADAMTS9	ADAM metallopeptidase with thrombospondin type 1 motif, 9
AIDS	Acquired Immune Deficiency Syndrome
AMP	Adenosine monophosphate
apM1	Adiponectin
AT	Adipose tissue
ATP	Adenosine triphosphate
BMI	Body mass index
CAMK1D	Calcium/calmodulin-dependent protein kinase ID
CDC123	Cell division cycle 123 homolog
CRP	C-reactive protein
CTLA-4	Cytotoxic T-lymphocyte associated 4
CTNNB1	Catenin (cadherin-associated protein), beta 1
CVD	Cardiovascular disease
dHPLC	Denaturing high performance liquid chromatography
DM	Diabetes mellitus
DN	Diabetic nephropathy
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetate
ERK	Extracellular regulated mitogen-activated protein kinase
FFA	Free fatty acid
FPG	Fasting plasma glucose
GAD, antiGAD	Glutamic acid decarboxylase, Glutamic acid decarboxylase autoantibodies
GCK	Glucokinase
GDM	Gestational diabetes mellitus
GLUT	Glucose transporter
Grb2	Growth receptor-binding protein 2
GWA, GWAS	Genome-wide association, Genome-wide association study
HbA1c	Glycated hemoglobin
HDL	High-density lipoprotein

HLA	Human leukocyte antigen
HNF	Hepatic nuclear factor
HT	Hypertension
IA(IA-2,IA-2β)	Protein tyrosin phosphatase autoantibodies
IAA	Insulin autoantibodies
ICA	Islet cells autoantibodies
IDDM	Insulin dependent diabetes mellitus
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IGF2	Insulin-like growth factor 2
IGF2BP1	Insulin-like growth factor 2 mRNA-binding protein 1
IGF2BP2	Insulin-like growth factor 2 mRNA-binding protein 2
IGF-I	Insulin-like growth factor I
IGT	Impaired glucose tolerance
IkB	Inhibitor NFkB
IL	Interleukin
INS-VNTR	Insulin variable number of tandem repeats
IPF-1	Insulin promoter factor -1
IR	Insulin resistance
IRS	Insulin receptor substrate
IS	Insulin sensitivity
JAZF1	Juxtaposed with another zinc finger gene 1
kDa	kiloDalton
LADA	Latent autoimmune diabetes in adults
LD	Linkage disequilibrium
LDL	Low-density lipoprotein
LPS	Lipopolysacharide
miRNA, miR	MicroRNA
MODY	Maturity-onset diabetes of the young
MS	Metabolic syndrome
NDDG	National Diabetes Data Group

NDRD	Nondiabetic renal disease
NeuroD1	Neurogenic differentiation 1
NF-κB	Nuclear factor-kappa B
NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases
NIDDM	Non-insulin-dependent diabetes
NIH	National Institute of Health
NIK	NFκB inducing kinase
NOTCH2	Notch homolog 2
NRs	Nuclear hormone receptors
NTP	Nucleotide triphosphate
OGTT	Oral glucose tolerance test
OHA	Oral hypoglycemic agents
PCR	Polymerase chain reaction
PDK1, PDK2	3-phosphoinositide-dependent protein kinases
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PNDM	Permanent neonatal diabetes mellitus
PPAR	Peroxisome proliferator activated receptor
PPRE	Peroxisome proliferator response elements
PTPN22	Protein tyrosine phosphatase non-receptor type 22
RA	Rheumatoid arthritis
RBP4	Retinol binding protein 4
RFLP	Restriction fragment length polymorphism
RXR	9-cis retinoic acid-X receptors
SCAT	Subcutaneous adipose tissue
SLE	Systematic lupus erythematosus
SNP	Single nucleotide polymorphism
SUR1	Sulphonylurea receptor 1
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus

TCF7L2	Transcription factor 7-like 2 (T-cell specific, HMG-box)
TCR	T cell receptor
TG	Triglycerides
THADA	Thyroid adenoma associated
TNFα	Tumor necrosis factor α
TSPAN8	Tetraspanin8
TZD	Thiazolidinedione
UTR	Untranslated region
VAT	Visceral adipose tissue
VLDL	Very-low-density lipoprotein
VO_2max	Maximal aerobic capacity/maximal oxygen uptake
VSMCs	Vascular smooth muscle cells
WGA, WGAS	Whole genome association, Whole genome association study
WHO	World Health Organization
WTCCC	Wellcome Trust Case Control Consortium

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1 Introduction

1.1 History

In 1922, for the first time in history, 13 years old boy, Leonard Thompson, survived the ketoacidosis coma through application of "íletin" (later on renamed in insulin). This year represents the milestone for diabetes mellitus (DM) as the deadly disease became curable. Professor John James Richard Macleod and Frederic Grant Banting were awarded the Nobel Prize in Medicine 1923 for the discovery of insulin. They shared the reward with 2 colleagues, Charles Best and James Bertram Collip, who helped them to discover and purify insulin. Frederic Grant Banting realized that if he were to ligate the pancreatic ducts of dogs, thus preventing the damaging effects of pancreatic fluid, he could isolate and extract the islets of Langerhans to create an injectable treatment. The twenty-one-year-old Best had only finished the final examinations for his biochemistry degree a day before beginning work with Banting. Their work paid dividends when one of their canine subjects that laid near-death responded almost immediately to an injection of pancreatic extract by raising its head and wagging its tail (**Figure 1**).

Figure 1. Frederick G. Banting and Charles H. Best (University of Toronto Archives).

Historically, insulin for treating the patients with DM was isolated from animals, primarily pigs and cows.

In 1958 Frederic Sanger was awarded the Nobel Prize in Chemistry for his work on the structure of proteins, especially insulin. Early in the insulin era the clinical consequence of the immunological reactions to extracts of animal pancreas included local and systemic allergic reactions and lipodystrophy. The purification of insulin has progressed since the first step, that of crystallization [1] to "high purified" insulin by chromatography in 1971 [2] to avoid unwanted side effects. Since 1978 semi synthetic human insulin was available and a bit later biosynthetic human insulin is produced with recombinant DNA methods having the identical sequence and chemical composition as insulin produced in the human pancreas. From that time there are few companies producing lots of different insulin analogous (rapid- or short-acting) for the treatment of DM. Further there are also oral antidiabetic drugs for management of the disease, diet and physical exercise. So the endocrinologists have the opportunity to individualize partly the treatment for the particular patient and its metabolic conditions.

1.2 Prevalence of diabetes mellitus and its complications

The World Health Organization (WHO) estimates that more than 180 million people worldwide suffer from diabetes. According to the International Diabetes Federation (IDF) there are even more people affected, about 246 million people worldwide and it is expected to be 380 million by 2025 (**Figure 2 and Figure 3**). The information about the prevalence of DM is calculated from known prevalence data from some parts of the world. So from that reason there is probably a difference between WHO and IDF. In 2007, the five countries with the largest numbers of people with DM are India (40.9 million), China (39.8 million), the

United States (19.2 million), Russia (9.6 million) and Germany (7.4 million). In 2007, the five countries with the highest DM prevalence in the adult population are Nauru (30.7%), United Arab Emirates (19.5%), Saudi Arabia (16.7%), Bahrain (15.2%), and Kuwait (14.4%). By 2025, the largest increases in DM prevalence will take place in developing countries.

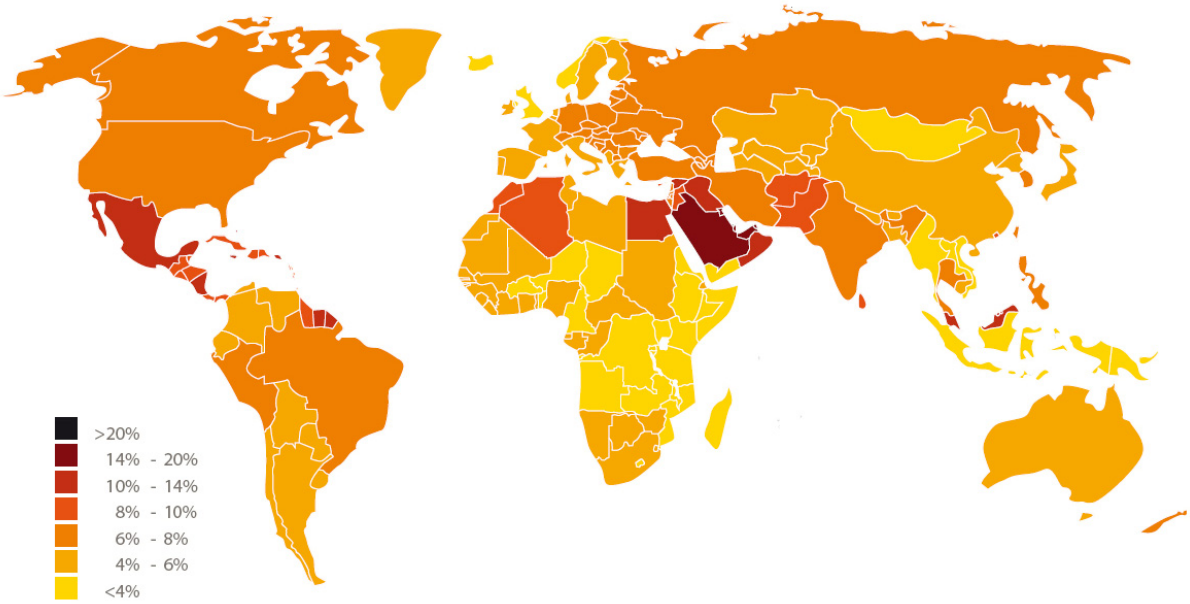


Figure 2. Prevalence estimates of DM in 2007 (Source: Diabetes atlas, 3rd ed., IDF 2006).

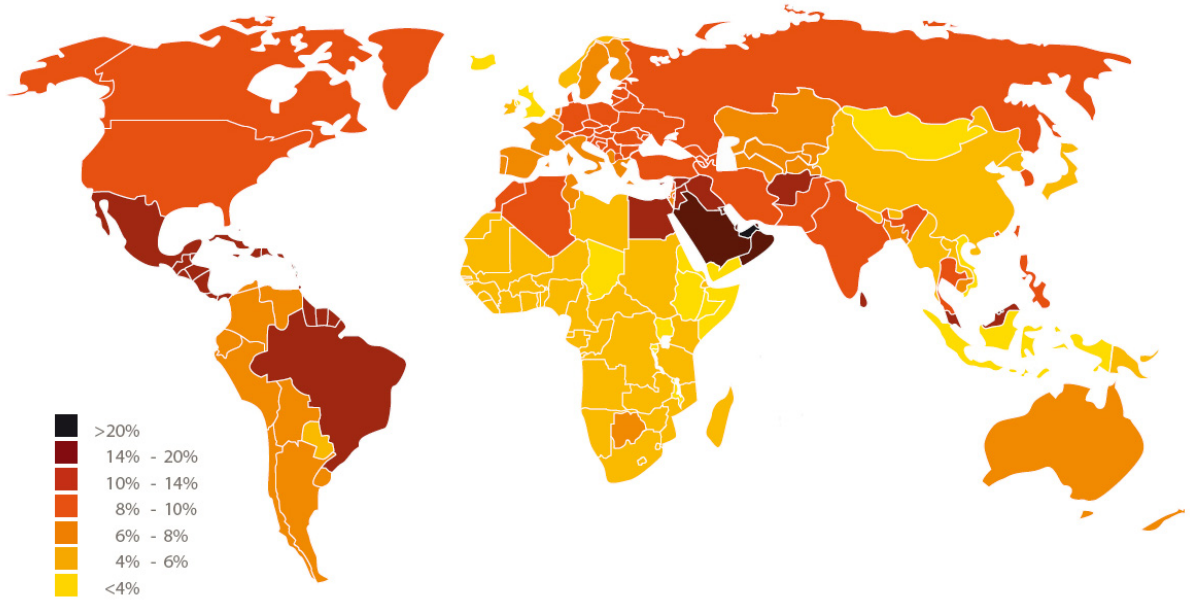


Figure 3. Prevalence estimates of DM in 2025 (Source: Diabetes atlas, 3rd ed., IDF 2006).

Each year 3.8 million deaths are attributable to DM. An even greater number die from cardiovascular disease (CVD) made worse by diabetes-related lipid disorders and hypertension (HT). Diabetes is the largest cause of kidney failure in developed countries and is responsible for huge dialysis costs. It is estimated that more than 2.5 million people worldwide are affected by diabetic retinopathy. Diabetic retinopathy is the leading cause of vision loss in adults of working age (20 to 65 years) in industrialized countries. CVD is the major cause of death in diabetes, accounting for some 50% of all diabetes fatalities, and much disability. It is very clear from these numbers that diabetes presents an epidemic that cannot be ignored. The challenge is that up to 80% of type 2 diabetes mellitus (T2DM) is preventable by adopting a healthy diet and increasing physical activity.

To meet the challenges of the epidemic of diabetes the medical doctors, patients and their relatives, scientists, all of them work together to manage this disease with all its complications the best way they are able.

There is one more serious epidemic connected to T2DM and it is obesity. 1.1 billion adults worldwide are overweight, and 312 million of them are clinically obese. If adjusted for ethnic differences, the prevalence is higher and 1.7 billion people would be classified as overweight. The WHO estimates for 2005, standardized for body mass index (BMI) ≥ 30 kg/m^2 and ages 15 -100 years, show a prevalence of 20.7% females and 18.5% males in the Czech Republic suffering from obesity. The rising levels of overweight and obesity, this "modern disease", drive the prevalence of chronic non-communicable diseases. There is very close connection among obesity, CVD and T2DM. In most developed countries, heart disease and stroke is the first and diabetes mellitus is the fourth leading cause of death, what is more deaths each year than AIDS. Obesity is almost invariably linked with hyperinsulinemia and as a consequence, the resistance to insulin-mediated glucose disposal develops. Insulin

resistance (IR) is considered as a core early abnormality in the pathogenesis of T2DM. Obesity, IR or DM together with other factors form a cluster of conditions referred to as insulin resistance syndrome. Originally termed as Syndrome X by Dr. Reaven, the syndrome has been assigned internationally as the dysmetabolic syndrome and the prevalence data clearly show that it is a large problem everywhere in the world; only in the U.S. it concerns 70-80 million people.

The epidemics of obesity and T2DM have emerged as the global public health issue of the 21st century. Excess weight gain and associated disorders may however be reversible. Through the regular physical exercise and the control of food intake, the patients can benefit from weight reduction. Even a 5% weight loss results in improvement of several metabolic parameters, mostly due to increased insulin sensitivity (IS). Diabetes and obesity are not an easy problem to tackle and it requires the action at the government level. Nevertheless without fundamental changes in national strategies in non-government sectors as food industry, the media and communities, the enormous costs of these health issues (direct, indirect and intangible) will be hard to reduce. Management of DM and its complications present a huge financial burden to society. Healthcare expenditures on diabetes are expected to account for 11.6% of the total healthcare expenditure in the world in 2010. About 80% of the countries are predicted to spend between 5% and 13% of their total healthcare on diabetes (**www.diabetesatlas.org/content/economic-impacts-diabetes**).

1.2.1 **Incidence of diabetes mellitus in the Czech Republic (www.uzis.cz)**

The number of treated diabetic patients steadily increases; the total number in 2008 was 774 thousand, almost 19 thousand more than in the previous year (2.4% increase). The number of patients treated only with diet decreases (210 thousand in 2008), whereas using

drugs grow up. The chronic diabetic complications are long-term around 27% in the group of treated patients. **Table 1** shows the percentage proportion of different types of DM with the numbers of patients suffering from DM in 2008.

Gender	Primary diabetes				Secondary diabetes		IGT
	T1DM		T2DM				
	number	%	number	%	number	%	number
Male	26 662	7.4	326 215	91.0	5 521	1.5	22 653
Female	27 812	6.7	382 632	92.2	4 719	1.1	27 966
Total	54 474	7.0	708 847	91.6	10 240	1.3	50 619

Table 1.Incidence of diabetes mellitus in Czech Republic according to different types.
T1DM – type 1 diabetes mellitus, T2DM – type 2 diabetes mellitus, IGT – impaired glucose tolerance

Treatment of DM in Czech Republic in numbers

27.1% of patients with T2DM were treated in 2008 with diet only. 47% of patients with DM were treated with oral hypoglycemic agents (OHA), 16.5% with insulin and 9.3% with the combination of OHA and insulin.

It is predicted that today there are still approximately 200 thousand undiagnosed diabetic patients in Czech Republic.

1.3 Classification and characterization of diabetes mellitus

1.3.1 Etiopathogenesis diabetes mellitus

DM is a complex multi-factorial group of metabolic diseases characterized by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, or action, or both. The main problem is that the chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. The most severe

are the blood vessels chronic complications, especially atherosclerosis. Lots of patients suffer from diabetic retinopathy with potential loss of vision, diabetic nephropathy (DN) leading to renal failure, peripheral neuropathy with risk of foot ulcers, amputations, and Charcot joints; and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms and sexual dysfunction. These complications of diabetes can present a big healthy issue for both, medical doctors and patients themselves for significant part of their life.

The latest classification of DM is based on current etiology knowledge **(Table 2)**. Some forms of DM are well characterized in terms of specific etiology or pathogenesis, but the underlying etiology of the most common forms remains still mostly unclear. The disorders grouped together under the term of diabetes differ markedly in pathogenesis, natural history, response to therapy, and prevention. Such heterogeneity has had important implications not only for treatment of patients with diabetes but also for biomedical research. The etiology of diabetes is very broad and varies from purely genetic (rare monogenic diabetes, e.g. MODY) to purely behavioral basis (e.g. T2DM due to obesity). Nonetheless, more common forms of diabetes are polygenic (polygenic/common diabetes). It represents a complex interaction of genetics and environment.

Diabetes mellitus	
Type 1 diabetes mellitus	autoimmune
	idiopathic
Type 2 diabetes mellitus	mostly insulin resistant
	mostly insulin deficient
Gestational diabetes mellitus	
Other specific types	see **Table 4**
Impaired glucose homeostasis	
Impaired fasting glucose	
Impaired glucose tolerance	

Table 2. Etiologic classification of DM.

The recent classification system for diabetes and related states of hyperglycemia has focused on etiopathogenesis whenever possible. The vast majority of cases of diabetes fall into two broad etiopathogenetic categories, type 1 and type 2 DM.

1.3.1.1 Type 1 diabetes mellitus

T1DM comprises approximately 5-10% patients with DM [3]. Previous terms for T1DM were insulin dependent diabetes or juvenile-onset diabetes, which correspond to the onset of disease in childhood and life dependence on insulin treatment. T1DM results from T-lymphocytes and macrophages mediated autoimmune progressive destruction of the insulin producing β-cells of the pancreas leading to absolute insulin deficiency [4]. The targeted autoimmune attack against β-cells of pancreas, not the other parts of the organ or other cell types, is due to aberrant expression of major histocompatibility systems molecules which makes the cells to become an object for immune response. This form is prone to ketoacidosis. The prediabetic stage can last even few years; during those the immune system attacks the β-cells. Nowadays we can distinguish between 2 subgroups of T1DM. One is named as **1A type** and it is possible to detect autoantibodies the other one is called **1B type – idiopathic type** of T1DM – no known autoantibodies can be detected. The etiology of 1B type of T1DM is not known yet, but it can be caused by some autoantibodies which we are currently unable to detect. Only a minority of patients with T1DM belongs into this category, most are of African or Asian ancestry [5]. This form is strongly inherited and it is know today that there is no human leukocyte antigen (HLA) association [5]. It does not include those forms of β-cells destruction or failure for which non-autoimmune specific causes can be assigned (e.g., cystic fibrosis).

Individuals at increased risk of developing 1A type of diabetes can be often identified by serological evidence of an autoimmune pathologic process occurring in the pancreatic islets and by genetic markers. Markers of the immune destruction of the β-cell include islet-cell autoantibodies (ICA), autoantibodies to insulin (IAA), autoantibodies to glutamic acid decarboxylase (GAD65), and autoantibodies to the tyrosine phosphatases IA-2 and IA-2β [6–14]. Antibodies to ICA, IAA, or GAD in a normoglycemic individual indicate a high likelihood for ultimate progression to T1DM [15]. At latest one but usually more of these autoantibodies are present in 85–90% of individuals when fasting hyperglycemia is initially detected [5]. The manifestation of the disease has few phases because the destruction of the β-cell mass may progress with different intensity. In this form of diabetes, the rate of β-cell destruction is quite variable, being rapid in some individuals (mainly infants and children) and slow in others (mainly adults) [16]. Individual with T1DM have low or undetectable levels of insulin and plasma C-peptide. The C-peptide is a short peptide which is produced during insulin synthesis when proinsulin is cleaved into its active form. Many individuals with form of type 1 diabetes eventually become dependent on insulin for survival and are at the risk of ketoacidosis. At the moment there are only 10% of physiological amount of β-cell mass there is clear clinical evidence of the disease with all the symptoms. T1DM can be diagnosed also in adulthood and then it is called latent autoimmune diabetes in adults (LADA) type of diabetes [16].

Genetic predisposition to T1DM

More than 85% of patients diagnosed with 1A type DM do not have family history of DM. However, there is clear familiar predisposition in type 1A DM. The concordance rate for

monozygotic is far less than 100%, with most studies placing the rate in the range of 25% to 50% [17]. The genetic part of the predisposition is about one third of the whole risk. For almost all autoimmune diseases researchers found some association with the genes of (HLA). So, there is no exception for T1DM which is having strong HLA associations, accounts for about half of the genetic susceptibility, with linkage to the DQA and DQB genes, and it is influenced by the DRB genes. These HLA-DR/DQ alleles can be either predisposing or protective [18-20]. Other important loci associated with T1DM include the insulin variable number of tandem repeats (INS-VNTR) [21], protein tyrosine phosphatase non-receptor type 22 (PTPN22) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) [22,23]. Extensive genetic research of human diseases, T1DM included, involves candidate gene association, linkage and for last approximately 4 years also genome-wide association studies (GWAS) (see Section 1.4.3.2.1). All to date known T1DM genetic associations have been detected on the basis of candidate gene approach. Some were confirmed via positional approach and GWA. Positional approach, so successful with monogenic Mendelian disorders, has resulted in the identification of 18 loci (named IDDM1-IDDM18) [24], of which almost all have turned out to be statistical artifacts due to underestimates of the sample size required for meaningful statistical power [25]. The same issues, the sample size and statistical power, have to be remembered for GWAS.

1.3.1.2 Type 2 diabetes mellitus

T2DM presents much more prevalent category, accounts for 90-95% diagnosed DM cases. This form was previously referred to as non-insulin-dependent diabetes (NIDDM), or adult-onset diabetes according to usually no need of insulin for survival and onset in

adulthood. The cause of this form of DM is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response **(Figure 4)**.

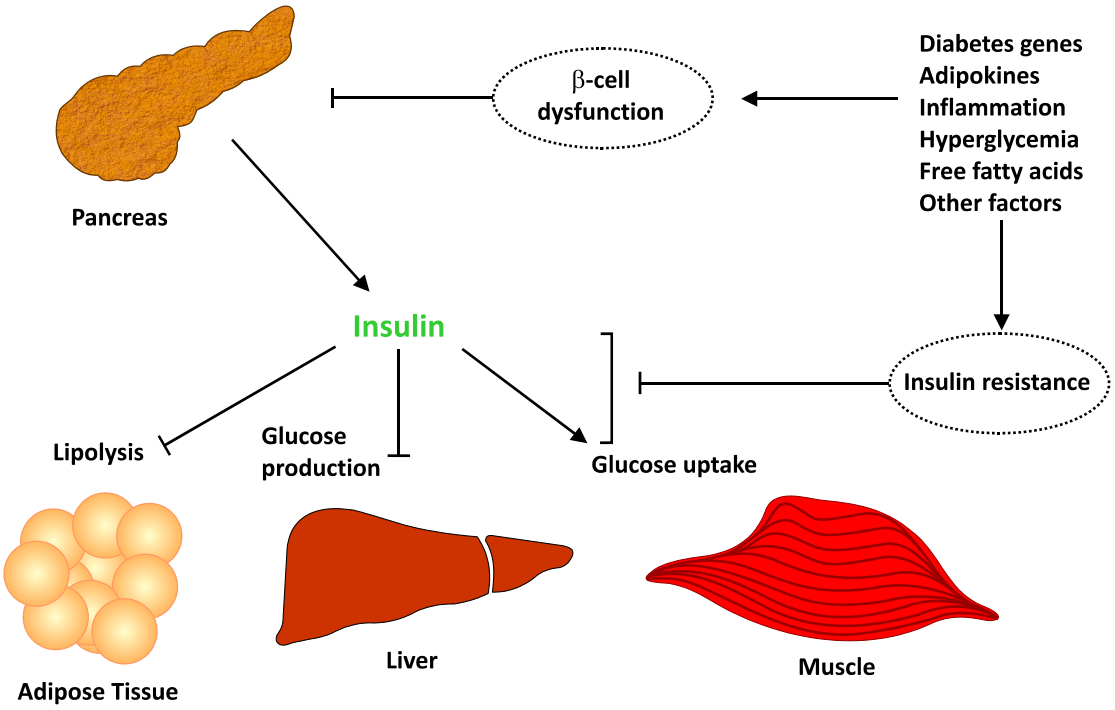


Figure 4. Insulin resistance and β-cell dysfunction.
(adapted from Stumvoll M et al., *Lancet* (2005), 365: 1333-46).

IR results in deficient metabolic effects of insulin, including the stimulatory effects of insulin on peripheral (predominantly skeletal muscle) glucose uptake and glycogen synthesis, suppression of glucose production by liver and the inhibitory effects of insulin on adipose tissue (AT) lipolysis. However the development T2DM appears to require an additional defect in insulin secretion [26]. The mechanisms of IR, as well as the cause of β-cell failure and insulin deficiency are complex and still not fully understood.

Defects in insulin action and impairment of insulin secretion frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of

the hyperglycemia. Although the specific etiologies of this form are not known, most of the patients suffer from obesity or may have an increased percentage of body fat distributed predominantly in the abdominal region. Obesity itself causes some degree of insulin resistance [27,28]. T2DM patients may have insulin values appearing normal or elevated. If their β-cell function is normal, individuals can compensate the IR, which is the cause of higher blood glucose levels, with higher insulin levels (hyperinsulinemia). Hence, some individuals who are suffering from IR may never progress to T2DM. Unfortunately it was shown by Despres with colleagues that the risk of atherosclerosis is apparently comparable in nondiabetic, IR individuals and those with T2DM [29]. More often the insulin secretion is defective in these patients and insufficient to compensate for insulin resistance. In some cases adequate glycemic control can be achieved with weight reduction, exercise, and/or oral glucose lowering agents. Treatment of hyperglycemia usually rapidly improves the IS but the blood glucose level is seldom restored to normal. Only some patients with T2DM need insulin treatment throughout their lifetime. Ketoacidosis seldom occurs spontaneously in this T2DM category; usually it arises only in association with the stress of another illness such as infection. No evidence for autoimmune destruction of β-cell occurs. The hyperglycemia develops gradually and may stay unrecognized for several years; however, as mentioned above such patients are at increased risk of developing macrovascular and microvascular complications. In the future the identification of specific pathogenic processes and genetic defects, which are still mostly unknown, will permit better subclassification of these patients. If we want to predict the risk of developing T2DM for a particular person there are currently a few known risk factor we should consider. They are summarized in **Table 3.** Genetic part of the risk for T2DM is described in Chapter 1.4.3.

Basic risk factors
Ancestry
Age (increases with age over 45)
Family anamnesis (T2DM, abdominal obesity)
Impaired glucose tolerance (IGT)/impaired fasting glucose (IFG)
Gestational diabetes mellitus (GDM) in anamnesis
Hypertension
Dyslipoproteinemia (low concentration of HDL-cholesterol and higher concentration of triacylglycerols)
Other risk factors
Undernourishment in the 1st year of the life
Malnutrition of the fetus before parturition
Life factors (can be influenced)
Overeating
Diet with high content of fat (low content of sacharides)
Lack of physical activity
Smoking

Table 3. Risk factors for development of T2DM.

1.3.1.3 Special types of diabetes mellitus

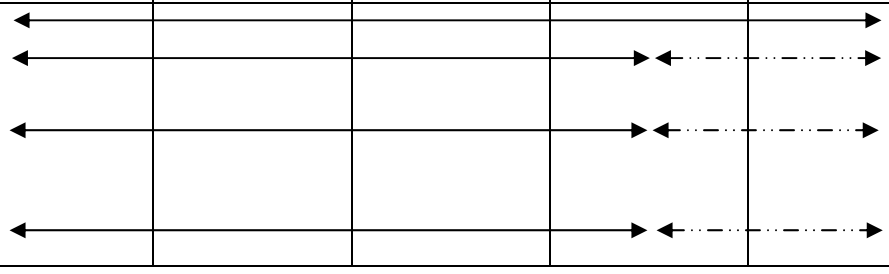
Other specific types of diabetes are those in which the underlying defect or disease process can be identified in a relatively specific way or those that have other distinctive, distinguishing features. This category encompasses a variety of types of diabetes secondary to other specific condition or associated with particular disease or syndromes with distinct etiology. The categories and many of other specific types of DM are shown in **Table 4**. These include genetic defects of β-cell function, which encompass several types of DM that are associated with specific monogenic defects (e.g. most often maturity-onset diabetes of the young (MODY) (see Chapter 3) or a mitochondrial variant Leu3243Ala, which leads to DM associated with deafness [30]).

Genetic defects of β-cell function
Maturity-onset diabetes of the young
Mitochondrial DNA, A3242G mutation
Genetic defects in insulin action
Type A insulin resistance
Leprechaunism
Others
Other genetic syndromes sometimes associated with diabetes
Down syndrome
Huntington disease
Others
Uncommon forms of immune-mediated diabetes
Insulin autoimmune syndrome (antibodies to insulin)
Anti-insulin receptor antibodies
Others
Disease of the exocrine pancreas
Pancreatitis
Trauma/pancreatectomy
Neoplasia
Cystic fibrosis
Hemochromatosis
Others
Endocrinopathies
Cushing syndrome
Glucagonoma
Hyperthyroidism
Others
Drug and chemical induced
Nicotinic acid
Glucocorticoids
Others
Infections
Congenital rubella
Cytomegalovirus
Others

Table 4.Other specific type of diabetes mellitus (more details can be found [31]).

1.3.2 Clinical stages

There are several clinical stages known for each type of DM. Diabetes usually progresses to more severe one but in some types of diabetes it can also be the opposite way around **(Table 5)**.

Stages / Types	Normoglycemia		Hyperglycemia		
			Diabetes mellitus		
	Normal glucose tolerance	Impaired glucose regulation	Not insulin requiring	Insulin requiring for control	Insulin requiring for survival
Type 1					
Type 2*					
Other specific types*					
Gestational diabetes*					

* In rare instances patients in these categories may require insulin for survival. The bold line presents the progression of the disease (in some cases it could be very slow progression) and where the line is dotted the stage can be influenced and get better with e.g. diet or exercise.

Table 5. Clinical stages and etiologic types of diabetes.

If we exclude permanent neonatal diabetes mellitus (PNDM) then initially, glucose regulation is normal and no abnormality of glycemia can be identified even if the individuals undergo an oral glucose tolerance test (OGTT). This stage is followed by a period of variable duration in which glucose regulation is impaired. They may have some abnormality of the fasting glucose concentration, or if they receive an OGTT, they may demonstrate impaired glucose tolerance. Once diabetes develops, glycemia may be controlled by lifestyle changes such as diet and increased physical activity in some patients, whereas in others insulin or oral hypoglycemic agents are needed for its control or to prevent ketosis and ketoacidosis. If insulin is required to prevent ketosis, such patients are designated as "insulin requiring for survival." In all forms of diabetes, there may be a remission in the extent of hyperglycemia. This is seen most frequently in patients with recent-onset T2DM but it may also be seen in T1DM, in which after short period of insulin treatment, there may be a variable period when insulin is no longer required for survival and glucose tolerance may improve – the so-called honeymoon period. Eventually such patients do need insulin treatment for survival [32]. All

subjects with diabetes can be classified according to clinical stage regardless of the underlying etiology of the diabetes. This applies to both men and women, independently of age.

1.3.3 **Diagnosis of impaired glucose homeostasis and diabetes mellitus**

Since 1965 WHO has published guidelines for the diagnosis and classification of DM. These were last reviewed in 1998 and were published as the guidelines for the Definition, Diagnosis and Classification of DM and its complications. Since then more information relevant to the diagnosis of DM has become available and also the more detailed knowledge about the connection between blood glucose level and complication of DM, so according to this information the criteria are reviewed and updated. In November 2005 a joint WHO and International Diabetes Federation (**www.idf.org**) Technical Advisory Group met in Geneva to discuss current WHO guidelines. After consideration of available data and recent recommendations made by other organizations, the Group made several updated recommendations (**www.who.int/diabetes/publications/Definition and diagnosis of diabetes_new.pdf**).

There are few more organization contributing to the discussion about collection, analysis, and dissemination of data on diabetes and its complications: the American Diabetes Association (ADA) (**www.diabetes.org**) and the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) (**www2.niddk.nih.gov**) which is part of the National Institute of Health (NIH) and the U.S. Department of Health and Human Services. In 1979 the National Diabetes Data Group (NDDG) published classification and diagnosis criteria used in the U.S. until 1997 [33]. An international Expert Committee working under the sponsorship of the ADA was established in May 1995 to review the scientific literature since 1979. The Committee assessed all the data and published a comprehensive review [5].

In clinical practice, establishing the diagnosis of diabetes is seldom a problem. Diabetes is characterized by either fasting glycemia or marked abnormalities of glucose tolerance, or both. When symptoms of hyperglycemia exist (thirst, polyuria, unexplained weight loss, etc.) a random plasma glucose concentration of ≥ 11.1 mmol/l (200 mg/dl) or a fasting plasma glucose (FPG) of ≥ 7.0 mmol/l (126 mg/dl) confirms the diagnosis. The main change in the diagnostic criteria for diabetes proposed by both ADA and WHO from their previous identical recommendation is the lowering of the diagnostic value of the fasting plasma glucose concentration to 7.0 mmol/l from the former level 7.8 mmol/l and above. For whole blood the proposed new level is 6.1 mmol/l and above, from the former 6.7 mmol/l. Venous plasma glucose should be the standard method for measuring and reporting glucose concentration in blood. If a blood sample is collected, plasma should be immediately separated, or the sample should be collected into a tube with preservatives prevent glycolysis. If whole blood glucose is used, the sample should be kept at 0 - 4 °C or centrifuged immediately, or assayed immediately. In recognition of the widespread use of capillary sampling, especially in under-resourced countries, conversion values for capillary plasma glucose are provided for post-load glucose values **(Table 6)**. Fasting values for venous and capillary plasma glucose are identical. If the fasting glucose concentration is in the diagnostic range for diabetes, an OGTT is not required for diagnosis. A confirmatory test should be performed because a diagnosis of diabetes carries considerable and lifelong consequences for the patient.

		glucose concentration mmol/l (mg/dl)			
		whole blood		plasma	
		venous	capillary	venous	capillary
DM	Fasting ***OR***	≥ 6,1 (110)	≥ 6.1 (110)	≥ 7.0 (126)	≥ 7.0 (126)
	2h post-glucose load	≥ 10.0 (180)	≥ 11.1 (200)	≥ 11.1 (200)	≥ 12.2 (220)
IGT	Fasting (if measured) ***AND***	< 6.1 (110)	< 6.1 (110)	< 7.0 (126)	< 7.0 (126)
	2h post-glucose load	≥ 6.7 (120) and < 10.0 (180)	≥ 7.8 (140) and < 11.1 (200)	≥ 7.8 (140) and < 11.1 (200)	≥ 8.9 (160) and < 12.2 (220)
IFG	Fasting	≥ 5.6 (100) and < 6.1 (110)	≥ 5.6 (100) and < 6.1 (110)	≥ 6.1 (110) and < 7.0 (120)	≥ 6.1 (110) and < 7.0 (120)
	2h post-glucose load	< 6.7 (120)	< 7.8 (140)	< 7.8 (140)	< 8.9 (160)

Table 6. Values for diagnosis of DM and other categories of hyperglycemia [34].

The ADA recognizes an intermediate group of subjects whose FPG is ≥ 6.1 mmol/l (110 mg/dl) but < 7.0 mmol/l (126 mg/dl) and has defined this group as having IFG. It has recently been suggested by the ADA that the FPG level to diagnose IFG should be reduced from ≥ 6.1 mmol/l (110 mg/dl) to ≥ 5.6 mmol/l (100 mg/dl). A further abnormal category is defined as having a plasma glucose ≥ 7.8 mmol/l (140 mg/dl) but < 11.1 mmol/l (200 mg/dl) at 2 h when an OGTT is used and is described as IGT. Where diagnostic difficulty exists, the precise diagnosis can be established with an oral glucose tolerance test (OGTT) using a 75 g anhydrous glucose load dissolved in water: a 2 h venous plasma glucose value ≥ 11.1 mmol/l (200 mg/dl) establishes the diagnosis of diabetes. The OGTT is not recommended for routine clinical use, but may be an important test for epidemiologic purposes and it is used in the screening for GDM.

Glycated hemoglobin (HbA1c) reflects average glycemia over the previous 2 - 3 months in a single measure which can be performed at any time of the day and does not

require any special preparation such as fasting. In certain cases, it gives equal or almost equal sensitivity and specificity to glucose measurement but the actual threshold value has differed between studies we can compare nowadays. Further there are aspects of HbA1c measurement which are problematic, e.g. anemia, pregnancy or uremia can influence the result. Furthermore lack of standardization and its unavailability in many parts of the word make it difficult to recommend it as a good alternative at this time. Therefore, currently HbA1c is not considered a suitable diagnostic test for diabetes or intermediate hyperglycemia.

1.4 Type 2 diabetes mellitus

1.4.1 Metabolic syndrome

The metabolic syndrome is a long-term process, with complex predisposition having genetic and environmental part, that can progress many years to manifestation and is involved in the pathophysiology of a large percentage of cases with T2DM and atherosclerosis [35-37] The term “metabolic syndrome” (MS) was originally coined in 1970s [38]. In 1988 Gerald M. Reaven proposed insulin resistance as the underlying factor and named the constellation of abnormalities Syndrome X. Reaven did not include abdominal obesity, which has also been hypothesized as the underlying factor, as part of the condition [39]. Nowadays MS is also known as metabolic syndrome X, syndrome X, insulin resistance syndrome or Reaven's syndrome. Recent criteria have defined MS as a cluster of symptoms, including large waist size (abdominal obesity), HT (130/85 mm Hg or greater), hyperglycemia, dyslipidemia (high levels of triglycerides (TG), low levels of high-density lipoprotein (HDL) cholesterol) and IR, all of which are commonly associated with the increased prevalence of obesity and T2DM [40] and make the issue even more complex . In essence, unlike diabetes or obesity, MS is not a specific disease but simply a cluster of factors which jointly put one at risk for developing a

cascade of detrimental CVD. The two primary approaches to optimal control risk factors associated with MS are lifestyle changes and medications. However, a confirmed status of MS is having clinical and therapeutic relevance because treating a single component may or may not rectify other components or reduce the prevalence of T2DM, obesity or MS related atherosclerosis. Patients with MS are at increased risk for developing CVD. The Diabetes Prevention Program demonstrated that either a rigorous regimen of diet and exercise or metformin can prevent or delay DM [41]. Diagnosing the MS in these patients would allow earlier treatment and thereby reduce the likehood of their developing CVD. Interrelated disorders of MS like T2DM or obesity have become a major worldwide health problem.

1.4.2 **Insulin resistance in pathogenesis of type 2 diabetes mellitus**

IR presents resistance to one or several insulin's biological functions, involves the suppressive effects of insulin to endogenous glucose production, the stimulatory effects of insulin on peripheral (predominantly skeletal muscle) glucose uptake and glycogen synthesis, and the inhibitory effects of insulin on adipose tissue lipolysis. It is generally accepted that IR plays a major role in the development of T2DM [42]. The mechanisms of IR, as well as the cause of β-cell failure and insulin deficiency are complex and that makes the treatment strategy very difficult. IR can develop in all insulin-responsive tissues, classically central (liver) and peripheral (AT and skeletal muscle), but also in the pancreas, kidney and brain among others. Hence IR is highly heterogeneous in terms of the primary causes, development or biochemical pathways. In normal conditions, skeletal muscle mass represents the largest tissue mass in humans and contributes the most to total body glucose disposal. The identification of the link between adiposity and impaired IS in skeletal muscle has proved difficult. Recently there are different opinions about the primary tissue of IR. In addition to

skeletal muscle some scientists speculate about AT as a primary site of IR, and some others about liver. More data are still expected to answer such question.

1.4.2.1 The role of skeletal muscle in insulin resistance

Skeletal muscle is quantitatively the most important tissue in systemic glucose homeostasis, because it accounts for approximately 80% of glucose disposal following glucose infusion or ingestion [26,43]. Thus decrease ability of insulin to stimulate glucose disposal by this tissue is of considerable importance to whole-body glucose homeostasis. Some very interesting findings about the role of skeletal muscle as a site of insulin action was obtain from experiments with transgenic mouse models. It was shown that inactivation of insulin receptor in skeletal muscle causes the changes in fat metabolism, but the animals do not develop hyperinsulinemia and diabetes [44,45]. Unlike some solution to compensate insulin receptor changes the targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance [46,47]. It was shown in mice lacking the insulin receptor in muscle that both the contraction-activated pathway [48,49] and the insulin-like growth factor I (IGF-I) signaling pathway [50] can compensate for the ablation of insulin signaling. The former, which activates adenosine monophosphate (AMP)-activated protein kinase and thereby stimulates translocation of glucose transporter, remains intact [51]. There is a considerable amount of interesting but partial results from in vitro or animal experiments, but it seems the whole-body studies of glucose metabolism in humans are needed for further understanding of metabolic disorders associated with alteration in glucose homeostasis [52]. In 1998 a new surgical biopsy procedure to obtain rectus abdominal skeletal muscle specimens under general anesthesia was presented [53]. For

example, with this methodological advance it is now possible to do detailed studies of insulin action on intracellular events in human skeletal muscle.

1.4.2.2 The role of adipose tissue in insulin resistance

Obesity and IR are strongly related. Recently, three theories apply to explain the contribution of AT into the development of obesity-linked diseases, the theory of "portal paradigm"/"portal/visceral fat theory" [54-56], "endocrine paradigm" [57,58], and "ectopic fat storage syndrome"/ "ectopic fat deposition" theory [59,60]. These theories are discussed in more detail in Section 1.4.2.2.2 and 1.4.2.2.3 below. Although an enormous progress has been done regarding better understanding of the mechanisms underlying obesity-linked IR during past decade, none of these theories is universally accepted. New data are still expected to answer such questions as: "Is IR the consequence of obesity with respect to regional adiposity, or is it IR that contributes to obesity and visceral fat development?" "Fatty acids, hyperinsulinemia, and IR: which comes first?" [61]

Previously, the biological function of AT was considered to be the long-term repository for energy excess, having metabolic activities such as lipogenesis (storage of free fatty acids (FFA) in the form of TG) and lipolysis (release of FFA and glycerol from the TG). Recently, it has become clear that AT is an endocrine organ producing hormones, adipokines and other peptides. Sustained imbalance between energy intake and energy expenditure favoring positive energy balance leads to obesity. Obesity has genetic and environmental background. Increasing adiposity activates local, portal and systemic effects on inflammation in IR-states. Increased FFA amounts derived from enhanced AT-lipolysis (together with adipokines, drained from visceral adipocytes) delivered to the portal system contribute to

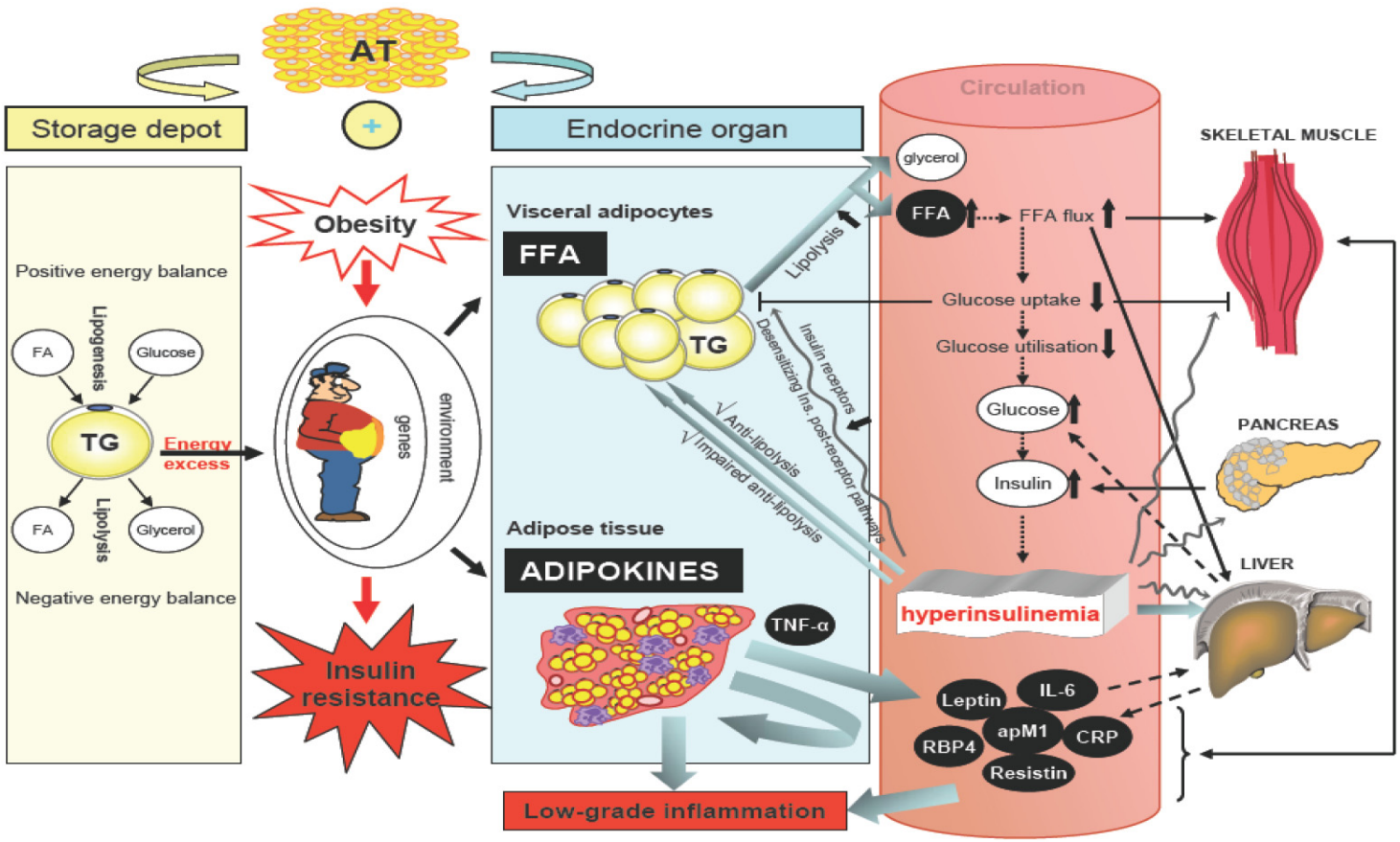


Figure 5. Role of adipose tissue in the development of insulin resistance.

AT-adipose tissue, IR-insulin resistance, FFA-free fatty acid, TG-triglyceride, TNF-a-tumor necrosis factor alpha, IL-6-interleukin 6, apM1-adiponectin, RBP4-retinol binding protein, CRP-C-reactive protein

hepatic inflammation and IR. Increased production of several adipokines (cytokines and chemokines) from AT causes local inflammation and IR. Sustained production of pro-inflammatory and decreased release of anti-inflammatory cytokines by AT, liver or associated immune cells creates a systemic low-grade inflammatory state that promotes IR at the periphery (skeletal muscle and other tissues) **(Figure 5)**.

1.4.2.2.1 Obesity

Inappropriate energy balance between the food intake and consumption can be the cause of obesity and increased mass of normally functioning AT may produce in obese increased concentrations of FFA in the blood, which reduces glucose utilization in muscle and AT. Too much of FFA can result in systemic IR.

1.4.2.2.2 Fatty acids as candidate mediators of insulin resistance

The inhibiting effect if insulin on the release of FFA from AT is halted in condition of IR. Elevated fasting and postprandial levels of free fatty acids (FFAs) are typical hallmark of obesity and other IR states [62,63]. The first and earliest hypothesis of "portal paradigm"/"portal/visceral fat theory" highlights the central role of visceral AT (VAT), adipocyte and FFAs in the development of IR. It is based on two lines of investigations, an increased lipolytic activity of visceral adipocytes and a venous drainage of intraperitoneal VAT [54,64]. According to several studies, visceral adipocytes are more resistant to the anti-lipolytic effect of insulin and together with enhanced sensitivity to catecholamines' lipolytic action, they feature a high release of FFAs [65-69]. Intraperitoneal VAT drains into portal vein and hence there is a direct FFA flux into the liver [64], which in turn may modify hepatic lipid metabolism (increase TG synthesis and VLDL), impair insulin-inhibition of hepatic glucose production and result in hepatic IR [70,71]. In 1963, Randle and colleagues

postulated the concept of substrate competition (FFAs and glucose) to explain the adverse effect of FFAs at periphery, the so called glucose fatty-acid or Randle cycle [72]. They suggested that in skeletal muscle, which is the major site of insulin-mediated dietary glucose uptake (approximately 75%) [73], chronically available FFA become preferred substrate over glucose.

However, several issues that do not support the role of VAT and FFAs in the control of the whole-body IS were raised. Surprisingly, the generally accepted notion of increased FFA levels in insulin-resistant obese subjects was recently argued [74] referring to the study that demonstrated fasting FFA concentrations not to be increased in this population [75]. Another line of evidence that cast doubt on the portal fat hypothesis is the question of FFAs origin in the systemic circulation. It has been shown that subcutaneous adipose tissue (SCAT) remains the predominant contributor of systemic FFAs and accounts for approximately 70% of total FFAs in the circulation in lean subjects (lower amounts are seen in obese when compared to lean) [76,77]. If the role for VAT is presumed, one would expect a substantially higher contribution of VAT into systemic FFA concentrations.

It remains unclear if FFA elevation is the primary event causing IR and its compensatory consequence, hyperinsulinemia like an effort to regulate FFA outflux efficiently.

1.4.2.2.3 Adipokines as candidate mediators of insulin resistance

The second theory “endocrine paradigm” was developed together with the hypothesis of “ectopic fat storage syndrome”. Based on the endocrine function of AT, the “adipocentric view” of the pathogenesis of IR has emerged, and implies bioactive molecules secreted by AT as the main culprits in IR. Research on adipocytes has revealed that they produce and secrete

a variety of bioactive substances, named “adipocytokies or adipokines”: these include growth factors, cytokines and complement factors. It has become clear that AT is an endocrine organ that may affect the function of other organs by secreting a variety of adipokines [78]. The importance of adipokines is highlighted by the fact that AT is one of the largest organs in the body [79] and adipokines released from adipocytes flow easily into the systemic circulation [80]. The most studied adipokines are: adiponectin, leptin, resistin, visfatin, apelin, TNF-a, IL-6, retinol binding protein 4 (RBP4) and others.

1.4.2.2.4 Environmental factors of insulin resistance

In addition to metabolic issues which are discussed in this thesis there are also environmental factors which play a role in IR. Factors such as poor nutrition (high-fat diet) or inactivity (lower oxygen consumption i.e. VO2max) or both also seem to have a major effect on the development of peripheral insulin resistance [81-83].

1.4.3 Genetics of type 2 diabetes mellitus

T2DM presents genetically, pathophysiologically and phenotypically heterogeneous group of metabolic disorders sharing glucose intolerance. The combination of genetic, environmental and social factors (**multifactorial disease**) contributes to a manifestation of the disease and nowadays it is clear that not only one or one major gene but many genes are involved in T2DM susceptibility (**polygenic disease**). The increased prevalence in families (familial aggregation) is often summarized in terms of the sibling relative risk (λs: the ration of disease prevalence in the sibling of affected compared with that of general population) and according to Framingham Offspring Study [84], the risk of T2DM manifestation for sibling of one affected parent is 3.5 times higher in comparison to general population, the risk of sibling of both affected parents is 6.1 x higher. There is one stronger tool in genetics which can be

used to prove the genetic component of the predisposition, and it is the different concordance rates between monozygotic and dizygotic twins. It is estimated 34% for monozygotic twins and 16% for dizygotic twins for T2DM [85]. The huge differences in prevalence for separate ethnic groups (40% in Pima Indians) are clearly consistent with the genetic component susceptibility. The last evidence for genetic component of T2DM is presented through the theory of "thrifty genotype" [86], which supposes the existence of evolutionary preferential genotype allowing the maximum storage of energy. Nowadays we have the permanent abundance of the food and lack of physical activity so it happened that from advantageous genotype became disadvantageous ones. Based on modeling of the risk of T2DM in relatives of patients [87], Rich concluded that best fit model incorporated only a "few genes" with a moderate effect, superimposed on a polygenic background.

Two main approaches have been followed to identify genes for multifactorial forms of T2DM: A) candidate genes studies and B) positional candidate genes studies including whole-genome screens.

1.4.3.1 Candidate-gene association studies

The main idea of this type of studies is to compare the frequency of candidate gene polymorphisms between unrelated patients and control individuals. Candidate genes can be chosen from their known or presumed function which suggests that disturbance of their role in physiology might contribute to disease pathogenesis, from human or animal models or from positional cloning. There has not been a high success rate from the candidate gene studies (more than 200 candidate genes) carried out to date [88]. Only 2 candidate-genes for association with T2DM have stood the test of the time. The Pro12Ala variant in *PPARγ* gene and the Glu23Lys variant in *KCNJ11* are both common polymorphisms shown in lots of

studies to influence risk of T2DM. Their effect sizes are only modest; each copy of the susceptible allele increases the risk.

1.4.3.1.1 Genes involved in insulin signaling

Insulin action at the molecular level is the result of a complex network of signaling events (Figure 6). The principal events of insulin signaling cascade with the stimulatory effect on glucose uptake and cell proliferation [89,90], are illustrated in **Figure 6**. After insulin binds to the α-subunit of the insulin receptor, the tyrosine kinase activity of the insulin receptor β-subunit is activated and several key proteins within the cell are phosphorylated, including receptor itself, the insulin receptor substrates (IRS) and Shc [91-94]. Six IRS isoforms have been identified till now, IRS1-6 [95]. The activated insulin receptor phosphorylates major IRS isoforms, IRS1 and IRS2 on their tyrosine residues. IRSs possess over 20 potential tyrosine residues that can act as docking sites for downstream signaling proteins [96]. After IRSs phosphorylation, the signal is conducted downstream via binding of Src homology 2 (SH2) domain-containing downstream signaling proteins (PI3K, SHC, SHP2, Fyn, Grb2 among others) to tyrosine phosphorylated residues. Among the best studied SH2 proteins are adaptor molecules, such as the regulatory subunit p85 of the enzyme phosphatidylinositol 3-kinase (PI3K) and growth receptor-binding protein 2 (Grb2). These signaling molecules can associate with IRS-1 and activate two main signaling pathways, the PI3K and the ras-mitogen activated protein kinase (MAPK) pathway through several events. These events involve recruitment of PI3K heterodimer complex that consists of a regulatory subunit of approximately 85 kDa and a catalytic subunit of 110 kDa (p85-p110) [97] to the plasma membrane, thus generating lipid second messenger phosphatidylinositols. PI3K has been implicated as a key signaling transducer in insulin-mediated glucose transport in skeletal

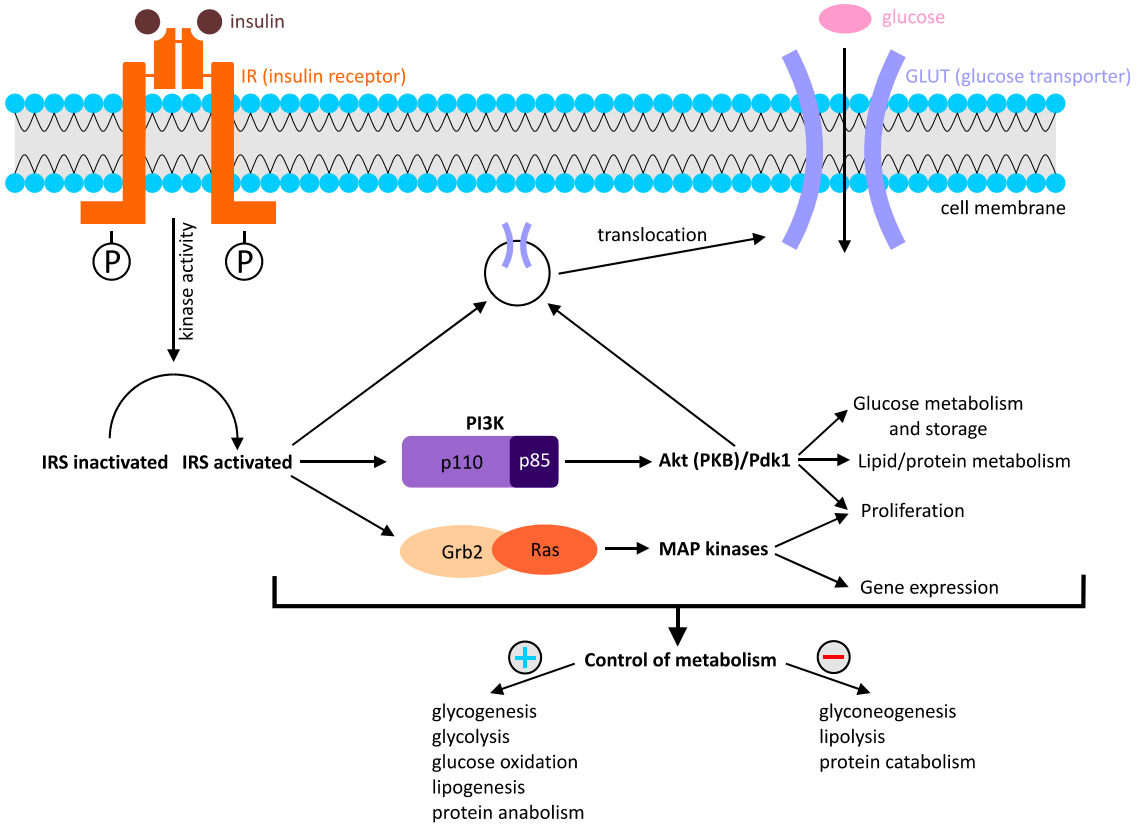


Figure 6. Insulin signaling - two main signaling pathways: PI3K and the ras-mitogen activated protein kinase (MAPK) pathway.

muscle and adipocytes [98-100] and there are at latest eight different isoforms of the regulatory subunit. Six are derived by alternative splicing and two other isoforms are derived from separate genes [94]. Thus, each subunit might have a specific function dependent on its specific affinity to IRS proteins and ability to regulate PI3K activity. It is also possible that each isoform participates in specific subcellular compartmentalization [94]. Downstream molecules with PH domains such as 3-phosphoinositide-dependent protein kinases (PDK1 and PDK2) can bind to PIP3, and further phosphorylate and activate protein kinase B (PKB)/AKT or atypical protein kinase C (aPKC) [95]. This process promotes most of the metabolic actions of insulin, e.g. translocation of vesicles containing glucose transporter 4

(GLUT4) from the intracellular pool to the plasma membrane ensuing glucose uptake in adipocytes and skeletal muscle. A potential target responsible for the GLUT4 translocation is Rab-GTPase-activating protein AKT substrate of 160 kDa (AS160). However, the downstream signals of PI3K are not well documented. In addition to PI3K pathway, other alternative pathways seem to be required for insulin-stimulated glucose uptake. The signaling molecule Grb2, which exerts large interaction with IRS1, is constitutively associated with Sos, the guanine nucleotide exchange factor for plasma membrane-bound Ras [101]. Docking of Grb2 to IRS proteins recruits Sos to Ras, resulting in activation of its GTPase [102] and can activate as well serine/threonine kinase cascade known as the MAPK pathway that promotes mitogenic effects of insulin (cell growth and differentiation) by some of the four MAPKs (ERK, JNK, p38, ERK/Big MAPK 1), most probably through extracellular regulated mitogen-activated protein kinase (ERK) pathway.

Genetic alterations in the insulin signaling genes

Naturally occurring mutations of the insulin receptor are rare, and the phenotype, when present, is usually a syndrome of extreme IR, such as leprechaunism. In other syndromes of insulin resistance, insulin receptor abnormalities remain the exception [103].

IRS1 was found to have multiple natural polymorphisms from which some occur more significantly in type 2 diabetic patients than in controls. These include the G972R, S892G, G818R, and A513P variants [104,105]. Few more polymorphisms and mutations were detected in the *IRS-1* gene. It was found they are associated with obesity and T2DM in some populations, but it was confirmed many times that none of these is a major contributor to genetic susceptibility to obesity or T2DM. Interestingly they get very close to *IRS* gene with

association resulting from GWAS so, we can speculate about the connection to the IRS function [106].

A common polymorphism of the regulatory subunit p85 of PI3K, Met326Ile, is having probably only a minor impact on signaling events; however, in combination with variants in other genes encoding signaling proteins, this may have a functional impact on early insulin signaling and IS [107,108].

1.4.3.1.2 Peroxisome proliferator activated receptors

Nuclear hormone receptors (NRs) are important transcriptional regulators involved in widely diverse physiological functions such as control of embryonic development, cell differentiation, and homeostasis [109,110]. These molecules are extremely important in medical research since a large number of them are implicated in diseases such as cancer, diabetes, or hormone resistance syndromes and that is the reason they become often targets for new drugs. This large superfamily of NRs comprises form six subfamilies; peroxisome proliferator activated receptors (PPARs) belong to the group C in the largest subfamily 1 (NR1C) [111]. Since the discovery of the first PPAR by Issemann and Green in 1990 [112], the role of this class of NRs in normal physiology and pathophysiology has become progressively studied. PPARs play a key role in regulating inflammation, lipoprotein metabolism, and glucose homeostasis and that is why it is very important to understand in details the biochemical pathways they are included and try to find out their potential implications for the treatment of governable diseases. Drugs for the treatment of T2DM and associated diseases such as obesity, dyslipidemia, or hypercholesterolemia are targeted at peroxisome proliferator activated receptors (PPARs) as many links were suggested between the excess of AT and PPARs [113].

A trio of PPAR isotypes encoded by separate genes, PPARα (NR1C1), PPARβ/δ (NR1C2, NUC-1, FAAR) and PPARγ (NR1C3) (with two subforms PPARγ1 and PPARγ2) have been identified to date [111]. These three isoforms share 60% to 80% homology in their ligand- and DNA-binding domains [114]. PPARs great potency, different ligand-activation profiles and diverse cell, tissue and organ distribution gives them a broad range of physiological functions **(Table 7)**.

PPAR isoform	Expressed
PPARα	**Brow AT, liver**, kidney, cardiac tissue, skeletal muscle, VSMCs, endothelial cells, monocytes/macrophages, T-lymphocytes
PPARβ/δ	Numerous tissue (AT, skin, skeletal muscle, cardiac tissue,…)
PPARγ	**AT**, colon, VSMCs, endothelial cells, macrophages, T-lymphocytes

Table 7. Localization of PPARs expression (higher expression is marked with bold font)

AT – adipose tissue, VSMCs – vascular smooth muscle cells

Despite an intensive research, our understanding of PPARs roles is far from being complete. The functions we know about them today are summarized in **Figure 7**. PPARα is believed to participate in fatty acid uptake (β- and ω-oxidation) mainly in the liver and heart. PPARβ/δ is the most ubiquitously distributed isotype, involved in fatty acid oxidation in muscle. PPARγ is highly expressed in AT to facilitate glucose and lipid uptake, stimulate glucose oxidation, decrease free fatty acid level and ameliorate insulin resistance.

PPARs are ligand dependent transcriptional regulators which heterodimerize with a retinoid X receptor and bind to peroxisome proliferator response elements (PPREs) in the promoters of target gene [115] **(Figure 8)**. In the absence of ligands, PPARs can repress transcription through the recruitment of co-repressors, or by other mechanisms such as by antagonizing other transcription factors, e.g. NFκB or activator-protein [116].

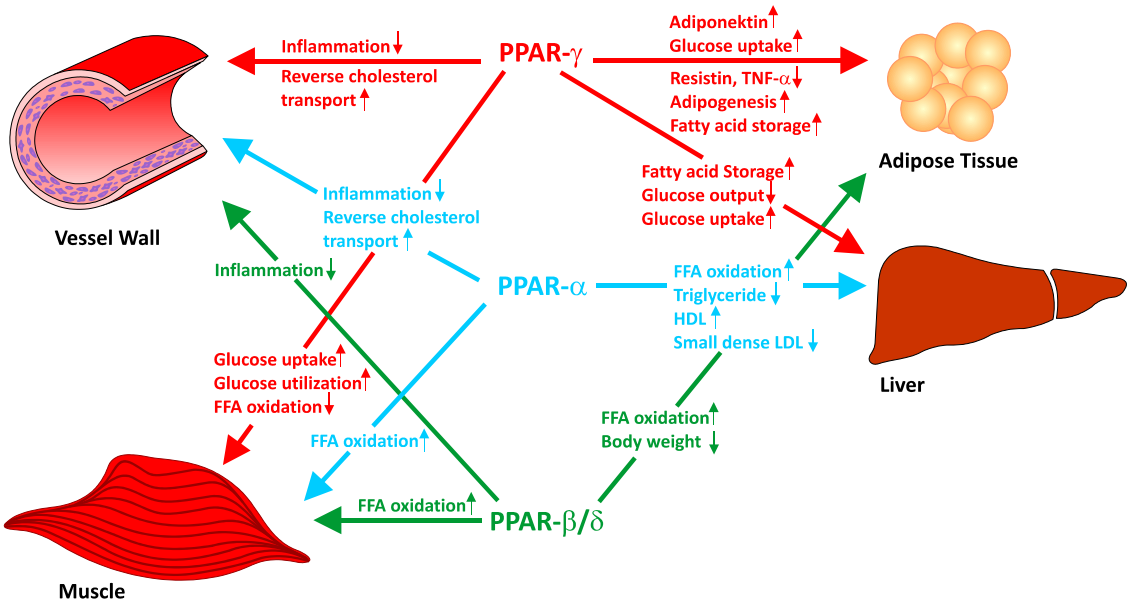


Figure 7. Effect of PPAR isoforms (PPAR-a, PPAR-b/d, PPAR-g) on AT, liver, muscle and vessel wall. (adapted from Blaschke F et al. *Arterioscler Thromb Vasc Biol* (2006) 26: 28-40).

After identification of natural ligands like fatty acids (FA) and FA-derived metabolites as endogenous ligands for all three of the PPARs, the prevalent view on their function is that they are implicated in energy homeostasis and act as lipid sensors. Only two classes of synthetic PPAR activators are in clinical use with some limitations, thiazolidinediones (TZDs) targeting PPARγ and fibrates as ligands for PPARα. They are used for the treatment of T2DM, pre-diabetic IR and hyperlipidemia to improve IS and plasma lipid profile [117-120]. PPARγ plays a key role among the genetic factors to influence IS [121]. PPARγ has been identified as a functional receptor for TZD class of insulin-sensitizing drug [122]. The first TZD was troglitazone but was withdrawn from the market because of its rare but detrimental

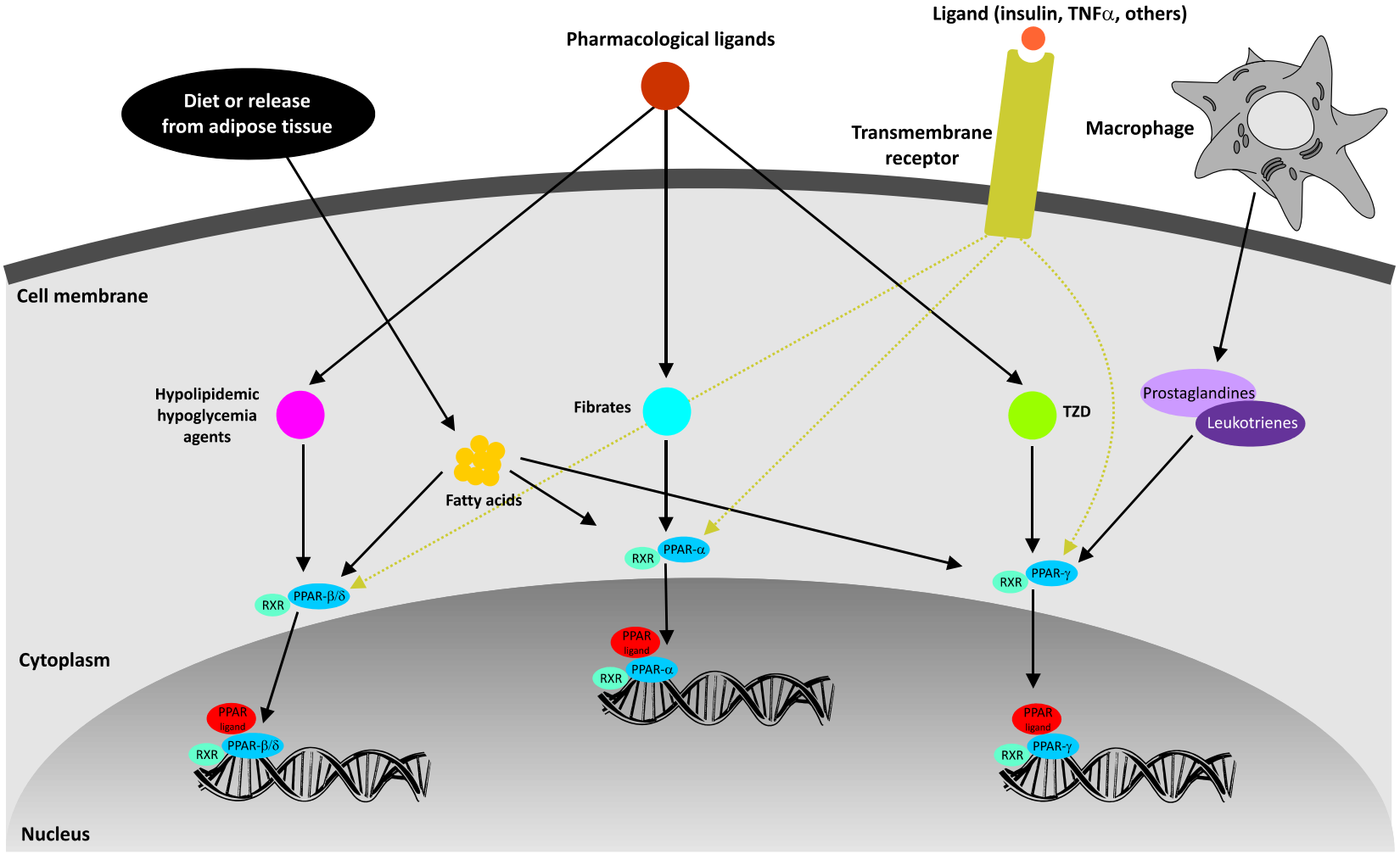


Figure 8. PPAR pathway.

hepatic side effects (hepatotoxicity) [123]. At present, two other TZDs are clinically available, rosiglitazone and pioglitazone that are not toxic to the liver [124], but have some other side effects such as weight gain and edema [125,126]. Ligands for PPARβ/δ are currently under clinical development, and there is a first recent study reporting that pharmacological PPARβ/δ agonist GW501516 was administered to healthy man and enhanced fat clearance in the circulation. Recently, synthetic dual PPARs (a single ligand activating both γ and α) and pan PPARs (activating all, α, γ, β/δ) are also emerging [127].

However, issues of safety and clinical indication remain undetermined for use of PPAR agonists for the incidence of heart failure [125] and carcinogenesis (we can see sometimes in animal experiments). We have to wait for more data not to cause more harm than benefit. Taken together, the identification of specific, more efficacious and safer PPAR agonists as potential candidates for treatment is still being a big challenge.

Genetic alterations in the PPARγ

Two rare mutations, heterozygous nucleotide substitutions CCG to CTG corresponding to Pro467Leu and GTG to ATG resulting in Val290Met in ligand-binding domain of PPARγ have recently been found to be associated with severe IR and DM in addition to hypertension [128]. Another amino acid variant identified in a German population, Pro115Gln, was found exclusively in obese subjects. It seems the Pro115Gln mutation in PPARγ2 accelerates the differentiation of adipocytes and may cause obesity [129]. A much more frequent amino acid polymorphism, affecting the transcriptional activity, has been described in PPARγ2 (Pro12Ala) [130,131] (more information about this polymorphism in chapter Results).

1.4.3.1.3 NFκB

Nuclear factor-kappa B (NFκB) was initially identified as transcription factor bound to enhancer of immunoglobulin κB light chain gene of B lymphocytes [132]. The five members of the mammalian NFκB family, RelA/p65, cRel, RelB, p50 (a processing product of p105), and p52 (a processing product of p100), exist in unstimulated cells as homodimers or heterodimers bound to inhibitory proteins (IκB) [133]. The most abundant in almost all cell types is p65/50 heterodimer. The DNA binding function as well as dimerization, and association with the inhibitory proteins (IκB) is due to a highly conserved N-terminal Rel homology domain, which share all of the NF-κB/Rel proteins. Upon stimulation they are released from inhibitory subunits and the homo- or heterodimers are translocated into the nucleus, where they promote transcriptional activation of target genes **(Figure 9)**. The mechanism by which the activity of NF-κB is terminated remains poorly understood. To date most efforts in this have focused on mechanisms that involve new synthesis of IκBs [133]. The NFκBs thus control the expression of wide variety of genes participating in the regulation of immune and inflammatory responses, carcinogenesis, cell growth, proliferation, survival and apoptosis. The response depends on different extracellular stimuli, able to activate the NFκBs. There is classical signaling pathway, triggered by agonists such as tumor necrosis factor α (TNFα), IL-1β, lipopolysacharide (LPS) or T-cell receptor (TCR) leading through NEMO (IKKα/β and NFκB essential modulator), which phosphorylates IκBα bounded to the p65/p50 heterodimer. The alternative pathway, called non-canonical is involved in processes of adaptive immunity, B-cell maturation and secondary lymphoid organogenesis, and depends on NIK (NFκB inducing kinase) and IκB kinase α (IKKα) resulting in dimerization and activation of p52/relB heterodimer.

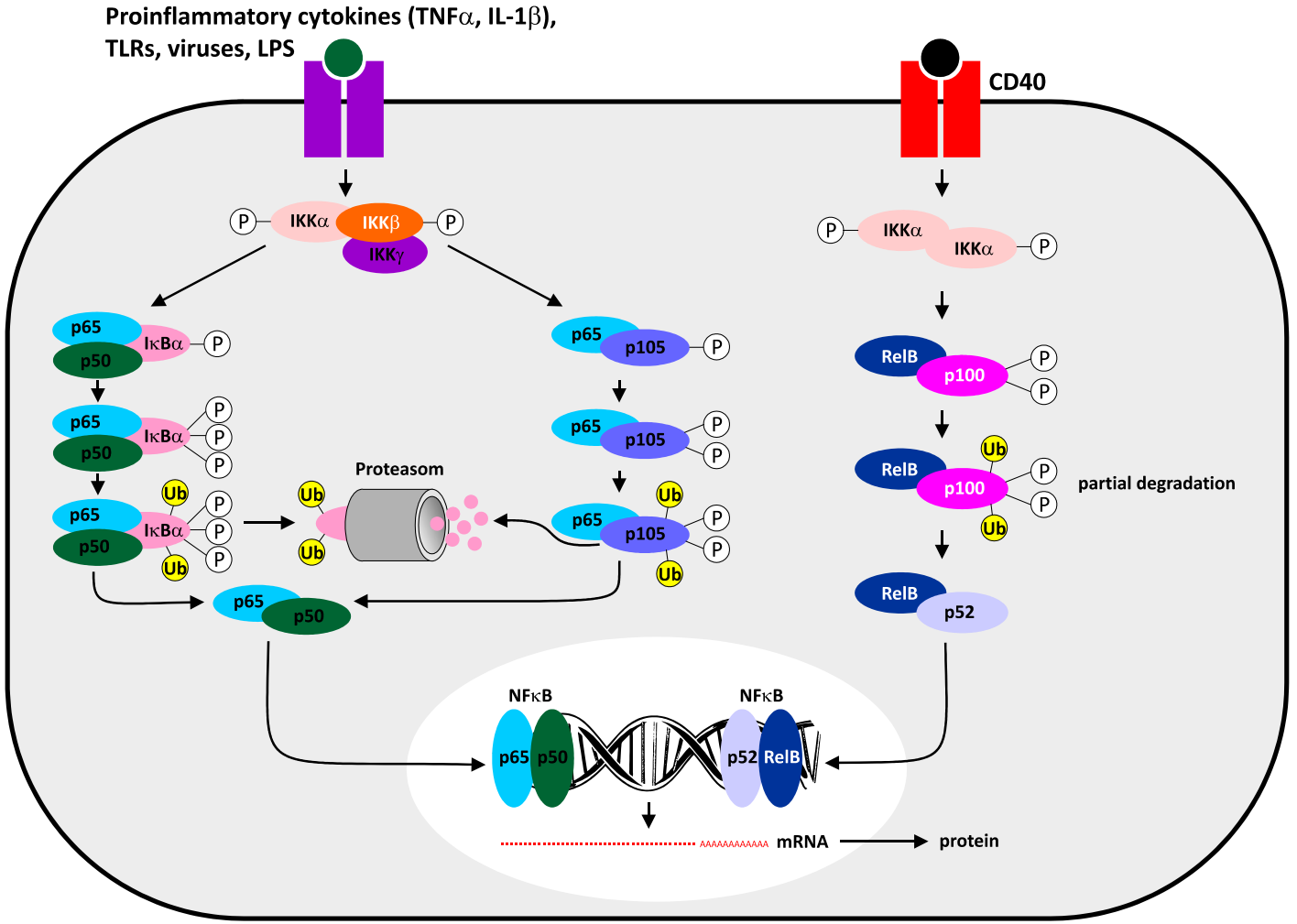


Figure 9. NFκB pathway.

As mentioned above the NFκB promotes the expression of many different genes, e.g. cytokines, chemokines, enzymes, adhesion molecules, so for that reason lots of recent studies have investigated the role of NFκB in the pathogenesis of various human diseases including neurological disorders, inflammatory diseases, carcinogenesis, and atherosclerosis [134-140]. In several studies NFκB has been suggested to be a potential predisposition factor in type 1 diabetes and NFκB induced β-cell destruction has been confirmed [141-144]. The possible link between NFκB and the development of insulin resistance and type 2 diabetes has also been discussed [145-150]. In addition, the association of NFκB with renal disease has been investigated [151-153].

1.4.3.1.4 SUR1/Kir6.2

Among many candidate genes of impaired insulin secretion the gene coding adenine nucleotide-sensitive potassium channels (K_{ATP}) was the most studied one because of his role in coupling glucose metabolism to insulin release. The K_{ATP} channels of β-cells consist of two subunits: sulphonylurea receptor 1 (SUR1, the gene symbol *ABCC8*) and potassium inward rectified channel *Kir 6.2* subunit (the gene symbol *KCNJ11*). Insulin secretion is initiated by closure of the channels and inhibited by their opening [154]. Both Kir6.2 and SUR1 are required for correct metabolic regulation of the channel: ATP closes the channel by binding to Kir6.2, and magnesium nucleotides (Mg-ADP and Mg-ATP) stimulate channel activity by interacting with SUR1. Sulfonylureas stimulate insulin secretion by binding to SUR1 and closing K_{ATP} channels by an ATP-independent mechanism[154], which can be uses for patients with T2DM or PNDM with mutation in the genes, which comprise the channel. Rare mutations in both subunits have been observed in persistent hyperinsulinemic hypoglycemia

of infancy [155,156]. Few polymorphism in SUR1 and *Kir 6.2* could also play a role in T2DM, but the evidence is controversial [157-161].

1.4.3.2 Positional approach

1.4.3.2.1 Genome-wide association study

A genome-wide association study (GWA study, or GWAS) - also known as whole genome association study (WGA study, or WGAS) - is an approach that has developed rapidly in the first decade of the 21st century following completion of the Human Genome Project in 2003. GWAS approach could be used after advances in genotyping technology and reductions in cost. These made it feasible to conduct large-scale GWAS that genotype many thousands of single nucleotide polymorphisms (SNPs) in thousands of individuals. Needed tool for the design and analysis of GWAS presents The International HapMap Project (**hapmap.ncbi.nlm.nih.gov**). The goal of the International HapMap Project is to develop a haplotype map of the human genome, the HapMap, which will describe the common patterns of human DNA sequence variation. In other words the aim of the International HapMap project has been developing a dense genome-wide map of SNPs and characterizing the linkage disequilibrium (LD) among them. About 10 million SNPs exist in human populations, where the rarer SNP allele has a frequency of at least 1%. Theoretically, researchers could look for these regions by genotyping 10 million SNPs. However to do the study in such wide range is currently too expensive. The HapMap study which will identify from 200,000 to 1 million tag SNPs provide almost as much mapping information as the 10 million SNPs (**hapmap.ncbi.nlm.nih.gov/whatishapmap.html.en**). This substantial cost reduction will make such studies feasible to do. Most chromosome regions have only a few common haplotypes (each with a frequency of at least 5%), which account for most of the variation

from person to person in a population. A chromosome region may contain many SNPs, but only a few "tag" SNPs can provide most of the information on the pattern of genetic variation in the region. A tag SNP represents SNP in a region of the genome with high linkage disequilibrium (the non-random association of alleles at two or more loci). It is known that any two unrelated people are the same at about 99.9% of their DNA sequences, the remaining 0.1% is important because it contains the genetic variants that influence how people differ in their risk of disease or their response to drugs. GWAS studies require two groups: cases group suffering from the disease and control group. The theory of this approach is about chromosome regions in which the two groups differ in their haplotypes frequencies. These might contain genes affecting the disease. The associated genetic variations are then considered as pointers to the region of the human genome where the disease-causing problem is likely to reside.

This type of studies has of course some limitations. Most genetic variations are associated with the geographical and historical populations in which the mutations first arose. This ability of SNPs to tag surrounding blocks of ancient DNA (haplotypes) underlies the rationale for GWAS. However, because of this, studies must take account of the geographical and racial background of participants - controlling for what is called population stratification. As the peoples of the world have migrated and inter-married over many generations, these geographical variations also become broken down and mixed over time. The HapMap is expected to develop a public resource that will help researchers find genes associated with human disease, and responses to drugs and environmental factors. Researches mostly agreed such studies are very expensive but could be useful in finding genetic variations through whole-genome that contribute to common, complex diseases, such as asthma, cancer, diabetes, heart disease and mental illnesses, which are affected by many genes and

environmental factors investigated in a non-hypothesis-driven manner. But in the other hand some others are worried about misinterpretation of the results, false positive and false negative findings [162]. Lots of associated loci have been indentified in genes not previously suspected of having a role in the disease under study, and some in genomic regions containing no known genes. Once new genetic associations are identified they have to be confirmed in other independent studies. The GWA approach is new powerful strategy but it seems very clear that researchers have to think carefully about the design, interpretation, application and limitation in the case of these studies [163-165]. After results confirmation scientists can try to use the information to develop better strategies to detect, in the future, hopefully, to treat or prevent the disease.

Since 2007, GWAS have rapidly increased the number of T2DM-associated loci. The first major GWAS success is the identification of four loci associated with T2DM: *TCF7L2* that had been previously implicated in this disease, a second locus includes the zink transporter *SLC30A8* gene that is expressed only in insulin-producing beta cells, and two linkage disequilibrium blocks that contain genes potentially involved in β-cell development or function (IDE-KIF11-HHEX and EXT2-ALX4). These results were published by Robert Sladek *et al*. in a study searching for T2DM variants [166].

Few months later in the same year, the Wellcome Trust Case Control Consortium (WTCCC) carried out GWAS for 7 diseases: bipolar disorder, coronary artery disease, Crohn's disease, hypertension, rheumatoid arthritis, T1DM and T2DM [167]. This study was successful in uncovering many new genes underlying these diseases. The authors confirmed the association for previously identified loci playing role in T2DM: PPARγ (rs1801282), KCJN11 (rs5219) and TCF7L2 (rs7903146). There are many supplementary results to WTCCC study. The feature of GWA approach is to generate lots of new data with no need for

the initial hypothesis because the SNPs cover actually whole genome. The results have to be always confirmed with other studies.

In 2009 Zeggini E. *et at* published meta-analysis of GWA data and large-scale replication identifies additional susceptibility loci for T2DM [168]. At least six new loci with robust evidence for association were detected: one in the intron 1 *JAZF1* (juxtaposed with another zinc finger gene 1) gene encodes a transcriptional repressor of NR2C2; region between *CDC123* (cell division cycle 123 homolog [*S.cerevisiae*]) and *CAMK1D* (calcium/calmodulin-dependent protein kinase ID) genes = (*CDC123/CAMK1D*), *TSPAN8* (tetraspanin8)/*LGR5*, exon 24 of *THADA* (thyroid adenoma associated), region near *ADAMTS9* (ADAM metallopeptidase with thrombospondin type 1 motif, 9) and introns 5 of *NOTCH2* (Notch homolog 2 [Drosophila]) gene.

GWAS use high-throughput genotyping technologies. Since 2005, nearly 100 loci for as many as 40 common diseases have been identified [163]. Many genes are connected to the disease, but many others not, and some in genomic regions containing no known genes. Some other interesting results of known genes: SNP rs13266634, Arg325Trp variant in the pancreatic beta-specific-zinc transporter SLC30A8 showed evidence for T2DM association. SLC30A8 transports zinc from the cytoplasm into secretory vesicles [169,170], where insulin is stored as a hexamer bound with two Zn2+ ions before secretion [171]. Variation SLC30A8 may affect zinc accumulation in insulin granules, affecting insulin stability, storage, or secretion. IGF2BP2 is a paralog of IGF2BP1, which binds to the 5 untranslated region of the insulin-like growth factor 2 (IGF2) mRNA and regulates IGF2 translation [172]. And there are more data, but we have to remember the limitations of GWAS and always think about false-positive and false-negative results.

1.4.3.2.2 TCF7L2

Very strong association of T2DM with variants of the transcription factor 7-like 2 gene (TCF7L2) gene was identified after following up the linkage peak on chromosome 10q in Icelandic individuals with T2DM and controls in the year 2006 [173]. Common intronic variants of TCF7L2 are strongly associated with DM in all racial groups. A microsatellite, DG10S478, within intron 3 of the TCF7L2 (formerly TCF4) was associated with T2DM ($p = 2.1 \times 10^{-9}$). This was replicated in a Danish cohort ($p = 4.8 \times 10^{-3}$) and in a US cohort ($p = 3.3 \times 10^{-9}$). Compared with non-carriers, heterozygous and homozygous carriers of the risk alleles (38% and 7% of the population, respectively) have relative risks of 1.45 and 2.41 [173]. A common variant of TCF7L2, when present in two copies, is associated with an approximate 2-fold risk of T2DM. TCF7L2 variants were strongly correlated with each other and represent one risk allele that is present in approximately 28% of control subjects and approximately 36% of T2DM patients [174].

TCF7L2, also known as TCF-4, is a nuclear receptor for CTNNB1 (previously known as β-catenin), which in turn mediates the canonical WNT signaling pathway [175]. The gene product of TCF7L2 is a high mobility group box-containing transcription factor previously implicated in blood glucose homeostasis. The identified variants are in the introns so the fundamental question if there are any functional consequences remain unanswered. The influence of those variants may still lead to differences in protein stability or gene expression. There are several known mechanisms for the involvement of WNT signaling in both insulin secretion and action, as well as in cell differentiation and maturation. It is thought to act through regulation of proglucagon gene expression in enteroendocrine cells via the Wnt signaling pathway. Subsequent studies have consistently replicated the association of TCF7L2

variants with T2DM for different ethnic groups [166,176-182]. The scientists agree on genetic component predisposition to T2DM in spite of imperfection of above mentioned studies.

1.4.3.3 MicroRNAs

Since the initial discovery in *Caenorhabditis elegans* in 1993, microRNA (miRNA)-dependent gene regulation has been widely investigated in various eukaryotic organisms [183,184]. miRNAs are single stranded non-coding small RNA molecules (21-23 nucleotides) involved in post-transcriptional control of gene expression probably over 30% of human genes [185,186]. Considering the prediction the each miRNA can regulate more than 200 different transcripts, it is not very easy to understand whole complexity [187]. miRNAs have been implicated to play a role in many diseases, including diabetes. Recently, several specific miRNAs have been proved to have an impact on regulation of glucose and lipid metabolism [188]. A major player that emerged as a significant mediator of insulin release and thereby of glucose homeostasis is the pancreatic islet specific miRNA-375 [189]. MiRNA-375 has been shown to regulate glucose-stimulated insulin secretion in a negative manner [187]. miRNA-143 was reported to play a role in adipocytes differentiation [190]. And there are much more indentified miRNAs included in insulin synthesis (miR-9,miR-34a/146, miR124a, miR-30d), glucose metabolism (miR-145?, miR-29a), lipid metabolism (miR-122, miR-14,miR-103/107, miR-278) and diabetic complications (miR-133, miR-1, miR-320, miR-192, miR-377) [191].

To conclude the chapter: “Genetics of T2DM” we can summarize that numerous published reports have identified association between T2DM and common genetic variants in human populations; but until today there are only a few variants of genes, which have been

consistently replicated across populations and with large sample sizes. Among these are the Pro12Ala (rs1801282) variant in PPARγ2 [192], the Glu23Lys (rs5210) variant in the potassium channel gene *KCNJ11* [193], and several variants in the Wnt-receptor signaling pathway member *TCF7L2* [173].

1.5 Maturity onset diabetes of the young

Maturity-onset diabetes of the young (MODY; MIM# 606391) is a genetically and clinically heterogeneous form of DM, characterized by an autosomal dominant inheritance, early onset non-insulin-dependent diabetes mellitus, and by a primary defect in the pancreatic β-cells function [194]. This type of diabetes was first described by Tattersall and Fajans in 1974 – 1975 [195,196]. The typical clinical descriptions for MODY is: a form of diabetes affects more members within family, coupled with simple pattern of inheritance, early-onset of the disease. MODY is the most common form of monogenic diabetes, accounting for an estimated 1-2% of all cases of diabetes in Europe [197,198], but is often misdiagnosed as type 1 or type 2 diabetes, which can be a pity for the patient. MODY is probably the first area of diabetes in which molecular genetics has played a clear clinical as well as research role.

Our understanding has been transformed by identifying the genes involved in MODY. Until now, 6 types of MODY diabetes have been identified depending on the gene causing the disease [199]. Each gene involved in MODY has its own specific clinical and physiological characteristics [200] **(Table 8)**. The first gene to be indentified was glucokinase (*GCK*) [201-204], obvious candidate gene from its known role as the pancreatic glucose sensor [205], followed by hepatocyte nuclear factor-4alpha (HNF-4α) (the gene symbol *HNF4A*) [206] and HNF-1α (the gene symbol *TCF1*) [207]. Mutations in the different transcription factors result in clinical heterogeneity. β-cell transcription factor genes are important in the

Type of MODY **Gene symbols**	**MODY1** ***HNF4A***	**MODY2** ***GCK***	**MODY3** ***HNF1A (TCF1)***	**MODY4** ***IPF1***	**MODY5** ***HNF1B (TCF2)***	**MODY6** ***NeuroD1***	**MODY X** **Unknown**
Chromosomal location	20q13.12	7p13	12q24.31	13q12.2	17q12	2q31.3	Unknown
Frequency in U.K.	3%	14%	69%	< 1%	4%	-	12%
Gene Accession no.	NM_000457.3	NM_000162.2	NM_000545.4	NM_000209.2	NM_000458.1	NM_002500.2	Unknown
Linkage to T2DM	Yes	No	No	Yes	No	No	Unknown
Onset of hyperglycemia	Adolescence, early adulthood	From birth	Adolescence, early adulthood	Early adulthood	Adolescence, early adulthood	Adolescence, early adulthood	Unknown
Severity of hyperglycemia	Mild to severe	Mild DM or IGT	Progressive, may be severe	Mild DM	Mild to severe	Mild to severe	Unknown
Microvascular complications	Frequent	Rare	Frequent	Unknown	Unknown	Unknown	Unknown
Other features	Low plasma triglycerides	Reduce birth weight	Low renal threshold, frequent need for insulin	Pancreatic agenesis in homozygotes	Renal cysts, proteinuria, renal failure, genital malformations		

Table 8. Details of MODY genes.

pathophysiology of the β-cell, with mutations in *HNF4A* (MODY1), *TCF1* (MODY3), insulin promoter factor -1 (IPF-1) (the gene symbol *IPF1)* (MODY4), HNF-1β (the gene symbol *TCF2*) (MODY5), and *NeuroD1/BETA2* (MODY6), all resulting in early-onset type 2 diabetes. The most often are MODY2 and MODY3 the other types of MODY are quite rare. The relative prevalence of distinct MODY subtypes differs substantially in studies in various populations [197,208,209], mutations in *GCK* representing from 8 to 63% and *HNF1A* mutations from 13 to 64% of all subjects with MODY [210]. There are other patients with MODY in whom the genetic defect is still unknown. This group of patients we called MODY X. The identification of new mutations which are the cause of MODY is still fruitful research area.

According to clinical characteristics it is as well possible to divide MODY into two groups. First one is diabetes of transcription factors (HNF-1α, HNF-4α, HNF-1β, IPF-1 and NeuroD1) mostly including about 75% of patients with MODY depends on population. Patients with mutations in *HNF1A* have normal glucose tolerance in early childhood and usually present with symptomatic diabetes in their late teens or early adulthood. They show increasing hyperglycemia and treatment requirements with frequent microvascular complications. The underlying defect is progressive β-cell failure, with the early lesion characterized by failure to increase insulin secretion with increasing glucose levels. Patients with *HNF4A* and *IPF1* mutations show a similar clinical picture to *HNF1A* although diabetes may be diagnosed later. Second one is diabetes caused with heterozygous inactivating mutation in *GCK* gene, MODY2, mostly including about 14% of patients. Patients with heterozygous inactivating mutations of *GCK* gene have mild fasting hyperglycemia throughout life, and rarely require medication or develop microvascular complications. *GCK*

encodes the glucokinase enzyme, which acts as the pancreatic glucose sensor. So, the β-cell dysfunction is characterized by reduced sensing of glucose by the pancreas.

A precise molecular genetic diagnosis often changes management, since patients with *GCK* mutations rarely require pharmacological treatment and *HNF1A/4A* mutation carriers are sensitive to sulfonylureas [211] or insulin. The scientists with medical doctors and patients are trying to study and describe the relationship between genotype and phenotype to understand better the underlying molecular genetics. This effort should help to particular families who could gain after genetic tests the information about treatment options and future prognosis.

2 Aims

The aim of this work was to study some molecular aspects of genetic predisposition to T2DM and its monogenic forms. We searched for correlations of genotypes with many clinical and laboratory markers and tried to evaluate their significance. The consequences for possible changes in the treatment some particular patients were under discussion. Especially for those suffering from monogenic forms we were able to reach better compensation of DM, or at latest the same compensation for more acceptable therapy.

We studied the associations of chosen candidate genes, *PPARγ,* NF-κB, its inhibitor IκB, known polymorphisms and T2DM including diabetic complications. We used the association study approach to find out if there is any association between available genetic variants among our patients and T2DM with its phenotypic abnormalities (changed levels of total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides, further the presence of diabetic complications).

1. Single nucleotide polymorphism **Pro12Ala of *PPARγ-2* gene** for its involvement in insulin resistance, type 2 diabetes and for its effect on lipid levels.

2. Two polymorphisms of the NFκB family genes: single nucleotide **polymorphism A/G in the 3'UTR region of the *NFKBIA* gene**, coding inhibitory protein IκB and **CA repeat polymorphism of the *NFKB1* gene,** coding p105 subunit of NFκB. The role of both was investigated in connection to type 2 diabetes, insulin resistance, diabetic complications or atherosclerosis.

The second part of my work was designed as "clinical-genetic study". Our effort in this part of work was focused to better understanding which patients are suitable for genetic

testing. We cooperate with medical doctors who try to pick up according to available clinical criteria the patients who are "candidate" for monogenetic form of DM or are somehow not clear with their diagnosis of DM type to see if they fit at the end to MODY or PNDM group.

As the most important bonus for us from this testing was the opportunity to sometimes completely change the treatment for few individuals included to our study.

3. We aimed at specifying the genetic etiology of unexpected finding of asymptomatic hyperglycemia in a cohort of children and adolescents. Those patients may suffer from presymptomatic progressive pancreatic β-cell dysfunction when a rapid and effective diagnostic action is required. This diagnostic action comprises from 2 parts: first of all medical examination including biochemical laboratory tests and gaining family anamnesis is done and second part: the **blood collection, DNA isolation and genetic examination.** One of our aims was to **find out the proportional representation of different types of DM (T1DM, MODY, PNDM)** for this group of patients.

4. We examined the prevalence of mutations in ***HNF4A, GCK, TCF1*** in a large cohort of patients and their family members with the clinical MODY diagnosis.

3 Methods

3.1 Subjects

3.1.1 For the study of the polymorphism Pro12Ala of *PPARγ2* gene

Czech patients with T2DM (characterization: age > 35 years, C-peptide > 200 pmol/l, antiGAD < 50 ng/ml). The patients were chosen by their diabetologists from 2 Centers: Internal Clinic of Faculty Hospital Královské Vinohrady, Prague 10 and Dr. Jan Březina ambulance in Mělník.

3.1.2 For the study of the polymorphism A/G in the 3'UTR region of the *NFKBIA* gene and CA repeat polymorphism of the *NFKB1* gene

The group of patients with T2DM (n = 211), were subdivided into three groups based on their renal status. The first group of patients (n = 50) included persons with nondiabetic renal disease (NDRD). Diseases in this group included atherosclerotic renal disability, glomerulonephritis, focal segmental glomerulosclerosis, vascular nephrosclerosis, as well as inflammatory tubulointerstitial nephritis and chronic pyelonephritis. The second group of patients (n = 118) consisted of persons with DN. The third group (n = 78) consisted of patients who were excluded from groups 1 and 2 but were able to meet the following criteria: duration diabetes 15 years, normoalbuminuria (albumin 20mg/day), and were not using angiotensin-converting enzyme inhibitors, angiotensin II receptor antagonists, diuretics, or nonsteroidal anti-inflammatory drugs. All subjects were chosen on the basis of biochemical and clinical characterizations (Table 1 in the included article). Additionally, SLE patients (n = 152) were included in the genotyping of NFKBIA polymorphism with a group of healthy controls (n = 138). Both groups were Czech and Slovak origin. The affected group included 90% of women with an average age of 40 years, an average SLE duration 17.5 years.

3.1.3 For the study of an asymptomatic fasting hyperglycemia and testing of MODY genes

Children and adolescents with asymptomatic fasting hyperglycemia, who were referred by general pediatricians, pediatric endocrinologists or pediatric departments of local hospitals to the Department of Pediatrics of the 3rd Medical Faculty in Prague for elucidation of asymptomatic fasting hyperglycemia.

Suspected MODY subjects: All probands included in the study had positive family history a fasting plasma glucose (p-glucose) > 6.1 mmol/l. After an overnight fast, a blood sample was taken for measurements of p-glucose, serum C-peptide and glycosylated hemoglobin (HbA1c). The assigned normal range of HbA1c was 4.4 – 6.4%. All were young.

3.2 Preparation of genomic DNA

Genomic DNA was isolated from peripheral blood anticoagulated with ethylenediaminetetraacetate (EDTA) using a commercially available kit Qiagen DNA blood isolation kit (Qiagen, Hilden, Germany). The DNA samples were stored at -20 ^{o}C.

3.3 Polymerase chain reaction (PCR)

PRC mix: PCR buffer 1x, $MgCl_2$ 1.5mM, dNTP 200 μM, primer 0.2 μM each,

Amplitaq gold polymerase 0.35U, DNA template 100 ng

PCR conditions generally used:

1. 94 ^{o}C 3 min
2. 94 ^{o}C 30 s, 60 ^{o}C 30 s, 72 ^{o}C 30 s, number of cycles 35
3. 72 ^{o}C 9 min

Gene	Forward primer	Reverse Primer
PPARγ2 – exon (ex) 2	GACAAAATATCAGTGTGAATTACAGC	GTATCAGTGAAGGAACCGCTTTCTG
NFKB1	CTTCAGTATCTAAGAGTATCCT	CAAGTAAGACTCTACGGAGTC
NFKBIA	GGCTGAAAGAACATGGACTTG	GTACACCATTTACAGGAGGG
HNF1A pro 1	AGCCAGCACTGTTCTTGG	AGGGACAGGGAGCTATG
HNF1A pro 2	TCCCATCGCAGGCCATAGCTC	CCGTCTGCAGCTGGCTCAGTT
HNF1A ex1a	GAGGCGGCTAGCGTGGTGGA	CCATTGGGCAGCTCAGC
HNF1A ex1b	GAAGGCCCCCTGGACAAGG	CCCTCTAGGCTCTCCTGGGA
HNF1A ex2	CCCTTGCTGAGCAGATCC	CCACCTATGAGTTAGGGGAGA
HNF1A ex3	GGGCAAGGTCAGGGGAATGGA	CAGCCCAGACCAAACCAGCAC
HNF1A ex4	CAGAACCCTCCCCTTCATGCC	GGTGACTGCTGTCCATGGGAC
HNF1A ex5	GGCAGACAGGCAGATGGCCTA	GCCTCCCTAGGGACTGCTCCA
HNF1A ex6	TGGAGCAGTCCCTAGGGAGGC	GTTGCCCCATGAGCCTCCCAC
HNF1A ex7	GGGGGCCCAGCTGATTC	CAACCTCTATCATCATCTCCTGCT
HNF1A ex8	GAGGCCTGGGACTAGGGCTGT	CTCGTCACAGGCCGAGGGAG
HNF1A ex9	CCTGTACAGAGCCCCTCACC	CGGACAGCAACAGAAGGGGTG
HNF1A ex10	GTACCCCTAGGGACAGGCAGG	ACCCCCCAAGCAGGCAGTACA
GCK prom	ATGGGGATGGAGGCTCTTTG	TGTGGGGCTTAGTGTCCTTC
*GCK*ex1a	TCCACTTCAGAAGCCTACTG	TCAGATTCTGAGGCTCAAAC
GCK ex1b	GGGGCAGAGTATTTTGAGCAG	TGCCCCCAGCCTTAGTTTTG
GCK ex1c	CTCCACATCTACCTCTCCAG	AGGGGCTGAGGAGAGGAACA
GCK ex2	ATGGCGTGTGGGGGAGAT	TCGGGCTGGCTGTGAGTC
GCK ex3	TAATATCCGGCTCAGTCACC	CTGAGATCCTGCATGCCTTG
GCK ex4	GTGCCCTGAGGAATAGCTT	TACATTTGAAGGCAGAGTTC
GCK ex5	TCCAGATATGTTAGCAGCCA	GGAGAAAGGCAGGCAGTG
GCK ex6	CCAGCACTGCAGCTTCTGTG	GAGCCTCGGCAGTCTGGAAG
GCK ex7	AGTGCAGCTCTCGCTGACAG	CATCTGCCGCTGCACCAGAG
GCK ex8	GCCCTCCCTCGTGCCTGCTG	TCGCCCTGAGACCAAGTCTG
GCK ex9	ACTGTCGGAGCGACACTCAG	CTTGGAGCTTGGGAACCGCA
GCK ex10	GTCGACTGCGTGCAGGGCGC	TGTGGCATCCTCCCTGCGCT
KIR6.2 1	TGTAAAACGACGGCCAGTCCGAGAGGACTCTGCAGTGA	CAGGAAACAGCTATGACCCACCAGCGTGGTGAACACGT
KIR6.2 2	TGTAAAACGACGGCCAGTGAAAGGCAACTGCAACGTGG	CAGGAAACAGCTATGACCTAGTCACTTGGACCTCAATG
KIR6.2 3	TGTAAAACGACGGCCAGTCTGTGTCACCAGCATCCAACT	CAGGAAACAGCTATGACCTGATGATCATGCTCTTGCGG
KIR6.2 4	TGTAAAACGACGGCCAGTTCAGCAAGCATGCGGTGATC	CAGGAAACAGCTATGACCACGCCTTCCAGGATGACGAT
KIR6.2 5	TGTAAAACGACGGCCAGTCTACCATGTCATTGATGCCA	CAGGAAACAGCTATGACCCTGTGGTCCTCATCAAGCTG
KIR6.2 6	TGTAAAACGACGGCCAGTGCTGAGGAGGACGGACGTTAC	CAGGAAACAGCTATGACCCCACATGGTCCGTGTGTACACACG

Table 9. Primers used for amplification of *PPARγ2*, *NFKB1*, *NFKBIA*, *HNF1A*, *GCK*, *KIR6.2*

3.4 Restriction fragment length polymorphism (RFLP)

The BseLI restriction endonuclease was used for digestion of PPARγ2 product. We changed the sequence of DNA with the reverse primer and we prepared the digest site for this inexpensive restriction enzyme. The digestion was done at 55^{o}C for 1 h.

Genotyping of the *NFKBIA* point variation (A/G) polymorphism was performed using 10 ul of product, which we digested with HaeIII at 37°C overnight.

3.5 Fragment analysis method

We performed these analyses on ALFexpress fragment analyzer (Amersham Pharmacia Biotech, Uppsala, Sweden) with ALFwin software, for the detection of polymorphic alleles in the *NFKB1* gene.

3.6 Mutation detection

All exons, the intron-exon boundaries and promotor region of *HNF4A, GCK, HNF1A* were screened using denaturing high performance liquid chromatography (dHPLC) as previously described [212]. The nature of identified mutations was established by direct nucleotide sequencing using BigDye Terminator v3.1Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) according to manufacture's instructions. The sequencing was performed on an ABI PRISM® 3100-Genetic Analyzer (Applied Biosystems, Foster City, USA). Mutations were confirmed using a second, independent amplification of the affected part of gene and re-sequenced the following day.

For more details about used methods see the attached articles.

4 Results

4.1 Genetic association studies for chosen polymorphisms, Pro12Ala in *PPARγ2* gene, CA repeat polymorphism of *NFKB* gene and A/G point variation in 3´UTR region of *NFKBIA* gene.

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The frequency of alleles of the Pro12Ala polymorphism in PPARγ2 is different between healthy controls and patients with type 2 diabetes. Pintérová D, Černá M, Kološtová K, Novota P., Čimburová M., Romžová M., Anděl M. *Folia Biologica. 2004;50:153-156.*

Commentary to the first article

The article deals with association study on 133 unrelated Czech patients with T2DM and 97 control subjects (blood donors). The aim was to confirm or refuse hypothesized protective role of the Ala12 genotype of PPARγ2 gene in the pathogenesis of T2DM, and furthermore, the effect of the Pro12Ala polymorphism on selected phenotypic features related to lipid metabolism. The study sustained the association of Ala allele with decreased risk for developing type 2 diabetes when significant difference in allele frequencies was observed between patient and control groups (13.9% vs. 21.4%, p=0.022). In other hand we did not see any significant difference in the proportion of the different genotypes at codon 12, between the group of patient and the control group and no correlation between the levels of total cholesterol, HDL and LDL cholesterol, triglycerides and BMI. The data suggest that the Pro12Ala polymorphism of PPRγ2 gene is some role in the pathogenesis of T2DM.

♠♠

NFkB and its inbihitor IkB in relation to type 2 diabetes and its microvascular and atherosclerotic complications. Romzova M, Hohenadel D, Kolostova K, Pinterova D, Fojtikova M, Ruzickova S, Dostal C, Bosak V, Rychlik I, Cerna M. *Human Immunology. 2006;67:706-713.*

Commentary to the second article

We performed association study of genetic variants in the genes coding NFκB and its inhibitor for 246 patients with DM and with or without DN and for 159 control subjects. Both, NFKB1 (4q24, gene for p105 of NFκB) and NFKBIA (14q13, gene coding IκBα), polymorphisms were tested in T1DM as well as T2DM. Patients with T1DM were divided according to the age: adults and children (under the 18th year of the life), LADA patients (first diagnosed after the age 35), type 2 diabetics (Czech and German origin) with different extent of renal damage. The role of NFκB in immune responses, via activation of different genes, has been recently reviewed. It must be stressed that in addition to obvious inflammatory diseases (rheumatoid arthritis, systemic lupus erythematosus (SLE)), a low degree inflammation is increasingly considered important in other disorders such as T2DM, obesity or atherosclerosis. Additionally, 152 patients with SLE were genotyped for NFKBIA polymorphism.

We found no statistically significant differences in allele or genotype frequencies among type 2 diabetic patients with renal disease, diabetic patients without renal disease and control group ($p > 0.05$). We identified 15 out of 18 previously described alleles of CA repeat polymorphism [142] (ranging in size from 114 to 142bp – corresponds to 12-26 CA repeats) for NFKB1 **(Table 10)**. The longest alleles (144-154bp), identified among UK subjects, were

not found in our population. The shortest alleles (114-122bp) were found in T1DM and control groups. The most frequent alleles were A3 with size of 124bp (23.2%) and A9 with length of 136bp (35.3%). **We identified 43 genotypes** and found **no significant difference in genotype frequencies of diabetic groups compared to ones in control group. The difference in allele frequencies, compared to healthy controls, was observed only in the group of adult T1DM patients.** The frequency of A7 allele (size 132bp, 20 repeats) was significantly increased with relative risk value of 1.88 (OR = 10.69, P<0.01). **The NFKB1**

Group			**Control**	**T2DM**			**T1DM**		**LADA**
Allels	**Lenght (bp)**	**Repeats**	**n = 139**	**With DN n=117**	**Without DN n=78**	**NDRD n=50**	**Adults n=67**	**Children n=55**	**n=34**
A01	114	12 CA	0.4						1.47
A02	116	13 CA							1.47
A03	118	14 CA							2.94
A1	120	15 CA						0.9	
A2	122	16 CA	0.7				1.49		
A3	124	17 CA	**22.7**	**20.08**	**18.59**	**22**	**23.13**	**19.1**	**20.58**
A4	126	18 CA	5.8	6.84	5.77	7	5.97	10	2.94
A5	128	19 CA	3.6	2.14	0.64	1	2.23	1.9	2.94
A6	130	20 CA	8.6	11.54	12.82	7	11.19	4.5	14.7
A7	132	21 CA	4.3	3.84	1.93	3	13.43*	0.9	2.94
A8	134	22 CA	9	6.84	7.05	7	12.68	10.9	14.7
A9	136	23 CA	**37**	**41.88**	**44.87**	**48**	**24.6**	**38.4**	**27.9**
A10	138	24 CA	3.6	4.7	3.21	4	2.47	4.5	4.41
A11	140	25 CA	2.9	2.14	2.56	1	0.74	8.1	4.41
A12	142	26 CA	1.4		2.56				
*statistical significance, bolded frequencies present most frequent alleles in particular group									

without DN	patients with diabetic nephropathy	T1DM	patients with type 1 DM
with DN	patients with diabetic nephropathy	T2DM	patients with type 2 DM
NDRD	patients with non-diabetic renal disease	LADA	latent autoimmune diabetes in adults
C	control group		

Table 10. Distribution of NFκB1 allele frequencies between different diabetic groups

genotyping revealed previously **confirmed association** of the NFκB/Rel gene family **with autoimmune diabetes**. As mentioned above we found increased frequency of A7 allele in adult type 1 diabetic patients. This result may mean that NFκB signaling pathway is involved in the pathogenesis of the autoimmune process seen in type 1 diabetes mellitus. The mechanism, however, is different from type 2 diabetes.

The NFKBIA genotyping results are shown in **Figure 10**. **Significant difference in the frequencies of AA genotype** was observed **between the T2D groups** (1, 2 and 3) **and the control group** with relative risk value of 1.38 (OR=2.81, P< 0.001). In **LADA group** was also observed **significant increase** in the **frequency of AA genotype** with relative risk value of 2.23 (OR =2.68, P<0.001). There is evidence that heterozygous genotype AG is protective for diabetes onset in adulthood, according to the results in the groups of LADA (non-significantly) and adult T1D (RR= 0.56, OR =0.44, P<0.01) patients. The groups of SLE and RA patients had significantly changed allele distribution comparing to control subjects, in both groups with relative risk of 1.4 (OR=2.08, P=0.01). We observed significant increase in the frequency of homozygous AA genotype when compared to the controls in all groups of type 2 diabetics, but also in group of LADA patients. **However RA and SLE groups had significantly increased incidence of A allele comparing to the control group.** On the basis of these findings we suggest that NFκB may participate in increased oxidative stress, insulin resistance and (or) improper immune responses via its sustained activation caused by variation in 3' UTR region of the gene encoding its inhibitor IκB, however the mechanism of this action remains to be investigated in functional studies.

In fine of the first part, we can conclude that we believe in the role of Pro12Ala polymorphism in PPARγ2 gene in the pathogenesis of T2DM (Ala allele frequency 13.91% vs

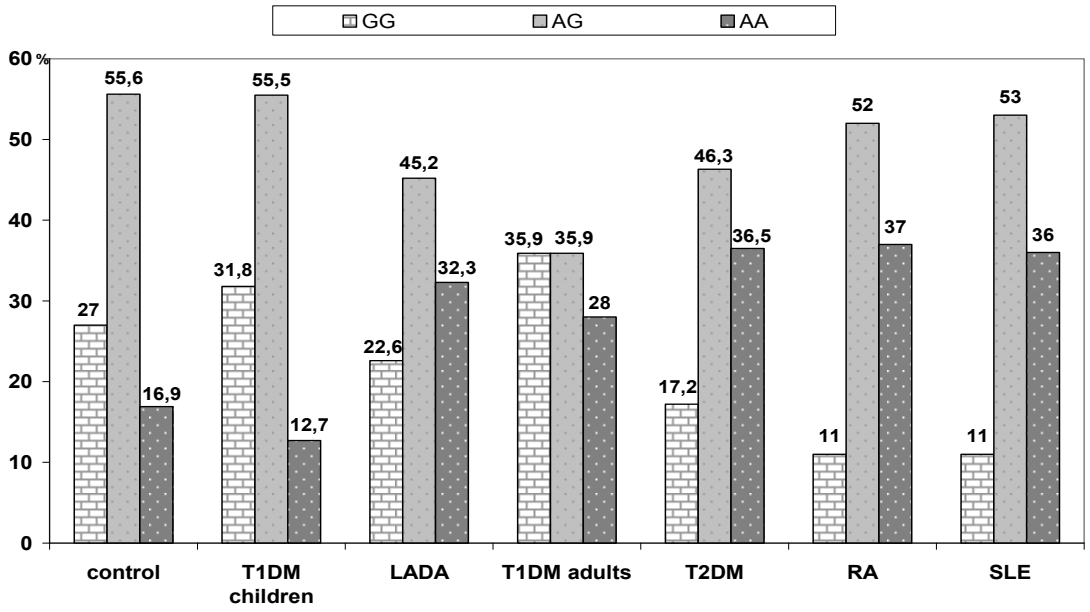

Figure 10. Frequencies of NFKBIA genotypes in tested groups of patients with different diseases

21.43% in patients and controls). In the second association study we detected significantly higher frequency of AA genotype of NFKBIA for LADA and T2DM groups of patients in comparison to control group. This genotype represents the risk factor for diabetes itself but with no connection to renal damage. The role of NFKBIA gene coding IκB in pathogenesis of diabetes suggests its function in regulation of gene expression not only during immune responses but in metabolic processes, too.

4.2 Testing for mutations in known MODY genes and mutations in *KCNJ11* causing PNDM.

♠♠♠

Aetiological heterogeneity of asymptomatic hyperglycaemia in children and adolescents. Feigerlová E, Prhuhová Š, Dittertová L, Lebl J, Pinterová D, Kološtová K, Černá M, Pedersen O, Hansen T. *Eur J Pediatr 2006;165:446-452.*

Commentary to the third article

This study deals with 82 children and adolescents (38 males, 44 females, aged 2 months – 18.5 years, median 13.1 years) who were referred by general pediatricians, pediatric endocrinologists or pediatric departments of local hospitals to the Department of Pediatrics of the 3rd Medical Faculty in Prague for elucidation of asymptomatic fasting hyperglycemia. None of the patients suffered from polyuria/polydipsia/weight loss or was ketotic at initial examination. The baseline evaluation included family history, fasting glycemia, fasting serum insulin and C-peptide, HbA1c. All individuals were tested with OGTT and for mutations in *GCK* and *HNF1A*. Those with positive family history of DM in first-degree but negative results for mutation search in *GCK* and *HNF1A* were screened for mutations in *HNF4A*. The *KCNJ11* gene was analyzed in all children younger than 2 years.

We conclude that 48% (39 patients from our group) suffered from monogenic form of DM. 35 children carry heterozygous inactivating mutation in *GCK* (MODY2 patients) and the early identification prevent them from future investigations and maybe "overtreatment". 2 were MODY3 and 1 MODY1 patient. It is known that MODY3 (*HNF1A*) patients are very sensitive to low dose sulphonylurea.

One child suffers from PNDM. We identified this boy with mutation R201H (first time described in subject with PNDM earlier in 2004 [213]) of the *Kir6.2* gene encoding a subunit of the ATP-sensitive potassium-channel in 2004 and since this year some preliminary reports suggest that patient with mutation in *Kir6.2* may profit from therapy with OHA (glibenclamid) instead of insulin. At the time of first examination he was 18 days old and came for prolonged neonatal jaundice. He was found to be mildly hyperglycemic (8.7 mmol/l). At re-investigation in 2.5 months his glycemia was 13,6 mmol/l. At admission, he had permanent glycosuria and blood glucose 22.6 mmol/l with no signs of ketoacidosis. The

level of C-peptide was 183 pmol/l and autoantibodies antiGAD were negative. He was started with insulin and his metabolic control was very good. At his age 3.5 years we found out the cause of his DM so he was recommended for glibenclamid therapy instead of insulin and his compensation was very good with OHA, too. 11 children progress to T1DM and 9 had IGT or DM of undetected origin. We are quite sure that in different hospitals and different populations, the results might differ largely. However for our medical centre we found out the distribution of different types of DM among children coming for asymptomatic hyperglycemia.

♠♠♠♠

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

Commentary to the fourth article

Screening for glucokinase (GCK) mutations in subjects with clinical characteristics of maturity-onset diabetes of the young (MODY) allows to distinguish between patients with a benign metabolic condition (*GCK* mutation positive, clinical diagnosis MODY2) and those with a higher risk of progressive hyperglycemia associated with more prevalent and severe diabetic complications (*GCK* mutation negative). We screened for *GCK* mutations in 92 Czech probands fulfilling classical MODY criteria. The probands were recruited from pediatricians and endocrinologists from the entire Czech Republic. We identified 15 different missense mutations in 27 patients, all within exons 2-10 **(Table 11).** Six missense mutations, R250C, L315H, F316V, F419L, I436N and A454E were novel. Interestingly, 3 missense mutations (E40K, C220X, G318R) which were originally identified in the Czech population

as novel in a previous study [214] were also identified in unrelated probands in the present study cohort **(Table 11)**. Some of the identified *GCK* missense mutations are located near putative functional domains: R250C was found in the close vicinity of a putative glucose binding site, whilst F419L was detected near a putative MgATP binding site and could thus affect binding kinetics [215]. Five of the novel missense mutations co-segregated with hyperglycemia in family members carrying the mutation identified in the probands. For the novel R250C variant, family members were not available for co-segregation studies. All codons, which are changed by the 6 novel mutations, are conserved in the genome of human, mouse, rat and chimpanzee and we found none of these mutations in 50 unrelated healthy Czech Caucasian subjects. Therefore we assume that the mutations are novel disease-causing mutations. Furthermore, we detected 5 previously reported *GCK* polymorphisms and 4 novel variants, a silent variant in exon 10, a promoter variant and 2 intron variants.

The clinical characteristics of patients with *GCK* mutations and those without mutation in *GCK* are compared in **Table 12**. The treatment of hyperglycemia with diet was more frequent ($p<0.001$) in the group of probands with mutations in *GCK* compared to other MODY probands and they had a significantly lower frequency of diabetic complications ($p=0.02$). None of the patients with mutation in *GCK* was treated with insulin ($p<0.001$). Moreover, *GCK* mutation carriers had a lower level of glycosylated hemoglobin ($p=0.02$).

In conclusion, we identified 29% carriers of *GCK* mutations among Czech MODY probands confirming that mutations in *GCK* are a common cause of MODY in the Czech population. Even more importantly for the MODY patients carrying *GCK* mutations the good news are that this diagnostic tool also has major implications for therapeutic decision making and counseling about lifelong diabetes prognosis.

Location	Codon/nt	Nucleotide change	Designation (#)	References
		Mutations		
Exon 2	36	CGG(Arg)→TGG(Trp)	R36W (1)	[216]
Exon 2	40	GAG(Glu)→AAG(Lys)	E40K* (8)	[214]
Exon 3	72	GGG(Gly)→AGG(Arg)	G72R (1)	[217]
Exon 4	157	GAA(Glu)→AAA(Lys)	E157K (1)	[210]
Exon 5	175	GGA(Gly)→AGA(Arg)	G175R (1)	[218]
Exon 5	188	GCT(Ala)→ACT(Thr)	A188T (1)	[219]
Exon 6	220	TGC(Cys)→TGA(Stop)	C220X* (2)	[214,220]
Exon 7	250	CGC(Arg)→TGC(Cys)	R250C[b] (1)	unpublished
Exon 8	315	CTC(Leu)→CAC(His)	L315H[a] (2)	unpublished
Exon 8	316	TTC(Phe)→GTC(Val)	F316V[a] (1)	unpublished
Exon 8	318	GGG(Gly)→AGG(Arg)	G318R* (4)	[214]
Exon 9	383	TCG(Ser)→TTG(Leu)	S383L (1)	[220]
Exon 10	419	TTC(Phe)→CTC(Leu)	F419L[a] (1)	unpublished
Exon 10	436	ATC(Ile)→AAC(Asn)	I436N[a] (1)	unpublished
Exon 10	454	GCG(Ala)→GAG(Glu)	A454E[a] (1)	unpublished
		Polymorphisms		
promotor	-30	G→A	(32)	[221]
promotor	-71	G→C	(2)	unpublished
IVS2-12		C→T	(1)	[218,222]
Exon 6	215	TAC(Tyr)→TAT(Tyr)	Y215Y (2)	[217]
IVS8+18		G→A	(1)	[217]
INS9+8		C→T	(2)	[218]
IVS9+49		G→A	(2)	unpublished
INS9+8, INS9+49		C→T G→A	(2)	unpublished
Exon 10	441	TCG(Ser)→ TCA(Ser)	S441S (1)	unpublished

Table 11. Mutations, silent mutations, intronic variants and polymorphism in *GCK* among 92 Czech patients with clinical MODY.

GCK gene - GenBank accession number, AF041012-22
number of families in brackets
* mutations which were first found in Czech population
[a] new mutation which co-segregated with diabetes within the family
[b] data about family members not available

	Subjects without mutation in *GCK*	Subjects with *GCK* mutations	p value
Number	65	27	
Sex (W/M)	37/28	17/10	ns
Age (years)	24 ± 2	20 ± 2	ns
Age at diagnosis (years)	17 ± 1	16 ± 2	ns
BMI (kg/m^2)	22.2 ± 0.7	21.8 ± 0.9	ns
Fasting p-glucose (mmol/l)	9.0 ± 0.6	7.1 ± 0.2	ns
HbA_{1c} (%)	6.5 ± 0.2	5.7 ± 0.2	0.02
Fasting serum C–peptide (pmol/l)	598 ± 62	518 ± 47	ns
Diabetic complications neuropathy/nefropathy/retinopathy	7/ 5/ 6	1/ 0/ 0	0.02
Current therapy (without/diet/OHA/insulin)	7/ 25/ 7/ 26	2/ 22/ 3/ 0	ns/ <0.001/ ns/ <0.001

ns – not significant

Table 12. Clinical characteristics of 92 probands with clinical MODY stratified into groups with or without a *GCK* mutation.

In fine of the second part, we conclude that making a diagnosis of monogenic diabetes is important as it can have a dramatic effect on the treatment a patient should receive: *GCK* MODY patients need no treatment; *HNF1A* MODY patients are very sensitive to low dose sulphonylurea; and patients with neonatal diabetes due to *Kir6.2* mutations, despite being insulin dependent, can discontinue insulin and be well controlled on high dose sulphonylurea tablets.

5 Discussion

First of all we have been focused on the role of previously identified polymorphisms and their associations with T2DM itself or including kidney complications, either DN or nondiabetic renal disease.

Our findings support the minor role of Pro12Ala PPARγ2 polymorphism in predisposition to T2DM. The results showed that the frequency of Ala12 variant of the PPARγ2 gene is higher in the control group than in the group of patients. This can be explained by the fact, that the proline to alanine substitution in the codon 12 in the PPARγ2 is affecting the transcriptional activity [130,131]. In one of the first reports, the Ala12 allele was significantly associated with lower BMI and improved IS, with Ala12 carriers having four times less risk of DM [131]. In our study we were not able to confirm the association with BMI. We have demonstrated that the polymorphism is not associated with BMI and changed lipid levels. Another study, published on Danish population, indicated that the Pro12Ala polymorphism might have divergent modulating effect on BMI, being associated with higher BMI in obese subjects and lower BMI in the control group [223]. Studies from other populations have not always been able to confirm the association of Pro12Ala polymorphism with T2DM; most have shown a tendency to a lower risk of DM among Ala12 carriers [131,192,224,225], but some studies have inconsistent results [226-228]. Some authors compared more clinical characteristics and the Pro12Ala polymorphism between type 2 diabetic subjects and control subjects or type 2 diabetic subjects with and without the Ala12 variant. They found associations of the Pro12Ala polymorphism with changed concentrations of total cholesterol [225,229] and LDL-cholesterol [229]. From our analysis of clinical characteristics, there is no significant difference in the BMI or lipids levels. So it seems that

for studying a role of the Pro12Ala polymorphism of PPARγ2 gene, in the genetic background of dyslipidemia, much larger studies are needed.

Further in our research we focused on another transcription factor NFκB and its inhibitor (NFKBIA) in patients with T2DM, with or without kidney complications, and SLE patients. NFKBIA genotyping resulted in the finding of association between homozygous AA genotype and T2DM. No differences in allele or genotype frequencies between type 1 diabetic children and controls were observed, however there was an increase in the frequency of AA genotype in LADA patients. These findings suggest that NFκB can play a role in the pathogenesis of diabetes mellitus, although the mechanism of its action in type 1 differs from that in type 2. The main event leading to either total or progressive lost of pancreatic β-cells in T1DM and T2DM is apoptosis. It has been described in the recent literature that main pathway resulting in β-cells death leads via activation of NFκB, but it is believed that it is just a "common final pathway" for both types of diabetes [230,231].

The stimuli, triggering apoptosis in pancreatic β-cells, as proven in several studies, differs in T1DM from those in T2DM. There is evidence that in T1DM the production of cytokines (namely IL-1β and TNF-α) by immunocompetent cells infiltrating the pancreatic islets induces activation of NFκB pathway in pancreatic β-cells leading their apoptotic destruction, whereas in T2DM the lost of β-cells is slower and in contrast to T1DM occurs mostly by necrosis, which attracts the scavenging macrophages as a consequence. Therefore it is hypothesized that β-cells dysfunction in T2DM, caused by overexposure to glucose and free fatty acids, is NFκB independent. Further functional studies are necessary to investigate the role of nuclear factor in the T2DM pathogenesis.

The results regarding NFKBIA genotyping had also revealed the association of the Rel/NFκB family gene with other autoimmune diseases such as RA and SLE and disapproved

our previously published findings on specific relation of NFκB to T2DM. In both, SLE and RA groups of patient significant differences in the allele and genotype frequencies were observed comparing to healthy controls. Previously has been found that in patients in early, but also late stage of RA is elevated expression of NFκB in synovial tissue [232]. In the joints activated NFκB triggers the expression of pro-inflammatory mediators and adhesion molecules, thus participate on the RA pathogenesis. The involvement of NFκB in the pathogenesis of RA and other chronic inflammatory diseases was investigated also in animal models, where inhibition of NFκB has led to decreased production of inflammatory cytokines and suppression of disease process [233,234].

The role of NFκB in the immune responses is indisputable and recent studies suggest its central function in other chronic inflammatory and autoimmune diseases, such as asthma, inflammatory bowel disease, ulcerative colitis and atherosclerosis. The therapeutic strategies in mentioned diseases are all oriented on the same goal - suppression of NFκB activation. A number of anti-inflammatory and anti-rheumatic drugs such as antioxidants, glucocorticoids, aspirin and salicylates, non-steroidal anti-inflammatory drugs, immunosuppressants and gold compounds have been found to act as potent blockers of NFκB pathway [235]. The selection of appropriate inhibitors however is dependent on the ethiopathogenesis of specific disease. The complexity of NFκB signaling offers broad opportunities to develop specific inhibitors acting via different mechanisms.

NFKBIA genotyping showed a significant increase in the frequency of the AA genotype in the T2DM groups (RR=1.38, OR=2.81, P< 0.001), LADA group (RR=2.23, OR =2.68, P<0.001), non-significantly in adult T1D patients, but also increase in the A allele frequency in RA and SLE groups (RR=1.4, OR=2.08, P=0.01). The presence of association in these diseases raises a question of common factor implicated in the ethiopathogenesis of all

aforementioned diseases. Additionally, after dividing T2DM patients into the groups with different renal conditions, we observed association of AA genotype only with non-diabetic renal diseases and not diabetic nephropathy. We also found significantly decreased frequency of AG genotype in this group. We assume that the differences in the findings of association among T2DM groups issue form the various background of complications present in these groups. The non-diabetic renal diseases included mainly atherosclerotic kidney changes. From this point of view the AA genotype seems to be predisposition factor for atherosclerosis and the AG genotype renders protection against it.

Furthermore we used the latest genetic knowledge to screen patients, who were coming with asymptomatic hyperglycemia or were candidates for monogenic form of DM. We used our results to try to settle better compensation of their diabetes. This part of our work actually presents kind of personalized medicine. It is very important for this approach to have very good collaboration with clinicians, who are choosing the patients for genetic testing. It saves lots of money and time in the quantity of diabetic patients.

Within the cohort of 82 children and adolescents with asymptomatic hyperglycemia, we identified 35 mutation carriers in the glucokinase gene (MODY2) and 3 patients with mutations in genes encoding transcription factors: one case of MODY1, two cases of MODY3 and one infant with a KCNJ11 mutation causing PND. Thus, in 39 patients (48% of the study cohort) the randomly found hyperglycemia led to the disclosure of a single gene defect condition, MODY2 being the most common. Among the mutation-negative subjects, 11 (13%) developed T1DM, 9 (11%) had IGT/DM of unknown cause and 23 (28%) were glucose tolerant. Molecular genetic testing was used to make a diagnosis nearly 50% of the patients, who suffer from monogenic diabetes. The results had a significant effect on the treatment. These patients should receive: glucokinase MODY patients need no treatment; HNF1α

MODY patients are very sensitive to low dose sulphonylureas; and patients with neonatal diabetes due to Kir6.2 mutations, despite being insulin dependent, can discontinue insulin and be well controlled on high dose sulphonylurea tablets. The challenge for diabetologists is to use clinical skills to detect these monogenic patients whose care will be greatly helped by molecular genetic testing that follow the treatment changes.

Finally, we screened 92 MODY patients for mutations in *GCK* and identified 15 different missense mutations in 27 patients. Six of the mutations R250C, L315H, F316V, F419L, I436N and A454E were novel. Five of these mutations co-segregated with diabetes within the family suggesting that the variants are new disease-causing mutations. We were not able to prove the co-segregation of R250C. However, this codon is conserved in healthy Czech population as well in the human, mouse, rat and chimpanzee genome. Thus, we conclude that all of these 6 mutations are likely to be novel MODY2 causing variants. The 3 mutations E40K, C220X and G318R previously identified among Czech MODY subjects [214] were in the presents study found in 8, 2 and 4 apparently unrelated probands, respectively. Thus, it appears that some founder mutations may be more prevalent in the Czech.

The prevalence of known types of MODY differs in reports from various European populations. Mutations in *GCK* were described to be a common cause of MODY in France [218,236] and Italy [210] whereas mutations in *TCF1* (MODY3) predominated in the UK [237,238], Denmark [222], Scandinavia [217] and Germany [209]. We observed a relative prevalence of 29% of MODY2. The present high relative prevalence of MODY2 compared to some other European studies might reflect not only a specific genetic background but also the mode of recruitment, since most of the probands in the present investigation were recruited by pediatricians. Our findings highlight the concept that molecular diagnostic methods in clinical

practice may help to verify a diagnosis. Even more importantly for the MODY patients carrying *GCK* mutations the good news are that this diagnostic tool also has major implications for therapeutic decision making and counseling about lifelong diabetes prognosis.

6 Conclusions and perspectives

In this chapter I would like to conclude the situation about the research of DM and mention some interesting and possible directions for future research.

DM with all diabetic complications is serious and costly disease. The complex web of susceptibility factors - genetic, environmental, social and psychological – that contribute to individual risk of developing DM means that most predisposing genetic variants will have probably only a modest marginal impact on disease risk [239]. Much has been made over the past decade of the potential for genetics to advance our understanding of the pathogenesis of DM. With each subsequent identification of new gene, which is associated with diabetes, there are new opportunities coming to re-evaluate our knowledge about current understanding to pathogenesis of DM and its complications. The majority of genetic studies to date have simply had insufficient power to uncover these reliably. That is the reason why research groups are coming together to set up huge Consortia to gain more powerful studies and make the science useful for the diabetic patients.

Division into the two largest diagnostic categories, type 1 and type 2 of DM, is usually made on the basis of simple clinical criteria such as age of onset, whether the patient is obese, the presence of autoantibodies and ketoacidosis. Into which of these two categories a young adult is classified will make a fundamental difference to their management as they will either be treated with diet and exercise and then go on to OHA or they will immediately start on insulin. Despite the clear different therapeutic outcomes, there are for some cases limited clinical guidelines on how to diagnose these two major subgroups or how to recognize who have neither type 1 nor type 2 diabetes but have a defined genetic etiology. Since the candidate gene approach and positional cloning, newly for 3 years also the screening of whole genome (GWAS) are included in the studying of DM the monogenic forms of DM (MODY,

PND) have been classified and very well described. It could happen for some patients, who have difficulties to be diagnosed for the type of DM that those with their medical doctors have to deal with treatment rather than diagnosis. In such cases any information about the patient and his/her disease is very useful. Considerable advances have been made in our understanding of the genetics of type 1 and type 2 diabetes, but gene variants only predispose to these polygenic conditions and cannot be used in diagnostic testing. In contrast, in monogenic diabetes a mutation in a single gene causes diabetes, so genetic testing can potentially have an important role in the diagnosis. This becomes important if it helps explain associated clinical features, anticipate the patient's prognosis and alter treatment decisions.

GWAS are very powerful and extremely challenging for good cooperation, high-throughput, money consuming and need of an excellent statistician. This approach is generating lots of data, some are known associations from the past but lots of them are completely new. According to my opinion it is important to study very careful the results to avoid all false positives from GWAS as well as loosing gene which are below detection threshold of linkage studies. Currently one of the tasks is to identify the genes that are responsible for the linkage peaks detected by genome screens. The linkage peaks for complex diseases are rather large, being often defined on the basis of probabilities. Hundreds of genes may be placed in a linkage interval, and prioritizing the analysis of these genes may be misleading. The aim of this effort is to genetically classify all cases of DM. Geneticists all over the world are studying complex diseases, like DM but unfortunately the progress is painfully slow. After the complete classification/diagnosis the new era of prediction and attempts at prevention of disease may begin.

T2DM is very closely associated with obesity and IR. These topics present other huge possibilities for further studies. Both, T2DM and obesity are epidemic in the world so it could

be very useful to study these entities together in their context. Interestingly the obese AT is a source of numerous other proteins, known and unknown awaiting to be explored, that may carry messages to the rest of the body and influence IS. Recently there is quite new hypothesis that obesity is a low-grade systemic inflammatory disease. Low-grade inflammation may have an important impact on insulin signaling in insulin target tissues and be a driver of IR. It seems appropriate to notice that this hypothesis is still being only a hypothesis and there is a great deal of research ahead to elucidate several key questions, which drive researchers to straits so far. A better understanding of this very complex issue at different levels (genetic, molecular, physiological) and from different points of view (clinical, social, environmental) is a beating board for improving or opening new therapeutic strategies (pharmacologic or non-pharmacologic) to prevent and/or treat diabetes and diabetes-related conditions.

MicroRNAs belong to a recently identified class of small non coding RNAs that have been widely implicated in several physiological processes and thereby it can be very fruitful to study them in association with pathogenesis of DM, obesity and other diseases. The problem could be the complexity because each miRNA can targeted several genes and one gene can be regulated by several miRNA [185] that makes from miRNA quite difficult target.

Our effort during these past years was to contribute to better understanding of genetic predisposition to T2DM and its complications. Further we applied the current genetic knowledge in clinical practice, due to we could change the treatment of some patients.

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ATTACHED ARTICLES

Original Articles

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

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

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

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

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

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

Material and Methods

Subjects

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

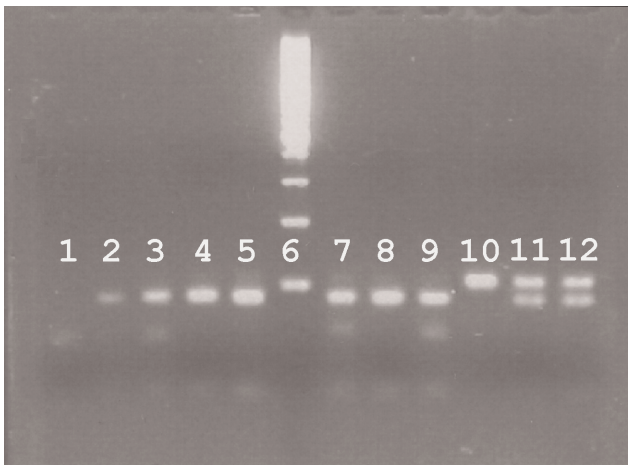

Fig. 1. Electrophoresis of PCR products after restriction on 2% agarose gel. Line 1: negative control. Lines 2,3,4,5,7,8,9: homozygotes for the Pro/Pro genotype. Line 6: DNA marker. Line 10: homozygotes for the Ala/Ala genotype. Lines 11,12: heterozygotes for the Pro/Ala genotype.

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

Genetic analysis

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

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

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

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

Statistical methods

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

Results

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

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

Table 1. Calculated P values for genotype frequencies

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

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

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

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

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

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

n.s. – not significant

Discussion

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

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

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

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ELSEVIER

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

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

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

ABBREVIATIONS

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

INTRODUCTION

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

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

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

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

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

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

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

MATERIAL AND METHODS

Subjects

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

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

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

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

Written informed consents were obtained from all participants.

Genotyping

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

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

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

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

[a] Unavailable data.

NFKBIA

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

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

NFKB1

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

Statistical Analysis

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

RESULTS

Inhibitor of NFKBIA

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

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

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

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

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

[a] Statistical significance.

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

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

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

NFKB1

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

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

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

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

		Czech Republic		Germany	Czech Republic				United Kingdom		Denmark	Spain				Australia		
NFKB1 alleles	Length (bp)	C $n = 39$	T2DM $n = 73$	T2DM $n = 122$	C $n = 57$	T1DM $n = 67$	LADA $n = 34$	JDM $n = 55$	C $n = 222$	T1DM $n = 434$	T1DM $n = 229$	C $n = 200$	RA $n = 197$	SLE $n = 181$	CD $n = 311$	C $n = 109$	C (BC) $n = 102$	BC $n = 102$
A01	114	0.4					1.47									2.94		
A02	116						1.47									2.94	0.45	
A03	118						2.94										0.98	1.96
A1	120							0.9	0.45									0.98
A2	122	0.7				1.49			0.45	1.6	0.87		0.5	0.4			24[b]	25[b]
A3	124	22.7[b]	21.9[b]	18.1[b]	23.7[b]	23.13[b]	20.58[b]	19.1[b]		1.15	0.44	0.76	0.26	0.3		23.5[b]	11.8	6.37
A4	126	5.8	3.4	8.2	10.5	5.97	2.94	10		6.2	22.71[b]	21.83[b]	17.56[b]	19.27[b]	19.9[b]		0.49	1.98
A5	128	3.6	0.7	2	4.4	2.23	2.94	1.9		4.8	5.24	9.64	8.25	9.04	9.7		4.4	8.3
A6	130	8.6	10.3	13.1	7.9	11.19	14.7	4.5		5.8	1.31	2.28	2.21	2.24	1.5	14.7	3.9	2.45
A7	132	4.3	2.7	3.3	1.75	13.43[ab]	2.94	0.9	0.45	5.8	9.17	4.57	7.25	6.35	6.8	2.94	8.8	13.7
A8	134	9	7.5	6.6	8.8	12.68	14.7	10.9	19.8[b]	6.2	4.59	6.09	8.75	7.34	9.7	14.7[b]	37.8[b]	34.8[b]
A9	136	3.7[b]	43.2[b]	43[b]	36[b]	24.6[b]	27.9[b]	38.4[b]	9.9	14.9	10.26	11.68	10.2	10.8	12.1	2.94	3.4	1.96
A10	138	3.6	5.5	3.3	2.6	2.47	4.41	4.5	2.7	17.5[b]	34.93[b]	32.99[b]	36.28[b]	34.67[b]	28.6[b]	2.94	3.9	2.45
A11	140	2.9	2.7	2	2.6	0.74	4.41	8.1	9.9	10.6	3.093	5.08	3.5	4.27	5.8	5.88		
A12	142	1.4	2.1	0.4	1.75				2.25	7.6	5.68	4.82	4.74	4.92	4.4			
A13	144								11.26	5.3	0.44	0.25	0.5	0.4	1.5			
A14	146								28.38[b]	3.9	0.44							
A15	148								7.21	6.7								
A16	150								5.41	1.6								
A17	152								0.9	0.23								
A18	154								0.9									

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

[a] Statistical significance found.

[b] Most frequent alleles in particular population.

DISCUSSION

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

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

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

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

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

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

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

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

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ORIGINAL PAPER

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Aetiological heterogeneity of asymptomatic hyperglycaemia in children and adolescents

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

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

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

Introduction

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

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

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

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

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

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

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

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

Subjects and methods

Subjects

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

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

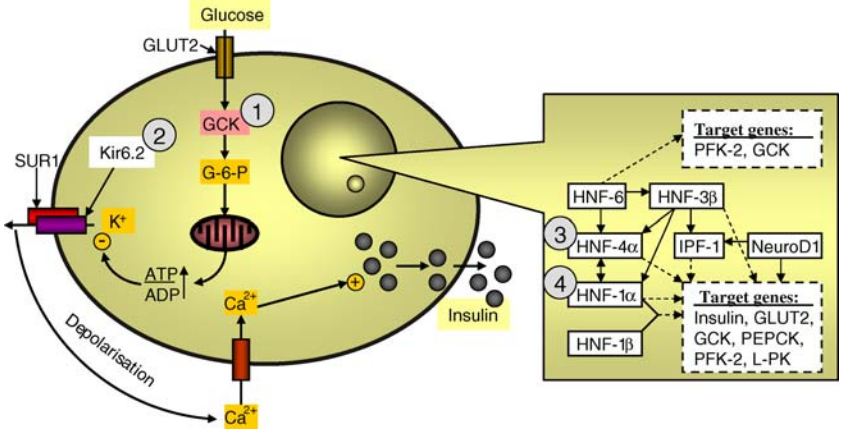

Fig. 1 Main pathways regulating insulin secretion in β-cells (*left*) and the current concept of β-cell transcriptional regulation network (*right*). Glucose molecules enter β-cells via the GLUT2 membrane transporter. The cytoplasmic enzyme glucokinase (GCK) senses the glucose concentration and initiates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

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

Study protocol

Baseline investigations

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

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

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

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

Clinical follow up

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

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

Ethics

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

Testing procedures

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

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

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

Routine laboratory assays

Plasma glucose

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

C-peptide and insulin

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

HbA_{1C}

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

Genetic analyses

Preparation of genomic DNA

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

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

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

Analysis of the KCNJ11 gene

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

Definition of diagnostic subgroups

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

Statistics

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

Results

Monogenic forms of hyperglycaemia

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

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

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

Type 1 diabetes mellitus (pre-manifest phase)

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Normal glucose tolerance

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

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

Summary of clinical laboratory data in diagnostic subgroups

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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Letter to the Editor

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

To the Editor:

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

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

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

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

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

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