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**GENETIC PREDISPOSITION TO DIABETIC
NEPHROPATHY**

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PhD Thesis

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1. INTRODUCTION TO DIABETIC NEPHROPATHY (Literature Review)

1.1. *Basic Aspects of Diabetic Nephropathy*

1.1.1. Definition and Epidemiology

Diabetic nephropathy is kidney disease associated with long-standing hyperglycemia, first described in 1936 by C. Wilson and P. Kimmelstiel as intercapillary glomerulonephritis (1). The main features of diabetic nephropathy include the nephrotic syndrome with excessive filtration of protein into the urine (proteinuria), high blood pressure (hypertension), and progressive impairment of kidney function. In severe cases DN leads to kidney failure, end-stage renal disease (ESRD) with the need for chronic dialysis or kidney transplantation.

Clinically, diabetic nephropathy is characterized as the presence of proteinuria > 0.5 g per 24h, referred to as overt nephropathy or clinical proteinuria. In the early 1980s, several European studies revealed that small amounts of albumin in the urine, usually undetectable by conventional methods, were predictive of the later development of proteinuria in both type 1 and type 2 diabetic patients (2-4). This stage of renal involvement was termed microalbuminuria or incipient nephropathy.

Proteinuria occurs in 15–40% of type 1 diabetic patients, with a peak of incidence at around 15 years of diabetes duration (5-7). The cumulative incidence of proteinuria in type 2 diabetic patients is more variable, ranging from 5 to 20% (6,8-10). There is, however, evident racial/ethnic variability in the prevalence of nephropathy. It is much higher among African Americans, Asians, Hispanics, and Native Americans (especially Mexican-Americans) than Caucasians (11). In Pima Indians diabetic nephropathy has been observed in about 50% of all diabetic patients.

Increased prevalence of type 2 diabetes and prolonged life expectancy of these patients in the last decade has led the number of diabetic patients that had progressed to the end staged renal disease (ESRD) to renal replacement therapy. In the US, diabetic nephropathy accounts for about 40% of new cases of ESRD. About 20–30% of patients with type 1 or type 2 diabetes develop symptoms of nephropathy, but in type 2 diabetes, a considerably smaller fraction of them progress to ESRD. However, because of the much greater prevalence of type 2 diabetes, such patients represent over half of all the patients currently starting the dialysis program.

1.1.2. Stages and Clinical Course

Approximately 25% to 40% of type 1 diabetic patients ultimately develop diabetic nephropathy, which progresses through 5 clinically characterized stages. In type 2 diabetes the clinical stages are less easily defined (12).

Stage I – Hyperfiltration, Hypertrophy

Stage I is found in the patients with newly diagnosed diabetes and it is characterized by 20 – 40% increase in glomerular filtration (GF) and increase in capillary permeability leading to microalbuminuria (MAU) and development of renal hypertrophy. Therapy with insulin leads to decline in MAU and GF.

Stage II – Latent Diabetic Nephropathy

It is a clinically silent phase with persistent hyperfiltration and hypertrophy. The GF remains elevated or returns to normal, but glomerular damage starts to manifest. The low or above-normal levels of albumin are present in the urine (30 mg/day, or 20 μ g/min). This is the earliest clinical evidence of nephropathy, referred to as microalbuminuria. Some authors consider this stage for stage III, and patients with microalbuminuria are defined as having incipient nephropathy. After 2 – 4 years the typical histopathological changes start to develop. Improved glycemic control decreases GF in this phase also (13-15).

Stage III – Incipient Diabetic Nephropathy

Albuminuria 30-300 mg/day (20-200 μ g/min), glomerular basement membrane (GBM) thickening and almost unaffected GF are typical signs of this phase. About 20% of patients with microalbuminuria develop incipient nephropathy within 6-12 years on standard diabetic care, although up to 50% of diabetic patients may not develop microalbuminuria et all. Hypertension typically develops during stage 3. Except for being a predictor of manifestation and progression of nephropathy (mainly in type 1 diabetes), albuminuria is a marker of greatly increased cardiovascular morbidity and mortality, especially in patients with type 2 diabetes. Thus, screening for microalbuminuria on yearly basis in all diabetic patients is important for detection of possible vascular disease and for aggressive intervention in order to reduce all cardiovascular risk factors (e.g., lowering of cholesterol levels, antihypertensive therapy, cessation of smoking, exercise, etc.) (16,17).

Stage IV – Overt Nephropathy

Without specific interventions, 80% of subjects with type 1 diabetes who develop sustained microalbuminuria progress to the stage of overt nephropathy or clinical albuminuria. Over a period of 10–15 years the amounts of albumin in the urine exceed 300mg/day or 200µg/min and hypertension develops along the way in almost all patients. Glomerular damage continues and the filtering ability of kidneys begins to decline steadily, with urinary albumin excretion (UAE) increased at a rate of 10–20% per year. The glomerular filtration rate (GFR) decreases about 10% annually. Glycemic compensation and tight blood pressure control slow down the progression, otherwise this stage leads to renal insufficiency, acceleration of hypertension and development of nephrotic syndrome with simultaneous progression of other diabetic complications, such as diabetic retinopathy, neuropathy or diabetic foot. A higher proportion of type 2 diabetic individuals is found to have microalbuminuria and overt nephropathy shortly after the diagnosis because diabetes is actually present for many years before it is diagnosed. Without specific interventions, 20–40% of type 2 diabetic patients with microalbuminuria progress to overt nephropathy, but 20 years after onset of overt nephropathy, only 20% will progress to ESRD.

Stage V – End Stage Renal Disease

Once overt nephropathy occurs, without specific interventions, GFR gradually falls over a period of several years at a rate that is highly variable from individual to individual (2–20 ml/min per 1 year) and renal replacement therapy is needed (i.e., hemodialysis, peritoneal dialysis, kidney transplantation). ESRD develops in 50% of type 1 diabetic individuals with overt nephropathy within 10 years and in 75% by 20 years. Survival of dialysis patients with diabetic ESRD comparing to non-diabetic patients is much lower due to cardiovascular risk and insufficient diabetes control (18).

1.1.3. Differential Diagnosis

The personal history, physical examination, laboratory evaluation, and imaging of the kidneys are basic tools of differential diagnosis in diabetic nephropathy. It is aimed at proving the persistent albuminuria higher than 30 mg/day, exclusion of other renal disease and proving the presence of diabetes. Three methods can be used to confirm the presence of disease and its follow up: chemical and microscopic examination of urine and urine sediment, assessment of proteinuria and screening for determinants of renal functions.

The first mentioned approach uses diagnostic strips to examine urine features: pH, presence of red blood cells, leucocytes, proteins, glucose, bilirubin, nitrites, and ketone bodies. The test for the presence of proteins is positive only in the IV stage of nephropathy according to Morgensen. Test for the glucose in the urine is only for orientation and do not serve to follow the diabetes compensation.

Very important for diagnosis of nephropathy is microalbuminuria assessment. Microalbuminuria is present if urinary albumin excretion is > 30 mg/24h, equivalent to 20 g/min in timed specimen, or 30 mg/g creatinine in random sample. In type 2 diabetic patients a test for the presence of microalbuminuria should be performed at time of diagnosis (19), because of the difficulty in precise dating of the onset of type 2 diabetes. If the absence of previously demonstrated microalbuminuria is observed, the test should be performed annually. In type 1 diabetes screening of individuals begins in 5 years after disease diagnosis because microalbuminuria rarely occurs within shorter period of type 1 diabetes duration (19). Screening for microalbuminuria is preferably performed by measurement of the albumin-to-creatinine ratio in random spot collection, because it is easy to carry out in office settings and is accurate (20). Morning collections are best because of the known diurnal variation in albumin excretion. Microalbuminuria measurement can also be done by 24-h urine collection with creatinine, allowing the simultaneous measurement of creatinine clearance, or timed collection (e.g., 4-h or overnight). For such screening the standard hospital laboratory assays for urinary protein are not sufficiently sensitive, therefore specific assays are needed. Microalbuminuria screening is important for the early diagnosis of diabetic nephropathy, and institution of angiotensin-converting enzyme (ACE) inhibitor or angiotensin receptor blocker (ARB) therapy and blood pressure control as a prevention of diabetic nephropathy.

The third tool, which helps to determine right diagnosis of diabetic nephropathy, is the measurement of specific markers of renal functions such are: creatinine, GF, cystatin C and urea. Creatinine is the end product of muscle metabolism and is elevated in several pathological situations. Its levels are significantly increased when GF drops under 50 %, therefore is not very suitable for detecting the incipient nephropathy. GF is estimated by measurement of clearance of creatinine, which is substance that is not reabsorbed back and only 5% is excreted by tubules (it increases with progression of renal insufficiency). Thus is a good marker for assessment of glomerular function. There are several formulas for counting the GF from creatinine values. GF is basic examination in patients with nephropathy, because in initial phases it is elevated in 25- 50% of type 1 diabetic till 5 years from diabetes diagnosis and more than 45% of type 2 diabetics at the time of diagnosis. It is a consequence of hyperfiltration and microalbuminuria; nevertheless it

stabilizes with progression to stage of persistent microalbuminuria. It was proved that moderate decrease in GF can be identified by measuring of cystatin C, unglycosylated protein, and inhibitor of cystein proteases, which is fully reabsorbed and metabolized in proximal tubules. The progression of renal insufficiency to renal failure in late stages of nephropathy can be determined by concentrations of urea, which are increased in diabetic nephropathy (21).

In the special situations such as proteinuria over 1 g/24 h, renal impairment in the absence of retinopathy, or unexplained hematuria, renal biopsy is recommended to perform (22). Although the criteria for renal biopsy are not well established in type 1 diabetes the reason may be the presence of proteinuria in patients with short diabetes duration, especially in the absence of diabetic retinopathy (23). In patients with type 2 diabetes, the criteria are less clear. Non-diabetic renal complications are common in proteinuric type 2 diabetic patients, but the proportion varies according to the criteria used to perform the biopsy and to the ethnic origin. The diagnosis of diabetic nephropathy is easily established in long-term type 1 diabetic patients (>10 years of diabetes duration), especially if retinopathy is present. Typical diabetic nephropathy is also likely to be present in proteinuric type 2 diabetic patients with retinopathy. However, diagnostic uncertainty exists in some patients with type 2 diabetes since the onset of diabetes is unknown and retinopathy is absent in a significant proportion (28%) of these patients (24). The presence of symptoms during urination suggests urinary tract disorders such as obstruction, infection, or stones. Skin rash or arthritis may indicate systemic lupus erythematosus or cryoglobulinemia. Presence of risk factors for parenterally transmitted disease may raise the suspicion of kidney disease associated with HIV, hepatitis C, or hepatitis B. History of proteinuria and/or hypertension during childhood or pregnancy may suggest other glomerulonephritis. Also, family history of kidney disease may indicate the presence of polycystic kidney disease or other genetic diseases (25). Imaging of the kidneys, usually by ultrasonography, should be performed in patients with the family history of polycystic kidney disease or when symptoms of urinary tract obstruction, infection, kidney stones are present (25).

1.2. Pathophysiology of Diabetic Nephropathy

1.2.1. Renal Structural Damage

Diabetes causes typical histopathological changes in kidney structure, first described by Kimmelstiel and Wilson (1), presented by increase in mesangial matrix and GBM thickening, followed by tubular and interstitial changes. Macroscopically an early feature of nephropathy in

both experimental and human diabetes is enlargement of whole kidney due to combination of tubular hypertrophy and interstitial expansion (26,27), probably as a result of hyperglycemia and fluid reabsorbing. Furthermore, renal scarring and papillary necrosis may occur, mainly in women, as a consequence of urinary tract infections (28). Finally atherosclerosis of renal arterioles and renal artery sclerosis may follow (29).

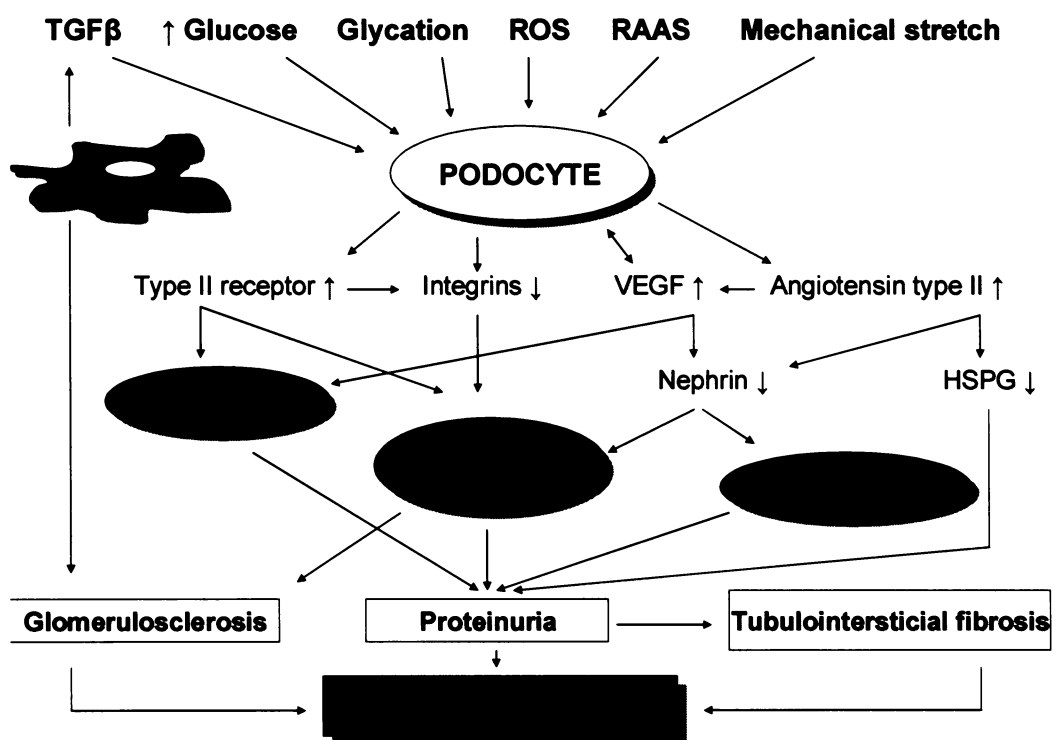
Diffuse mesangial expansion is a distinctive mark of diabetic nephropathy (30). Kimmelstiel-Wilson nodules, which are areas of extreme mesangial accumulation observed in 40–50% of patients developing proteinuria may be also present (1). Mesangial matrix expansion correlates with both, proteinuria and deterioration of renal function. Accumulation of matrix in the mesangial area reduces the capillary surface area available for filtration, thus it contributes to the progressive loss of renal function (31,32). The result of advanced glomerulopathy is hyalinized glomerulus with no obvious capillary loops. Increase in matrix is secondary to a combination of its excess production and decreased degradation. Also composition changes from normal to abnormal with elevated collagen IV and reduced proteoglycans (heparan sulfate) (33).

Another conspicuous feature of diabetic glomerulopathy, however not the specific one because it occurs irrespective of nephropathy status, is GBM thickening due to alterations in its architecture and composition (34). It would seem that thickened GBM participates in more effective barrier to the filtration of proteins, but it is in fact more porous to proteins (35). The biochemical composition changes in several aspects. The increase in hydroxylation of amino acids (lysine, proline, glycine), multiplication of disaccharide bounds and reduction in laminin and heparan sulphate content has been observed (36,37). The loss of charge selectivity in the GBM rises due to reduction in negatively charged proteoglycans (38), allowing passage of positively charged proteins (albumin). This action partially explains the proteinuria (39). The final barrier separating plasma proteins from the vasculature is the slit diaphragm of podocytes (glomerular visceral epithelial cell). The changes in podocytes are variable. There is certain reduction in number of podocytes and widening of pedicels (40), which appears with onset of microalbuminuria, however it does not fully correlate with degree of proteinuria (41). During development of nephropathy total lost of podocytes may be reached. The remaining podocytes have to compensate the lost by widening of pedicels (42). Taken together, all mentioned alterations of the GF barrier, composed of the glomerular endothelium, GBM, and podocytes result in the reduction of GFR and proteinuria (*Figure 1*).

The harmful effect of proteinuria is observed also downstream, in tubules, presented by nephron atrophy and interstitial fibrosis (43,44). Tubular interstitial alterations reflect the degree of renal damage, although the structural and functional changes do not correlate. Persistent

hyperglycemia impairs the tubular transport leading to haemodynamic unbalance and worsening of renal functions. Thickening of tubular basement membrane (TBM) is present, the most striking in proximal tubules (45). Interstitial fibrosis may be observed in later stages of diabetic nephropathy, and it correlates with GFR (46). It may be accompanied by chronic inflammatory infiltration of T lymphocytes and macrophages (47). Furthermore, hyaline arteriosclerosis is early manifestation of diabetic nephropathy, corresponding to glomerular impairment. Hypertension and hyperlipidemia worsen this condition by narrowing the lumen of arterioles resulting in the accelerated renal vascular hypertension.

Figure 1: Mechanism of podocytes injury and development of proteinuria in diabetic nephropathy



Wolf, G.: From the Periphery of the Glomerular Capillary Wall Toward the Center of Disease. Podocyte Injury Comes of Age in Diabetic Nephropathy. Perspective in Diabetes, VOL. 54, June 2005

In addition to typical features of nephropathy, induced by diabetic environment, non-diabetic renal lesions can be found in diabetic patients. They occur more often in type 2 diabetes. Possible reason for this is the presence of atherosclerosis, or the urinary tract infections typically

present in these patients. The age may also play a role in the development of non-diabetic lesions. The most typical non-diabetic renal diseases found in diabetic patients are: membranous glomerulopathy, focal segmental glomerulosclerosis, or IgA nephritis (48) (other are listed in *Table 1*).

Table 1: List of primary, non-diabetic renal diseases present in diabetic patients

Glomerulonephritis	Non-glomerular diseases
Endocapillary – post infectious	Amyloidosis
Membranous	Myeloma
IgA nephritis	Cortical necrosis
Focal segmental proliferative	Tubular necrosis
Mesangial proliferative	Interstitial necrosis
Membrane proliferative	Papillary necrosis
Lupus nephritis	Atherome embolisation
Minimal change disease	Hypertensive nephrosclerosis
	Sarcoidosis

1.2.2. Risk Factors

Epidemiological (49) and familial studies (50-54) have demonstrated that genetic susceptibility together with environmental factors contribute to the development of diabetic nephropathy in patients with both type 1 and type 2 diabetes. The main exogenous potentially modifiable factors in genetically susceptible individuals are sustained hyperglycemia and hypertension (18,55-60). Other putative risk factors are glomerular hyperfiltration, smoking, dyslipidemia, proteinuria levels, and dietary factors (31,56,59,61-72).

1.2.2.1. Metabolic Factors

Diabetic metabolic milieu, characterized by hyperglycemia, elevated levels of glucagon and growth factors and variable insuline levels has evident impact on microvascular complications (73,74). The majority of patients with diabetes escape renal failure, although some histological

damage occurs in their kidneys. Therefore, it appears that in humans hyperglycemia is necessary, but not sufficient to cause renal damage that leads to kidney failure.

Experimental studies show that in diabetes the rate of matrix and GBM components synthesis is significantly accelerated, resulting in increased collagen formation. This may be due to overexpression of glucose transporter 1 (GLUT1) as a result of hyperglycemia, but the pathophysiological role of GLUT1 in the nephropathy is not clear yet (75-77).

Another deleterious effect of hyperglycemic is the reduction in the production of glycosaminoglycans, glycoproteins rich in sulfonyl groups, composed of uronic acid and aminosacharide dimmers. In many tissues they are components of extracellular matrix. In kidneys they are responsible for creating the negative charge on GBM (mainly heparan sulfate, and minor component sialic acid) and regulation of its permeability (78). In diabetes, hyperglycemia causes reduced synthesis of both heparan sulfate and sialic acid, leading to loss of negative charges and fusion of foot processes. These actions contribute to the development of diabetic nephropathy and are described in the Steno hypothesis (79).

Glucose can also react with circulating structural proteins, giving the rise to advanced glycosylation end products (AGEs), in the reaction called non-enzymatic glycosylation (80-82). The products of this reaction, AGEs, affect the GBM compounds by creating the cross-links, leading to decreased sensitivity to proteases resulting in their accumulation and depositing (83). AGEs may cause alterations in the macrophage removal system and interfere with mesangial clearance mechanisms leading to mesangial expansion and subsequently glomerular occlusion (84-86).

In the alternative metabolic pathway of glucose, the polyol pathway, glucose is transformed into sorbitol. This reaction is catalyzed by aldose reductase (ALR), an enzyme present in the glomerular, tubular and mesangial cells. Accumulation of sorbitol as a result of increased availability of glucose leads to reduced intracellular concentration of myoinositol. These changes contribute to diabetic complications by impairment of cellular osmotic regulation. However, the long-term human studies with use of ALR inhibitors in diabetic patients did not prove its effect on the development of diabetic nephropathy (87).

1.2.2.2. Haemodynamic Factors

The pathogenesis of haemodynamic changes leading to diabetic hyperfiltration is multifactorial. There is a whole scale of potential mediators and mechanisms. Except for metabolic factors connected to hyperglycemia, the proofs exist that diabetic hyperfiltration is

caused by unbalance between vasoactive factors controlling afferent and efferent arteriolar tonus (88).

Atrial natriuretic peptide (ANP) controls sodium homeostasis and vascular permeability. Its effect on renal arterial system is dilatation of afferent and constriction of efferent arterioles. Its level is elevated in experimental diabetes (89), which suggests that it may be responsible for increase exchangeable sodium levels and hyperoxoalemia (90,91). In fact, it was really showed that sodium restriction leads to normalization of haemodynamic changes in experimental diabetes (92,93).

Another potent dilator of afferent arteriole is nitric oxide (NO), however some vasodilatation action on efferent arteriole was also observed. Its elevated levels in kidneys may be responsible for increased GF. It is produced by enzyme NO synthase (NOS), which has three forms (NOS1, 2, 3). Inhibition of NOS decreases GF in diabetic rats with hyperfiltration (94-96). Recent studies identified endothelial NOS (NOS 3, eNOS) as a causal enzyme responsible for overproduction of NO in diabetic kidneys. Nevertheless, it may be also a consequence of high concentration of ROS present in diabetic kidneys (94,95,97). Other studies suggest the role of neuronal NOS (NOS1, nNOS) in diabetic hyperfiltration (98,99), pointing at its modulation of negative tubuloglomerular feedback loop. NO acts also as a regulator of other factors implicated in the pathogenesis of nephropathy. For example, it modulates activity of vascular endothelial growth factor (VEGF) (100), but also participates in the signaling through transforming growth factor β (TGF- β) (101).

Interesting is the role of insulin, NO-dependent vasodilator, in the haemodynamics of kidneys. Study on streptozocin induced diabetic rats proved that hyperfiltration is more evident in heavy hyperglycemia combined with insulin treatment (102,103).

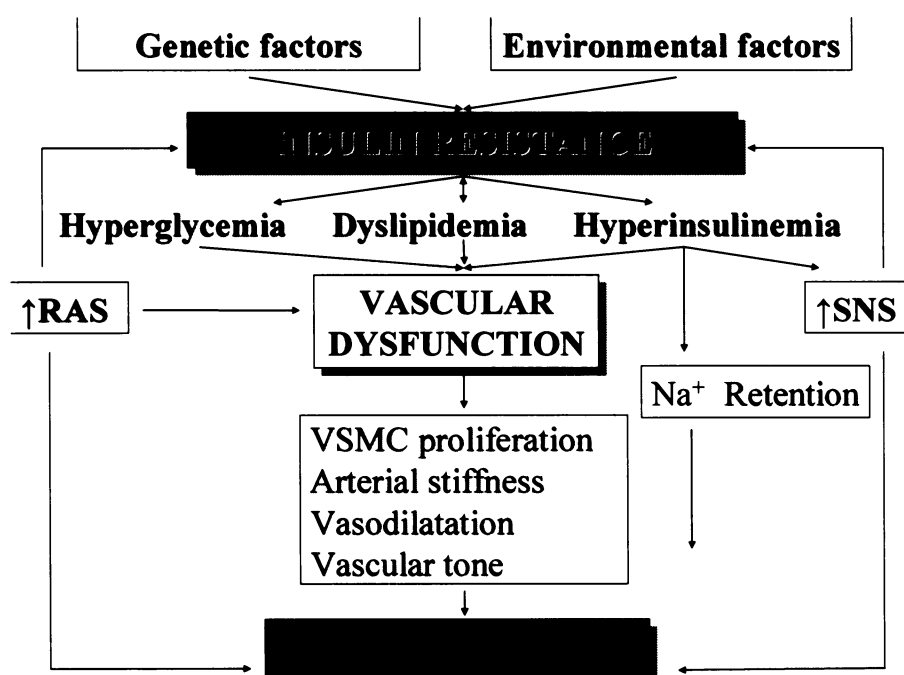
In early stage of nephropathy enhanced excretion of prostaglandins has been observed. Its inhibition has led to restoration of renal haemodynamics in type 1 diabetes (104,105), but also in diabetic models (106-108).

1.2.2.3. *Hypertension*

Hypertension (HT) commonly occurs with diabetes and obesity and is up to three times more frequent in diabetic patients than in non-diabetics (109). It significantly increases the risk for microvascular as well as cardiovascular disease (CVD). On the other hand hypertensive patients are 2.5 times more susceptible to develop type 2 diabetes than the normotensive counterparts (110). Frequency of hypertension development differs greatly in both types of diabetes. In type 1

diabetes, hypertension is uncommon in the absence of renal disease. The onset of hypertension usually starts in the stage of incipient nephropathy, and with the manifestation of microalbuminuria, 60-70% of the patients develop hypertension. In the stage of renal failure all patients are hypertensive (111). In contrast, in type 2 diabetes, the prevalence of hypertension before onset of microalbuminuria is 71% and rises to 90% with its development (112). In United Kingdom Prospective Diabetes Study, 39% of newly diagnosed type 2 diabetic patients had hypertension and 24 % of the hypertensive group had microalbuminuria (113). In type 2 diabetes, hypertension usually clusters with the other components of the cardio-metabolic syndrome such as central obesity, insulin resistance, microalbuminuria, dyslipidemia, increased inflammatory status and hyper coagulation.

Figure 2: Putative mechanisms of hypertension in diabetes



Sameer, N.: Pathogenesis of Hypertension in Diabetes, *Rev Endocrin Metabol Dis* 2004; 5:221–225

The pathogenesis of hypertension in diabetes is complex and involves multiple factors (Figure 2). The genetic involvement is also discussed in the pathogenesis of hypertension in both types of diabetes. In type 1 diabetes, the presence of hypertension in the parents is an independent risk factor for developing nephropathy and hypertension comparing to the patients from non-

hypertensive family (114,115). Genetic predisposition for essential hypertension is associated on the other hand with insulin resistance and development of type 2 diabetes. There is more often prevalence of hypertension and insulin resistance in offspring of type 2 diabetic patients (116,117). The typical changes playing role in the pathogenesis of hypertension are sodium retention and extracellular volume and endothelial dysfunction.

A key role in the pathogenesis of hypertension in type 1 and type 2 diabetic patients plays sodium retention in kidneys (90). It is related to glucose induced glomerular hyperfiltration leading to increased sodium uptake in proximal tubules. Elevated body sodium and retention of water caused by hyperinsulinemia stimulate the tubular sodium transport (118-120). Increased water retention and raised body sodium content are assumed to be a result of anti-natriuretic effect of insulin observed in normal subjects, hypertensive patients, and insulin-resistant obese individuals (121). Suppression of ANP activity in diabetic patients may also play a role in sodium retention, leading to decreased tubular response and extracellular fluid volume expansion, which may promote increased cardiac output and blood pressure (90,122). The ANP system normally has a protective effect against the development of hypertension due to its natriuretic and vasodilator effects (123,124).

Vascular dysfunction plays a crucial role in the pathogenesis of hypertension in diabetes. Increased peripheral resistance to insulin and insulin like growth factor-1 (IGF-1) signaling, plasma volume expansion and elevated contractility of vascular smooth muscle cells (VSMC), as the response to vasopressor stimuli, noradrenalin and angiotensin II, has been observed in diabetic patients with hypertension. Major factors affecting the vascular tree in diabetes are thus insulin resistance and hyperglycemia. Hyperglycemia inhibits endothelial cell repair and induces apoptosis, which promotes the inflammatory process, impairs vasodilatation, and increases the likelihood of plaque formation and blood pressure elevation (125). Both glucose and insulin increase endothelin secretion (126). Insulin-induced endothelin secretion may promote insulin resistance by interfering with insulin signaling in VSMC (127).

Increased glucose levels promote structural and functional changes in VSMC by inducing cell proliferation, increase of oxidative stress and suppression of NOS activity (128,129). The increase in oxidative stress, in addition, can be caused by binding of AGEs to VSMC and inhibiting the anti-proliferation effects of NO, which may facilitate the progression of hypertension (130).

The renin–angiotensin–aldosterone system (RAAS) may also contribute to promoting oxidative stress and endothelial dysfunction in diabetes (110,131). Activation of RAAS is associated with increased reactive oxygen species (ROS) formation. ROS impair vasodilatation

by decreasing NO availability (132). Interruption of RAAS by administration of ACE inhibitors or ARBs improves insulin sensitivity and decreases the progression of type 2 diabetes in hypertensive patients (131,133).

Insulin has a growth-promoting effect on VSMC, mediated by several pathways including induction of IGF-1 expression in VSMC (134), stimulation angiotensin II and up-regulation of angiotensin type 1 receptors (AT1R) (135). Insulin and IGF-1 can induce vascular relaxation through effects on VSMC divalent ion metabolism. Reduction of Ca^{2+} concentration and stimulation of the Na^{+}/K^{+} adenosine triphosphatase (ATPase) pump cause vascular relaxation. Also reduced tissue Mg^{2+} contributes to decrease insulin-stimulated glucose uptake, and may increase vascular resistance. Thus, increased VSMC Ca^{2+}/Mg^{2+} ratio may play an important role in hypertension associated with insulin resistance (118,133,136,137).

1.2.2.4. *Cardiovascular Complications*

Cardiovascular diseases are the most severe macrovascular complications of diabetes, especially in type 2 diabetes and they include: myocardial infarction, angina pectoris, coronary artery bypass graft and percutaneous transluminal coronary angioplasty (138,139). Diabetes mellitus is a major risk factor for developing CVD (140). The presence of diabetic nephropathy, especially in the stage of incipient microalbuminuria, greatly enhances this risk, which is in type 1 diabetic patients 40 times higher (141,142). Rising cardiovascular morbidity and mortality correlate with increase of other risk factors such as dyslipidemia or arterial hypertension. Although there are no significant results from studies about the effect of antihypertensive treatment on lowering cardiovascular risk in type 1 diabetic patients, hypertension is considered for major input on increased CVD associated death in type 2 diabetes (16,143,144). It was demonstrated that intervention with antihypertensive agents, particularly angiotensin type 2 receptor (AT2R) agonist, has led to reduced incidence of cardiovascular events in type 2 diabetic patients (145).

The prevalence of CVD differs depending on the ethnic background (139). In Pima Indians, where the prevalence of type 2 diabetes is high, the incidence of CVD is relatively small. However, it is higher in newly diagnosed type 2 diabetics and women. This is probably due to late diagnosis of diabetes. Important aspect in the pathology of CVD is susceptibility to atherosclerosis, typically occurring in subset of diabetic patients. This may be explanation for ethnic differences in prevalence of the CVD. Other pathophysiological elements are present even before onset of diabetes. Impaired glucose tolerance, increased BMI, hypertension, hyperlipidemia

and insulin resistance occur in patients that consequently develop diabetes. Pathological mechanisms leading to CVD are complex, and include all of aforementioned factors associated with diabetes. They are together known as metabolic syndrome (146). In patient with metabolic disorders, microalbuminuria is the strongest predictor of cardio-renal impairment (147-149), followed by smoking, high blood pressure and serum cholesterol levels (150). The correlation between microalbuminuria and degree of cardiovascular risk has been demonstrated in many studies (147,151-153).

1.2.2.5. Lipid Disorder

Diabetic dyslipidemia, also called atherogenic, is characterized by three abnormalities: increased very low density lipoprotein (VLDL), formation of small low density lipoprotein (LDL) particles and decrease in high density lipoprotein (HDL) cholesterol levels.

Together with hypertension and hyperglycemia, dyslipidemia is considered to be implicated in the pathogenesis of diabetic nephropathy and other vascular complications of diabetes, however it is not clearly established whether impaired lipid metabolism is a cause or a consequence of diabetic nephropathy. It is known that hyperglycemia and insulin insufficiency cause abnormalities in lipid metabolism, but also that proteinuria accelerates lipid disorder in diabetic patients and greatly increases the risk of ischemic heart disease. Furthermore, with increasing plasma concentrations of triacylglycerols (TAGs), lipoproteins and LDL cholesterol more rapid progression of nephropathy to ESRD has been observed (154).

Most of the diabetic patients with dyslipidemia are insulin resistant and in some of them elevated plasma apolipoprotein B (ApoB) concentration is also present (155,156). Therefore the impairment of lipid metabolism is thought to promote the processes of atherosclerosis and increase the risk of CVD related death in diabetic patients. Whether or not dyslipidemia participate on the development of cardiovascular events, its pathological and clinical features differ between both types of diabetes.

In type 1 diabetes the activity of lipoprotein lipase is increased as consequence of insulin deficiency, which leads to reduced degradation of TAG containing lipoproteins (157). Insufficient metabolic compensation of hyperglycemia participates on the development of triacylglycerolemia and increased ability of LDL cholesterol to oxidize and thus become atherogenic (158). Microalbuminuria has been proven to be associated with increased dyslipidemia, which is thought to be caused by loss of lipoprotein due to proteinuria. Thus, it appears that poor glycemic control

and proteinuria are two independent determining factors in development of hyperlipidemia in type 1 diabetes.

In type 2 diabetic patients the elevation of LDL cholesterol and lipoproteins as well as dropout of HDL cholesterol are present even when the hyperglycemia is well compensated and no proteinuria is present. Dyslipidemia appears already before clinical manifestation of type 2 diabetes and poor glycemic conditions, obesity and proteinuria only worsen it. Hyperlipidemia is probably primary defect resulting from insulin resistance. Thus it is not out of the question that hyperlipidemia participates on the development of diabetic nephropathy and decreased HDL cholesterol level observed in type 2 diabetic patients is an independent risk factor of cardiovascular complications such as ischemic heart disease.

1.2.2.6. Eye Complications

Diabetic retinopathy (DR) is diabetic complication, which histopathology is based on capillary damage with typical loss of endothelial cells and pericytes, deposition of glycoproteins in capillary basement membrane and increased vascular permeability (162).

Two distinct forms of diabetic retinopathy can be distinguished: proliferative and nonproliferative. The second is found in the 90 % of all diabetics and is typical for senior type 2 diabetic patients. It has slower progression and it is divided into three stages (mild, moderate, and severe). During its progression more and more blood vessels become blocked. Retinopathy usually does not evoke vision loss at this stage. The capillary walls may lose their ability to control the passage of substances between the blood and the retina. Fluid can leak into the macula – the part of the eye where the focusing occurs. Then the macula swells with the fluid and condition called macular edema appears. The vision blurs and subsequently can be lost entirely. Although nonproliferative retinopathy usually does not require treatment, macular edema must be treated. Fortunately treatment is usually effective in stopping and sometimes reversing the vision loss.

The proliferative retinopathy is stage when the obliteration of capillaries reaches certain degree and ischemic retina releases agents, which are stimulating local neovascularization. New vessels can grow either by the optical nerve, or by the big blood vessels. They incline to bleed (hemophthalmus) and may cause the corrugation and retina avulsion. In the case of severe retinal ischemia neovascularization of iris may occur (rubeosis iridis), resulting in very painful and hardly treatable neovascular glaucoma and eventually blindness. Proliferative retinopathy develops after 15 years of diabetes duration in 25% of type 1 diabetics and 15% of type 2 diabetic patients.

There is very close relationship between DR and DN in both epidemiology and pathogenesis. It is known that while almost all patients with kidney impairment have also retinopathy (159,160), approximately one third of patients with proliferative lesions on the retina will never develop microalbuminuria (161). However proteinuria is not considered to be a predictive factor for development of DR, it may predict the progression of existing lesion to its proliferative form. The incidence of both diseases differs. The retinopathy increases progressively with diabetes duration and reaches 100% prevalence after 15-20 years. The incidence of nephropathy rises between 5 – 15 years and then the risk of nephropathy declines, thus only third of patients eventually develop nephropathy (163). In Joslin Clinic study 80 % of diabetic patients with persistent proteinuria had proliferative retinopathy, while only 25 % of diabetics developed retinopathy in absence of proteinuria (164). Retinopathy occurs in less than 1% per year, but its incidence rises to 10 – 15% when proteinuria develops. Results of other epidemiological studies point at relationship between development of retinopathy and degree of metabolic control, but its progression is dependent on the compensation of high blood pressure (165,166). Klein *et al* has presented in his study that risk factor for transformation to proliferative form of retinopathy is presence of hypertension, whereas development of macular edema is associated with proteinuria (167).

The common pathological mechanism, leading to both diabetic complications, may present microangiopathic damage of capillaries of affected organs, kidneys and eyes. Capillary blood flow in both retina and renal glomerular system positively correlates with levels of glucose (168). Endothelial cells of the vessels in retina and kidneys are also affected by diabetic milieu. The production of vasorelaxants is decreased and on the contrary the synthesis of vasoconstrictors is enhanced (169-173). Also the production of extracellular matrix components by endothelium is increased, resulting in basement membrane thickening and impairment of vessels function in both retinopathy and nephropathy (80,174-178). Endothelial ability to synthesize and regulate fibrinolysis and coagulation was also suggested to be implicated in the common pathological events leading to development of microangiopathic complications of diabetes (178).

1.3. Genetic Risk Factors

1.3.1. Diabetes Mellitus

Diabetes mellitus (DM) is one of the most common endocrine disorders affecting almost 6% of the world's population. This chronic metabolic disorder affects the metabolism of

carbohydrates, protein, fat, water, and electrolytes, leading to structural changes in tissues of many organ systems in the body, especially those of the vascular system. The prevalence of DM is on the increase (179) and according to estimates the number of patients with DM will in 2025 reach 300 millions (180,181).

Two major forms of the disease are distinguished: type 1 and type 2. The pathophysiology of both forms is incompletely understood, but it has now been widely accepted that both genetic and environmental factors play a contributing role in the development of both forms of DM.

1.3.1.1. Type 1 Diabetes: Epidemiology and Genetics

Type 1 diabetes (T1D) is an autoimmune disease characterized by specific destruction of the insulin-secreting β -cells of the pancreatic islets. The susceptibility to type 1 diabetes is determined by interactions of multiple genes with unknown environmental factors (182).

The incidence rate of type 1 diabetes varies with age and rarely occurs before the first 6 months of age (183). It begins to rise sharply at about 9 months of age, continues until age 12 – 14 years, and then declines (184). Accumulated data on the incidence of type 1 diabetes during the last 20 years showed major ethnic and geographical differences in its prevalence. The highest risk for type 1 diabetes is among white population and even within the white population the variability is almost as large as that observed among all races. The lowest incidence of type 1 diabetes is in Japan, the Caribbean, and southern Europe, while the highest is in the Scandinavian countries (especially Finland) (185). Bruno *et al* observed significantly higher incidence of type 1 diabetes in males compared to females (186), but in some countries (Poland, Canada, Italy) sex difference in the incidence of type 1 diabetes was not observed (187-189). This may be due to the influence of sex hormones or differential exposure to environmental contaminants. From other factors that may influence the incidence of type 1 diabetes there are: seasonal changes (higher in autumn and winter (190,191), nutrition uptake during neonatal period and early infancy (consumption of cow's milk protein) (192,193), viruses (coxsackie B4 and congenital rubella have been reported in 20–30% of new cases of type I diabetes) (194,195) and presence of other autoimmune diseases (Addison's disease, pernicious anemia or autoimmune thyroid disease) (196). All of these examples support the role of environmental factors in the etiology of diabetes.

A number of studies have shown that the destructive process in autoimmune diabetes specifically directed against pancreatic islet β -cells, is mediated by T cells, can last for years before the appearance of clinical symptoms and is characterized by the presence of circulating autoantibodies to β -cell antigens (197). These include insulin autoantibodies (IA), glutamic acid

decarboxylase (GAD) autoantibodies, and antibodies against the islet cell antigen 512 phosphatase (IA-2), of which only insulin ones are β -cell specific. IA occur more in DR4 haplotype patients and are useful if measured prior to administering exogenous insulin. GAD antibodies persist the longest following diagnosis and are useful in confirming autoimmune etiology in long-standing cases. The presence of more than one type of antibody, highly predictive of disease, occurs years before clinical manifestations. More than 70% of the newly diagnosed type 1 diabetic patients have islet cell antibodies that serve as markers of disease, primarily in the first year of diagnosis, without playing a role in β -cell destruction. Those patients can be subclassified as autoimmune (type 1a). On the other hand, small percentage of type 1 diabetic patients lacks any evidence of autoimmunity – presence of antibodies (type 1b). It is unclear what leads to deregulation of autoimmunity in type 1 diabetes. It is hypothesized that some imbalance between effector T cells and regulatory T cells exists (198,199).

Genetic factor in type 1 diabetes consists of multiple susceptibility genes, with a major locus encoded by human leukocyte antigen (HLA) region on chromosome 6 (191,200,201), and an undefined number of loci with minor effect (202). HLA class II, DQ and DR genes have been consistently reported to be associated with type 1 diabetes in multiple ethnic groups. Around 95% of type 1 diabetic patients carry HLA-DR3, HLA-DR4, or both. The relative risk of type 1 diabetes conferred by DR3 is 7 times, by DR4 9 times and by both DR3 and DR4 is more than 14 times that of normal (203). In addition to HLA, several non-HLA loci have been shown to contribute to the disease susceptibility (204-207). Despite of the large number of loci mapped to the genome, only a limited number of genes have been identified as genes responsible for susceptibility conferred by these loci. Among these are the insulin gene (INS), cytotoxic T-lymphocyte-associated protein 4 (CTLA4), small ubiquitin-like modifier 4 protein SUMO4 and protein tyrosin phosphatase (PTPN22) encoding lymphoid tyrosine phosphatase (LYP) (208-212). Most of these genes were identified in Caucasian populations, thus it is yet to be confirmed whether these genes are the susceptibility genes for type 1 diabetes also in other ethnic groups.

1.3.1.2. Type 2 Diabetes: Epidemiology and Genetics

Type 2 diabetes (T2D) is metabolic disease with chronically elevated glucose levels, characterized by two main abnormalities: impairment of insulin secretion and decrease in insulin sensitivity (213). Undiagnosed or poorly controlled, leads to complications such are blindness, renal insufficiency, amputation of lower extremities, heart disease, or stroke. Approximately 150 million people worldwide are affected by type 2 diabetes, with expectancy to double in next 20

years (214). Two factors are participating on clinical picture of type 2 diabetes: environment and genetics.

A large body of studies refers about the role of environmental factors in the development of type 2 diabetes. It has been reviewed that ethnicity may play important role. The prevalence of type 2 diabetes differs in people with different ethnic origins in Africa or Asia (215). Other studies demonstrate the importance of residence on the prevalence of diabetes mellitus. The role of geographical location in the development of type 2 diabetes is supported for example by the fact that Japanese living in Brazil, or in Hawaii and Los Angeles have higher prevalence of type 2 diabetes compared to those in Japan (216,217). The difference between the prevalence of diabetes in the urban and rural setting may be due to influence of junk food supply in the rural versus urban setting, and possibilities of physical activity. These are another factors influencing insulin sensitivity and glucose tolerance in type 2 diabetes (218). In a recent study performed on an African American population in the United States, was observed that the prevalence of diabetes increases with the degree of inactivity and obesity (191,217,219). Obesity is present in about 80% of type 2 diabetic patients and has been implicated as one of the risk factors for type 2 diabetes (220-222). Body mass index is directly associated with increased risk of type 2 diabetes in many ethnic groups (191). Severe and prolonged stress associated with modern life style may also be one of the environmental factors associated with glucose intolerance and may hence increase the risk of type 2 diabetes by activation of the adrenal hormones, notably the glucocorticoids, that have been observed to cause glucose intolerance (223). Also drugs such as corticosteroids and some oral contraceptive steroids may cause glucose intolerance and type 2 diabetes in susceptible individuals (224).

Several reports point at attributes that are not result of environmental influence, supporting the role of genetic factors in the pathogenesis of type 2 diabetes. The differences in the prevalence of various ethnics and races are such examples. Extremely high prevalence of type 2 diabetes was observed in Pima Indians of Arizona, also in Hispanic minorities (Cubans in Florida and Mexican Americans in the border states, but does not approach that of Indians (225). In European populations, the prevalence of type 2 diabetes is less than half of the prevalence observed in American populations (226). The prevalence of type 2 diabetes in Saudi Arabia is similar to that obtained in US populations, while in Central Asia the prevalence among men, aged 50 years and over, is similar to that in Europe.

Genetic susceptibility plays a crucial role in etiology and manifestation of the disease, however very little is known about specific genetic variants. The important evidence for the role of genetic involvement are results of twin studies, performed in USA and Denmark, showing high

concordance rate for monozygotic twins (55%), comparing to dizygotic (15%) (227,228). Comparison of the phenotypic characteristics of the disease has also given more information on the nature of genetic factors in this disease. For example studies on Asian Indians with type 2 diabetes showed that they are more insulin resistant than are Caucasians with type 2 diabetes, even when the degree of obesity is comparable (229). This suggests that there may be distinct subtypes of type 2 diabetes in different ethnic groups depending on the phenotype of the diabetes gene. Based on the role of genetic factors, type 2 diabetes can be divided into two forms: monogenic and polygenic (230,231). First form is a consequence of single gene mutations, has high penetrance, severe clinical picture and the environment only slightly influences its pathogenesis. The genes, identified for monogenic form of type 2 diabetes, cause either severe defect in insulin secretion: hepatocyte nuclear factors 4 α and 1 α (HNF-4 α , HNF-1 α), glucocinase, insulin promoter factor-1 (IPF1), neurogenic differentiation 1 (NEUROD1) (232-238), or they participate in decreased insulin sensitivity: peroxisome proliferator-activated receptor γ (PPAR γ) and v-akt murine thymoma viral oncogene homolog 2 (AKT2) (239,240). It is believed that polygenic type 2 diabetes results from inheritance of set of susceptibility genes, each partially contributing in the pathogenesis of the disease. Only the effect of these genes together with environmental factors triggers the disease. Genome-wide linkage mapping for type 2 diabetes provided a number of different susceptibility loci, among them 1q, 1q, 8p, 10q, 12q and 20q (241-243). However, so far, only the chromosomal locus 2q37, with the gene calpain 10 (CAPN10), has been a successful example of positional cloning in identifying a susceptibility locus and the gene for type 2 diabetes (244,245). The other genes that showed the most consistent results on the susceptibility for polygenic type 2 diabetes are: PPAR γ gene (246-248), polymorphism of KCNJ11 gene (resp. Kir6.2 gene, coding subunit of ATP-sensitive K⁺ channel) (249-252) and HNF4A gene(253-256).

1.3.2. Genetics of Diabetic Nephropathy

Today, substantial evidence exists for the role of genetic factors in the pathogenesis of diabetic nephropathy, although the inheritance pattern is not known yet. A plausible genetic model may be one or two major genes with a dominant or recessive effect interacting with environmental factors, or a few genes with a moderate effect in a similar interaction (257). Diabetic nephropathy thus may represent a typical complex disease such as diabetes.

1.3.2.1. *Inherited Susceptibility*

1.3.2.1.1. **Incidence studies**

The prevalence of nephropathy rises with prolonged duration of diabetes but levels out at around 15 years after diabetes diagnosis, after which it declines substantially, in contrast to diabetic retinopathy, where the incidence rate is linear (5,49,258,259). It is less easy to demonstrate it in type 2 diabetics, where the onset of the disease is not clearly defined. It is thus apparent that only subset of diabetic patients are at risk of having renal complications. After 25 years of diabetes duration, the lifetime risk for developing overt nephropathy and ESRD is low in the diabetic patients with normal UEA. This observation can be interpreted to imply that some genes either expose the subject to or protect the subject from diabetic nephropathy during a certain time-span (257).

1.3.2.1.2. **Familial Clustering and Heritability**

Familial clustering of diabetic nephropathy presents strong evidence for genetic involvement. Seaquist *et al* observed elevated UAE in 83% of diabetic siblings of patients with diabetic nephropathy, whereas among siblings with a normal UAE, only 17% had nephropathy (260). Although this observations were doubt and considered to be a result of sharing the same environment (261), later studies have confirmed this finding, despite the considerably lower degree of familial clustering was found (50,262,263). There also appears to be familial clustering of certain risk factors in diabetic nephropathy. Hypertension (264,265), type 2 diabetes (266), and insulin resistance (267) have been spotted more often in parents of type 1 diabetic patients with overt nephropathy than in those with normal UAE. Similarly, the parents of patients with diabetic nephropathy had higher mortality and more CVD, although these findings are not consistent (266,268-271).

1.3.2.1.3. **Ethnic Variation**

Substantial differences in the prevalence of diabetic nephropathy have been observed between various ethnic groups. The incidence of diabetic renal disease in Native Americans: Pima, Navajo, Winnebago and Omaha Indians is 7-fold higher than in the Caucasian population (272). Also African-Americans have a 5-fold increased risk for developing ESRD compared to

Caucasian type 2 diabetic patients, which cannot be explained by differences in glycemic control or in diabetes duration (273). The increased risk in African-Americans may, however, be a marker of an inherited susceptibility to development of renal disease, rather than to development of diabetic nephropathy. Similarly, among Jews with type 1 diabetes, the non-Ashkenazi are at higher risk for nephropathy development than other Jewish groups (274).

1.3.2.1.4. The Role of Gender

Due to the lack of large prospective studies, the role of gender is not entirely examined. Because of the excess cardiovascular mortality in both diabetic and non-diabetic males, results of prevalence studies may be biased, influencing potential difference between males and females (275). There is evidence that in males the prevalence of overt nephropathy as well as ESRD is higher (5,276). It is also supported by an 18-year follow-up study from Denmark in which male gender was an independent predictor for microalbuminuria (6).

1.3.2.2. Genetic Analyses of Complex Traits

The search for genetic markers that predict risk for diabetic nephropathy is important for several reasons however the model of inheritance in diabetic nephropathy was not established definitively yet. It is implied that it is rather a complex trait with several genes, each playing only a modest role in the disease process, than a result of classic Mendelian recessive or dominant inheritance caused by a variation in a single gene at a single locus. There are two major approaches that may identify the susceptibility genes for diabetic nephropathy: linkage analysis and population-based association (case-control) studies often referred to as candidate gene studies. These methods were design to define a group of subjects who are predisposed to nephropathy and thus may help to established new therapeutic approaches by revealing the pathways leading to the pathogenesis of the disease.

1.3.2.2.1. Linkage Analysis Studies

Linkage analysis approaches fall into two main classes: parametric (or model-based) and nonparametric (or model-free) methods. Traditional linkage analysis (model-based) requires that mode of inheritance, penetrance, phenocopies, and gene frequencies are known (277).

The inheritance pattern of complex disorders includes multiple gene and environment interactions is not understood therefore nonparametric methods are preferred. This approach usually is performed on rather high number of sibling pairs or larger pedigrees. In a genome-wide scan, markers randomly spread over the entire genome cover all chromosomes are used. This method requires no previous knowledge of the putative gene. Only a few studies have used a whole genome approach, such as scan of micro-satellite markers, in the assessment of genetic susceptibility to diabetic nephropathy. A whole genome linkage analysis using families of Pima Indians showed susceptibility loci for diabetic nephropathy on chromosome 3, 7, and 20 (278). Another linkage study using discordant sib-pairs of Caucasian families with type 1 diabetes identified a critical area on chromosome 3q (279). The chromosomal regions identified as susceptibility loci for diabetic nephropathy are listed in *Table 2*.

Table 2: List of susceptibility loci for DN identified by genome-wide linkage analyses

Chromosomal regions	Candidate genes	Study by
1q21	AT	(Hansen)
3q21	ATR1	(Moczulski, Imperatore)
7q		(Imperatore, Bowden)
- 7q35	ALR, TCR, NOS3	
- 7q21	PAI1	
9q		(Bowden, Imperatore)
11q23		(Hansen)
18p22.3-23	CNDP1	(Vardarli, Jansen)
20q		(Imperatore)

1.3.2.2.2. Association Studies

This method requires identifying of genetic variations or polymorphisms in the candidate genes that may alter the function or expression of proteins involved in the disease process. The most frequently used design is a case-control study. The frequency of gene variation in subjects with a disease (cases) is compared to the frequency of those without disease (controls). Appropriate collection of a large number carefully characterized cases and controls are thus needed to avoid false positive or negative results. In order to overcome these problems, alternative

designs have been developed. Family-based association analysis has been established as a standard test of a genetic association. Common method to analyze how the putative disease allele is transmitted to the patients from their heterozygous parents the transmission disequilibrium test (TDT) is used (280). It requires no control group, since the genotypes of the patient's parents are known. If it is transmitted in significantly more often than in half of the cases, the disease allele is associated with the trait. The finding of allelic association in such cohorts not only confirms a population association, but also places the susceptibility locus to within a region of approximately 2mb. Chromosome regions of this size may be small enough to allow physical mapping and, potentially, the identification of causative polymorphisms and specific gene mutations.

1.3.2.2.3. Candidate Genes Studies

Due to the difficulty in enrolling a sufficient number of sibling pairs concordant or discordant for diabetic nephropathy, and high mortality rates of diabetic patients with ESRD, studies on the genetics of diabetic nephropathy have been almost entirely association studies. Due to the several factors suggested to be involved in the pathogenesis, numerous candidate genes have already been studied in patients with diabetic nephropathy. These have included genes that have previously been studied in hypertension, type 2 diabetes, CVD, glucose and fat metabolism and immune system. None of the found associations of gene variants with diabetic nephropathy have been convincingly and undisputedly replicated in other populations or in prospective studies.

1.3.2.2.3.1. The Renin-Angiotensin-Aldosterone System

The genes of the RAAS have drawn special attention in diabetic nephropathy since it is well known that ACE inhibitors effectively reduce proteinuria beyond the reduction of blood pressure levels (281).

The insertion/deletion (I/D) polymorphism of the **ACE** gene is the most dealt one during the recent years, in the studies showing rather inconsistent results. The polymorphism is characterized by the insertion (I) or deletion (D) of a 256bp segment of DNA (282). Patients with the II genotype have low level of serum ACE, whereas DD carriers have the highest level (ID carriers being intermediate). Individuals with the DD genotype may have a higher risk of CVD than other genotypes. Two small studies of patients with diabetic nephropathy have suggested a positive association between this complication and the D allele (283,284). A number of subsequent larger studies have, however, shown no association between nephropathy and the D

allele, one study even suggesting that the association is in fact between nephropathy and the I allele (13,285,286). The probable explanation of these confusing data is that polymorphism within the ACE gene may exert an effect on the natural history of diabetic nephropathy and diabetic patients with nephropathy and a certain ACE genotype have more rapid deterioration in renal function, or that ACE genotype modulates the impact of therapeutic agents on the progression of nephropathy. A trend towards reduced renal protection with ACE inhibitors according to the ACE genotype has been observed in DN patients, but also in non-diabetic renal failure (287,288). The fact of no current ACE gene analysis in the clinical management of diabetic nephropathy suggest that it has either minor or none role in the pathogenesis of nephropathy.

Another important gene studied in the RAAS is the angiotensinogen (AT) gene, associated with both hypertension and CVD. A positive association appeared for the TT genotype of the M235T polymorphism in a TDT study in male subjects (289). However the others results were also contradictive in regard to this polymorphism (*Table 3*).

The C allele of the A1166C polymorphism of the **AT1R** gene, linked to essential hypertension, has been examined in diabetic nephropathy, partly because of the study by Moczulski *et al* (279), showing a strong linkage in the region containing this gene. However, association studies have mainly been negative for the AT1 gene. Other studied genes of the RAAS are summarized in the *Table 2*.

No association of **bradykinin 1 receptor** with nephropathy in a Caucasian cohort of both type 1 and type 2 diabetic patients was confirmed (290). Interestingly, in a Danish cohort, a positive association appeared between a polymorphism in the **rennin** gene and diabetic nephropathy (291). This finding has not been replicated in other populations.

Table 3: Candidate genes in the RAAS studied in DN patients

Gene	Polymorphism	Positive association Reference	No association Reference
ACE	I/D	(292-304)	(305-317)
ACE	PstI	(318)	
AGT	M235T	(289,317,319)	(292,307,311,315,320-322)
AT1	A1166C		(279,317,322-326)
AT1	T573C	(327)	
Bradykinin 1 receptor	G699C	(290)	
Renin	BglI	(291)	

1.3.2.2.3.2. Genes Related to Blood Pressure

Since elevated blood pressure is a characteristic feature in patients with DN, genes involved in blood pressure regulation have drawn much attention (summarized in *Table 4*).

ANP plays a central role in the regulation of blood pressure, sodium homeostasis, and vascular permeability (287,328), and significant associations between DNA polymorphisms at the pronatriodilatin (**PND**) gene (encoding the precursor molecule of ANP) and hypertension have been reported (13,58,282-286,288,328-331). Moreover, ANP could be involved in the regulation of GF and hyperfiltration seems to participate in the pathogenesis of glomerular injury (332-334). Serum ANP levels have also been reported to be elevated in diabetic patients with micro- and macroalbuminuria (335,336). A group from Heidelberg examined the HpaII polymorphism of intron 2 of the ANP gene and found no association with diabetic nephropathy in either type 1 or type 2 diabetes (337). In contrast, a group from Pisa found a positive association between polymorphism in the PND gene and nephropathy in type 1 patients (338). They examined two polymorphisms (C708T and Scal), reporting an association with each of them. The C708T result was unconvincing since the association was seen only in patients with incipient, rather than established, nephropathy. In comparison, the progression was much more convincing for the Scal polymorphism, with which the A1/A2 allele frequencies were 20% and 80% respectively in healthy controls and 6% and 94% in type 1 diabetic subjects with overt nephropathy. These results suggest a possible role of PND gene in conferring protection from nephropathy and microvascular damage in type 1 diabetes, however, replication of these data is awaited.

A positive association appeared between the endothelial **NOS** gene and advanced diabetic nephropathy in an American cohort examined by both case control and family design (339-341). NO mediates local vasodilatation, antagonizes platelet aggregation and inhibits VSMC proliferation. In response to stimuli such as hypoxia or shear stress the VSMC synthesize NO from L-arginine by an enzyme known as NO synthase. A deletion of one of five nucleotide repeats in the intron 4 in the gene coding endothelial form of this enzyme has been reported to be associated with coronary heart disease and has recently been more often found in patients with diabetes and advanced nephropathy (31,342,343). Hence, the gene appeared to promote the progression of nephropathy in those with established disease.

Although the **α -adducin** gene has been associated with essential hypertension, in a type 1 diabetic cohort from Ireland and Northern Ireland no association existed between this gene and diabetic nephropathy or hypertension (344). One potential candidate from genes regulating blood pressure is the dopamine receptor **DRD3** gene, since disruption of this gene in mice results in a

renin-dependent form of hypertension (345,346), however no studies thus far have explored its role in diabetic complications.

Table 4: Candidate genes related to blood pressure studied in DN patients

Gene	Polymorphism	Positive association Reference	No association Reference
α -adducin	Gly460Trp		(344)
ATP1 A1	Bgl II		(347)
β 3 adrenergic receptor	Trp64Arg		(348)
Epithelial sodium channel β	Arg564X	(349)	
G-protein β 3	C825T	(350)	(351,352)
eNOS	4a/b	(341)	(339,340)
Nitric oxide 2A	CCTTT-repeat	(353)	
Nitric oxide 2A	promoter		(339)
Pronatriodilatin (ANP)	Scal	(354)	
Pronatriodilatin (ANP)	T2238C	(355)	
Pronatriodilatin (ANP)	HpaII (intron 2)		(356)
SA HT associated homologue	PstI		(351)

1.3.2.2.3.3. Genes Related to Cardiovascular Diseases

Due to the high prevalence of early atherosclerosis and cardiovascular complications in DN patients, genes associated with CVD have frequently been studied as candidate genes for nephropathy, as shown in *Table 5*.

Methylenetetrahydrofolate reductase (**MTHFR**) is a key enzyme of homocysteine metabolism. A deficiency of MTHFR, leading to hyper-homocysteinaemia, homocysteinuria and hypomethioninaemia, has been detected in patients with CVD. In type 2 diabetes, the C677T polymorphism of the MTHFR gene has been studied by several groups in regards to diabetic nephropathy. A positive association with nephropathy came from Japan. Also in the study from Russia a significant difference between type 1 diabetics with and without nephropathy for homozygosity of the TT allele (21.6% versus 5.6%) has been reported, however, the study was

small (only 51 subjects with nephropathy) (357,358). On the other hand Savage's group found no association between the C677T polymorphism and nephropathy.

A quite similar constellation exists for the e2, e3, and e4 alleles of the apolipoprotein E gene (**ApoE**), with two positive (359,360) and two negative (361,362) studies. ApoE is a major protein compound of lipoproteins mediating the hepatic uptake of lipoproteins and reverse cholesterol transport. It occurs as three isoproteins: ApoE3 – the wild type with normal function, ApoE2 – with reduced affinity for the apoE receptor and ApoE4 – with increased binding of the receptor. These are encoded by three codominant alleles e2, e3 and e4 localized on chromosome 19. Polymorphism of the ApoE gene is associated with coronary artery disease in diabetic and non-diabetic populations. Since CVD is common in DN patients, the ApoE gene was considered a candidate susceptibility locus to this complication. However, there continues to be controversy on the association in the literature, the ApoE gene remains an area of interest for future analysis.

Interestingly, a functional Leu7Pro polymorphism of the prepro-neuropeptide Y gene (**PNPY**) has been associated with carotid atherosclerosis, serum LDL cholesterol levels, triglycerides, and blood pressure in Finnish non-diabetic subjects (363-365). The Leu7Pro has also been associated with both retinopathy and carotid atherosclerosis in Finnish type 2 diabetic patients (366,367). Its role in late complications in type 1 diabetic patients has, however, not yet been elucidated.

Table 5: Candidate genes related to cardiovascular disease studied in DN patients

Gene	Polymorphism	Positive association Reference	No association Reference
Apolipoprotein E	e2,e3,e4	(359,360)	(361,362)
β 3-integrin	PIA1/PIA2		(368)
β -fibrinogen	G455A		(369)
Methylenetetrahydrofolate reductase	C677T	(370,371)	(372-374)
Plasminogen activator inhibitor-1	4G/5G		(310,375)
Paraoxonase 1 (PON1)	Several		(376)
Paraoxonase 2 (PON2)	Several		(377)
Werner syndrome helicase	C/R		(310)
von Willebrand factor	T789A, A138T		(378)

1.3.2.2.3.4. Genes Related to Immunology and Glucose Metabolism

The major histocompatibility complex (MHC) on chromosome 6 encodes **HLA**, which contribute to the susceptibility to type 1 diabetes. These genes have also been postulated as having a role in susceptibility to microvascular disease (379). Twin studies in type 1 diabetes show a concordance for diabetic retinopathy status, especially in those twins with the DR3/4 genotype (287). However, a recent association study using large cohorts of type 1 diabetic patients with and without nephropathy showed no positive or negative associations with HLA markers (328).

The insulin (**INS**) gene lies within the susceptibility locus for diabetes. This gene has also been implicated in premature atherosclerosis and has thus been examined as candidate for diabetic nephropathy. Raffel *et al* found a positive association between polymorphism in the **INS** gene and type 1 diabetic patients with nephropathy (380), a finding not yet consistently replicated (381). A few other studies dealing with genes related to diabetes and glucose metabolism have been performed, as shown in *Table 6*.

ALR has been suggested to contribute to diabetic microangiopathic complications and polymorphism of this gene has been reported to be strongly associated with nephropathy in type 1 diabetes. **ALR** is the first enzyme in the polyol pathway. It catalyses the NADPH-dependent reduction of hexose sugars to their corresponding polyols (alcohols). Under hyperglycemic conditions the cells produce significant quantities of the sorbitol, resulting in a cascade of metabolic abnormalities leading to hypoxia and tissue ischemia. A group from the UK found a strong association in Caucasian patients between a CA repeat polymorphism in **ALR** gene and diabetic nephropathy (382). Interestingly, this positive association was later repeated in an American cohort (383), but not in another cohort of Caucasian patients from the UK (384).

There has been focus on polymorphism within the human paraoxonase (**PON1**) gene, involved in the protection of LDL from oxidation. Animal studies suggest its role in the atherosclerosis. The polymorphism in the promoter region of the human **PON1** gene has been associated with highly significant differences in the serum concentration and activity of **PON1**. Regarding diabetic complications, one study has showed a significant difference in **PON1** activity between patients with and without overt proteinuria and another association of L54 allele with diabetic retinopathy(385,386), however, in Krolewski's group, none of the three polymorphisms of the **PON1** gene in a large cohort of nephropathy subjects was associated (376).

Table 6: Candidate genes related to diabetes, immunology and glucose metabolism

Gene	Polymorphism	Positive association Reference	No association Reference
Genes related to diabetes and immunology			
Complement C4	C4AQ0		(387)
ENPP1/PC-1	K121Q		(296)
Human leukocyte antigen	Several		(381)
Hepatocyte nuclear factor 1 b	E260D, Q378Q		(388)
Insulin	Ins+/-		(381)
Insulin	5'end	(380)	
Genes involved in glucose metabolism			
AGE-receptor 1	Several		(389)
AGE-receptor 2	Several		(389)
AGE-receptor 3	Several		(389)
AGE-receptor (RAGE)	Several		(389)
Aldose reductase	(CA)n	(383,390,391)	(384,392,393)
GLUT1	XbaI	(394,395)	(396)

1.3.2.2.3.5. Genes Related to Glomerular Structure

Although the basic structure of the glomerulus and its proteins are well characterized, the genes regulating these proteins and glomerular function are poorly known. Only a few candidate genes have been studied regarding structural proteins, as seen in *Table 7*.

Heparan sulphate proteoglycan (HSPG) is an important part of the GBM. It consists of a central core protein to which anionic polysaccharide chains (heparan sulphates) are linked and thereby contributes to the integrity and negative charge of the GF barrier as well as the composition of the GF product, indirectly. It has been postulated that a loss of heparan sulphate from the GBM may be a major pathogenic factor in diabetic nephropathy (79). A study in a combined Danish-British cohort with respective 260 + 397 patients showed a positive association between BamHI polymorphism of the **HSPG** gene and UAE (397). This finding has not been

confirmed, although it supports the Steno hypothesis that a defect in the GBM leads to loss of charge selectivity and subsequent proteinuria (79).

An allelic variant of another GBM gene, **decorin**, has been associated in a longitudinal study with a 6.5 year follow-up with a slower progression of nephropathy (398). Dysfunction of the glomerular actin cytoskeleton, a component of the glomerular podocyte layer, occurs in diabetic nephropathy (399). Interestingly, a positive association was recently found in two distinct Irish cohorts between diabetic nephropathy and **caldesmon**, one of the genes coding the actin cytoskeleton (400).

Table 7: Candidate genes related to glomerular structure studied in DN patients

Gene	Polymorphism	Positive association Reference	No association Reference
Caldesmon	-579A>G	(400)	
Collagen IV	A1 HindIII		(401)
Decorin	179/183/185	(398)	
Heparan sulphate	BamHI	(397)	

1.3.2.2.3.6. Other Genes

A few other genes mostly coding cytokines and growth factors have been studied for association with diabetic nephropathy, as seen in *Table 8*.

Preliminary studies suggested an association between **IL-1** markers distributed across the region on chromosome 7q35 and nephropathy, but once again, there are contradictory data. Most recently, Loughrey *et al* reported an association between nephropathy and the T105C polymorphism in exon 5 of the IL-1B gene (402). They found, however, no association with the IL1RN*2 allele (encoding the IL-1 receptor antagonist), which had been implicated in two previous reports (403,404). In contrast, Tarnow *et al* found no significant association between diabetic nephropathy and polymorphism within the studied region (405).

Several polymorphisms of the **TGF- β** gene have been characterized in two candidate gene studies regarding this gene and diabetic nephropathy. However, the results are again rather conflicting (406,407). A recent study found a positive association between variants in the vascular endothelial growth factor gene and diabetic nephropathy in type 1 diabetic patients from the UK

(11). Interestingly, this association was further strengthened when analyzed together with polymorphisms in the ALR gene, suggesting a gene-gene interaction.

Table 8: Genes related to growth factors and enzymes studied in DN patients

Gene	Polymorphism	Positive association Reference	No association Reference
Interleukin-1A	C/T -889		(402,405)
Interleukin-1B	ex5 T/C	(402)	(405)
Interleukin-1R1	ex1B T/C		(402,405)
Interleukin-1RN	intr2(86bp repeat)		(402,405)
N-acetyltransferase	fast/slow	(408)	
Nuclear factor kappa B	A1-A18		(409)
Transforming growth factor – 1	Several		(406)
Transforming growth factor – 1	Thr263Ile	(407)	
VEGF	D/I -2549	(11)	

1.4. Prevention and Treatment of Diabetic Nephropathy

The treatment of diabetic nephropathy is based on the effective reduction or elimination of risk factors for diabetic nephropathy such are: hypertension, hyperglycemia, dyslipidemia or smoking. The prevention of the progression from micro- to macroalbuminuria, the decline of renal function in patients with macroalbuminuria, and the occurrence of cardiovascular events should be included in the therapy strategies.

1.4.1. Glycemic Control Effect

The effect of strict glycemic control on the progression of diabetic nephropathy is still controversial. In patients with type 2 diabetes, very few studies analyzed have been done. In the Diabetes Control and Complications Trial, intensive treatment of diabetes reduced the incidence of microalbuminuria by 39% (16). In the UKPDS, a 30% reduction of the risk for the development of microalbuminuria was observed in the group intensively treated for hyperglycemia (17). Moreover, in the Kumamoto Study, intensive glycemic control also reduced the rate of

development of micro- and macroalbuminuria (410). Therefore, intensive treatment of glycemia aiming at HbA1c <7% should be pursued as early as possible to prevent the development of microalbuminuria. Although the effects of strict glycemetic control on the progression of diabetic nephropathy are not firmly established, it should be pursued in all these patients. Some oral antidiabetic agents seem to have beneficial effect in the prevention of renal complications in type 2 diabetes (411,412). Rosiglitazone, has been shown to decrease UAE in patients with type 2 diabetes (412). Metformin should not be used when serum creatinine is <1.5 mg/dl in men and <1.4 mg/dl in women due to the increased risk of lactic acidosis (413). Sulfonylureas and their metabolites are eliminated via renal excretion and should not be used in patients with decreased renal function. Thus, most type 2 diabetic patients with diabetic nephropathy should be treated with insulin.

1.4.2. Blood Pressure Control, Antihypertensive Intervention

In patients with type 1 diabetes, hypertension is usually caused by underlying diabetic nephropathy and typically becomes manifest about the time that patients develop microalbuminuria. In patients with type 2 diabetes, hypertension is present at the time of diagnosis of diabetes in about one-third of patients. In general, the hypertension in patients with both types of diabetes is associated with an expanded plasma volume, increased peripheral vascular resistance, and low renin activity. Both systolic and diastolic hypertensions markedly accelerate the progression of diabetic nephropathy, and aggressive antihypertensive management is able to greatly decrease the rate of GFR fall (329). Appropriate antihypertensive intervention can significantly increase the median life expectancy in patients with type 1 diabetes, with a reduction in mortality from 94 to 45% and a reduction in the need for dialysis and transplantation from 73 to 31% 16 years after the development of overt nephropathy. Numerous studies have demonstrated that treatment of hypertension, irrespective of the agent used, produced a beneficial effect on albuminuria (414). One of the strategies of treatment of hypertension is RAAS blockade by using the ACE inhibitors or ARBs. The renoprotective effect of ACE inhibitors and ARBs is independent of blood pressure reduction (414,415) and may be related to decreased intraglomerular pressure and passage of proteins into the proximal tubule (416). These drugs decrease UAE and the rate of progression from microalbuminuria to more advanced stages of diabetic nephropathy. Therefore, the use of either ACE inhibitors or ARBs is recommended as a first-line therapy for type 1 and type 2 diabetic patients with microalbuminuria, even if they are normotensive. Mogensen *et al* has demonstrated, almost 30 years ago, that treatment of

hypertension reduced albuminuria and GFR in proteinuric patients (417). He also developed the new concept of dual blockade of the RAAS. ACE inhibitors and ARBs interrupt the RAAS at different levels, and the combination of these classes of drugs may have an additive effect on renoprotection. Also other studies have demonstrated that the combination of ACE inhibitors and ARBs had a synergistic effect in blood pressure and UAE reduction in diabetic patients with nephropathy and this dual blockade is more effective in reducing UAE than maximal recommended doses of ACE inhibitors alone (418).

1.4.3. Dietary Modifications, Protein Restrictions

According to a metaanalysis of five studies including a total of 108 patients, dietary protein restriction reduces hyperfiltration and intraglomerular pressure, retards the progression of diabetic nephropathy in patients with diabetes (14,419). The general consensus is to prescribe a protein intake of approximately 0.8 g/kg/day (less than 10% of daily calories) according to the adult Recommended Dietary Allowance (RDA) in the patient with overt nephropathy. However, it has been suggested that once the GFR begins to fall, further restriction to 0.6 g /kg/day may prove useful in slowing the decline of GFR (19). On the other hand, nutrition deficiency may occur in some individuals and may be associated with muscle weakness.

1.4.4. Dyslipidemia

The effect of antilipidemic agents on progression of diabetic nephropathy is still unknown. So far, there have been no large trials analyzing whether the treatment of dyslipidemia could prevent the development of diabetic nephropathy or the decline of renal function. However, there is some evidence that lipid reduction might preserve GFR and decrease proteinuria in diabetic patients (420). The desired level of LDL cholesterol for diabetic patients in general is <100 mg/dl and <70 mg/dl for diabetic patients with CVD (421). Several studies discussed beneficial effect of statins on the reduction of the cardiovascular events by 25% and GFR decline independent of cholesterol levels in patients with diabetes (422).

1.4.5. Anemia

Anemia has been considered a risk factor for progression of renal disease and retinopathy (423). It has been related to erythropoietin deficiency (424). Anemia may occur in patients with

diabetic nephropathy even before the onset of advanced renal failure (serum creatinine 1.8 mg/dl). It is recommended to start erythropoietin treatment when hemoglobin (Hb) levels are below 11 g/dl. The target Hb levels should be 12–13 g/dl, and the potential risk of elevation of blood pressure levels with erythropoietin treatment should be taken into account (423).

1.4.6. Multifactorial Intervention

Patients with microalbuminuria frequently have other complications, such as hypertension or dyslipidemia. Importance of multifactorial intervention, consisted of a stepwise implementation of lifestyle changes and pharmacological therapy has been investigated by the Steno-2 study in 160 microalbuminuric type 2 diabetic patients (425). It included a low-fat diet, a 3-5 times a week light-to-moderate exercise program, a smoking cessation program, prescription of ACE inhibitors or ARBs and aspirin. The intensively treated group had significantly decreased risks for developing: macroalbuminuria (61%), retinopathy (58%) and autonomic neuropathy (63%).

1.4.7. New Potential Therapeutic Strategies

The procedures described above might not be effective in some patients with diabetes, thus novel therapeutic strategies are necessary. High doses of thiamine and its derivative benfotiamine have been shown to retard the development of microalbuminuria in experimental diabetic nephropathy, probably due to decreased activation of PKC (protein kinase C), decreased protein glycation, and oxidative stress (426). Treatment with a PKC- β inhibitor (ruboxistaurin) normalized GFR, decreased UAE, and ameliorated glomerular lesions in diabetic rodents (427). In rat model of diabetes-induced glomerulosclerosis, administration of a modified heparin glycosaminoglycan prevented albuminuria, glomerular and tubular matrix accumulation and TGF- β mRNA overexpression (428). Very few studies have been conducted in humans. Glycosaminoglycan Sulodexide significantly reduced albuminuria in micro or macroalbuminuric type 1 and type 2 diabetic patients (429). Pimagedine, a second-generation inhibitor of advanced glycation end products, reduced urinary protein excretion and the decline in GFR in proteinuric type 1 diabetic patients in a randomized, placebo-controlled study (430).

2. AIMS OF THE STUDY

The aim of this work was to investigate new genetic markers which are implicated in the pathogenesis of diabetic nephropathy by using association study design. This approach identifies genetic variants, which correlate with phenotypic abnormalities.

We tested several polymorphic regions to identify new candidate genes contributing to initiation or progression of diabetic nephropathy.

1. Single nucleotide polymorphism **Pro12Ala of PPAR γ -2 gene** for its involvement in insulin resistance, type 2 diabetes and for its protective effect on diabetic nephropathy.
2. Two polymorphisms of the NF κ B family genes: single nucleotide **polymorphism A/G of the NFKBIA gene**, coding inhibitory protein I κ B and **CA repeat polymorphism of the NFKB1 gene**, coding p105 subunit of NF κ B. The role of both was investigated in connection to type 1 diabetes and NF κ B induced pancreatic β -cell destruction, as well as in type 2 diabetes, insulin resistance, diabetic complications or atherosclerosis.
3. Trinucleotide **CTG repeat polymorphism of the CNDP1 gene**, coding serum carnosinase. Hypothesis of new candidate gene from chromosomal region identified as susceptibility locus for diabetic nephropathy was tested. Functional analysis was performed to correlate the effect of polymorphisms on the protein function and to confirm protective effect of the gene product on hyperglycemia induced renal damage.

3. METHODS

3.1. *PPAR* γ

Subjects

DNA samples were obtained from 133 unrelated Czech patients with type 2 diabetes (age > 35 years, C-peptide > 200 pmol/l, antiGAD < 50 ng/ml). The control group consisted of 97 healthy individuals, recruited from the Blood Transfusion Department of Faculty Hospital Kralovske Vinohrady, Prague, Czech Republic. Informed consents were obtained from all subjects.

Genotyping

DNA was isolated from collected EDTA peripheral blood and extracted on the column with silicate gel membrane (Qiagen, Hilden, Germany). The part of exon 2 containing codon 12 was amplified (247). The BseLI restriction endonuclease was used for digestion of 106bp fragment of polymerase chain reaction (PCR), performed at 55°C for 1 h.

Statistical Analysis

To confirm the difference between groups of genotypes, the χ^2 analysis was used. The influence of the genotype on the clinical parameters was estimated by the ANOVA test. P value < 0.05 was considered significant.

3.2. *NF* κ *B*

Subjects

The study of polymorphisms in the *NFKBIA* and *NFKB1* genes involved 211 type 2 diabetic (T2D) patients, 267 individuals with type 1 diabetes (T1D) and 159 ethnically matched control subjects. T2D patients were divided into 3 groups according to the different renal condition – with diabetic nephropathy, with non-diabetic renal diseases (mainly on atherosclerotic basis) and without diabetic nephropathy or other kind of renal damage. Together 122 German and 189 Czech T2D patients were collected. T1D patients, chosen according to current World Health Organization (WHO) definition, were subclassified into 3 groups: T1D children (juvenile type of autoimmune diabetes, age of onset maximum 20 years old – average 7.7) T1D adults (juvenile type of autoimmune diabetes with age of onset minimum 20 years old) and LADA patients (latent

autoimmune diabetes in adults – defined as autoimmune diabetes diagnosed in people aged 30-50 years, but with presence of antibodies to glutamic acid decarboxylase and minimum of 6 months without insulin therapy after diagnosis of disease, with age of onset 25 years old – average 47).

Additionally, 152 patients with systemic lupus erythematosus (SLE) (90% of female and 10% of males with average age of 47, average duration of SLE being 17.5 years), and 153 samples from individuals with rheumatoid arthritis (RA) (average age at diagnosis 44.98 and average age 57.4; females 77.14% and males 22.86%) were collected for NFKB1A genotyping.

All subjects were of Caucasian descent, residing in the Czech Republic, Slovak Republic, and Germany. Patients were recruited from Center for diabetes, metabolism and nutrition research, Department of Pediatrics, 2nd Internal Clinics and Nephrology Outpatient Clinic, Faculty Hospital Kralovske Vinohrady in Prague and Private Diabetology Outpatient Clinic in Prague, Czech Republic; German samples were obtained from 5th Medical Department of the University Clinic in Mannheim, Germany. Rheumatologic and SLE patients came from Institute of Rheumatology in Prague, Czech Republic and Institute of Rheumatology in Piestany, Slovak Republic. The control group came from blood donors recruited from the Blood Transfusion Department of the Faculty Hospital Kralovske Vinohrady in Prague, Czech Republic. None of the healthy control subjects were taking any anti-inflammatory or immunosuppressive medication. Written informed consents to participate in the study were obtained from all participants. The biochemical and clinical characterizations are shown in the *Table 9* and *Table 10*.

Table 9: Clinical characterization of different groups of type 1 diabetic patients (T1D, n=267)

Ethnicity	Czech				
	Groups	T1D children <i>n</i> =179	T1D adults <i>n</i> =75	LADA <i>n</i> =31	Control <i>n</i> =153
Features					
Female [%]	60	52	44	*	
Age at disease onset [yrs]	7.7 (1- 16)	22.1 (20 - 45)	47 (25 - 64)		
Duration of DM [yrs]	4.9 (1 -16)	17 (1 - 50)	15 (3 – 32)		
Insulin therapy	yes	yes	yes	no	
Fasting C peptide [pmol/L]	*	*	478 (4,4 – 1522)	*	
Anti-GAD [ng/ml]	*	*	392 (5 - 2800)	*	

Table 10: Clinical characterization of type 2 diabetic patients (T2D; n=274) with different renal complications

Groups	Czech			German	
	NDRD n=61	with DN n=33	no DN n=40	with DN n=84	no DN n=38
Features					
Women [%]	40	40	45	46	53
Average age [yrs]	73	68	53	64	57
Duration of DM [yrs]	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	*	142+-21,6	134,8+-21,6
Mean diastolic BP	83+-12,7	93+-11,6	*	79+-10,9	79+-12,0
BP Amplitude	59+-15,74	73+-20,8	*	74+-11,11	68+-11,51
History of MI [%]	16	24	*	26	31
History of stroke [%]	13	7	*	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
HbA1C[g/l]	*	*	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 Creatinine[μ mol/l]	169,5+-64	171+-89,4	103,9+-21,8	*	*

Genotyping

DNA was isolated from collected EDTA peripheral blood and extracted on the colony with silicate gel membrane (Qiagen, Hilden, Germany).

NFKBIA polymorphism genotyping

The NFKBIA A/G point variation polymorphism was tested by using the restriction fragment length polymorphism (RFLP) method. The PCR reaction was performed in total volume of 20 μ l with specific primers. For restriction digestion was used HaeIII to cleave amplification product at 37 °C for 1 hour. Following genotypes were identified: the wild-type GG (424bp), the

variant AA (306bp and 118bp) and the heterozygote AG (424bp, 306bp and 118bp fragments) (431).

NFKB1 polymorphism genotyping

Testing of the CA repeat polymorphism of NFKB1 gene was performed by fragment analysis method with fluorescently labeled primers. ALFexpress analyzer and ALFwin software (Amersham Pharmacia Biotech, Upsala, Sweden) were used for the detection of polymorphic alleles in the NFKB1 gene. 16 polymorphic alleles were identified in the size range from 114 to 144bp, which corresponds to 10-26 CA repeats.

Expression Profiling

An independent group of T1D patients (children and adults) and their sex- and age-matched controls were chosen for the expression study. 28 T1D adult patients, (with average age 36.4 years \pm 11.5SD) and 55 T1D children patients (with average age 11.4 years \pm 8.2SD) were included in the expression study. The control groups was matched by presence of specific HLA-DRB1*04 allele. The analysis was performed by real time (RT) PCR method. Antigen presenting cells were obtained from peripheral blood by immunomagnetic separation with Dynabeads (DynaL HLA-class II, 210.04, Dynal, Oslo, Norway). RNA was extracted using the RNA blood mini kit (Qiagen, Hilden, Germany). The quantitative real time RT-PCR was performed using the Taqman® PCR Kit on the ABI PRISM 7000 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). The cDNA was amplified using specific primers for HLA-DRB1*04 designed by Primer express® as well as of Taqman®MGB. Testing of NFKB1 expression was done with assay on demand set of primers and probes. As an internal control the human beta actin was used (Applied Biosystems, Foster City, CA, USA). The $2^{-\Delta\Delta Ct}$ (2-delta delta Ct) method was applied for relative quantification (432).

Statistical Analysis

To determine significant differences in genotype and allele frequencies of the NFKB1A and NFKB1 genes, genotype and allele distributions were compared between affected and control population using the χ^2 analysis, followed by the Bonferroni correction for multiple comparisons. P value $<$ 0.05 was considered significant

The results from real time PCR were compared between the groups by one-way ANOVA testing and also by nonparametric Kruskal-Wallis statistics. The observed groups were also compared by Dunn's multiple comparison tests. P value $<$ 0.05 was considered significant.

3.3. CARNOSINE

Subjects

Type 1 and type 2 diabetic patients divided into 3 examined groups were recruited for gene testing study. Group 1 was presented by trios compound of 45 individuals with type 2 diabetes (trio was defined as 1 patient and his 2 closest relatives). Group 2 included 140 type 2 diabetics. Group 3 included 57 individuals with type 1 diabetes. All 3 groups together consisted of two types of diabetic patients: with (145 patients) and without (107 patients) diabetic nephropathy. Informed consents were obtained from all subjects. Demographic data are given in *Table 11*.

Table 11: Clinical characterizations of type 1 and type 2 diabetic patients, as well as type 2 diabetic trios from different ethnic groups

Ethnicity	German		German, Czech, Arabic, Dutch		German, Dutch	
	Group 1 (trios with T2D)		Group 2 (T2D)		Group 3 (T1D)	
Groups	with DN <i>n</i> =37	without DN <i>n</i> =8	with DN <i>n</i> =77	without DN <i>n</i> =63	with DN <i>n</i> =21	without DN <i>n</i> =36
Features						
Age [yrs]	68.2±10.5	64.3±9.3	61.8±10.6	65.4±11.6	48.0±11.7	41.9±11.3
Female	26/11	4/4	38/39	30/33	11/10	13/23
Retinopathy [%]						
-proliferative	56.8	50	58.4	4.8	95.2	5.6
-nonproliferative	37.8	25	37.7	15.9	4.8	11.1
-none	5.4	25	3.9	79.4	0	83.3
Arterial hypertension [%]	79.2	87.5	70.1	46	90.5	2.8
Diabetes duration [%]	12.5±7.0	23.1±8.0	14.9±8.6	22.3±6.9	25.5±10.7	23.8±8.1
A1C [%]	7.6±0.7	7.4±1.2	7.5±2.0	7.3±1.7	7.0±1.5	7.5±1.3

Genotyping

DNA was isolated from collected EDTA peripheral blood and extracted on the colony with silicate gel membrane (Qiagen, Hilden, Germany).

Sequencing

CNDP1 exons were amplified using intronic primers (MWG-Biotech, Ebersberg, Germany) and sequenced using ABI 3100 capillary sequencer (Applied Biosystems, Darmstadt, Germany). Sequence analysis of the CNDP2 gene was performed by Syngene (Qormi, Malta). PCR genotyping of different polymorphisms was followed by further analysis: RFPL and fragment analysis method using an ALF-Express sequencer (Pharmacia-Biotech, Freiburg, Germany), TaqMan SNP genotyping, using ABI Prism 7000 (Applied Biosystems, Darmstadt, Germany), DHPLC analysis (denaturing high-performance liquid chromatography) (Transgenomic, Berlin, Germany).

Functional Studies

Measurement of enzymatic activity in serum

Serum samples from 45 healthy volunteers were used for investigation of carnosine and carnosinase activity in serum. Carnosine concentrations were assayed by fluorometric determination and separated by DHPLC according to Schonherr (433). Serum carnosinase activity was correlated with genotypes of CTG repeat polymorphism (leucine repeat, CNDP1 gene).

In vitro study – cell culture

To investigate the effect of L-carnosine on the increased production of extracellular matrix proteins and TGF- β 1 in hyperglycemic conditions, human SV 40 – transformed mesangial cells and podocytes were used (434,435). All cell lines were cultivated in uncoated culture flasks in Dulbecco's modified Eagle's medium (PAA, Coelbe, Germany) supplemented by 10% FCS (Greiner, Frickenhausen, Germany), insulin-transferin selenium (in all concentrations of 5 ng/ml), epidermal growth factor (5ng/ml), and penicillin/streptomycin (10units/ml, Sigma, St. Louise, MO). Methods of RNA isolation and real time PCR on human glomeruli; CNDP1 antibodies, indirect immunofluorescence and immunohistochemistry were used for analyses.

Statistic Analysis

Statistical analyses were performed with SAS V8.02. Fisher's exact test and chi-square analyses were performed to test the association. The Mantel-Haenszel method was applied to multiple population data analysis. The Wilcoxon rank-sum test was used for sample comparison. P value < 0.05 was considered significant.

4. RESULTS

4.1. *PPAR* γ

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor family of ligand-dependent transcription factors, expressed in many organs (436-442). PPARs heterodimerize with the retinoid X receptor (RXR) and bind to specific peroxisome proliferator response elements (PPREs), direct hexameric DNA motif in the regulatory region of target genes, thus regulate their transcription (443-445). The natural ligands, that bind to PPARs and activate them, are free fatty acids (FFA) or their derivatives (446).

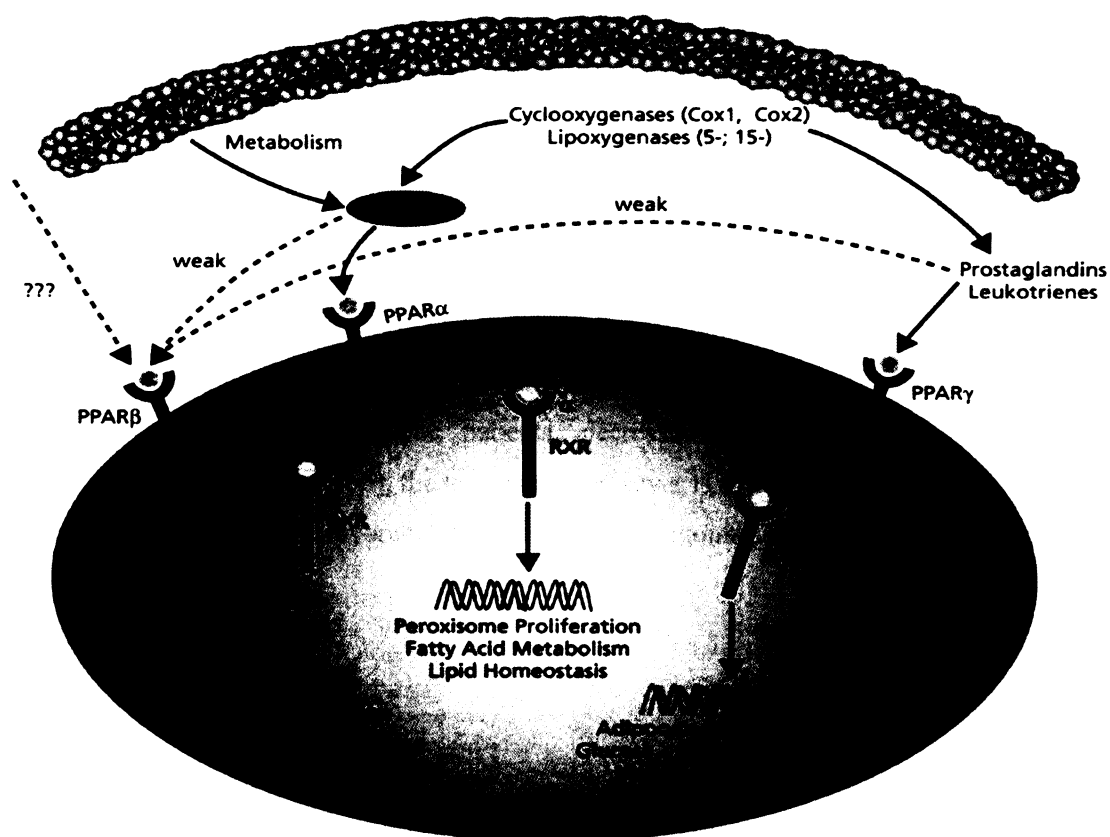
Three different PPARs were identified: PPAR α , PPAR β (also called δ , NUC-1 or FAAR) and PPAR γ (Figure 3).

PPAR α is mainly presented in organs with intensive fatty acids metabolism, such are liver, brown adipose tissue, heart and kidney, where it has important role in nutrient metabolism – positive (FA oxidation, gluconeogenesis) and negative regulation (amino acid metabolism) (447). Potent synthetic ligands of PPAR α are fibrates, a group of drugs that improves blood lipid parameters.

PPAR β is present in numerous tissues, but little is known about its function yet. Several reports suggest its role in regulation of lipid metabolism in nerve cells (448), and FA-controlled differentiation of pre-adipocytes, pointing at possible implication in the pathogenesis of obesity.

PPAR γ is the most studied isotype of PPARs. Three splice variants of PPAR γ has been described: γ 1, γ 2, γ 3 (449). They are highly expressed in adipose tissue, playing crucial regulatory function on adipogenesis. They also regulate genes related to lipid and carbohydrate metabolism, cell differentiation and proliferation (442). PPAR γ plays very important role in glucose homeostasis and insulin sensitivity discovered with the finding of PPAR γ agonist called thiazolidinedions (TZD). These drugs decrease insulin sensitivity in peripheral tissues and thus improve responsiveness to insulin and disposal of glucose (450,451). PPAR γ is also found to be expressed in macrophages, which suggests its anti-atherosclerotic and anti-inflammatory function (452).

Figure 3: PPAR Signaling Pathway



Fajas, L., et al., Peroxisome proliferator-activated receptor γ : from adipogenesis to carcinogenesis. J. Mol. Endocrinol., 27, 1-9 (2001)

In recent studies the role of PPARs in the pathophysiology of type 2 diabetes and its renal complications is vexed. Especially the beneficial effect of different PPAR agonist and antagonist, which modulate renal function on patients with diabetic nephropathy (442,453,454). From the large number of discussed functions of PPAR γ its role in insulin resistance, hyperinsulinemia and hyperglycemia have clear significance. Despite the crucial therapeutic use of PPAR γ agonist in the anti-diabetic and antihypertensive treatment, as well as its beneficial effect on renal glomerular function, the actual role of PPAR γ in the pathogenesis of type 2 diabetes, insulin resistance or diabetic nephropathy is yet undisclosed. The genetic studies have already described several mutations in PPAR γ gene to determine its role in pathophysiology of mentioned diseases, however obtained data were contradictory.

4.1.1. Commentary to Original Paper 1

The article dealt with association study on 135 type 2 diabetic patients and 97 control subjects. The aim was to confirm or refute hypothesized protective role of the Ala12 genotype of PPAR γ 2 gene in the pathogenesis of type 2 diabetes, 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 control and patient groups (13.9% vs. 21.4%, $p=0.022$). However no correlation between the levels of total cholesterol, HDL and LDL cholesterol, triglycerides and BMI with the polymorphism was detected. The data suggest that the Pro12Ala polymorphism of PPAR γ 2 gene is playing certain role in the pathogenesis of type 2 diabetes. Two recent papers indicated the association of studied polymorphism with microalbuminuria in type 2 diabetic patients, decreased risk to develop diabetes-related nephropathy respectively (455,456), the mechanism however is so far unexplained. These findings are also supported by known favorable effects of PPAR γ agonist, TZD, on microalbuminuria in early stages of nephropathy.

4.1.2. Original paper 1

**THE FREQUENCY OF ALLELES OF THE PRO12ALA POLYMORPHISM IN PPAR GAMMA 2 IS
DIFFERENT BETWEEN HEALTHY CONTROLS AND PATIENTS WITH TYPE 2 DIABETES**

*Pinterova D, Cerna M, Kolostova K, Novota P, Cimburova M, Romzova M, Kubena A, Andel M.,
Folia Biol (Praha). 2004;50(5):153-6.*

Original Articles

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

(PPAR γ 2 / 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 PPAR γ 2 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.

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). PPAR γ is activated by naturally occurring fatty acids and fatty acid derivatives (Debril and Renaud, 2001). The biomolecular action of PPAR γ 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). PPAR γ activation is linked to an increased differentiation of preadipocytes to adipocytes. There are three already known forms of PPAR γ : PPAR γ 1, PPAR γ 2 and PPAR γ 3. 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 PPAR γ 2 (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 PPAR γ 2 gene with type 2 diabetes. There are two large studies suggesting a decreased risk of type 2 diabetes for the Ala12 genotype in PPAR γ 2 (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:

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

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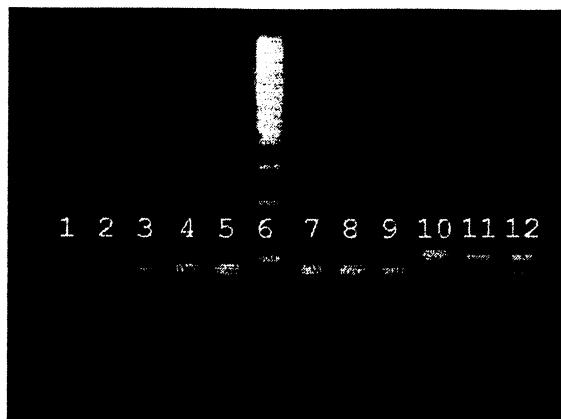


Fig. 1. Electrophoresis of PCR products after restriction on 2% agarose gel. Line 1: negative control. Lines 2,3,4,5,7,8,9: homozygotes for the Pro/Pro genotype. Line 6: DNA marker. Line 10: homozygotes for the Ala/Ala genotype. Lines 11,12: heterozygotes for the Pro/Ala genotype.

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 MgCl₂, 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 PPAR γ 2 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			Total
	Pro/Pro	Pro/Ala	Ala/Ala	
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 PPAR γ 2 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 \pm 9.6	64.6 \pm 10.0	66.3 \pm 9.0	n.s.
BMI [kg/m ²]	30.9 \pm 6.3	31.2 \pm 4.9	31.5 \pm 5.8	n.s.
Total cholesterol [mmol/l]	6.3 \pm 1.1	6.3 \pm 1.1	6.4 \pm 0.9	n.s.
HDL [mmol/l]	1.5 \pm 0.3	1.5 \pm 0.3	1.6 \pm 0.3	n.s.
LDL [mmol/l]	3.9 \pm 1.1	3.9 \pm 1.1	4.0 \pm 0.9	n.s.
Triglycerides [mmol/l]	2.7 \pm 1.2	3.1 \pm 1.7	2.4 \pm 1.8	n.s.

n.s. – not significant

Discussion

This study supports the hypothesis that the Pro12Ala polymorphism of the PPAR γ 2 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 PPAR γ 2 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 PPAR γ 2 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 difference in the BMI or lipid levels. It thus seems that for studying the role of the Pro12Ala polymorphism of the PPAR γ 2 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 PPAR γ 2 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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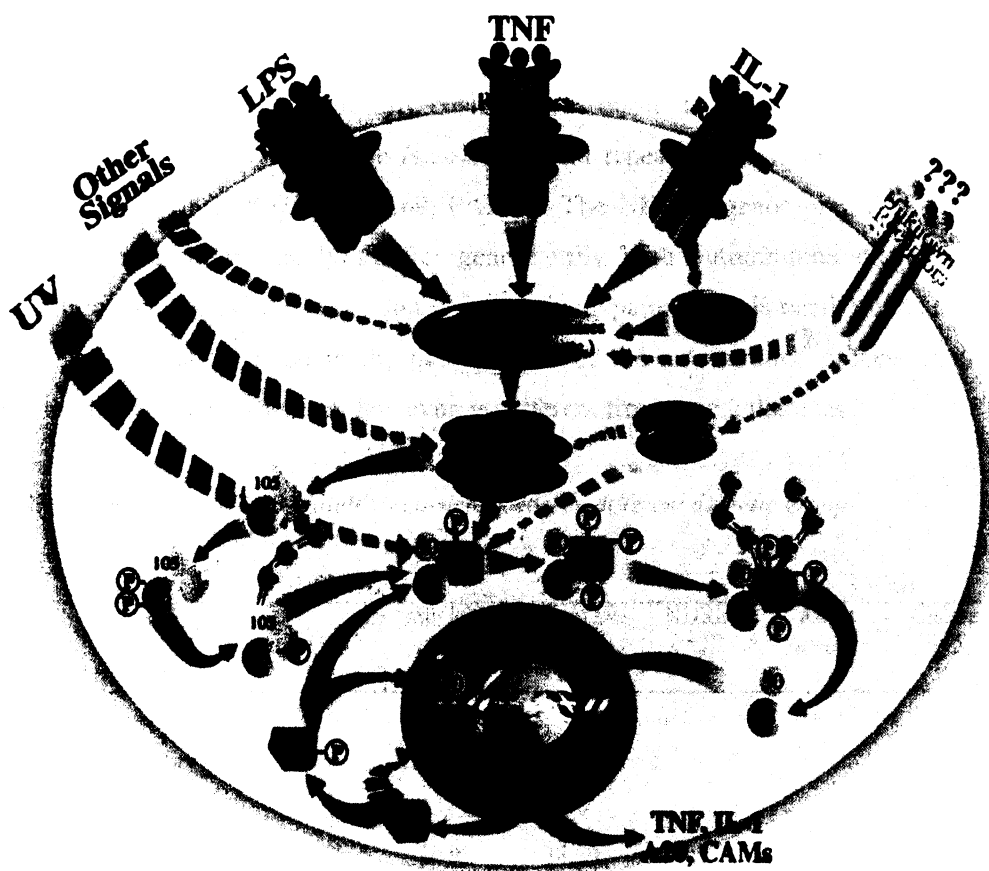
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4.2. *NFκB*

Nuclear factor kappa B (NFκB) was initially identified as nuclear factor bound to enhancer of immunoglobulin k light chain gene of B lymphocytes (457). Five different products fulfilling the NFκB functions are known: RelA/p65, cRel, RelB, p50 (a processing product of p105), and p52 (a processing product of p100). They exist either as homodimers or heterodimers. 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 NFκB/Rel proteins. The NFκBs are present in every cell type in an inactive form in the cytoplasm (458,459). Upon stimulation they are released from inhibitory subunits and transferred into the nucleus, where they promote transcriptional activation of target genes (*Figure 4*). The signal is eventually terminated by the new synthesis of *IκBs*. 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 (459-461). 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 (TNF α), IL-1 β , lipopolysaccharide (LPS) or T-cell receptor (TCR) leading through NEMO (IKK α/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 (IKK α) resulting in dimerization and activation of p52/relB heterodimer.

Recent studies have investigated the role of NFκB in the pathogenesis of various human diseases including neurological disorders, inflammatory diseases, carcinogenesis, and atherosclerosis (302,462-467). 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 (409,468-470). The possible link between NFκB and the development of insulin resistance and type 2 diabetes has also been discussed (471-476). In addition, the association of nuclear factor with renal diseases has been investigated (477-479).

Figure 4: Schematic representation of the main pathway of NFκB activation



<http://alfin2100.blogspot.com/search/label/cancer>

4.2.1. Commentary to the Original Papers 2, 3

We performed two studies on association of genetic variants in the genes coding nuclear factor and its inhibitor with diabetes mellitus and diabetic complications. Both NFKB1 (4q24, gene for p105 of NFκB) and NFKBIA (14q13, gene coding IκBα) polymorphisms were tested in groups of autoimmune diabetics (adults, children), LADA patients, type 2 diabetics with different extent of renal damage and SLE patients. In addition to these studies we genotyped the NFKBIA polymorphism in another autoimmune disease – RA (unpublished data).

The NFKB1 testing of diabetic groups is summarized in *Table 12*. We identified 15 out of 18 previously described alleles of CA repeat polymorphism (409) (ranging in size from 114

to 142bp – corresponds to 12-26 CA repeats). The longest alleles (144-154bp), identified among UK subjects, were not found in our population. The shortest alleles (114-122bp) were found in T1D 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 T1D 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 genotyping revealed previously confirmed association of the NFκB/Rel gene family with autoimmune diabetes. We found increased frequency of A7 allele in adult type 1 diabetic patients. This result points out 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.

Table 12: Distribution of NFKB1 allele frequencies between different diabetic groups

Group			CONTROL		T2DM			T1DM		LADA
			n=139	with DN n=117	without DN n=78	NDRD n=50	Adults n=67	Children n=55	n=34	
Allels	Lenght(bp)	Repeats								
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; highlighted frequencies present most frequent alleles in particular population

without DN patients with diabetic nephropathy

with DN patients without diabetic nephropathy

NDRD patients with non-diabetic renal disease

T1DM

T2DM

LADA

patients with type 1 diabetes mellitus

patients with type 2 diabetes mellitus

latent autoimmune diabetes in adult patients

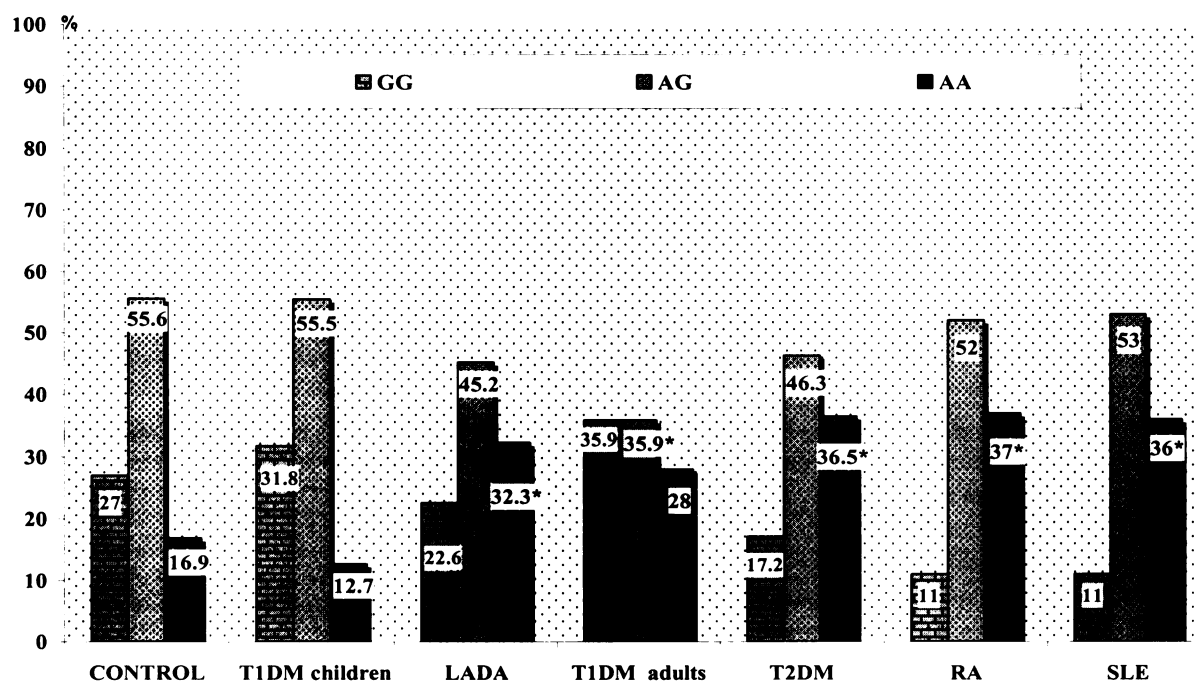
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controls

The NFKB1A genotyping results are shown in Figure 5. 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 NF κ B 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 I κ B, however the mechanism of this action remains to be investigated in functional studies.

Figure 5: Frequencies of NFKBIA genotypes in tested groups of patients with different diseases



4.2.2. Original paper 2

**NFKAPPAB AND ITS INHIBITOR IKAPPAB IN RELATION TO TYPE 2 DIABETES AND ITS
MICROVASCULAR AND ATHEROSCLEROTIC COMPLICATIONS**

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Hum Immunol. 2006 Sep; 67(9):706-13. Epub 2006 Jun 22.

NF κ B and Its Inhibitor I κ B in Relation to Type 2 Diabetes and Its Microvascular and Atherosclerotic Complications

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Marketa Fojtikova, Sarka Ruzickova, Ctibor Dostal,
Vladimir Bosak, Ivan Rychlik, and Marie Cerna

ABSTRACT: Nuclear factor κ B (NF κ B) is an important transcription factor that together with its inhibitor (I κ B) participates in the activation of genes involved in immune responses. We examined the CA repeat polymorphism of the NF κ B1 gene (encoding for NF κ B) and A/G point variation in the 3'UTR region of the nuclear factor kappa B inhibitor alpha (NFKBIA) gene (encoding for I κ B) 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 NF κ B1 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 NF κ B 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 NF κ B 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
HbA1c hemoglobin A1c
I κ B inhibitor of nuclear factor κ B
IKK- β I κ B kinase β

INF- γ interferon γ
NF κ B 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.

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

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 NF κ B 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, NF κ B is activated through phosphorylation of an inhibitor of NF κ B (I κ B). Phosphorylated I κ B is released from NF κ B/I κ B complex, allowing the translocation of NF κ B 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 NF κ B in the pathogenesis of various human diseases including neurologic disorders, immune deficiency, carcinogenesis, and atherogenesis. In addition, the possible link between NF κ B and the development of insulin resistance and type 2 diabetes has also been suggested [3,5-8].

The NF κ B transcription factor complex has two alternative DNA binding subunits, nuclear factor kappa B p 105 subunit (NF κ B1) and NF κ B2. The gene coding for NF κ B1 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 NF κ B1 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 NF κ B is associated with the development of diabetic microvascular complications [12,13], we examined the CA repeat polymorphism of the NF κ B1 gene in relation to diabetic nephropathy.

The gene coding for I κ B (NF κ BIA) has been mapped to chromosome 14q13, and A/G point variation in the 3'UTR region of NF κ BIA has been detected. We also examined single nucleotide polymorphism of the I κ B 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 NF κ BIA polymorphism in a second disease, systemic lupus erythematosus (SLE), to confirm or refute a specific association between NF κ B and diabetic complications or diabetes itself.

MATERIAL AND METHODS

Subjects

The study of polymorphisms in the NF κ BIA and NF κ B1 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 NF κ BIA 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	*	142 ± 21.6	134.8 ± 21.6
Mean diastolic BP	83 ± 12.7	93 ± 11.6	*	79 ± 10.9	79 ± 12.0
BP amplitude	59 ± 15.74	73 ± 20.8	*	74 ± 11.11	68 ± 1.51
History of MI (%)	16	24	*	26	31
History of stroke (%)	13	7	*	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
HbA1c (g/L)	*	*	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	*	*

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

* 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 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

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

* 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 %)

NFKB1 alleles (bp)	Czech Republic			Germany			Czech Republic			United Kingdom			Denmark			Spain			Australia		
	C	T2DM	T2DM	T2DM	T2DM	LADA	JDM	C	T1DM	T1DM	T1DM	T1DM	C	RA	SLE	CD	C	C (BC)	BC		
	n = 39	n = 73	n = 122	n = 57	n = 67	n = 34	n = 55	n = 222	n = 434	n = 229	n = 200	n = 311	n = 109	n = 197	n = 181	n = 311	n = 102	n = 102	n = 102		
A01	0.4					1.47		0.45					2.94				2.94	0.45	1.96		
A02						1.47												0.98	0.98		
A03						2.94												0.98	0.98		
A1							0.9											0.45	0.45		
A2	0.7	21.9 ^b	18.1 ^b	1.49	23.13 ^b	20.58 ^b	19.1 ^b	0.45	1.6	0.87	0.5	0.4						24 ^b	25 ^b		
A3	22.7 ^b	3.4	8.2	5.97	2.94	2.94	10	0.45	1.15	0.44	0.76	19.27 ^b	23.5 ^b	0.26	0.3	19.9 ^b	11.8	6.37	6.37		
A4	5.8	3.4	8.2	5.97	2.94	2.94	10	0.45	6.2	22.71 ^b	21.83 ^b	9.7		17.56 ^b	19.27 ^b	9.7	4.4	1.98	1.98		
A5	3.6	0.7	2	2.23	2.94	2.94	1.9	0.45	4.8	5.24	9.64	9.7		8.25	9.04	9.7	4.4	8.3	8.3		
A6	8.6	10.3	13.1	11.19	14.7	14.7	4.5	0.45	5.8	1.31	2.28	1.5	14.7	2.21	2.24	1.5	3.9	2.45	2.45		
A7	4.3	2.7	3.3	1.75	13.43 ^{ab}	2.94	0.9	0.45	5.8	9.17	4.57	6.8	2.94	7.25	6.35	6.8	8.8	13.7	13.7		
A8	9	7.5	6.6	12.68	14.7	14.7	10.9	19.8 ^b	6.2	4.59	6.09	9.7	14.7 ^b	8.75	7.34	9.7	37.8 ^b	34.8 ^b	34.8 ^b		
A9	3.7 ^b	43.2 ^b	43 ^b	24.6 ^b	27.9 ^b	27.9 ^b	38.4 ^b	9.9	14.9	10.26	11.68	12.1	2.94	10.2	10.8	12.1	3.4	1.96	1.96		
A10	3.6	5.5	3.3	2.47	4.41	4.41	4.5	2.7	17.5 ^b	34.93 ^b	32.99 ^b	28.6 ^b	2.94	36.28 ^b	34.67 ^b	28.6 ^b	3.4	2.45	2.45		
A11	2.9	2.7	2	0.74	4.41	4.41	8.1	9.9	10.6	3.093	5.08	5.8	5.88	3.5	4.27	5.8	3.9	3.9	3.9		
A12	1.4	2.1	0.4	1.75				2.25	7.6	5.68	4.82	4.4		4.74	4.92	4.4					
A13								11.26	5.3	0.44	0.25	1.5		0.5	0.4						
A14								28.38 ^b	3.9	0.44											
A15								7.21	6.7												
A16								5.41	1.6												
A17								0.9	0.23												
A18								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 = systemic 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 NF κ B1 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 NF κ B1 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 NF κ B1 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 NF κ B1 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 NF κ B 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 NF κ B 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 NF κ B 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 I κ B. Aberrant I κ B may not bind to NF κ B 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 IKK β /I κ B α /NF κ B pathway [5,23,24]. NF κ B-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 I κ B in the pathogenesis of type 2 diabetes mellitus.

The IKK β /I κ B α /NF κ B 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 NF κ B 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 NF κ B1 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 I κ B/NF κ B are implicated in the pathogenesis of these two diseases.

Several reports on the association study about the CA repeat polymorphism of the NF κ B1 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 NFKB1A polymorphism and DN, we assume that NF κ B 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 NFKB1A polymorphism to be associated with autoimmune diabetes mellitus (Kolostova *et al.*, article in press). We suggest a dual mechanism for NF κ B participation in their pathogenesis. Additional functional studies are necessary for further investigation.

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4.2.3. Original paper 3

**HLA, NFKB1 AND NFKBIA GENE POLYMORPHISM PROFILE IN AUTOIMMUNE DIABETES
MELLITUS PATIENTS**

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

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Abstract

▼
 Type 1 diabetes mellitus (T1DM) is one of the long-time studied autoimmune disorders. The triggering of the autoimmune process has been ascribed to various genes active in the regulation of the cytokine gene transcription including the Rel/NF- κ B gene family. In our study the gene polymorphism of HLA class II, NFKB1 (nuclear factor of kappa light polypeptide gene enhancer in B-cells 1) and NFKBIA (inhibitor of nuclear factor kappa B) was tested. Patients were divided into the subgroups in relation to the disease type: T1DM in children, T1DM in adults, and Latent Autoimmune Diabetes in Adults (LADA). HLA-DRB1*04 and HLA-DQB1*0302 have been detected as risk factors for T1DM in adults and particularly in children ($P < 0.0001$, OR=22.9 and 46.5 respectively). HLA-DRB1*03 has been found as a single risk factor for LADA ($P < 0.0001$, OR=4.9). We detected 15 alleles for the *NFKB1*

gene polymorphism (CA-repeats) in the Czech population. The alleles were ranging in size from 114–142bp corresponding to 10–25 CA repeats. Frequency of the A7 allele of *NFKB1* gene has been significantly increased in T1DM adults ($P < 0.01$). There was no difference in A and a G allele frequency of *NFKBIA* gene between the control group and patients, but the association of the AA genotype of *NFKBIA* gene has been found for LADA ($P < 0.05$). Summarizing our results we concluded that there is a high probability of association of gene polymorphism from Rel/NF- κ B family with an autoimmune diabetes course. Due to the results obtained in the epidemiological study we have been looking also for the function significance of the genetic predisposition. No significant changes have been observed by real time PCR testing of HLA-DRB1*04 gene and *NFKB1* gene expression between T1DM diabetic group with different HLA, *NFKB1*, *NFKBIA* genetic background.

Abbreviations

▼

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

Introduction

▼
 Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disease characterized by a loss of tolerance towards own antigenic structures of beta-pancreatic cells. The destruction of beta cells subsequently leads to the loss of insulin production. T1DM is not a homogeneous disease, since several of its clinical features are different in children up to 6 years of age as compared to older patients (Csorba & Lyon, 2005). There are more factors, which trigger the autoimmune response in susceptible individuals; however, they are only partially known so far. One of the predisposing factors is the genotype background, ascribed mainly to genes of the *HLA*. Out of extensive genetic and epidemiological studies, the Caucasoid population is known to have a significant association of insulin-depend-

Table 1 Characteristics of the tested groups

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

. data not available.

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

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

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

A polymorphic dinucleotide CA microsatellite repeat, with 18 described alleles, has been identified close to the coding region

of the human NFKB1 gene. *NFKBIA* genotypes (A/G in 3'UTR) have been determined in previous studies as wild-type 424 bp; variant 306 and 118 bp; and heterozygote 424, 306 and 118 bp after restriction digestion (Curran et al., 2002).

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

Methods

▼

Subjects

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

Genotyping

DNA was extracted from peripheral blood leukocytes using of DNA blood mini isolation method (Qiagen, Hilden, Germany). Genotyping of the *NFKB1* dinucleotide repeat with use of fluorescently labeled primers was previously described by Ota et al. (Ota et al., 1999). Polymerase chain reaction (PCR) amplification was performed in a reaction mixture containing 25–50 ng genomic DNA, 0.5 mM each primer, 0.2 mM dNTPs, 1xPCR buffer, 2.5 mM MgCl₂ and DNA polymerase. PCR conditions were 4 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 52°C and 30 s at 72°C; followed by 5 min at 72°C. *NFKB1* alleles were then detected by

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

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

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

NFKB1A amplification was performed in a 20 µl final volume containing genomic DNA, 1xPCR buffer, 3.75 mM MgCl₂, 0.2 mM dNTPs, 0.5 mM each primer and DNA polymerase. PCR conditions were initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 30 s, with a final extension of 2 min at 72°C. Following amplification, 10 µl of product was digested with *Hae*III at 37°C overnight. Samples were then loaded into an ethidium bromide stained 2% agarose gel for genotype determination. Genotypes of 268 patients and 159 matched controls were determined as type (AA), 424 bp; variant (GG), 306 and 118 bp; and heterozygote (AG), 424, 306 and 118 bp (Curran et al., 2002).

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

Gene expression testing

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

Real time RT-PCR analysis

Peripheral blood with EDTA was collected by venipuncture, and APCs were immediately separated by immunomagnetic separation by Dynabeads (DynaL HLA-class II, 210.04, Dynal, Oslo, Norway). Total RNA was extracted using the RNA blood mini kit (Qiagen, Hilden, Germany). RNA was reverse transcribed by Taqman[®] real time PCR reagents. The quantitative real time RT-PCR was performed in duplicates using the Taqman[®] PCR Kit in 96-well microtiter plates on the ABI PRISM 7000 Sequence Detector Systems, according to the manufacturer's instructions (Applied

Biosystems, Foster City, CA, USA). The cDNA was amplified using specific primers for *HLA-DRB1*04* designed by Primer express[®] as well as of Taqman[®]MGB (*HLA-DRB1*04* gene forward primer 5'ACACCCGACCACGTTTCTTG 3', *HLA-DRB1*04* gene forward primer 5'TCCGTCCGTTGAAGAAATG 3', *HLA-DRB1*04* Taqman[®]MGB probe 6-FAM-CACTCATGTTTAACCTGCT). Testing of *NFKB1* expression was done with assay on demand set of primers and probes. As an internal control the human beta actin was used (Applied Biosystems, Foster City, CA, USA). The 2^{-ΔΔC_t} [2-delta delta C_t] method was applied for relative quantification (Livak & Schmittgen, 2001).

Statistical analysis

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

Results

The HLA association study

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

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

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

*significant P value (P<0.05)

association was comparable between these two groups. The results of the study just confirm the previous results from the HLA typing studies, but were needed in the relation to the further NFKB1 and NFKB1A polymorphism testing.

Distribution of NFKB1 polymorphism in Czech population

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

Distribution of 3' UTR NFKB1A polymorphism in Czech population

The genotype frequencies of the *NFKB1A* gene polymorphism in the control and patient groups are presented in Table 4. There was no difference in A and G allele frequency between the control group and patients, the difference was observed in distribution of genotypes between the patient groups. There is an evidence that the heterozygous genotype of *NFKB1A* is protective for diabetes onset in adulthood, according to the results in LADA group (nonsignificantly) and T1DM adults (RR = 0.56, OR = 0.44, P < 0.01, CI = 95%). In the mentioned groups the frequency of the heterozygotes is lowered and the frequency of the homozygous genotypes rises. A significant difference was observed for AA genotype in LADA group, with a relative risk value of 2.23 (OR = 2.68, P < 0.001, CI = 95%) The AA and GG genotype frequencies in T1DM adults were increased, but with the border significance.

Expression studies of HLA-DRB1*04 and NFKB1

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

*HLA-DRB1*04* gene expression was tested first among different groups of patients. The significant difference was found only in mRNA expression levels of *HLA-DRB1*04* between the group of T1DM children and the group of T1DM adults (P = 0.034). The expression of *HLA-DRB1*04* was significantly higher in T1DM adult patients. The comparison was done with two specific control groups (positive for *HLA-DRB1*04*). Since there was no difference between the control groups with different age, we compared the control groups as one control set. No difference in the expression of *HLA-DRB1*04* has been observed after analysis among different HLA class II, *NFKB1A* and *NFKB1* genotypes.

No difference in the expression of *NFKB1* gene has been observed among different types of diabetic patient groups and even after analysis based on the found HLA class II, *NFKB1A* and *NFKB1* genotypes.

Discussion

Development of type 1 diabetes requires coordinated expression of genes responsible for initiation and progression of the disease, what is regulated by small number of transcription factors including the Rel/NF- κ B family. In our study we have compared different groups of patients with autoimmune diabetes mellitus: 1) T1DM children 2) T1DM adults 3) LADA.

Based on the HLA - typing studies, we have concluded that *HLA - DR3* is significantly associated only with LADA (RR = 3.25) and children diabetes (RR = 1.51). *HLA - DQB1*0302* has three times stronger relative risk for diabetes in childhood (RR = 8.98) than for diabetes in adults (RR = 3.02). The results are in concordance with previous testing in Czech population (Cerna et al., 2003; Cinek et al., 2001; Novota et al., 2004). Surprisingly, there was only a border significance of *HLA-DQB1*0201* gene association in our T1DM children group. This is a difference from the results published in Czech population study by Cinek (Cinek et al., 2001). Generally, the found difference could be explained by different number of patients involved into the study what could change significance level.

Based on *NFKB1* and *NFKB1A* polymorphism studies, we have found out that A7 (132 bp) allele of *NFKB1* gene presents a risk

Table 4 Frequencies of NFKBIA genotypes in the tested groups

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

*significant P value (P<0.05)

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

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

*significant P value (P=0.034)

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

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

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

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

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

diabetic patients with different diabetes onset. We hypothesize that the increased *HLA-DRB1*04* mRNA expression could be a protecting factor in a group of T1DM adults.

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

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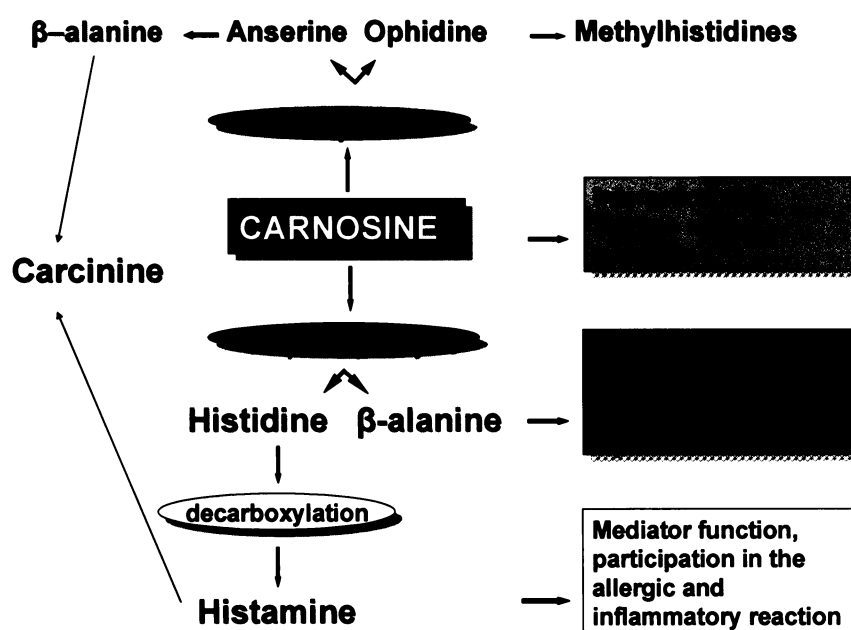
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4.3. CARNOSINE

Carnosine is simple dipeptide, β -alanyl-L-histidine with protective function additional to its anti-oxidant and free-radical scavenging roles (480-484). It extends cultured human fibroblast life-span (485,486), kills transformed cells, protects cells against aldehydes and an amyloid peptide fragment and inhibits in vitro protein glycation (formation of cross-links, carbonyl groups and AGEs) and DNA/protein cross-linking (487). Carnosine is an aldehyde scavenger and possible modulator of diabetic complications, atherosclerosis and Alzheimer's disease. In the central nervous system carnosine acts as neurotransmitter modulating synaptic processes but also appears to be involved in neuroprotection (488).

Figure 6: The metabolic pathway of Carnosine



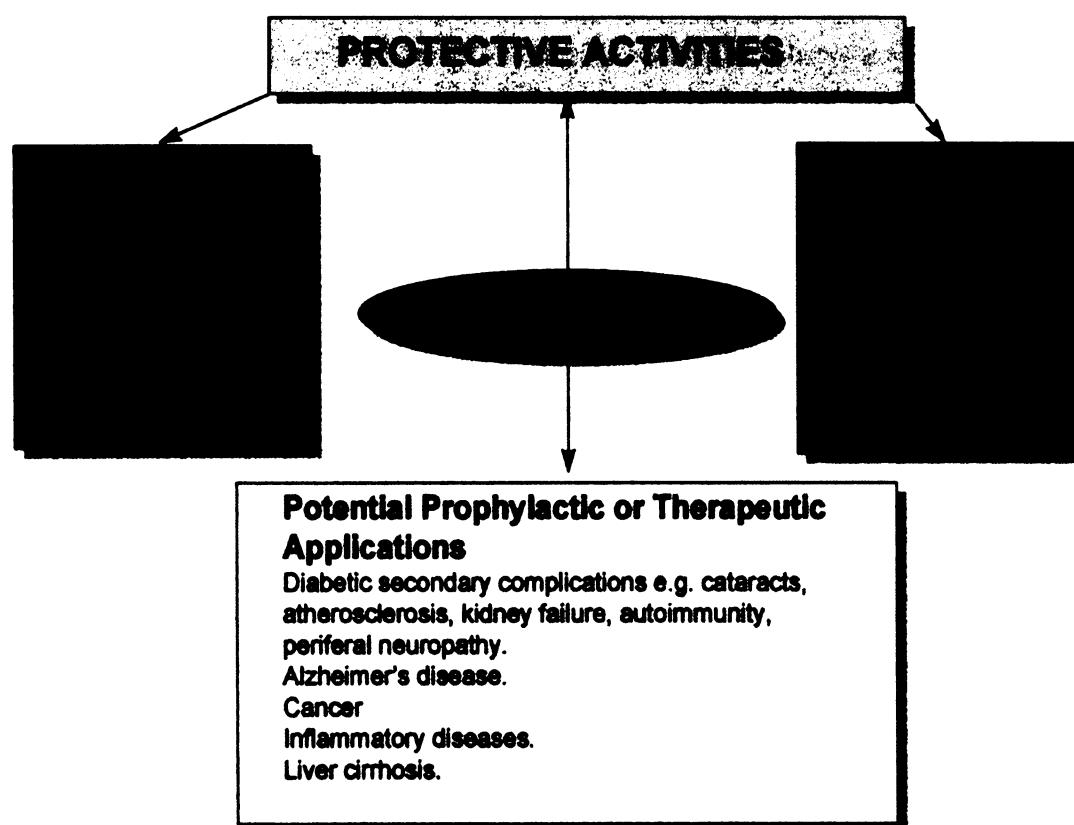
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Carnosine is synthesized by carnosine synthase (Figure 6) from β -alanine and histidine in many tissues (489) and is degraded by intra- or extracellular dipeptidases, also named carnosinases, all belonging to the large family of metalloproteases (490). Carnosinases exist in two isoforms. A cytosolic form acting as a nonspecific dipeptidase, previously named tissue carnosinase, is found in the liver, kidney, and spleen (491). A secreted form of carnosinase is found in serum (plasma), as well as in the brain and spinal fluid, is called serum carnosinase (492,493). These enzymes result

from different gene products and differ not only in their distribution but also in their enzymatic properties. Although under appropriate conditions both isoforms catalyze the hydrolysis of the dipeptide carnosine (β -alanyl-L-histidine) only serum carnosinase is able to hydrolyze homocarnosine (γ -aminobutyryl-L-histidine).

The recent studies has indicated and proved that carnosine and related compounds improved hyperglycemia, hyperlipidemia, and oxidation and improved glycemc control in diabetic animals (494,495). Also the protective effect of histidine and carnosine against copper induced oxidative damage of human LDL has been observed (494,496).

Figure 7: Summary of carnosine known and possible activities and potential applications



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4.3.1. Commentary to Original Paper 4

The paper on association of CNDP1 gene polymorphism with diabetic nephropathy revealed the role of new protective factor in development or progression of diabetic nephropathy. The study aimed to trace the candidate genes for diabetic nephropathy from the susceptibility locus on chromosome 18p22.3-23 (497). Two genes, related to the pathophysiology of microvascular complications, were found within this region. The carnosinase genes CNDP1 and CNDP2 code for serum and tissue carnosinases – enzymes belonging to the metalloproteases family, cleaving naturally occurring dipeptide carnosine. Complete sequence and association analysis of both genes detected several polymorphic variants, from which only D18S880 marker was significantly associated with diabetic nephropathy. This marker corresponds to trinucleotide (CTG=Leu) repeat polymorphism located in 5' coding region of CNDP1 gene corresponding to a leader sequence. The most associated with diabetic nephropathy were 6 and 7Leu allele, thus 5Leu allele was protective. The genotyping also showed that group of type 2 diabetics with nephropathy had significantly lower frequency of homozygous 5-5 genotype comparing to the group without nephropathy. The genotype frequencies were similar even among nephropatic patients with type 1 and type 2 diabetes. Different genotypes (5-5, 5-6, 5-7, 6-6, 6-7) of CNDP1 gene were compared to expected efficiency of cleaving and secretion of leader peptide of CNDP1 gene product. Sequence comparison was expressed as GvH score. A value > 0.0 represented functional leader peptide, the value below – 2.1 corresponded to nonfunctional peptide. Since the 7Leu allele showed GvH score of 0.83, whereas the GvH score for 5Leu allele was – 1.76 it suggests that 6 and 7Leu alleles were associated with higher enzyme activity, thus considered to be gain of function mutations. To confirm the correlation between CNDP1 genotypes and enzyme activity, the serum carnosinase activity was measured and plotted against genotype. The lowest enzyme activity was associated with homozygosity for 5Leu allele. The quantitative real-time measurement of CNDP1 expression in human glomeruli, and immunohistochemical staining using different antibodies revealed elevated expression of CNDP1 in all patients with diabetic nephropathy comparing to healthy controls. The protective role of carnosine, target of serum carnosinase, was also verified by experiments on cell cultures. Carnosine inhibited the effects of high glucose on human podocytes and mesangial cells by blocking the production of fibronectin, collagen IV and TGF- β 2, compounds of extracellular matrix produced by podocytes and mesangial cells.

In the conclusion, the study found association of CNDP1 gene polymorphism with diabetic nephropathy. The variants with the lowest number of Leu repeats (5) in both alleles are protective from diabetic nephropathy. Individuals with higher number of Leu repeats in CNDP1 (6, 7) had

increased serum carnosinase activity, thus increased degradation of carnosine, which is thought to protect renal cells against high level glucose effects.

This study found that the number of Leu repeats in the CNDP1 gene influences the susceptibility to diabetic nephropathy and point out the protective role of carnosine in diabetic complications and its possible therapeutic use (*Figure 7*).

4.3.2. Original paper 4

**CARNOSINE AS A PROTECTIVE FACTOR IN DIABETIC NEPHROPATHY: ASSOCIATION WITH A
LEUCINE REPEAT OF THE CARNOSINASE GENE CNDP1.**

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Carnosine as a Protective Factor in Diabetic Nephropathy

Association With a Leucine Repeat of the Carnosinase Gene *CNDP1*

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The risk of diabetic nephropathy is partially genetically determined. Diabetic nephropathy is linked to a gene locus on chromosome 18q22.3-q23. We aimed to identify the causative gene on chromosome 18 and to study the mechanism by which the product of this gene could be involved in the development of diabetic nephropathy. DNA polymorphisms were determined in 135 case (diabetic nephropathy) and 107 control (diabetes without nephropathy) subjects. The effect of carnosine on the production of extracellular matrix components and transforming growth factor- β (TGF- β) after exposure to 5 and 25 mmol/l D-glucose was studied in cultured human podocytes and mesangial cells, respectively. A trinucleotide repeat in exon 2 of the *CNDP1* gene, coding for a leucine repeat in the leader peptide of the carnosinase-1 precursor, was associated with nephropathy. The shortest allelic form (*CNDP1 Mannheim*) was more common in the absence of nephropathy ($P = 0.0028$, odds ratio 2.56 [95% CI 1.36–4.84]) and was associated with lower serum carnosinase levels. Car-

nosine inhibited the increased production of fibronectin and collagen type VI in podocytes and the increased production of TGF- β in mesangial cells induced by 25 mmol/l glucose. Diabetic patients with the *CNDP1 Mannheim* variant are less susceptible for nephropathy. Carnosine protects against the adverse effects of high glucose levels on renal cells. *Diabetes* 54:2320–2327, 2005

Diabetic nephropathy is one of the most severe complications of type 1 and type 2 diabetes. Diabetic nephropathy has become the leading cause of end-stage renal failure in the western world. In the U.S., the number of cases of end-stage renal failure due to diabetic nephropathy continues to increase at almost 10% per year (1).

There is a large body of evidence (2,3) of familial aggregation of diabetic nephropathy, and the existence of at least one genetic susceptibility factor seems likely. The most intensively studied genetic variant is the "D/I" polymorphism in the ACE gene on chromosome 17 encoding the angiotensin converting enzyme. Meta-analyses (4–6) have suggested a weak association between the presence of the D-allele and diabetic nephropathy.

In 2002, our group presented results of a family-based linkage study on 18 Turkish families with type 2 diabetes showing that a major susceptibility locus maps to chromosome 18q22.3-q23 (7). The locus was mapped between the markers D18S43 and D18S50 and was supported by a highly significant logarithm of odds score of 6.1. Linkage of diabetic nephropathy to 18q was confirmed by an affected sibpair analysis performed on Pima Indians and, very recently, by a large sibpair analysis of African Americans (7,8). We postulated that diabetic nephropathy might be caused by a dominant-acting mutation at this gene locus. Consequently, a recessive allele at this locus could protect carriers from the development of this complication. ZNF236 is the only gene within the 18q candidate region

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AGE, advanced glycation end product; ROS, radical oxygen species; TGF- β , transforming growth factor- β .

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that had previously been considered as a candidate gene for diabetic nephropathy (9). Since no mutations in the transcript of the gene were found, ZNF236 was subsequently excluded as a trait-causing gene (10). It therefore remained unknown which gene within the 18q region is responsible for the susceptibility of some diabetic subjects to develop diabetic nephropathy. We searched for genes related to the pathophysiology of microvascular disease and focused on the inhibition of oxidative stress and advanced glycation end products (AGEs). For example, AGEs have been found in glomeruli of diabetic animals and humans, the inhibition of AGEs is beneficial in experimental diabetic nephropathy, and AGEs have been shown to cause impaired proliferation of renal mesangial cells, a major feature of diabetic nephropathy development (11). The carnosinase genes *CNDP1* and *CNDP2* are such genes located in 18q22.3.

The enzyme carnosinase degrades the dipeptide carnosine (β -alanyl-L-histidine), while carnosine has been reported to inhibit the formation of AGE molecules (12). *CNDP1* encodes the secreted serum carnosinase, whereas *CNDP2* encodes tissue carnosinase, also known as cytosolic nonspecific dipeptidase (13). The genes are positioned adjacent to each other in a head-to-tail orientation. The aim of this study was to identify the causative gene on chromosome 18 and to gain insight in the mechanism by which the product of this gene could be involved in the development of diabetic nephropathy.

RESEARCH DESIGN AND METHODS

Sampling strategy for case-control study. We screened 5,500 patient records in different centers in Germany, The Netherlands, Qatar, and the Czech Republic. A complete list of participating centers can be found in the contributors section of the ACKNOWLEDGMENTS. To identify case and control subjects, screening of all patient records of a participating center was performed, and patients not fulfilling the inclusion criteria were excluded. The detailed sampling strategy, exclusion and inclusion criteria, and details on collected data are given in the addendum available from www.klinikum.uni-heidelberg.de/index.php?id=6,791.

Power analysis. Single type 2 and type 1 diabetic patients (designated groups 2 and 3, respectively) were recruited for exploratory testing of the sequence variant selected in the first phase (an exploratory search using group 1). Together, groups 2 and 3 consisted of 99 patients with diabetes and 98 patients with diabetic nephropathy. Power estimations had shown that such a sample size would be sufficient to confirm association with >80% power if the relative risk exceeds 2.5 (allele frequency 40–50%, significance level 5%).

Sequence analysis and genotyping. *CNDP1* exons were amplified using intronic primers (MWG-Biotech, Ebersberg, Germany) and sequenced using an ABI 3100 capillary sequencer (Applied Biosystems, Darmstadt, Germany). Sequence analysis of the *CNDP2* gene was performed by Synergene (Qormi, Malta). Techniques used for PCR-based genotyping of polymorphisms included restriction enzyme digest (rs2278161), fragment analysis using an ALF-Express DNA sequencer (Pharmacia-Biotech, Freiburg, Germany) (D18S58, rs10548323, rs3080862, DN31, rs10596720, D18S880, and D18S1161), TaqMan single nucleotide polymorphism genotyping using an ABI Prism 7000 (Applied Biosystems) (rs12965928 and rs2241509), denaturing high-performance liquid chromatography analysis (Transgenomic, Berlin, Germany) (rs1046407 and rs22778156), or sequence analysis (rs1559803, rs7237740, rs3764509, rs890332, rs4892247, and rs2887). Primer sequences are available on request. For D18S880, we used a standard PCR protocol with primers AGGCAGCTGTGTGAGGTAAC (forward) and GGGTGAGGAGAACATGCC (reverse). The annealing temperature was 60°C. Sequence analysis confirmed that a PCR product length of 167 corresponds to 5 CTG repeat units (five leucine codons).

Measurement of carnosine and carnosinase activity in serum. For measurement of serum-carnosinase activity, serum samples were withdrawn between 9:00 and 10:00 A.M., cooled on ice immediately, and stored at -80°C . A total of 150 μl carnosine (1 mg/ml in 50 mmol/l Tri-HCl buffer) (Sigma Aldrich Chemie, Deisenhofen, Germany) was added to 600 μl serum, divided into aliquots of 100 μl , and incubated at 30°C . The reaction was stopped after

5, 10, 15, 20, 30, and 40 min by adding 25 μl 10% 5-sulfosalicylic acid (Sigma Aldrich Chemie). After shaking, the samples were incubated at 4°C for 30 min and centrifuged at 13,000 rpm for 5 min to remove the precipitated proteins. Carnosine concentrations were assayed by fluorometric determination after derivatization with carbazole-9-carbonyl chloride. Separation was performed by liquid chromatography according to the method described by Schonherr (14).

In vitro studies

Cell culture. Human SV 40-transformed mesangial cells as well as human thermosensitive SV 40-transformed podocytes were used to investigate the influence of L-carnosine on the increased production of extracellular matrix proteins and transforming growth factor- β (TGF- β)1 and -2 under hyperglycemic conditions. SV 40-transformed, temperature-sensitive podocytes were characterized as previously described (15). These cells proliferate at the "permissive" temperature (33°C). After transfer to the "nonpermissive" temperature (37°C), they enter growth arrest and express markers of differentiated in vivo podocytes, including nephrin, podocin, CD2AP, and synaptopodin. Human SV 40-transformed mesangial cells were characterized as previously described (16). All cell lines were cultivated in uncoated culture flasks in Dulbecco's modified Eagle's medium (PAA, Coelbe, Germany) supplemented with 10% FCS (Greiner, Frickenhausen, Germany), insulin-transferrin selenium (all in concentrations of 5 ng/ml), epidermal growth factor (5 ng/ml), and penicillin/streptomycin (10 units/ml, all from Sigma, St. Louis, MO). Methods for indirect immunofluorescence, TGF- β 1 and - β 2, enzyme-linked immunosorbent assays, RNA isolation, and real-time PCR on human glomeruli, *CNDP1* antibodies, and immunohistochemistry are available from www.klinikum.uni-heidelberg.de/index.php?id=6,791.

Computer analyses and statistical tests. We used the PSORT II program (19) for functional analysis of 5' sequences (calculation of G. von Heijne [GvH] scores). Statistical analyses were performed with SAS V8.02 (PC version). Fisher's exact test and χ^2 analyses were performed to test for association within each population group. The Mantel-Haenszel test was used to analyze data obtained from multiple populations. The Wilcoxon rank-sum test was used to compare groups of samples. *P* values <0.05 were regarded as significant.

RESULTS

Patient data. A total of 5,500 patient files were screened to identify the patients included in this study. One hundred thirty-five patients with diabetic nephropathy and 107 diabetic patients without diabetic nephropathy were selected for the study. The most common cause for exclusion was microalbuminuria (~30%). An equal number of patients had normoalbuminuria but a diabetes duration of <15 years. Six percent had normoalbuminuria and a sufficient diabetes duration but were on an ACE inhibitor or angiotensin II receptor 1 antagonist, which they were not willing to stop for reevaluation of albuminuria. Four percent of macroalbuminuric and diabetic dialysis patients had to be excluded because they did not have diabetic retinopathy at the onset of renal disease. Other types of diabetes (e.g., gestational diabetes or diabetes due to pancreatic disease) led to the exclusion of 10% of patients. Another 10% were unwilling to participate or unable to give informed consent. Missing data were the reason for exclusion in only 6% of patients. For exact numbers on excluded patients and decision making on inclusion and exclusion see the addendum of this article available from www.klinikum.uni-heidelberg.de/index.php?id=6,791. If we compared the number of excluded patients per center, a similar pattern of exclusion was observed for each center. Demographic data and medication of the included patients are given in Tables 1 and 2.

Sequence analysis of coding regions. All 12 exons of the *CNDP1* gene, all 12 exons of the *CNDP2* gene, and the intronic regions flanking these exons were completely sequenced in three German patients with diabetic nephropathy and in two German patients without diabetic nephropathy after 15 years of diabetes. All patients had

TABLE 1
Demographic data of the examined groups*

	Group 1 (trios with type 2 diabetes)		Group 2 (type 2 diabetes)		Group 3 (type 1 diabetes)	
	With diabetic nephropathy	Without diabetic nephropathy	With diabetic nephropathy	Without diabetic nephropathy	With diabetic nephropathy	Without diabetic nephropathy
<i>n</i>	37	8	77	63	21	36
Age (years)	68.2 ± 10.5	64.3 ± 9.3	61.8 ± 10.6	65.4 ± 11.6	48.0 ± 11.7	41.9 ± 11.3
Sex (male/female)	26/11	4/4	38/39	30/33	11/10	13/23
Retinopathy (%)						
Proliferative	56.8	50.0	58.4†	4.8†	95.2†	5.6†
Nonproliferative	37.8	25.0	37.7†	15.9†	4.8†	11.1†
None	5.4	25.0	3.9†	79.4†	0†	83.3†
Arterial hypertension (%)	79.2†	87.5†	70.1†	46.0†	90.5	2.8
Diabetes duration (years)	12.5 ± 7.0†	23.1 ± 8.0†	14.9 ± 8.6†	22.3 ± 6.9†	25.5 ± 10.7	23.8 ± 8.1
A1C (%)	7.6 ± 0.7	7.4 ± 1.2	7.5 ± 2.0	7.3 ± 1.7	7.0 ± 1.5	7.5 ± 1.3
Ethnicity (<i>n</i>)						
German‡	37	8	42	18	2	21
Czech	0	0	11	36	0	0
Dutch	0	0	7	4	19	15
Arabic	0	0	11	5	0	0
Other	0	0	6	0	0	0

Data are means ± SD, unless otherwise indicated. *Shown are the demographic data of the three examined groups. Patients without retinopathy in the groups with diabetic nephropathy had biopsy-proven diabetic nephropathy. †Significant differences between the groups with and without diabetic nephropathy. ‡All German patients were of German extraction.

type 2 diabetes. Several polymorphic sequence variants were detected (Table 3), but no truncating or obviously obliterating mutations were identified.

Association analysis. We performed an explorative search for polymorphisms associated with the diabetic nephropathy trait upon the first group of patients: the “trio-group” (37 patients with type 2 diabetes and diabetic nephropathy and 8 diabetic control subjects). All polymorphisms listed in Table 3 were tested. The most significant association was found with marker D18S880, a trinucleotide repeat in *CNDP1* exon 2. Since this polymorphism lies in the 5' coding part of the transcript, the number of trinucleotide repeats is directly related to the number of leucine residues in the leader peptide of the carnosinase precursor: five, six, or seven leucines. The five-leucine allele was found on 88% of chromo-

somes in patients without diabetic nephropathy but had an allele frequency of only 59% in the diabetic nephropathy group. In contrast to diabetic nephropathy patients, most patients without diabetic nephropathy were homozygous for this allele ($P = 0.041$) (odds ratio 5.54 [95% CI 0.98–31.45]). The genotype frequencies are given in Table 4. Since the first individual found to be homozygous for the five-leucine allele came from the city of Mannheim, we designated this allele the “*CNDP1* Mannheim allele.”

The second patient group consisted of patients with type 2 diabetes with ($n = 77$) and without ($n = 63$) diabetic nephropathy from Germany, The Netherlands, Qatar, and the Czech Republic. In this group, we tested whether homozygosity for the *CNDP1* Mannheim allele was less frequent among patients with diabetic nephropathy.

TABLE 2
Medication of the patients

	Group 1 (trios with type 2 diabetes)		Group 2 (type 2 diabetes)		Group 3 (type 1 diabetes)	
	With diabetic nephropathy	Without diabetic nephropathy	With diabetic nephropathy	Without diabetic nephropathy	With diabetic nephropathy	Without diabetic nephropathy
<i>n</i>	37	8	77	63	21	36
Antidiabetic treatment						
Oral antidiabetics	28.6	12.5	18.3	44.1*	0	0
Insulin	57.1	50.0	60.0	18.6*	100	100
Combination	14.3	37.5	18.3	23.7	0	0
Other medication						
ACE inhibitor	43.5	25.0	57.6	23.7*	42.9	0.0*
AT1 antagonist	8.7	25.0	6.1	1.7	28.6	0.0*
Combination (ACE + AT1)	8.7	0.0	7.6	0.0*	0.0	0.0
β-Blockers	47.8	75.0	53.3	27.8*	33.3	0.0*
Statins	42.1	75.0	36.1	36.4	81.0	0.0*

Data are percent of patients on medication. Patients in the groups without diabetic nephropathy on ACE inhibitor or AT1 antagonist had undergone a 6-week washout phase before evaluation of albuminuria. *Significant differences between the groups with and without diabetic nephropathy.

TABLE 3
Selected polymorphisms in and near the *CNDP2* and *CNDP1* loci

Variant	Gene	Change	Allele most associated with diabetic nephropathy*	Odds ratio (95% CI)
D18S58	5' of <i>CNDP2</i>	CA repeat	149 bp	3.75 (0.45–31.26)
Rs10548323 ("DN33")	5' of <i>CNDP2</i>	Complex repeat	208 bp	1.40 (0.46–4.23)
Rs3080862 ("DN12")	5' of <i>CNDP2</i>	CA repeat	259 bp	2.53 (0.74–8.62)
Rs1046407	5' of <i>CNDP2</i>	C → T	C	2.33 (0.58–9.61)
Rs22778156	5' of <i>CNDP2</i>	C → T	C	1.86 (0.33–10.56)
Rs1559803	5' of <i>CNDP2</i>	T → A	T	2.94 (0.93–9.26)
Rs12965928	5' of <i>CNDP2</i>	C → T	C	1.57 (0.38–6.64)
DN31 (unpublished repeat. 17.9 kb 3' from rs22778156)	5' of <i>CNDP2</i>	TA repeat	341 bp	1.56 (0.52–4.72)
rs7237740 ("PM")	5' of <i>CNDP2</i>	C → T*	C	1.40 (0.46–4.23)
rs3764509	<i>CNDP2</i> (intron)	C → G	C	1.42 (0.43–4.76)
rs2278161 (Y126H)	<i>CNDP2</i>	A → G	A	1.79 (0.53–6.06)
rs2241509	<i>CNDP2</i> (intron)	T → A	T	1.08 (0.35–3.26)
rs890332	5' of <i>CNDP1</i>	T → C*	T	1.88 (0.43–8.26)
rs10596720 ("DN13")	<i>CNDP1</i> (intron)	CA repeat	179 bp	1.78 (0.36–8.85)
D18S880	<i>CNDP1</i>	CTG repeat	6–7 Leu	4.77 (1.01–22.5)†
rs4892247	<i>CNDP1</i> (intron)	T → C	C	1.80 (0.53–6.15)
D18S1161	<i>CNDP1</i> (intron)	CA repeat	96 bp	1.16 (0.37–3.64)
rs2887	<i>CNDP1</i> (3'UTR)	C → T	C	1.22 (0.22–6.76)

*Test for association on 37 patients with diabetic nephropathy and 8 patients with no sign of diabetic nephropathy and a duration of type 2 diabetes >15 years. †D18S880 is a three-allele repeat encoding five, six, or seven leucine residues. It is the only variant significantly associated with diabetic nephropathy in this study. Hence, this variant was selected for further investigations.

thy compared with patients without the complication. A significant difference was confirmed, with only 20 of 77 diabetic nephropathy cases being homozygous for the Mannheim allele compared with 25 of 63 cases without diabetic nephropathy ($P = 0.024$, single-sided Mantel-Haenszel test). The frequencies of the observed genotypes are shown in Table 4. There was no evidence of heterogeneity between the four populations.

In a third group, it was tested whether the predominance of the Mannheim allele in diabetic nephropathy-resistant patients also applies to type 1 diabetes. Homozygosity for the Mannheim allele was found in only 4 of 21 (19%) patients with diabetic nephropathy, compared with 15 of 36 (42%) case subjects without nephropathy ($P = 0.098$). Combined analysis of this group with the type 2 diabetic groups mentioned above showed an odds ratio of 2.56 (95% CI 1.36–4.84) ($P = 0.0028$). As shown in Table 4, the distribution of D18S880 genotypes is very similar among diabetic nephropathy patients with type 1

and type 2 diabetes; the Mantel-Haenszel test showed no evidence for heterogeneity.

Sequence comparisons on *CNDP1* leader peptide. The leader peptide at the NH₂-terminal end of the serum-carnosinase precursor protein has only little homology to the consensus pattern, as published by von Heijne (20). The GvH score, used to measure homology to the consensus, is the original weight-matrix score introduced by von Heijne subtracted by 3.5. The seven-leucine allele showed a GvH score of 0.83, whereas the *CNDP1* Mannheim allele showed a GvH score of -1.76 (Fig. 1A).

Measurement of serum carnosinase activity in normal volunteers. Serum-carnosinase activities and leucine repeat D18S880 genotypes were determined in 45 healthy volunteers. The enzyme activities (12–46 nmol · ml⁻¹ · h⁻¹, mean 26.6) were plotted against the genotype (Fig. 1B). There is a clear correlation between leucine repeat number and serum-carnosinase activity ($P = 0.018$). The lowest activity is associated with homozygosity for the five-

TABLE 4
Genotype frequencies

Genotype	Group 1 (trios with type 2 diabetes)		Group 2 (type 2 diabetes)		Group 3 (type 1 diabetes)		All groups	
	With diabetic nephropathy	Without diabetic nephropathy	With diabetic nephropathy	Without diabetic nephropathy	With diabetic nephropathy	Without diabetic nephropathy	With diabetic nephropathy	Without diabetic nephropathy
5–5	13 (35)	6 (75)	20 (26)	25 (40)	4 (19)	15 (42)	37 (27)	46 (43)
5–6	16 (43)	2 (25)	44 (57)	27 (43)	10 (48)	13 (36)	70 (52)	42 (39)
5–7	2 (5)	—	2 (3)	1 (2)	—	2 (6)	4 (3)	3 (3)
6–6	5 (14)	—	11 (14)	9 (14)	7 (33)	4 (11)	23 (17)	13 (12)
6–7	1 (3)	—	—	1 (2)	—	2 (6)	1 (1)	3 (3)
7–7	—	—	—	—	—	—	—	—
Total	37	8	77	63	21	36	135	107

Data are n (%).

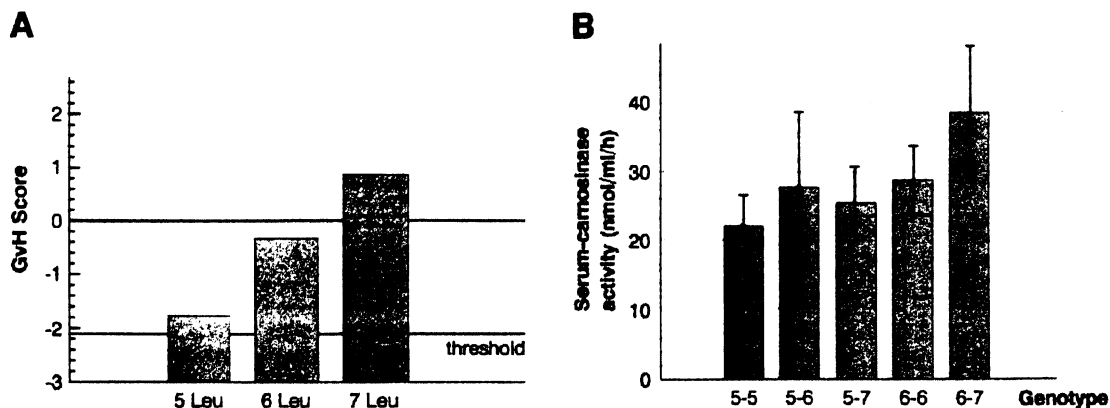


FIG. 1. A: The predicted efficacy of secretion and cleavage expressed as GvH score. This score quantifies the resemblance of 5' sequences to a leader peptide consensus. A value >0.0 is likely to represent a cleavable leader peptide. A sequence with a value below -2.1 is assumed to be nonfunctional as a cleavable leader peptide. Since the *CNDP1* gene product has to be secreted and cleaved to become a serum-carnosinase, the higher GvH scores suggest that the 6 Leu and 7 Leu alleles are gain-of-function mutations. B: The correlation between D18S880 genotype (leucine repeat length) and serum-carnosinase activity measured in 45 healthy control individuals. Shown are the mean enzyme activity and the SD for each observed genotype. The lowest activity was associated with homozygosity for the five-leucine allele ($P = 0.018$) (Wilcoxon rank-sum test).

leucine allele. This suggests that six and seven leucine alleles can be regarded as gain-of-function mutations associated with a higher enzyme activity. Despite the immediate stabilization of serum carnosine after blood withdrawal, the measured carnosine levels were very low. In the control subjects, the carnosine concentrations ranged from 0.08 to 0.81 $\mu\text{mol/l}$ (mean 0.33) and are close to our detection threshold.

Confirmation of the protective role of carnosine by cell culture experiments. Carnosine inhibited the effects of high glucose on cultured human podocytes (Fig.

2) and on cultured human mesangial cells (Fig. 3). The addition of carnosine blocked the glucose-induced increase in production of extracellular matrix components fibronectin and collagen type VI by podocytes and of TGF- β 2 in mesangial cells. TGF- β 1 production was not influenced by the addition of glucose or carnosine (data not shown).

CNDP1 expression in kidneys. Real-time measurement of *CNDP1* expression was performed on isolated glomeruli from four healthy kidneys and three diabetic nephropathy kidneys. The relative expression of *CNDP1* mRNA in

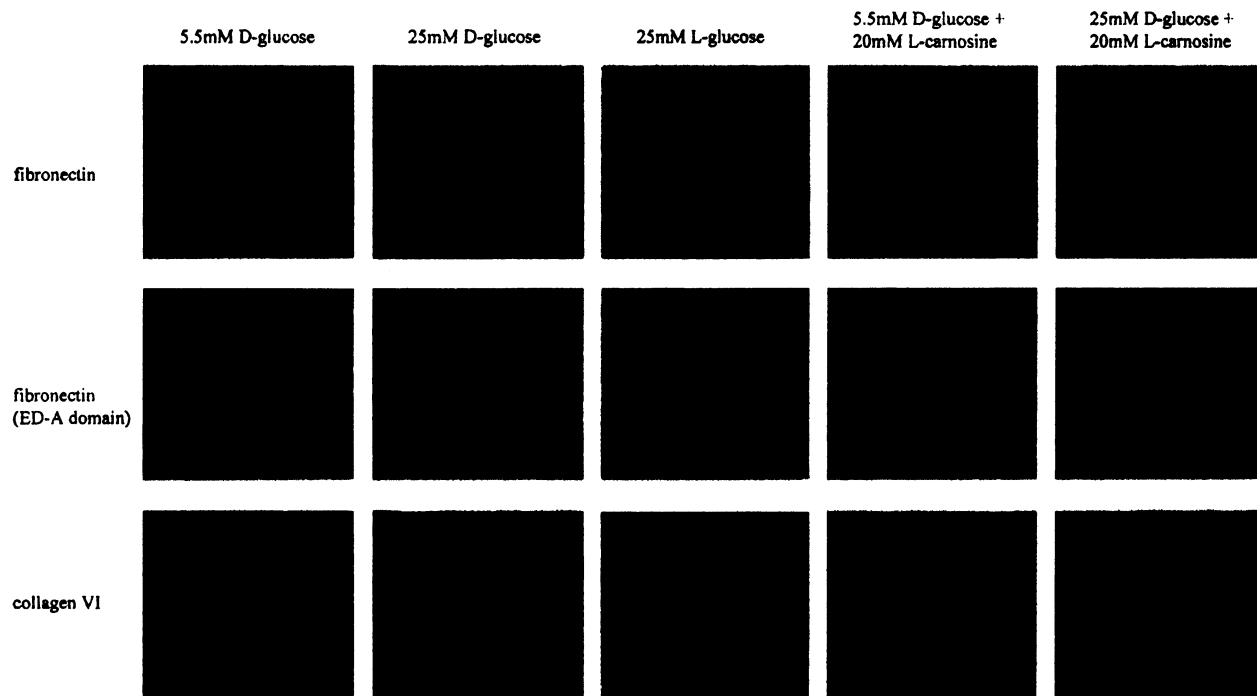


FIG. 2. Human thermosensitive SV 40-transformed podocytes were cultured at nonpermissive conditions (37°C) under various concentrations of D-glucose in the presence or absence of 20 mmol/l L-carnosine. A total of 25 mmol/l L-glucose was used as osmotic control. After 14 days, indirect immunofluorescence stainings were performed. The figure shows an increased production of fibronectin and collagen type VI in podocytes cultured with 25 mmol/l glucose compared with normal glucose (5.5 mmol/l). The panels on the right show that carnosine inhibits this increase.

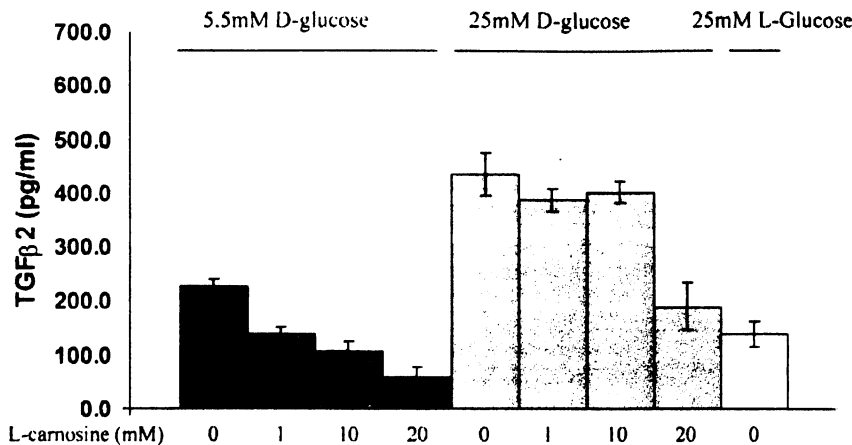


FIG. 3. Human SV 40-transformed mesangial cells were cultured under various concentrations of D-glucose in the presence or absence of 20 mmol/l L-carnosine. A total of 25 mmol/l L-glucose was used as osmotic control. After 10 days, the production of TGF- β 2 in the culture supernatant was assessed by an enzyme-linked immunosorbent assay technique. Three experiments per group were performed. In 5 mmol glucose, the decrease of the TGF- β 2 signal is significant with all three carnosine concentrations; in 25 mmol D-glucose, only 1 and 20 mmol/l L-carnosine lead to a significant decrease (Kruskal-wallis test modified according to Conover-Inman). All pairwise comparisons (P values) are available from www.klinikum.uni-heidelberg.de/index.php?id=6791.

glomeruli from patients with diabetic nephropathy was 1.0 (range 0.4–1.4) and 2.9 (2.7–3.4) in control glomeruli.

Human kidney specimens from seven patients with diabetic nephropathy and five from control individuals (three pretransplantation biopsies, two from patients after undergoing tumor-related nephrectomy) were stained for *CNDP1* using two different antibodies (C17E and Y18K). Both antibodies revealed a similar staining pattern for *CNDP1*. Representative stainings using C17E are depicted in Fig. 4. The stainings show *CNDP1* expression especially in podocytes and confirm an elevated expression in all kidneys from patients with diabetic nephropathy.

DISCUSSION

This study shows that a repeat expansion in the leader peptide of the *CNDP1* gene is associated with susceptibility for diabetic nephropathy in patients with type 1 and type 2 diabetes. Diabetic patients with two copies of *CNDP1* Mannheim, the gene variant with the lowest number of leucine repeats in the leader peptide, are less susceptible to diabetic nephropathy. Our data confirm that susceptibility for diabetic nephropathy is a dominant trait.

We stringently excluded from this study patients with an unclear cause of renal damage, such as patients with microalbuminuria, patients in which protein excretion was only determined while they were on ACE inhibitors or ATR1 blockers, and patients without renal symptoms with a duration of diabetes <15 years. The patients in this study

were white and from central and western Europe or the Middle East. These findings therefore confirm and extend earlier data obtained in Turkey, in North-American Pima Indians, and in African-American patients (7,8).

As we hypothesized, individuals with a higher number of *CNDP1* leucine repeats in the leader peptide had higher serum carnosinase activity levels. Our *in vitro* data strengthen the plausibility of our hypothesis: the substrate of the enzyme encoded by the *CNDP1* gene, carnosine, protects renal cells against the deleterious effects of high glucose levels. The carnosine concentrations used in the cell culture experiments are in the same order of magnitude as has been reported in tissues of long-lived species (12). The read-out parameters of the *in vitro* studies are relevant: the ED-A domains of fibronectin and collagen type VI are known to accumulate in human diabetic nephropathy (21–23), and several *in vitro* and *in vivo* studies (24–27) have implicated mesangial TGF- β as a key mediator in diabetic renal disease. In our experiments, glucose only induced TGF- β 2, not - β 1, in cultured human mesangial cells. Hill et al. (28) have suggested that TGF- β 2 is closely linked to fibrogenesis in diabetic nephropathy.

Carnosine, β -alanyl-L-histidine, was first described in 1900 and functions as a natural ACE inhibitor (29,30), a natural radical oxygen species (ROS) scavenger (31), an AGE breaker (32), and, thus, as a natural antiaging substance (29). The mitochondrial overproduction of ROS has been demonstrated to be fundamental in the vascular

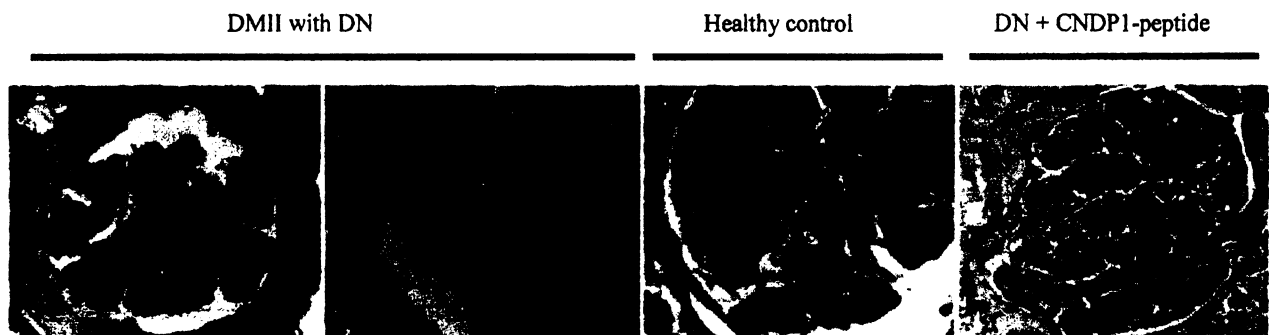


FIG. 4. Human kidney specimen from patients with diabetic nephropathy (DMII with DN) and control individuals (Healthy control) were stained for *CNDP1*. In the kidneys with diabetic nephropathy, there is increased staining in the glomerulus and in parietal epithelial cells. Representative stainings using the C17E antibody are depicted. A C17E antibody preincubated with the respective peptide was used as control (last panel).

pathobiology of diabetic microvascular complications (33). In light of the properties of carnosine, it is important to note that several studies have also shown that pharmacologic inhibition of the renin-angiotensin system effectively delays disease progression in patients with diabetic nephropathy (34,35), that binding of AGEs to the receptor for AGEs is thought to mediate the release of ROS in mesangial cells in kidneys with diabetic nephropathy (36), and that nondiabetic parents of children with diabetic nephropathy have been reported to die earlier than parents of diabetic children without nephropathy (37). Because AGEs induce the production of angiotensin II in mesangial cells and carnosine may function as an ACE inhibitor (30,36), our results are compatible with the notion that the renin-angiotensin system plays a crucial role in the pathogenesis of diabetic nephropathy (rev. in 38). It remains to be determined where in the cascade of AGE-receptor for AGE binding, ROS generation, activation of TGF- β -Smad signaling, fibronectin synthesis, and autocrine production of angiotensin II carnosine has its major effects. The effects of carnosine on life expectancy have been shown both in vitro (39) and in vivo (40), and the findings reported here may help to explain the shortened life expectancy in both patients with diabetic nephropathy and their relatives (37,41). Since carnosines have been reported to be generated in vivo by exercise (42), our findings provide a possible molecular basis for the previously reported beneficial effects of exercise on the incidence of diabetic nephropathy in patients with diabetes (43,44).

Although the association between *CNDP1* Mannheim and protection against diabetic nephropathy was robust in the four populations tested, patients with *CNDP1* Mannheim occasionally developed diabetic nephropathy. This indicates that additional modifying genes contribute to the genetic predisposition to develop diabetic nephropathy. A recent report by Bowden et al. (8) confirms the existence of four more loci, some of which may be restricted to African-American patients with diabetes. We assume that the occurrence of diabetic nephropathy is the net result of two counteracting mechanisms: protein glycation (mainly determined by glucose levels) and protective factors such as tissue carnosine content. It has been shown that in patients with type 1 and type 2 diabetes, there is a good correlation between diabetes regulation (as documented by HbA_{1c} [A1C] levels) and the chances of developing diabetic nephropathy (45,46). It is therefore conceivable that patients with *CNDP1* Mannheim may still develop diabetic nephropathy when blood glucose levels are very poorly controlled. Further clinical studies will be required to address this issue in more detail.

CNDP1 Mannheim was associated with lower serum carnosinase activity. However, we were unable to detect any meaningful serum carnosine levels; this is most likely explained by the high activity of serum carnosinase. It is unknown how polymorphisms in the *CNDP1* gene relate to carnosine levels in kidney tissues. Our study did not confirm the absence of *CNDP1* expression in kidneys, as suggested by Teufel et al. (13) but clearly showed *CNDP1* mRNA and protein expression in the glomerulus. The increased presence of the gene product in glomeruli with

diabetic nephropathy is compatible with a crucial role of this molecule in the pathogenesis of diabetic nephropathy.

We conclude that the number of leucine repeats in the leader peptide of the *CNDP1* gene is associated with susceptibility for diabetic nephropathy. The findings implicate carnosine as an important protective factor in diabetes. The value of the leucine repeat as a tool to determine the risk of a patient with type 1 or type 2 diabetes for developing diabetic nephropathy has to be investigated in a prospective setting. Our study suggests that carnosine or carnosine derivatives may possibly be used to design new therapeutic strategies to optimize renoprotection in diabetes.

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Contributors: Recruitment and screening of records of patients was performed by H.K., M.R., M.Z., and D.H., with assistance from M.C., I.R., S.J.L.B., and P.N. Participating centers and contact persons were Fifth Medical University Clinic, University Clinic at Mannheim, Germany; Diabetes Polyclinics, Dialysis Unit, and Kidney Transplantation Unit, University Clinic at Groningen, The Netherlands, (R.O.B. Gans); Diabetologist Practice, Speyer, Germany (T. Segiet); Diabetes Polyclinic, University Clinic at Heidelberg, Germany (A. Hamann); Medical Center, Leeuwarden, The Netherlands (J. Broekroelofs); Second Medical Department, Third Medical Faculty, Charles University Prague, Czech Republic (J. Psottova); University Clinic at Tübingen, Germany (T. Risler and B. Friedrich); Communal Clinic, Ludwigshafen, Germany, kidney biopsy records (R. Bergner); and Hamad Medical Corporation, Doha, Qatar. B.J., D.H., and F.V.D.W. were the lead investigators in this project and were principally responsible for the data analysis, interpretation, and preparation of this report. Together with the lead investigators, C.R.B. and H.-P.H. were involved in study design and logistical matters. E.D.H. and H.B. determined gene expression levels in kidneys. N.R. and J.M. performed the genetic analyses. V.P., N.R., and J.Z. were responsible for determination of carnosine levels and enzyme activities. The in vitro studies were performed by P.B., S.S., and B.A.Y., E.R., P.M., and M.A.S. provided the cell lines. C.F. provided statistical expertise.

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5. DISCUSSION

This work presented results from several studies investigating involvement of various genetic markers in the predisposition to diabetic nephropathy.

The first biomarker that has been tested for predisposition to T2D and its complications is transcription factor PPAR γ . There is a large body of studies with contradictory results about the association of Pro/Ala polymorphism in PPAR γ gene with type 2 diabetes, or its complications. Some has confirmed (498-500), while other has refuted the role of PPARs in pathogenesis of diabetes (501-504). However, favorable effect of thiazolidinediones, PPAR γ agonist, on insulin sensitivity suggests its involvement in T2D and leads to persistent interest of further investigation. Our study has not found the association of Pro/Ala polymorphism with neither T2D, nor its complications, although the small number of patients included in our study may Moreover, animal and in vitro studies have suggested the role of PPARs in the progression of diabetic nephropathy by suppression of mesangial cells expansion and reduction of extracellular matrix proteins expression (505,506). Furthermore, it has been shown that TZD have direct and beneficial effect on human glomeruli, by preventing glomerular hyperfiltration and albuminuria, independent on ability to increase glucose tolerance (507), which make them potential drugs in treatment of diabetic nephropathy. Therefore, it remains of our interest to continue in the investigation of PPAR γ involvement in diabetic renal disease.

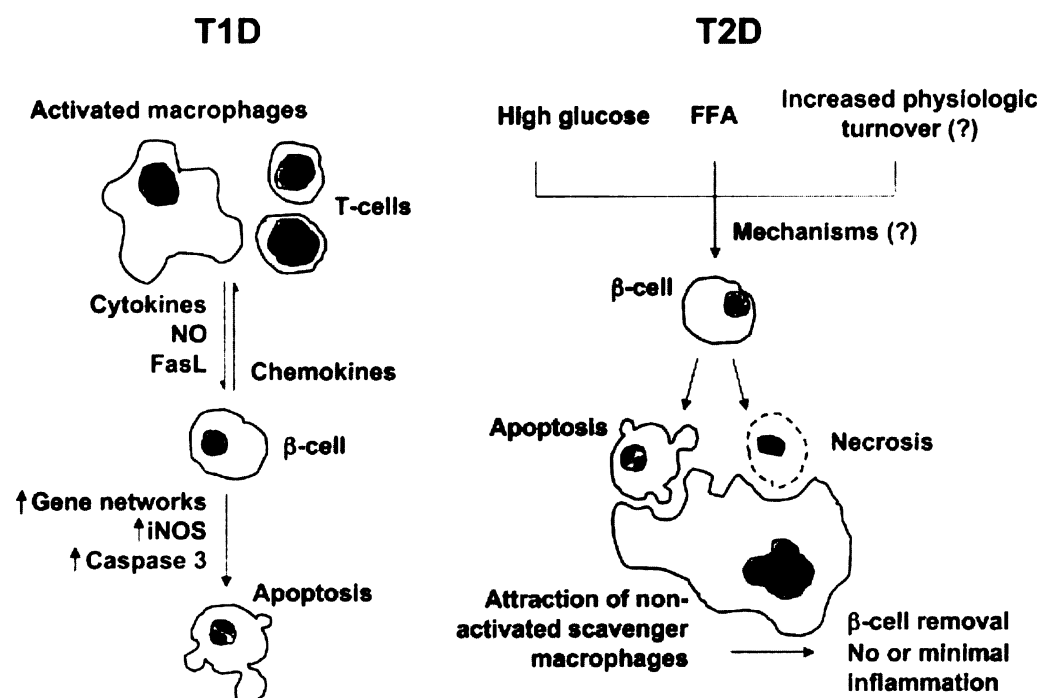
The results of two studies investigating as another candidate gene for DN transcription factor NF κ B, confirmed association of CA repeat polymorphism in NFKB1 gene with autoimmune diabetes already reported in UK study (409). Whereas in Czech patients was reported as the risk for type 1 diabetes allele A7 (132bp) in UK study it was allele A10 (138bp) (409). In Danish T1D patients, however, no association was detected (508,509). In patients with type 2 diabetes from Czech and German populations no association with NFKB1 polymorphism was found. We found divergences in NFKB1 allele distribution between control groups of our study (Czech) and other studies (UK, Danish) This was observed also in group of diabetic patients in Danish study, but not in group of diabetic patients form our study (Czech, German). The discrepancy of finding the risk alleles for type 1 diabetes in these studies and differences of allele distribution between control groups of different populations suggest that there was probably drift of NFKB1 alleles in various populations. This fact is also supported by genotyping of NFKB1 in following diseases: celiac disease, rheumatoid arthritis, systemic lupus erythematosus and breast

cancer in other studies (431,508-510). The differences in the allele frequencies can be, however, also explained by methodological differences between specific studies.

On the other hand the NFKBIA genotyping resulted in the finding of association between homozygous AA genotype and type 2 diabetes. 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 NF κ B is an important factor implicated 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 loss of pancreatic β -cells in T1D and T2D is apoptosis. It has been described in the recent literature that main pathway resulting in β -cells death leads via activation of NF κ B, but it is believed that it is just a "common final pathway" for both types of diabetes (511,512).

Figure 8: Overview of the putative sequence of events leading to β -cell death in animal models



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The stimuli, triggering apoptosis in pancreatic β -cells, as proven in several studies, differs in T1D from those in T2D. There is evidence that in T1D the production of cytokines (namely IL-

1β and $\text{TNF-}\alpha$) by immunocompetent cells infiltrating the pancreatic islets induces activation of $\text{NF}\kappa\text{B}$ pathway in pancreatic β -cells leading their apoptotic destruction, whereas in T2D the loss of β -cells is slower and in contrast to T1D occurs mostly by necrosis, which attracts the scavenging macrophages as a consequence. Therefore it is hypothesized that β -cells dysfunction in T2D, caused by overexposure to glucose and free fatty acids, is $\text{NF}\kappa\text{B}$ independent (*Figure 8*). Further functional studies are necessary to investigate the role of nuclear factor in the T2D pathogenesis.

The results regarding NFKBIA genotyping had also revealed the association of the $\text{Rel/NF}\kappa\text{B}$ family gene with other autoimmune diseases such as RA and SLE and disapproved our previously published findings on specific relation of $\text{NF}\kappa\text{B}$ to T2D. 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 $\text{NF}\kappa\text{B}$ in synovial tissue (513). In the joints activated $\text{NF}\kappa\text{B}$ triggers the expression of pro-inflammatory mediators and adhesion molecules, thus participate on the RA pathogenesis. The involvement of $\text{NF}\kappa\text{B}$ in the pathogenesis of RA and other chronic inflammatory diseases was investigated also in animal models, where inhibition of $\text{NF}\kappa\text{B}$ has led to decreased production of inflammatory cytokines and suppression of disease process (514,515).

The role of $\text{NF}\kappa\text{B}$ 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 $\text{NF}\kappa\text{B}$ 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 $\text{NF}\kappa\text{B}$ pathway (516). The selection of appropriate inhibitors however is dependent on the etiopathogenesis of specific disease. The complexity of $\text{NF}\kappa\text{B}$ signaling offers broad opportunities to develop specific inhibitors acting via different mechanisms. The inhibitors aimed at $\text{I}\kappa\text{B}$ degradation processes ($\text{IKK}\beta$ inhibitors, dominant-negative mutation of RelA , RelA antisense, $\text{NF}\kappa\text{B}$ decoy ODNs, super-repressor $\text{I}\kappa\text{B}$, 26S proteasome inhibitors) or transcriptional activation of $\text{NF}\kappa\text{B}$ have already been developed (517). A question of drug delivery, immunogenicity and the way of inflammatory cytokine inhibition still remains to resolve.

NFKBIA genotyping showed a significant increase in the frequency of the AA genotype in the T2D groups ($\text{RR}=1.38$, $\text{OR}=2.81$, $P<0.001$), LADA group ($\text{RR}=2.23$, $\text{OR}=2.68$, $P<0.001$), non-significantly in adult T1D patients, but also increase in the A allele frequency in RA and SLE groups ($\text{RR}=1.4$, $\text{OR}=2.08$, $P=0.01$). The presence of association in these diseases raises a

question of common factor implicated in the ethiopathogenesis of all aforementioned diseases. Additionally, after dividing T2D 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 T2D 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.

It is known from literature that in complex autoimmune diseases such as RA, SLE, or diabetes is increased prevalence of atherosclerosis and CVDs (518-523). The inflammatory nature of atherosclerosis has been characterized by infiltration of intima media by monocytes, macrophages and T lymphocytes, and by production of pro-inflammatory cytokines (524-527). Also animal models have confirmed that immunological reactions are involved in all stages of atherosclerosis (528-530). Important role in the development of atherosclerosis play interleukins, which are products of infiltrating immune cells, but also VSMC and epithelial cells of arterial walls. Among others, IL1- β and TNF- α are considered to be crucial in the development of inflammatory action. The mechanism of triggering the expression of adhesive molecules and chemokines is thought to be done via activation of NF κ B (531-533). The role of NF κ B in the pathogenesis of atherosclerosis supports also known anti-atherogenic effect of aspirin. Aspirin has been proven to inhibit T lymphocyte adhesion and expression of adhesive molecules by suppressing I κ B phosphorylation (534) leading to maintaining the nuclear factor inactive in the cytoplasm (535).

The last study investigating new DN predisposition markers brought interesting results. The association of CNDP1 trinucleotide (CTG=Leu) polymorphism with diabetic nephropathy was found. The CNDP1 gene encodes serum carnosinase, an enzyme cleaving naturally occurring dipeptide carnosine. Carnosine has been described to have a large number of biological effects, among which the most discussed are anti-senescing, anti-oxidant and free radical scavenging features (536). The study on CNDP1 has showed protective effect of carnosine on cultured renal cells (podocytes and mesangial cells) against high glucose levels. However, carnosine is characterized by its rapid degradation in blood, and inability to accumulate in tissues when exerted exogenous, thus its potential therapeutic use as a protective agent against toxic effect of hyperglycemia in the prevention or treatment of diabetic nephropathy is limited. Along with carnosine, other peptides, similar to those of carnosine, were found in the mammalian cardiac and skeletal muscles (537). These carnosine derivatives: N-acetylcarnosine, anserine, ophidine and

carcinine are products of carnosine transforming enzymes and dispose similar favorable detoxifying effect as Carnosine. Importantly, they are significantly less sensitive or fully insensitive to hydrolysis by carnosinases, therefore could present a future strategy in the development of new therapeutic agents in nephropathy treatment. Despite the beneficial effect of carnosine mimetic agents on renal cells, it is not clear yet whether it is possible to maintain their concentration at sufficient level. There are other nonspecific enzymes widely distributed in many human tissues that degrade a large number of dipeptides including carnosine derivatives (538). Consequently, another potential therapeutic approach is to design inhibitors of serum carnosinase, which would improve renal protection in the patients with higher carnosine expression due to presence of increased leucine repeats in CNDP1 gene. Vistoli *et al* recently published new homology model of human serum carnosinase, presenting its catalytic sites as new promising pharmacological target that will allow to design its competitive inhibitors as a therapeutic agents (539).

6. SUMMARY

Diabetic nephropathy is major microvascular complication leading to end stage renal failure and CVD associated death in diabetic patients, thus accounts for increased mortality and morbidity in these patients. Clinical definition of DN is presence of proteinuria over 0.5 g per 24h. It occurs in 15 – 30 % of type 1 diabetic patients after 20 years of diabetes duration, whereas prevalence in type 2 diabetes is more variable, ranging from 5 to 40 %. The fact that only subset of diabetic patients eventually develop DN despite long-term severe chronic hyperglycemia, together with the evidence of familial clustering of DN and various ethnic/racial prevalence of DN indicate hereditary predisposition to DN, independent from predisposition to diabetes mellitus.

The conception of combination of several “bad” genes and environmental factors, such as glycemic control, blood pressure control or hypertension, was established as model of DN inheritance. To reveal genetic markers, implicated in renal diabetic complications, two main strategies have been used in DN research – linkage analysis and population based association studies.

Present work shows new results of the investigation on DN predisposition markers. Several polymorphisms in the genes encoding transcription factor $\text{NF}\kappa\text{B}$ and its inhibitor $\text{I}\kappa\text{B}$, transcription factor $\text{PPAR}\gamma$ and serum dipeptidase Carnosinase were tested.

We found association of the number of leucine repeat in CNDP1 gene, encoding serum Carnosinase with DN and showed important protective function of Carnosine in the pathogenesis of DN. Although the association of tested polymorphism with DN revealed strong statistical significance in 4 tested populations and its protective effect against hyperglycemia has been proven on cultured renal cells, the study showed that some fraction of diabetic patients with protective CNDP1 allele have developed DN anyway. It implies that other modifying genes are important for developing DN. Nevertheless, Carnosine derivatives and Carnosinase inhibitors represent new potential therapeutic aims in the treatment of DN.

Testing polymorphisms of the genes for $\text{NF}\kappa\text{B}$, $\text{I}\kappa\text{B}$ and $\text{PPAR}\gamma$ showed no positive correlation with DN. However, we observed in several autoimmune diseases (T1D, SLE, and RA) and in type 2 diabetic patients statistically significant increase of the A allele frequency of $\text{I}\kappa\text{B}$ gene. Suggested connection between mentioned diseases, where the association with $\text{NF}\kappa\text{B}$ was found, is the atherosclerotic process that accompanies all of these diseases.

Similarly to $\text{NF}\kappa\text{B}$, the Pro/Ala polymorphism of $\text{PPAR}\gamma$ gene was also discovered to participate in etiopathogenesis of type 2 diabetes as a protective factor, however no correlation

with lipid levels or BMI was observed. This polymorphism was however observed to be protective for DN in several studies, thus PPAR γ could be one of the minor genes contributing to development of DN in type 2 diabetic patients.

This work brought new insights into genetics of DN and T2D. Carnosine has been described as new protective factor for DN and the transcription factors NF κ B and PPAR γ have also been proved to play role in the T2D pathogenesis, however not its complications. According to recent prognosis a massive increase in prevalence of type 2 diabetes and rising life expectancy will lead in next 10 years to doubling of prevalence of DN, thus the importance and urgency of revealing causal pathogenic genes for DN is evident not only for scientific and medical, but also for social and economic aspects.

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8. LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ACEi	Angiotensin converting enzyme inhibitors
AGE	Advanced glycation end products
AKT2	v-Akt murine thymoma viral oncogene homolog 2
ALR	Aldose reductase
ANP	Atrial natriuretic peptide
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
ARB	Angiotensin type 2 receptor blockers
AT1R	Angiotensin type 1 receptor
AT2R	Angiotensin type 2 receptor
ATPase	Adenosine triphosphatase
CAPN10	Calpain (Calcium-activated neutral proteinase)10
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CVD	Cardiovascular disease
DRD1-5	Dopamine receptors 1-5
EDTA	Ethylenediamine tetraacetic acid
ESRD	End stage renal disease
GAD	Glutamic acid decarboxylase autoantibodies
GBM	Glomerular basement membrane
GF	Glomerular filtration
GFR	Glomerular filtration rate
GLUT1	Glucose transporter 1
HDL	High density lipoproteins
HNF-4 α	Hepatic nuclear factor 4 α
HNF-1 α	Hepatic nuclear factor 1 α
HSPG	Heparan sulfate proteoglycan
IAA	Insulin autoantibodies
IA-2	Tyrosine phosphatase autoantibodies
IGF-1	Insulin-like growth factor 1
I κ B	Inhibitor of nuclear factor kappa B

IKK α	Inhibitor of kappaB kinase α
INS	Insulin
IPF1	Insulin promoter factor 1
IR	Insulin resistance
LADA	Latent autoimmune diabetes in adults
LDL	Low density lipoproteins
LPS	Lipopolysaccharides
LYP	Lymphoid tyrosine phosphatase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MTHFR	Methylenetetrahydrofolate reductase
NEMO	NF κ B essential modulator
NEUROD1	Neurogenic differentiation factor 1
NF κ B	Nuclear factor kappa B
NIK	NF κ B inducing kinase
NO	Nitric oxide
NOS	Nitric oxide synthase
PCR	Polymerase chain reaction
PON1	Paraoxonase 1
PON2	Paraoxonase 2
PPAR γ	Peroxisome proliferator-activated receptor γ
PPRE	PPAR Response Elements
Prepro-NY	Preproneuropeptide Y
PTPN22	Tyrosine-protein phosphatase non-receptor type 22
RFLP	Restriction fragment length polymorphism
RT-PCR	Real time PCR
RA	Rheumatoid arthritis
RAAS	Renin-angiotensin-aldosterone system
ROS	Reactive oxygen species
RXR	Retinoid X Receptor
SLE	Systemic lupus erythematosus
SUMO4	Small ubiquitin-like modifier 4
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TAG	Triacylglycerols

TBM	Tubular basement membrane
TCR	T-cell receptors
TDT	Transmission disequilibrium test
TNF- α	Tumor necrosis factor α
TRAF	TNF receptor associated factor
TGF	Transforming growth factor
TZD	Thiazolidinediones
UAE	Urine albumin excretion
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoproteins
VSMC	Vascular smooth muscle cell
WHO	World health organization

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10. LIST OF PUBLICATIONS

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