UNIVERSITY OF ZAGREB
SCHOOL OF MEDICINE

Matej Završnik

Polymorphisms of inflammatory genes as potential predictors of diabetic nephropathy in patients with type 2 diabetes

DISSERTATION

Zagreb, 2019.

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This dissertation was made at the Department of Endocrinology and Diabetes, University Clinical Center Maribor; Department of Internal Medicine, Murska Sobota; General Hospital Slovenj Gradec, Department of Internal Medicine, Slovenj Gradec and Institute of Histology and Embryology, Faculty of Medicine Ljubljana, University of Ljubljana, Slovenia.

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Abbreviations and Symbols

3'UTR	3 prime untranslated region	Hb	Hemoglobin
5'UTR	5 prime untranslated region	HDL	High-density lipoproteins
AGE	Advance glycemic end product	HIV-1	Human immunodeficiency virus type 1
AIDS	Acquired immune deficiency syndrome	HWE	Hardy-Weinberg equilibrium
AMP	Adenosine monophosphate	IBD	Inflammatory bowel disease
AMPK	Adenosine monophosphate-activated protein kinase	ICAM-1	Intercellular adhesion molecules-1
APC	Antigen presenting cell	IFN-γ	Interferon gamma
ATP	Adenosine triphosphate	Ig	Immunoglobulin
BMI	Body mass index	IL	Interleukin
CAM	Cell adhesion molecules	IL-1RA	IL-1 receptor antagonist
CHD	Coronary heart disease	JAK2	Janus kinase 2
CKD	Chronic kidney disease	JAM-A	Junction adhesion molecule A
CREB	cAMP response element–binding protein	LBRC	Lateral border recycling compartment
CVD	Cardiovascular disease	LD	linkage disequilibrium
DAMP	Danger associates molecular pattern	LDL	Low-density lipoprotein
DBP	Diastolic blood pressure	LET	Leukocyte endothelial transmigration
DF	Diabetic foot	LFA-1	Leukocyte function–associated antigen-1
DKD	Diabetic kidney disease		
DM	Diabetes mellitus	Mac-1	Macrophage-1 antigen
DN	Diabetic nephropathy	MAPK	Mitogen activated protein kinase ~~activation~~
DNeuro	Diabetic neuropathy		
DR	Diabetic retinopathy	MCP-1	Monocyte chemo-attractant protein-1
ECM	Extracellular matrix		
eGFR	Estimated glomerular filtration rate	MDRD	Modification of Diet in Renal Disease
ELISA	Enzyme-linked immunosorbent assay	MHC-II	Class II major histocompatibility complex
ERRγ	Estrogen-related receptor-γ	MI	Myocardial infarction
ESRD	End stage renal disease	MIP-1α	Macrophage inflammatory protein-1α
GF	Glomerular filtration		
GoKinD	Genetics of Kidneys in Diabetes		
GPCR	G protein coupled receptors		
GWAS	Genome-wide association study		

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NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells or nuclear factor kappa-B		T2DM	Diabetes mellitus type 2
			TG	Triglyceride
			TGF-β	Transforming growth factor beta
NK	Natural killer cell			
NLRP3	nucleotide-binding oligomerization do- main-, leucine-rich repeat and pyrin domain-containing 3 (NLRP3) inflammasome		Th1	Type 1 helper T cells
			Th2	Type 2 helper T cells
			TNF	Tumor necrosis factor
			TNF-α	Tumor necrosis factor alpha
			TYK2	Tyrosine-protein kinase
OR	Odd ratio		UACR	Urine albumin to creatinine ratio
PAMP	Pathogen associated molecular pattern			
			VCAM-1	Vascular cell adhesion molecule-1
PBMC	Peripheral blood monocyte cells			
			VLA-4	Very late antigen-4
PECAM-1	Platelet endothelial cell adhesion molecule-1			
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator-1α			
PKC	Protein kinase C			
PPAR	Peroxisome proliferator-activated receptor			
PPARGC-1α	Peroxisome proliferator-activated receptor-γ coactivator-1α			
RAAS	Renin-angiotensin-aldosterone system			
RANTES	Regulated upon activation normal T cell expressed and secreted			
ROS	Reactive oxygen species			
RXR	Retinoid X receptors			
SBP	Systolic blood pressure			
SLE	Systemic lupus erythematosus			
SNP	Single nucleotide polymorphism			
SOCS	Suppressor of cytokine signalling			
STAT	Signal transducer and activator of transcription			
T1DM	Diabetes mellitus type 1			

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1   INTRODUCTION

1.1   DIABETIC NEPHROPATHY AND INFLAMMATION

1.1.1   DIABETES MELLITUS

Diabetes mellitus (DM) is a group of common metabolic disorders, including hyperglycemia – a distinct feature. The most common types are type 2 diabetes (T2DM), with more than 90% of all cases, and the type 1 diabetes (T1DM). The metabolic dysregulation associated with DM causes multiple pathophysiologic changes in multiple organ systems that manifest themselves as a chronic complication of diabetes. Essentially, all chronic complications of DM are a pathology of small vessels called micro-vascular complications. The most common are diabetic nephropathy (DN), diabetic retinopathy (DR), diabetic neuropathy (DNeuro) and diabetic foot (DF) syndrome. DN is the leading cause of end-stage renal disease (ESRD) in the developed world, and increasingly more common in the developing world (1). A proposed new terminology is diabetic kidney disease (DKD) which comprises a larger pathology group because it encloses also kidney diseases of other etiologies that could occur in up to 20% of T2DM patients (2). Diabetes is the leading reason for ESRD, and about 40% of patients on renal replacement therapy have diabetes in the USA (3). Despite stabilizing the incidence of patients who develop ESRD due to DM, the number of patients in need of renal replacement therapy is increasing worldwide because of the rapid increase of T2DM prevalence (4, 5). The relentless increases in the incidence of T2DM are due to an increase in overweight and obesity, and the development of the metabolic syndrome as a consequence of sedentary life style and the abundance of high-calorie foods (6)

Diabetic nephropathy is usually a slow progressing complication of DM of both types that result in end-stage kidney disease (ESRD) in 10 to 20 years. According to current knowledge, ESRD develops only in one third of diabetic patients. Because of the markedly elevated cardiovascular risk there is survival competition between myocardial infarction and cerebrovascular insult, the main causes of death in T2D patients, and the progression of DN to ESRD (7, 8, 9). Hyperglycemia, hypertension, and hyperlipidemia are important factors in the pathophysiology of DN. In T1DM, hypertension develops as a consequence of DN, and usually appears at the time of microalbuminuria. About 30 to 40% of patients with T2DM have hypertension already at the time of diagnosis of diabetes. Renal vascular disease contributes to hypertension in about 20% of patients with T2DM and in up to 40% of those with overt DN (10). Increased blood pressure is in positive correlation with increased albuminuria, and represents an increased risk for progression of DN. All anti-hypertensive drugs decrease albuminuria, slow down the appearance of DN and improve survival. However, angiotensin converting enzyme inhibitors (ACEI) and angiotensin II receptor blockers (ARB) have a certain advantage, as in addition to the reduction of blood pressure they also work on the kidney to decrease intra-glomerular filtration pressure (11, 12, 13). The

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progression of kidney failure may be greater with dyslipidemia. Therapy with statins slows the progression of DN (14). Most of these risk factors are present in the majority of patients with T2DM, but the burden of DN is not shared equally between them. Not all patients with T2DM acquire DN, not even after many years of disease or with the presence of multiple risk factors and high level of hyperglycemia. On the contrary, some patients develope advance DN with relatively good regulated glycaemia, without or with few mild cardiovascular risk factors. There appears to be an inherent predisposition for DN. Certainly, this predisposition does not follow classical Mendelian rules because its inheritance is rather complicated. At present, even with recent genome-wide linkage studies, we do not know the combinations of genes that substantially contribute to this predisposition. In addition, we do not know which patients are significantly predisposed for the development of DN to concentrate therapeutic efforts (15, 16). Genetic predisposition is an important background, which can trigger the development of DN. Multiple number of genes, inter-genetic interactions and different levels of their regulations affect diabetes. This is not a significant change in the function of the same gene product, or distorted and non-functional proteins. On the contrary, there are minuscule changes in metabolism pathways due to small changes or variants in genetic information. However, a great number of such changes results in disturbed metabolism, fuel consumption and energy production with consequences in DM and its chronic complications.

DNA polymorphism is defined as a DNA sequence variation in the population with the frequency of a rare allele equal to one percent or higher. A single nucleotide polymorphism (SNP) is the substitution of one nucleotide, which can take place in the regulatory or coding sequence of genes. A synonymous substitution occurs when the change of a codon with SNP does not change the amino acid on the same place in the protein chain, and the amino acid sequence is not modified. Anyway, we are interested in SNPs with functional consequences (non-synonymous substitution). SNPs in the regulatory region (promoter, untranslated region (UTR)) can increase or decrease the transcription activity of the gene or influence in other ways the level of its product (17). In the coding region of the gene, SNPs can make different qualitative changes to the protein. There could be conformational changes with the exposure or hiding of some protein sequences or domains, which could change the interaction with other molecules. This can affect signal and metabolic pathways. All of this can affect disease susceptibility, progression or severity (18).

1.1.2   ALBUMINURIA AND CLINICAL DEFINITION OF DIABETIC NEPHROPATHY

Approximately one third of T1DM and T2DM patients develop DN, which is clinically silent in the beginning. In the clinical practice it is not known who will develop DN, which makes diagnosis and early treatment difficult. Persistent abnormal urine albumin excretion or albuminuria is the first marker of DN. Traditionally it is divided into microalbuminuria (urine albumin to creatinine ratio (UACR) 30-300 mg/g or 3.0-30 mg/mmol) and macroalbuminuria (UACR > 300mg/g, > 30 mg/mmol). When macroalbuminuria fully develops, the filtration barrier in glomeruli, which

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consist of an endothelial cell layer, glomerular basal membrane (GBM) and podocytes, is damaged to such an extent as to filtrate other proteins larger than albumins. Proteinuria is defined as an amount of more than 500 mg proteins in the urine over a period of 24 hours (19, 20).

An early DN is identified by persistent microalbuminuria, while overt DN is characterized by persistent macroalbuminuria or proteinuria, and usually with a declining glomerular filtration rate (GFR). The prevalence of microalbuminuria in T2DM is between 25% and 35%, and the prevalence of macroalbuminuria is between 3.4% and 20.5%, depending on the population based cross-sectional studies (21). In the United Kingdom Prospective Diabetes Study (UKPDS), after a nearly 15-year follow-up of patients with T2DM after the diagnosis of T2DM, 38% developed albuminuria, 28% had renal insufficiency with estimated GFR less than 60 mL/min/1.73 m^2, and 14% had albuminuria and renal insufficiency (22). The classic albuminuric pathway explains the progression of DN from normoalbuminuria to microalbuminuria, and further to macroalbuminuria and proteinuria. The classical course of nephropathy was determined on the basis of research in T1DM, where the beginning of the disease is well known. In these patients, DN can be detected as early as 5 years after diagnosis, but usually later, i.e. approximately between 10 and 15 years after the onset of T1DM. It is interesting that about 20 to 40% of microalbuminuric patients with T1DM will progress to proteinuria in 10 years; on the other hand, earlier studies showed a much higher percentage of progression. In the early stages of albuminuria, the regression of the disease, defined as a decrease in albuminuria by 50%, is possible in T1DM (23) and it was shown also in T2DM (24). Most probably, the natural course of DN has changed in the last several decades due to a better treatment of DM and arterial hypertension. About 20 to 25% of patients will return to normoalbuminuric levels, while the rest will remain microalbuminuric (25). The natural course of DN is divided in 5 stages. In the first phase, the glomerular hyperfiltration is at the forefront. Histological pictures show vasodilatation and glomerular hypertrophy. In the second phase, an increased thickness of the glomerular basal membrane and a slow increase of mesangium volume can be seen. Usually, persistent albuminuria is not seen in this phase. The third phase is marked with persistent microalbuminuria that often develops in the second decade of T1DM. An increase in albuminuria is connected to the progressive loss of podocytes and the development of glomerulosclerosis. A typical feature of the fourth phase is that the glomerular filtration rate (GFR) progressively declines and proteinuria develops. Usually, arterial hypertension, which results from the development of DN, is found in patients with T1DM. The decline in GFR can be fast, even in the range 10 to 15 ml/min/year without antihypertensive treatment. As a comparison, an average loss of GFR in healthy people over 40 years is about 0.75 to 1 ml/min/year. The ESRD represents the fifth phase of DN (12, 26, 27). These phases are quite typical in patients with T1DM. Although we are trying to transfer the pattern to T2DM, the entire process and distribution in stages is not so clear in patients with T2DM. Clinical measurements and histologic findings are not always equivocal. For example, one third of T2DM patients have an albumin excretion rate (AER), which

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is greater than observed in histological changes (28). Patients with T2DM could have kidney disease already at the diagnosis of DM because of hypertension, obesity, and ischemic changes. Histological changes described in T1DM patients are manly glomerular changes. In patients with T2DM the histological picture is much more variable, and changes in the tubulointerstitial compartment are also prominent. Similarly, a variable histological picture can be seen in patients with T1DM with non-classical / non-proteinuric phenotype of DN (27). In recent years it has been found that albuminuria is not necessarily a marker of DN. Different studies quote from 14 to 57% of patients with T2DM with declining renal function but without albuminuria. The exact reasons for the non-albuminuric phenotype of DN are not known. One of the possible influences is better therapy and a more optimal management of glycaemia, blood pressure and lipid values in patients with T2DM (26,27).

1.1.3   HISTOLOGICAL PRESENTATION OF DIABETIC NEPHROPATHY

Typical glomerular lesions, the hallmark of glomerulopathy in DN, consist of mesangial expansion, thickened glomerular basal membrane (GBM) and hyalinosis of afferent and efferent arterioles. Hyper-cellularity is present in the early stages of DN, but during the progression of the disease the accumulation of extracellular matrix (ECM) prevails, which may form nodular accumulations known as Kimmelstiel-Wilson nodules. Except for hyalinosis, these changes are not absolutely pathognomonic for DN (28, 29).

In the tubule-interstitial compartment, which occupies 90% of the kidney volume (tubular system, vasculature, and interstitial cells), tubular hypertrophy, i.e. the thickening of the tubular basement membrane, and interstitial infiltration with inflammatory cells represent early histological changes in DN. Progression of DN leads to tubule-interstitial fibrosis and tubule atrophy (30, 31).

1.1.4   PATHOPHYSIOLOGICAL CHARACTERISTICS OF DIABETIC NEPHROPATHY

The development and progression of DN depend on metabolic and hemodynamic changes. A basic fact is that there is no DN without hyperglycemia, which is a major driving force. Hyperglycemia causes abnormality in intracellular metabolism with oxidative stress and activations of downstream signalling with the production of pro-inflammatory cytokines, growth factors and profibrotic mediators (32). Molecular pathways, significantly modulated with hyperglycemia, contributing to the pathogenesis of DN are polyol, hexosamine and protein kinase C (PKC) pathways. Hyperglycemic conditions enable also a much greater formation of advance glycation end products (AGEs). AGEs are ligands for the receptor of AEGs (RAGE), which activates the signalling pathway that is strongly connected to inflammation and oxidative stress (33, 34, 35). This results in chronic inflammation, mitochondrial dysfunction, oxidative stress, the accumulation of extracellular matrix proteins, and tissue remodelling. Chronic activation of the key switches of proinflammatory responses, like PKC-β, nuclear factor kappa-light-chain-enhancer of activated B

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cells (or nuclear factor kappa-B, NF-κB), mitogen-activated protein kinases (MAPK), G protein-coupled receptors (GPCRs), and Toll-like receptors (TLRs) are induced (36, 37, 38, 39). Oxidative stress from an increased generation of reactive oxygen species (ROS), impaired mitochondrial function, and diminished antioxidant capacity further exacerbate the proinflammatory state, especially when NF-κB is activated by the mutual influence of ROS and PKC (40, 41). The main hemodynamic derangement is renal vasodilatation that appears first in cascade events of DN. Because of disturbed vascular auto-regulation and greater vasodilatation of afferent arterioles, central arterial pressure is transferred on the glomerular level. Consequently, intra-glomerular capillary pressure that leads to glomerular hyperfiltration and albuminuria is increased. Hyperfiltration also causes increased colloidal osmotic pressure that leads to an increased absorption of sodium in post-glomerular capillaries. The increased absorption of sodium is an element of arterial hypertension. Higher average blood pressure is transferred to glomeruli and the circle is complete (42). According to some researchers, hyperfiltration is one of the initiating factors for the development of DN. In the pathogenesis of DN, the activation of the renin-angiotensin-aldosterone system (RAAS) is very important. Angiotensin II causes vasoconstriction of efferent arterioles, which helps to further increase intra-glomerular pressure. It has many additionally influences, e.g. increases permeability of glomerular capillaries, stimulates the proliferation of glomerular and tubular cells, and the production of cytokines, ROS and oxygen stress (43). Angiotensin II is a stimulator of ECM production and induces the reabsorption of sodium in the proximal renal tubule (44, 45). Additionally, shear stress on endothelial and mesangial cells stimulates the up-regulation of angiotensin II, proinflammatory cytokines, and further derangement (46). In 1998, a hypothesis was developed that linked metabolic syndrome, insulin resistance and T2DM with innate immunity (47). Multiple studies have shown that chronic subclinical kidney inflammation is crucial in the development and progression of DN (48, 49, 50). The chronic subclinical low-grade silent inflammation is a background of DN. The inflammation is the initiator and cause of permanent kidney function decline. Diabetes mellitus, the atherosclerotic process and other chronic degenerative diseases, are basically inflammations. Today, mechanisms that connect obesity, metabolic syndrome with the development of glucose intolerance and diabetes mellitus are becoming better understood. Chronic microvascular complications, typical consequences of diabetes, are the consequences of chronic subclinical inflammation in diabetic milieu (51).

1.1.5   INFLAMMATION IN DIABETIC NEPHROPATHY

Acute inflammation is a response of the host organism to a foreign challenge like an invasion of pathogenic bacteria or viruses. Similarly, the acute inflammation is a reaction to noxious stress or tissue injury that could be "aseptic". The aim of the acute inflammation is to restore homeostasis and normal organ functions, with a subsequent repair of the damaged tissue structure. An inherent

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characteristic of acute inflammation is self-augmentation. To control excessive inflammation and the additional damage to the tissue, pro-resolving processes are initiated practically with the onset of acute inflammation. With the removal of pathogen or damaging noxious stimulus, pro-inflammatory mediators decline and pro-resolving factors increase. In the final stage of acute inflammation, cell debris and other component of necrotic tissue, including some inflammatory cells, are removed.

This makes space for cells that will repair tissue damage. Fibroblasts that originate from different sources proliferate and produce ECM. Furthermore, specific tissue cells are replenished from regional progenitor cells aiming to establish the original function of an organ (52, 53). When the harmful stimuli for inflammation are not eliminated and are chronically present, chronic inflammation develops. Chronic inflammation is not a persistent acute inflammation but has its distinct features. On the one hand, infiltrated cells generate local proinflammatory stimuli to drive chronic inflammation, while on the other hand parallel pro-resolving processes are initiated. Cytokines of both kinds, pro- and anti-inflammatory, are permanently secreted. Some kind of steady state is established where both pro-inflammatory factors and processes are intertwined with pro-resolving ones. This is typical also for the small-grade, subclinical or "sterile" inflammation found in diabetes (54, 55). Chronic inflammation usually leads to organ fibrosis and loss of function. In DN, chronic inflammation is in correlation with declining glomerular filtration. Continuous stimulation of pro-resolving processes in the long term, with derangement control lead to the accumulation of extracellular matrix (ECM) with insufficient degradation (56). Glomerular sclerosis and tubule-interstitial fibrosis develop. Experimental data showed that especially tubule-interstitial fibrosis is tightly connected to decreased renal function and the progress to ESRD (57).

With accumulated knowledge we begin to understand the initiation of inflammation in DN and inflammatory mechanisms, but not completely. Despite some decades of intensive research, it is still impossible to completely understand the pathophysiologic processes in DN. We need to consider that our knowledge is generally restricted to specific molecules and single pathways. At the same time, we know that multiple and parallel processes take place. For example, a signal could be transmitted through a usual signal (canonical or classical) pathway or an alternative one. The majority of processes in inflammation and metabolic pathways are intertwined. This creates very complex networks. In reality, despite immense accumulated data from research, there is a superficial understanding of intertwining multiple functions in the "micro cosmos" of a cell and its surroundings. Logically it is unavoidable to make relatively simplistic models of processes in live cells and tissues.

Typical for the inflammation in diabetic nephropathy, in contrast to other kidney inflammations, is the simultaneous inflammation in the glomerular and tubule-interstitial compartment (58). Anders

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and co-workers proposed 4 phases of inflammation in the kidney: initiation, amplification, progression and terminal stage (54).

1.1.5.1 Initiation phase

Noxious stimuli damage kidney cells. Damaged cells secrete inflammatory cytokines, and some of them become necrotic releasing strong stimuli for leukocyte invasion. An injury of any type of kidney cells could trigger acute inflammation. The inflammatory cytokines, like interleukin-1 (IL-1), IL-6, IL-8, IL-12, IL-18, etc., activate residential inflammatory cells (dendritic cells, tissue macrophage), parenchymal kidney cells (endothelial vascular and tubular epithelial cells, podocytes and mesangial cells) to start inflammation. Inflammation activates a range of inflammatory genes, and different kind of inflammatory cytokines are secreted. Chemotactic cytokines or chemokines, attract and guide leukocytes to the site of injuries.

The function of adhesion molecules in inflammation is to promote leukocyte transmigration across vascular walls and progress through tissue (54). How exactly do inflammations develop? As we now understand immune response, the onset of inflammation is the answer to an innate immune response to "danger molecules" or danger associated molecular pattern (DAMP) molecules. DAMP molecules are a group of heterogenic molecules that are released in the extracellular space with cell necrosis, such as histones, DNA, RNA, high mobility group box 1(HMGB1) from nucleus, adenosine triphosphate (ATP) and mtDNA from mitochondria and uric acid, RNA, heat shock protein (HSP) and S100 proteins from cytosol. Additionally, we know DAMP molecules that originate from the degradation of ECM (60). In normal physiological conditions those molecules never came into extracellular space. If they are present there, it is a sign of alarm and danger because they activate inflammation as a protective mechanism. Programmed cell death or apoptosis enables that these molecules are not released into extracellular space, because cell membranes stay intact with such cell removal (61).

Other forms of cell necrosis, like necroptosis, ferroptosis, pyroptosis and mitochondrial permeability transition with cyclophilin D, all release different DAMP molecules into extracellular space. DAMP molecules are ligands for pattern recognition receptors, such as toll-like receptors (TLRs), NOD (nucleotide-binding oligomerization domain)-like receptors and C-type lectin receptors (62). The TLR are pattern recognition receptors on cells membranes (TLR 1, 2, 4, 5, 6) and intracellular on the endosome membrane (TLR 3, 7, 8, 9). The activation of these receptors triggers signal events that produce active gene transcription factors and an increased expression of inflammatory cytokines (63). Inflammasomes, also pattern recognition receptors of innate immune system, are different cytoplasmic protein complexes with the common feature that they all finally activate the cysteine protease enzyme, caspase-1. The best-known and most common cytosol inflammasome NLRP3 (NLRP3: nucleotide-binding domain, leucine-rich-containing family, pyrin

domain-containing-3) recognizes many diverse stimuli (64). Dendritic cells are part of an innate immune system and are usually scattered in the tissue. They are professional antigen presenting cells and present antigens to lymphocytes and connect innate and adaptable immune systems. There are two types: myeloid-derived and plasmacytoid dendritic cells, both found in significant numbers in normal human kidneys. Plasmacytoid dendritic cells are somewhat less common, but secrete high levels of tumour necrosis factor-α (TNF-α) and are more pathogenic (58). Both types of dendritic cells are present in the tubule-interstitial compartment of mice kidney. Binding DAMP molecules on their innate immunity receptors trigger different signal pathways. The caspase-1, activated with inflammasomes, usually NLRP3, cleaves pro-IL-1β and pro-IL-18 in their active forms. The IL-1β and IL-18 are both strong pro-inflammatory mediators (65). The other signal pathways could be activated, for example, with the activation of TLR-4 or TLR-2, both abundant in kidney, phosphorylate and activate series of different kinases with the final result being the liberation of the components of NF-κB in cytosol. NF-κB, when in the nucleus, binds as a transcription factor to appropriate binding sequences in gene promoters. In that way, genes are upregulated for IL-6, IL-8, IL-12, CCL2, CCL5, Interferon-α, interferon-β, and also for IL-1β and IL-18 (60). Activated dendritic cells in contact with T cells from adaptive immunity can broaden inflammation to adaptive immune response. Similarly as dendritic cells, residential kidney macrophages can be activated with DAMP molecules. Residential macrophage can also function as antigen presenting cells, but their number in normal kidney is small. Additionally, an increased secretion of cytokines from activated cells influences leukocytes influx in damaged tissue and the augmentation of inflammation. Increased numbers of macrophages in glomeruli and tubule-interstitial compartments have been found in kidneys of T2DM patients (66). Macrophages are divided in two main groups, depending on the phenotype. Classically activated macrophages or M1 are typical proinflammatory macrophages. One of the most important stimuli for differentiation to M1 type is TNF-α. Because macrophages are not terminally differentiated cells, they proliferate at the inflammation site. Other different stimuli can also change phenotypes. In DN, the M1 type is predominant and it is connected with the injury of renal tissue. Moreover, macrophage M1 produces IL-1β, IL-18, IL-33, ROS with inducible nitric oxide synthase (iNOS), and inhibits T lymphocytes. The other type, also called anti-inflammatory macrophages or M2, can inhibit the activity of the majority of pro-inflammatory cells. M2 macrophages secrete IL-10, which is a strong opponent to TNF-α, and inhibit iNOS. M2 macrophages can improve cell survival and help repair damaged tissues. This phenotype of macrophages has not been well studied (58).

Adaptive immunity, especially lymphocytes, has its place in chronic renal inflammation. The group of T helper lymphocytes that can be divided into four basic subgroups, Th1, Th2, Th17 and Treg, is very important. All helper T cells have a membrane glycoprotein cluster of differentiation CD4 (CD4+). Again, T helper cells of the type 1 phenotype (Th1) have a pro-inflammatory function. They secrete a big quantity of interferon-γ (IFN-γ) and stimulate other pro-inflammatory cells. T

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helper cells with the second phenotype (Th2) have an opposite function compared to Th1: they secrete IL-4, IL-13, stimulate humoral immunity and are a strong inhibitor of differentiation to Th1 phenotype (53). T helper cells have an important role in autoimmune diseases. Regulatory phenotypes of T helper cells (Treg) are important in maintaining homeostasis and immune tolerance with the production of anti-inflammatory mediators, like IL-10, and transforming growth factor-β (TGF-β) (53, 67). From the physiological viewpoint it is important to keep a balance between different subtypes of T helper cells. In inflammation, the equilibrium between Th1 and Th2, as well as between Th17 and Treg, is compromised. In the inflammation in DN, Th1 and partly Th17 cells prevail. An increased production of IL-6, IL-8, IL-12, IL-17, TNF-α and INF-γ sustains chronic inflammation and macrophage infiltration (68). Imbalance was found between Th17 and Treg with lesser amounts of Treg. It is possible that lower inhibition of inflammation in DN manifests with increased M1 macrophages. Increased serum concentrations of IL-2, IL-6, IL-17, TNF-α, INF-γ and IL-10, but not IL-4 in T2DM patients support this assumption (67). Important structural cells in the glomerular space are vascular endothelial cells, podocytes and mesangial cells. All three types of cells are also important in glomerular inflammation. Podocytes are highly differentiated and specialized pericytes that build GBM and form the outer layer of the glomerular filtration barrier with interchanging digital projections (foot process) that are connected with "slit diaphragms" to enable the largest possible filtration area with selective permeability. In hyperglycemic conditions, this special structure of highly terminally differentiated podocytes is changed. Podocytopathy is the selective loss of podocytes through apoptosis or detachment from GBM. Foot process effacement changes the characteristics of the glomerular filtration barrier together with an increase in the thickness of GBM. Foot process widening and the denudation of GBM are in high correlation with albuminuria (69, 70). Further detachment leads to adhesions with glomerular parietal cells (internal lining of Bauman capsule) and the formation of segmental sclerosis, proximal tubular atrophy, and the formation of atubular glomeruli (71, 72). Similarly and probably simultaneously, diabetic milieu triggers inflammation in the tubulointerstitium compartment with damage to tubular epithelial cells. Moreover, hyperfiltration in the early phases of DN means additional injury to tubule epithelial cells with increased workload. Increased amounts of glucose, albumins and other filtrated substances in primary urine need to be resorbed having dramatically increased metabolic demand and the susceptibility of tubular cells. An increased reabsorption of glucose and sodium increased blood pressure together with the activated renin -angiotensin-aldosterone system (RAAS) (73). In addition to hemodynamic consequences of activated RAAS its activation brings the derangement of tubule-glomerular feedback (59). These mechanisms injure tubular cells that in response secrete inflammatory cytokines.

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1.1.5.2   Amplification phase

In the amplification phase, inflammatory cells that infiltrate injured tissues or are locally proliferated secrete increasing amounts of inflammatory cytokines that augment inflammation in self-reinforcement cycles. This is very typical for acute inflammation. However, in chronic inflammation these inflammatory phases are hard to distinguish because they pass smoothly from one to another. Anyway, when inflammatory cells go through activation and proliferation, mechanisms that inhibit inflammation and corresponding cytokines are simultaneously produced. These anti-inflammatory, pro-resolving mechanisms that prevent excessive inflammation and additional tissue damage are manifested with a small time lag in comparison to pro-inflammatory mechanisms.

1.1.5.3   Progression phase

The progression phase is probably the longest. Because of plenty ongoing harmful influences in diabetes and the repetitive release of DAMP molecules, the resolution of inflammation is not possible. Ongoing bouts of inflammation intertwined with its calming and repair mechanisms with fibrogenesis are a hallmark of chronic inflammation. Mesangial cells support capillary tufts and aid in the regulation of blood flow to the glomerular capillaries through contractile responses, similarly to smooth muscle cells in arterioles. Mesangial cells are rare examples of phagocytic cells deriving from smooth muscles and not monocytes. They have many other characteristics, like antigen presentation, responding to and secreting a number of cytokines that make them important in the local inflammation in the kidney (52). Mesangial cells, stimulated by inflammatory mediators, dramatically increase the production of ECM, which accumulate in glomerulus. They produce components of ECM, like collagen type IV, laminin, fibronectin, proteoglycans, hyaluronan, and glycoproteins. Interestingly, some components of ECM, like small leucine-rich proteoglycans, have the ability of stimulating, but as recent researches showed, also inhibiting inflammation (74). Focal fibrous tissue also accumulates in the places of denudated GBM, where GMB contact Bowman capsule and this can create crescent formed fibrosis. Additionally to abundant fibrotic tissue, an increased thickness of GBM, hyalinosis of vessels and tubule-less glomeruli were found during histological examinations of kidney biopsies.

Increased pressure in glomeruli because of ECM expansion exerts pressure on the vessels. Because of thrombosis, some vessels in glomeruli occlude and remain compensatory dilate. The filtration surface in glomeruli decreases and consequently glomerular filtration is decreased, which is seen as an increase in creatinine (75). The level of ischemia in the tubule-interstitial compartment increases because of occluded vessels also due to thrombosis. Under increased workload with a simultaneous increase of susceptibility due to ischemia, tubular epithelial cells are damaged, and some go into necrosis. These reverberate in an increased accumulation of inflammatory cells in the tubulo-interstitium compartment. Macrophages and lymphocytes further increase inflammation and

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activate fibroblast (76). The origin of fibroblast is not exactly known; they are residential cells and proliferate at the site of inflammation. Part of them comes from the bone marrow through circulation (fibrocytes), or through epithelial-mesenchymal and endothelial-mesenchymal transition (50). The situation in the tubulo-interstitium compartment is similar to inflammation in glomerular space, ECM accumulation and progression to fibrosis. Proximal tubular epithelial cells (PTEC) assume a proinflammatory and profibrotic role during proteinuria. Stimulated PTEC produce and secrete different chemokines and cytokines that lead to macrophage infiltration and inflammation in the interstitial compartment. An increased filtration of growing factors and locally increased levels of angiotensin II may stimulate PTEC to change their phenotype and become myofibroblasts by epithelial-mesenchymal transition that contributes to interstitial fibrosis and tubular atrophy (77). Increased interstitial pressure enhances vessels rarefaction and further deteriorates ischemia. Hypoxia in tubule-interstitium causes tubular cell necrosis or apoptosis and leads to tubular atrophy. Tubular resorption worsens, which definitively influences and brings to a timely decline in the glomerular filtration rate. Accumulated experiences in observing DN demonstrated that even though glomerular lesion importantly determinates renal function during most of natural history, tubulointerstitial fibrosis strongly determinates the decline of glomerular filtration rate (78). Renal fibrosis is a kind of stress-induced wound healing characterized by an excessive formation and accumulation of ECM. This is the final part of inflammation, but not only in DN; it leads to ESRD in all kinds of protracted renal diseases. Interestingly, renal fibrosis is the best predictor of survival in renal disease of different etiologies and it is not completely understood how glomerular injury causes tubule-interstitial fibrosis. Anyway, in DN the inflammation that drives fibrosis is already present in both compartments. In chronic inflammation leukocytes produce growth factors that stimulate mesangial cells and interstitial fibroblast to upgrade the secretion of ECM components (55). In DN there is a preponderance of M1 and less of M2 phenotype macrophages. M2 macrophages are anti-inflammatory with an important role in fibrosis with the secretion of TGF-β and IL-10. The change from the M1 to M2 phenotype happens in DN. However, overstimulation of M2 macrophages in the long term is detrimental because of the excessive production of TGF-β and ECM (79). Macrophages additionally stimulate fibroblast with the platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1) and galactin-3. Fibrotic process is also stimulated by angiotensin II, endothelin-1 and integrin receptors. Integrin receptors connect ECM with residential cells (55, 56). Chronic inflammation is a perpetual drive for ECM formation with an abundant formation of profibrotic mediators, especially growth factors. Simultaneously, an inappropriate degradation of ECM, which is the function of matrix metalloproteinases (MMP), is a sign of its metabolism dysregulation (80, 81). TGF-β, a prototypical hypertrophic and fibro-genic cytokine, is a master regulator of ECM accumulation in DN. Macrophages are the main source of TGF-β production in DN. Additionally, overexpression of TGF-β has been demonstrated in mesangial cells and podocytes. TGF-β increased the production of

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main ECM components like type I and IV collagen, fibronectin, and laminin. Degradation of ECM decreased with the inhibition of MMP and the up-regulation of protease inhibitors such as plasminogen activator inhibitor-1 (PAI-1). Nearly all mediators and signalling pathways participating in DN could stimulate TGF-β: hyperglycemia, AGEs, oxidative stress, PKC, angiotensin II, mechanical stretch, and the activation of mitogen-activated protein kinase (MAPK) pathway (80, 82). TGF-β up-regulates the expression of Glucose transporter-1 (GLUT-1) in mesangial cells that accelerate the progression of metabolic abnormalities with an increase of intracellular glucose. TGF-β is a contributing element in the apoptosis of podocytes that promotes albuminuria. Tubular epithelial cells, which transdifferentiate into myofibroblasts and exacerbate fibrosis, are stimulated by TGF-β (83). TGF-β is also involved in NADPH oxidase mediated oxidative stress. Neutralizing anti-TGF-β antibodies, antisense TGF-β oligo-deoxy-nucleotides or knocking off the *SMAD3* gene (SMAD3 is the main signal transducers for the receptors of the TGF-β) prevent glomerular hypertrophy, the accumulation of ECM components, lessen glomerular and tubulointerstitial fibrosis and preserve GFR. Increased urine excretion of TGF-β was demonstrated in patients with diabetes and in animal models (84). Interestingly, women with T1DM have a lesser risk of developing DN because of the protective effects of 17β-estradiol that partly regulates TGF-β expression and signalling (85). The connective tissue growth factor (CTGF or CCN2) is an extracellular matrix-associated heparin-binding protein, a member of the CCN family (ECM-associated proteins are involved in intercellular signalling). TGF-β is a potent stimulator of CTGF, which plays a role in cell adhesion, migration, proliferation, angiogenesis, skeletal development, tissue wound repair, and is critically involved in fibrotic diseases. Many effects of TGF-β seem to be mediated by CTGF. It is overexpressed in glomerular epithelial, mesangial cells, podocytes and PTEC as a response to hyperglycemia, AGEs, mechanical strain, inflammatory cytokine and TGF-β in DN (86). In diabetic patients with microalbuminuria and overt proteinuria, increased *CTGF* mRNA levels correlate with the amount of UAER in animal biopsy samples. Also, the amount that is excreted in urine is greater than that expected from plasma concentrations (87). Interestingly, degradation products of ECM function as DAMP molecules with the activation of pattern recognition receptors. In this way, the circle is complete. Renal inflammation and fibrosis are tightly bound processes (56).

1.1.5.4 Terminal phase

Abundant fibrosis in glomeruli forms the Kimmelstiel-Wilson nodules in the terminal stage of DN. Histologically we found fibrosis and atrophic tubules in tubule-interstitial place. Despite the decrease in leukocytes infiltration, the process of fibrous formation continues. Myofibrocytes enable the connective tissue to contract what can finally lead to shrunken kidneys. At the end, unremitting inflammation and fibrosis destroy renal function.

1.2 ADHESION MOLECULES

Cell adhesion molecules (CAM) are cell surface glycoproteins involved in the adhesion of cells to cells and cells to the Extracellular Matrix. This feature is essential for the structure of organs and their functions.

They have many roles, e.g. in differentiation, proliferation, migration and cell death. Because they are involved in important processes, from cell migration during embryogenesis, leukocyte diapedesis and homing, wound healing, to cancer progression and metastasis, they are referred to as "the glue of life" (88). Adhesion molecules are transmembrane protein receptors that consist of 3 main parts: extracellular, intra-membrane and intra-cellular. The extracellular part has a binding site for ligands and for the formation of dimers. The intracellular tail binds to the cytoskeleton and has a role in the transmission of signals that could be classically "out-side in" after binding a ligand or "inside-out", like as signal for integrin activations. The binding of identical CAM from opposite cell is a homophilic, while binding other types of CAM is a heterophilic interaction (89). Adhesion molecules are divided in five main groups: integrins, selectins, catherins, mucins, and the immunoglobulin (Ig) superfamily of cell adhesion molecules (88). Three members of the selectins group are L-, E- and P-selectins that beside cell-cell contact participate in cell activation by intracellular signalling. On the surface of endothelial cells there are P- and E-selectins, mediating initial, low-affinity adhesions with complex sialylated carbohydrate complexes on leukocytes. On the contrary, L-selectins are expressed on leukocytes and ligate sialomucins on endothelial cells. Integrins are heterodimeric cell membrane proteins that form one of more than 30 dimeric combinations from 18 alpha and 8 beta subunits. They are the only group that mediates connection to ECM, because other groups of CAM mediate only cell-cell connections (90). Additionally, in the resting state they are in a low-affinity (bent) state and after cell stimulation ("inside-out" signal) a high-affinity (extended) state is achieved through conformational change (91). In inflammation three important integrins are expressed on leukocytes: leukocyte function–associated antigen-1(LFA-1 or αLβ2), very late antigen-4 (VLA-4 or α4β1 or CD49d/CD29), and macrophage-1 antigen (Mac-1 or αMβ2 or CR3). LFA-1 binds intercellular adhesion molecule-1 (ICAM-1), and VLA-4 binds vascular cell adhesion molecule-1 (VCAM-1, CD106) on endothelial cells. Mac-1 binds inactivated complement C3b, important in opsonized-mediated phagocytosis, and also ICAM-1 to endothelial cells (53).

The Ig superfamily of cell adhesion molecules, which includes ICAM-1 and VCAM-1, these molecules interact with integrins, selectins, and each other. These molecules are vital elements in inflammation, because without them no infiltration with inflammatory cells is possible. In patients with DN, the increased expression of adhesion molecules on endothelial cells is one of the first events in hyperglycemia-mediated endothelial dysfunction (92).

1.2.1 INTERCELLULAR ADHESION MOLECULE-1 (ICAM-1)

The intercellular adhesion molecule-1, also known as Cluster of Differentiation 54 (CD54), is a transmembrane glycoprotein, and one of the five ICAM molecules (ICAM-1 to ICAM-5). It is a member of the Ig supergene family and a ligand for 2 integrin molecules present on leukocytes, integrin LFA-1 (αLβ2 or CD11a/CD18) and Mac-1 (αMβ2 or CD11b/CD18) (93). ICAM-1 has an important role in the immune system, as one of the key molecules in the leukocyte endothelial transmigration (LET) and in the interaction between T cell and antigen-presenting cell, where it functions as a co-activating signal (53). ICAM-1 is composed of five extracellular Ig-like domains, a transmembrane domain, and a short cytoplasmic tail. The ICAM-1 domain 1 interacts with the first domain of the integrin LFA-1 expressed on all circulating leukocytes. Domain 3 of ICAM-1 binds with Mac-1, primarily expressed on myeloid cells (neutrophils, monocytes and macrophages) (93, 94 ,95, 96). Leukocyte recruitment is a process that consists of three steps: (1) selectins-dependent leukocytes rolling and tethering on the stimulated endothelium, (2) firm leukocytes adhesion and the arrest of leukocytes on the endothelium mediated by ICAM-1 and leukocyte interleukins, and (3) transmigration of leukocytes through intercellular junction (diapedesis), which requires PECAM-1 (97). The binding of ICAM-1 with LFA-1 and Mac-1 is important for the crawling of neutrophils on endo-luminal surface before endothelial transmigration (94). The ICAM-1 dimeric structure interacts 1.5 to 3 times more vividly with LFA-1 than the monomer (95, 98). It is expressed at a very low level on endothelial cells and several other cells (leukocytes, fibroblasts, epithelial cells, and keratinocytes). However, with the beginning of inflammation and endothelial cell activation a sharp increase in expression is detected in the time span of one hour to eight hours, while the increased expression persists for several days (99). The expression of ICAM-1 is increased after stimulation with inflammatory cytokines (IL-1, TNF-α) or with bacterial lipopolysaccharide, oxygen radicals and hypoxia (93, 99, 100). ICAM-1 expression is regulated primarily through gene transcription. Its up-regulation is stimulated by a variety of inflammatory mediators, including pro-inflammatory cytokines (IL-1, TNF-α, and IFN-γ), hormones, cellular stress (retinoic acid, elements of oxidative stresses) and virus infection. Activation with NF-κB is particularly important and many different stimuli converge on this molecule. The down-regulation of ICAM-1 expression is mediated by anti-inflammatory cytokines (TGF-β, IL-4, and IL-10) and glucocorticoids (101).

In the body, ICAM-1 is expressed on cell membranes (mICAM-1) and as soluble intercellular adhesion molecule-1 (sICAM-1) that represents a circulating form of ICAM-1, and is found in the plasma, extracellular fluid, liquor, and saliva. The sICAM-1 exists as a monomer or as a dimer (102). The binding activity of dimeric sICAM-1 to LFA-1 is several times more potent than the monomeric form (103). Interestingly, we do not know if sICAM-1 binds Mac-1 because there have not been any reports so far (104). Serum levels of sICAM-1 are affected by race, sex, age and other

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factors (105, 106, 107, 108, 109). Moreover, sICAM-1 serum levels are affected by different disorders and environmental factors, such as hypertension, levels of cholesterol, triglycerides, fibrinogen and homocysteine, and smoking (109). Inflammation is a common denominator of pathological states with increased levels of sICAM-1. In T2DM, levels of sICAM-1 are elevated (106, 110, 111, 112). The levels of sICAM-1 additionally increase with the development of diabetic microvascular complications (113). Concentrations of sICAM-1 increased in T2DM patients with the progression of albuminuria and DN (112, 113, 114, 115), but not in all the studies (116, 117, 118). Concentrations of sICAM-1 were increased in in the renal tissue of DN animal models (119, 120, 121, 122). In ICAM-1-knockout mice or with the inhibition of ICAM-1 after the induction of DM, less renal injuries were demonstrated (120, 123). An increased expression of ICAM-1 was demonstrated on glomerular endothelial cells in diabetic experimental animals. Agents with anti-inflammatory effects, such as erythromycin, methotrexate, and tiazolidinedione, improved experimental DN through the inhibition of ICAM-1 expression on glomerular cells and subsequent macrophage infiltration. All three drugs suppressed endothelial cell activation through the inhibition of NF-κB activation (124, 125, 126).

1.2.1.1 The rs5498 polymorphism of the *ICAM1* gene

The human gene *ICAM1* (intercellular adhesion molecule 1 gene) for ICAM-1 is located on the short arm of chromosome 19 (19p13.2–). It spans over 15,781 base pairs and consists of 7 exons and 6 introns. The non-synonymous rs5498 polymorphism, with the change in the structure of ICAM-1, is most probably functional (127, 128). The rs5498 (rs5498:A>G, p.Lys469Glu), located in exon 6, changes the amino acid lysine on codon 469 to glutamic acid (**A**AG to **G**AG). The minor allele G (469E) is associated with higher levels of sICAM-1 (127). In the genome-wide association study (GWAS) in the Women's Genome Health Study, an association between the rs5498 polymorphism and elevated sICAM-1 was found (129). Diabetic proliferative retinopathy and significantly elevated sICAM-1 levels in T2DM patients were associated with the GG genotype of the rs5498 polymorphism (rs5498:GG) in the Caucasian population (130). In a meta-analysis of 7 studies, Su and co-workers found a significant association between the rs5498 major A allele [rs5498:A] and diabetic microvascular complications. An ethnic stratified subgroup analysis confirmed this association for the Asian, but not for the European population. Although just two studies with DN were included, a significant association between the risk of DN and the A allele [rs5498:A] was found in the recessive statistical model (131). In a Swedish population, the rs5498 polymorphism was associated with the pathogenesis of T1DM. The frequencies of the A allele [rs5498:A] increased in the direction of diabetic nephropathy; however, no statistical significance was reached (132). A larger study with increased power and similar design on the Genetics of Kidneys in Diabetes (GoKinD) population showed a possible protection from the development of DN in T1DM women carrying the minor G allele [rs5498:G] (133).

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1.2.1.2   The rs1799969 polymorphism of the *ICAM1* gene

Polymorphism rs1799969 (rs1799969:G>A, p.Gly241Arg) is located in exon 4, where amino acid glycine (G) changes to arginine (R) in codon 241 (**G**GG to **A**GG). This polymorphism is potentially functional. An association was found between the minor allele A of rs1799969 [rs1799969:A] and lower concentrations of sICAM-1 in the Women's Genome Health Study (128, 134, 135, 136). In the GWAS study, an association between the rs1799969 polymorphism and the concentration of sICAM-1 was confirmed (137). Increased cell surface expression of mICAM-1 was found in the [rs5498:G;rs1799969:G] (469Glu/241Gly) genotype when Holder and co-workers analysed polymorphisms, rs5498 and rs1799969 (138). In the field of DN two studies found no association between rs1799969 and DN in the Swedish and the GoKinD T1DM populations (134, 135).

We investigated an association between DN in patients with T2DM and rs5498, as well as rs1799969 polymorphism of the *ICAM1* gene. Additionally, we looked for a possible association between serum levels of ICAM-1 and rs5498 or the rs1799969 polymorphism in our population with T2DM.

1.2.2   PLATELET ENDOTHELIAL CELL ADHESION MOLECULE-1 (PECAM-1)

Inflammatory processes play a central role in the development and progression of DN. Inflammation is characterized by leukocyte infiltration at every stage of renal involvement and by an increased expression of adhesion molecules, chemokines, and proinflammatory cytokines (139). Increased levels of cell adhesion molecules in T2DM were shown to be strongly associated with both DN and cardiovascular disease (CVD) complications and mortality (140). Platelet endothelial cell adhesion molecule-1 (PECAM- 1) is a multifunctional vascular cell adhesion molecule and a signalling molecule of the Ig superfamily that is expressed on the surface of circulating leukocytes, some type T-cells and platelets. PECAM-1 is also highly expressed on endothelial cells and is practically the most abundant endothelial cell surface receptor with approximately 1-2 x 10^6 molecules per cell (141). The 130 kDa glycoprotein, spanning across 738 amino acids, consists of three parts depending on the plasma membrane. As it is typical of members of the Ig superfamily, the biggest extracellular part consists of six Ig-like homolog domains (IgD1 – IgD6). The smallest is the single transmembrane domain with 19 amino acids, while the cytoplasmic tail consist of 118 amino acids residues (52, 142). The extracellular part is encoded by exon 3 to 8, while the transmembrane part by exon 9. The cytoplasmic tail, which has a complex structure and functions, is encoded by exons from 10 to 16 (143). In PECAM-1 molecules, nine complex N-linked carbohydrate chains contribute 30% of its molecular mass. Three of them are located at distal extracellular part (IgD1 and IgD2), which is important for the interaction with other extracellular molecules (144). It is an important component of endothelial cell intercellular junctions (145). The molecules of PECAM-1 are on the apical, endo-luminal part

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of endothelial cell membranes, but the greatest concentration is found at the lateral borders. The extracellular domains of PECAM-1 mediate homo- and heterophilic interactions. The homophilic interactions can occur via the first and second N-terminal IgD (146). These two domains are in contact with the same two distal IgD from the adjacent cell in a face-to-face antiparallel pattern. Because PECAM-1 molecules are packed together in this cell membrane region, they have additional contacts with each other that make this structure and adhesion strong (147, 148). Interestingly, isolated PECAM-1 monomers have relatively little affinity for each other. Because circulating blood cells express monomers of PECAM-1 on its cell surfaces, PECAM-1 causes no aggregation between blood cells in circulation (148). Additionally, the PECAM-1 molecules have no interaction with integrin adhesion molecules because their structures lack integrin binding sequences (149). The main components of intercellular adhesive junction, a complex structure that firmly connects adjacent cells, are components of tight junctions, components of adherens junctions and interconnected PECAM-1 molecules. This structure is not static. It is constantly remodelled in response to different influences, like shear stress and leukocytes diapedesis (150). After firm adhesion and leukocyte crawling on the surface of endothelial cells looking for intercellular contact, leukocytes squeeze through adjacent endothelial cells. The re-modulation of intercellular adhesive junctions enables this passage. PECAM-1, CD 99 and junction adhesion molecule A (JAM-A) form together a lateral border recycling compartment (LBRC) which enable the circulation of these receptors between endothelial cell surface and cytoplasm to enable the transposition of these molecules on the endothelial cell lateral border ahead of leukocyte passage. This is important in enabling homologues PECAM-1/PECAM-1 adhesive interactions between leukocyte and endothelial cells in the process of transmigration (148). When PECAM-1 was blocked with antibodies or soluble recombinant PECAM, the transmigration of leukocytes was not efficient (151). Monoclonal antibodies, whose epitopes map to an extracellular Ig-like domain 1, selectively block monocyte migration through the endothelial junction (152). PECAM-1 on leukocytes promotes chemokine mediated directional migration of leukocytes to inflammatory sites. The interactions with PECAM-1 receptors triggers signalling events that lead to the activation of integrins on leukocytes, which are important for tight adhesion of leukocytes on activated endothelial cells (151). Despite lacking its own enzymatic activity, the cytoplasmic tail of PECAM-1 is involved in the transduction of various cellular signals. Its complex structure functions as scaffold for contacting different adaptor molecules that initiate several signalling pathways (146, 153). Beside the important function in the maintenance of cell adherent junctions, vascular permeability and enabling diapedesis, PECAM-1 takes part in the cytoskeleton rearrangement, sensing of shear stress, angiogenesis, cytoprotection and the control of transcriptional activities. As a signalling adhesion molecule, PECAM-1 serves various pro-inflammatory and anti-inflammatory functions, and thus plays a crucial role in the regulation of vascular inflammatory response (141, 153). Pro-inflammatory functions of PECAM-1 comprise the facilitation of leukocyte

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transendothelial migration and the transduction of mechanical signals in endothelial cells originating from fluid shear stress, whereas PECAM-1 anti-inflammatory effects include the dampening of leukocyte activation, suppression of pro-inflammatory cytokine production, and the maintenance and restoration of vascular barrier integrity (145).

1.2.2.1 The rs668 (rs281865545) polymorphism of the *PECAM1* gene

The rs688 tag was merged into rs281865545 (rs281865545:G>C, c.+373G>C, p.Leu125Val). PECAM-1 is encoded by a 75kb *PECAM1* (*platelet and endothelial cell adhesion molecule 1*) gene that is located near the end of the long arm of chromosome 17 (17q23) and comprises 16 exons (154). Previous studies have reported the existence of 16 different SNPs within the human *PECAM1* gene, but only 3 have been associated with disease and commonly studied. These SNPs are p.Leu125Val, p.Ser563Asn, and p.Gly670Arg (154, 155). We will focus on polymorphism rs668 of the *PECAM1* gene. The rs668 polymorphism (rs281865545:G>C) causes changes in codon 125 from **G**TG to **C**TG and a mutation of leucine to valine (p.Leu125Val). (Leu125Val - numbering based on protein sequence including the 27 amino acid signal peptide; c.+373G>C - numbering based on *PECAM1* transcription start site) (154). The rs668 polymorphism, encoded in exon 3, is located in extracellular IgD1, which is responsible for homologous PECAM-1/PECAM-1 adhesive interactions. Goodman and co-workers demonstrates that different PECAM-1 genotypes can alter the level of monocyte binding to endothelial cell, and that the heterozygous expression of a polymorphic protein may lead to an altered function (156). The rs668 polymorphism has been studied in coronary artery disease (CAD). In the German population this polymorphism may increase the risk of atherosclerosis, but not myocardial infarction (157). Allelic frequencies of rs668 were different in patients with CAD and controls in northern Italy (158). Frequencies of the rs668 polymorphism were not significantly different between patients with acute myocardial infarction and controls in a Sicilian population (159). However, the results of the association genetic studies published so far have not been unequivocal (160). Our study group was earlier able to confirm an association between the rs668 polymorphism and myocardial infarction in subjects with T2DM in a Slovenian population (161). Wei and co-workers demonstrated that the rs668 polymorphism in first extracellular Ig-like domains is associated with severe coronary artery stenosis in the Chinese population. These patients, homozygous for this polymorphism, had higher plasma levels of soluble PECAM-1 (162). The same positive association with CAD was confirmed in Asian Indians (163). The rs668 polymorphism has been shown to be also related to ischemic stroke (164), atherosclerotic cerebral infarction (165), bronchial asthma (166), deep vein thrombosis (167), and sepsis (168). Only one article was found in relation to diabetes and diabetic chronic complication. A study from Japan presented no difference in the distribution of genotypes and alleles in patients with DR and rs668 polymorphism (169). No study of diabetic nephropathy in any type of DM was found.

1.3   INTRODUCTION TO CHEMOKINES

Chemokines are an integral part of inflammation because they direct leukocytes to the place of inflammation. They are important as activators of integrins enabling a firm attachment of leukocytes to endothelial cells. A chemokine concentration gradient that established around the inflammation spot functions as guide for crawling leukocytes (170). Chemokines are small proteins, 8-10 kD, they share structural similarities and homology. Depending on the arrangements of cysteine residues in protein N-terminal they are divided in 4 families. The chemokines of our interest, CCL2 (monocyte chemo-attractant protein-1, (MCP-1)) and CCL5 (regulated upon activation normal T cell expressed and secreted, (RANTES)), belong to the CC (C-C motif) chemokine family or β-chemokine. The CC denotes that between two adjacent cysteine residues there are no other intercalating amino acid residues, as it is the case in other chemokine families. Some of the chemokines are constantly secreted (homeostatic), but in terms of inflammation only those that are rapidly induced and secreted (inflammatory) are important. The family of CC chemokines activates monocytes, eosinophils, basophils and lymphocytes, but they do not act on neutrophils (170,171). Inflammatory chemokines could be produced in practically any cell after stimulation with IFN-γ, IL-1, IL-4, bacterial and virus products. Endothelial cells produce and store chemokines in granules for rapid release upon stimulation (171). Further, endothelial cells transport chemokines from the underlying tissue to apical, endoluminal membranes with the help of chemokine receptors. Additionally, chemokines as basic proteins are attached to negatively charged extracellular glycosaminoglycans that build concentration gradients in the tissue and prevent their free diffusion. Chemokines embedded in the glycosaminoglycan layer on endoluminal endothelial surfaces enable a persistent concentration gradient despite blood flow (170). While attached on glycosaminoglycan, the oligomerisation of chemokines takes place, which function as secondary depot with a slow release of chemokines (172).

Chemokines receptors are part of a big family of G protein coupled receptors (GPCR). The basic structure consists of an extracellular N terminal, seven transmembrane domains and a relatively short cytoplasmic tail arranged along the internal surface of the plasma membrane. The cytoplasmic tail is important for activating several signalling pathways that act from cell activation to directed cell movement. Despite receptor redundancy in binding different chemokines, the receptors bind only chemokines from one chemokine subfamily and they bear their names after these, like CC receptors (CCR). For example, the CCL2 chemokine has only one receptor, a receptor for MCP-1 or CCR2, but CCR2 has also other high affinity ligands, like CCL7, CCL8 and CCL13, all from the same chemokine family of CC chemokines (173). These receptors also transport chemokines across the cell or internalize them for degradation and control of their extra-cellular concentration. The function of decoy receptors, where the binding of chemokines does not activate signals, is the clearance of chemokines (170). Mutations in chemokines or chemokines

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receptor genes may influence homeostasis maintenance. Well known is the mutation in the coding region of the receptor for CCL5 (CCR5); the rs333 polymorphism (NM_000579.3:c.554_585del32) renders this protein non-functional. Homozygotes of this CCR5 32-base pair deletion are resistant to human immunodeficiency virus type 1 (HIV-1) infection because this protein is used as a co-receptor by invading the virus (174, 175). On the other hand, the clinical course of encephalitis caused by the West Nile virus is more severe because homozygotes with CCR5 32-base pair deletion are less efficient in clearing this virus.

Supposing that small mutations, like SNP, can subtly affect the function of chemokines, we tried to find out if some potentially functional polymorphisms in the chosen chemokine genes have any association with DN in our studied population.

1.3.1 REGULATED UPON ACTIVATION, NORMAL T-CELL EXPRESSED AND SECRETED (RANTES) OR CHEMOKINE LIGAND 5 (CCL5)

Chemokine ligand CCL5, first known as RANTES, is a small protein of 68 amino acids. It belongs to the biggest group of chemokines, called C-C motif family or β chemokines (171). It is produced and secreted by many different cell types, like monocytes, macrophages, platelets, eosinophils, fibroblasts, endothelial and epithelial cells. Besides binding to its main receptor (CCR5), CCL5 as ligand binds also to other receptors, such as CCR1, CCR3, CCR4 and G protein coupled receptor 75 (GPR75) (176, 177). The CCL5 is highly susceptible to proteolysis by dipeptidyl peptidase 4 and cathepsin G, which deletes the first eight or more amino acid residues on the N terminal that lead to the elimination of chemotactic function (178). CCL5 has great tendency for oligomerisation, from dimers to long rod shaped polymers, and in such structures CCL5 is partly protected from rapid degradation. However, binding to the receptor requires a monomeric form of CCL5. Chemokines like CCL5, embedded in the endothelial cell surfaces coated by a glycocalyx, form something like a "road sign" for leukocytes to show where to exit vasculature and go in the direction of the inflammation (179, 180).

The CCL5 participates in the activation of T cells, respiratory burst in eosinophils, and degranulation of basophils that play a role in allergic diseases (181). Activated platelets release CCL5 from α-granules together with CXCL4. In leukocyte-endothelial transmigration, CCL5 enhances firm adhesion of leucocytes on endothelial cells (182). On the one hand, it is important for immune response against tumour, but on the other it promotes the progression of the malignant process and stimulates the spread of tumour with metastasis (183, 184, 185). Together with two other β-chemokines, macrophage inflammatory protein-1α (MIP-1α, CCL3), and MIP-1β (CCR4), CCL5 has antiviral capacities as it was demonstrated in the experiments with HIV replication. These three chemokines are ligands for the CCR5 receptor and compete for receptor occupancy with HIV-1. The HIV glycoprotein (gp120), from macrophage tropic (M- tropic) HIV-1 viruses, binds to CD4, its main receptor, and to co-receptor CCR5 on host cells. Binding CCR5 with its

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natural ligand prevents M-tropic viruses from entering the host cell (171, 186). After binding CCL5 to the receptor, the receptor complex is internalized, which allows the virus to enter the cell. Increased activities of CCL5 have been studied in different diseases, like HIV, infectious hepatitis, allergic diseases (bronchial asthma, atopic dermatitis), autoimmune diseases (rheumatoid arthritis, systemic lupus erythematosus (SLE)), atherosclerosis, and CAD (176, 177, 187).

In diabetic milieu, the increase in the secretion of chemokines is the result of factors like hyperglycemia, the formation of AGEs, oxidative stress, the activation of RAAS, tubular protein overload, other inflammatory cytokines and growth factors (188, 189). Levels of *CCR5* and *CCL5* mRNA increased within some hours after exposure to inflammatory stimuli, like TNF-α, IFN-γ, viruses or lipopolysaccharides (190). The expression of the CCL5 gene in activated mesangial and tubular cells is increased with the transcription factor NF-κB, which is activated through increased ROS and stimulated by PKC activity in diabetic conditions (191, 192). CCL5 expression is simply and quickly regulated in monocytes and fibroblasts (193). On the other hand, its expression in other cells is very complex, depending on many different signals and transcription factors (194, 195, 196). Among lymphocytes, only T cells secrete CCL5, usually with a 3 to 5 days delay after cell activation. This delay is necessary for the differentiation into cytotoxic and helper T cells, but also allows the expansion of inflammation before it is reduced (181).

In kidney tissues of experimental animals, the decreased expression of *CCL5* mRNA was found to correlate with the reducing number of infiltrating monocytes. Tubular epithelial cells induce the expression of CCL5 after contact with infiltrating leukocytes (197). A Met-RANTES, which differentiates from CCL5 in the first amino acid residue methionine, is a receptor antagonist that blocks CCL5 signalling and induces only weak internalization of CCR5 receptors (198). The application of cyclosporine with Met-RANTES in chronic allograft nephropathy decreases inflammatory cell infiltration, glomerulosclerosis, tubulointerstitial fibrosis, reduces albuminuria and pro-inflammatory cytokines mRNA in comparison to animals that were not treated with Met-RANTES (199).

Increased CCL5 secretion with a retroviral gene transfer technology additionally increases macrophages and the subpopulation of T lymphocyte cell infiltration (200). Unexpectedly, recent reports revealed that the blocked production of CCL5 in experimental animals led to a paradoxically increased infiltration of kidney tissue and increased tissue damage in RAAS dependent renal hypertension and fibrosis (201). This indicates that the regulation and function of CCL5 are more complex as previously thought.

In people with impaired glucose tolerance and patients with T2DM, serum levels of CCL5 are higher than in healthy people. This was found in the Cooperative Health Research in the Region of Augsburg Survey S4 in South Germany (202). Participants with impaired glucose tolerance in the Finnish Diabetes Prevention Study in an intensive treatment group with elevated serum CCL5

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levels had an increased risk for the development of T2DM (203). In overt DN an increased concentration of CCL5 was found in serum and urine in comparison to patients with T2DM without DN. In a study of Wu and co-workers, the concentration of CCL5, as well as CCL2 and others cytokines, in the urine was higher than in the plasma which indicates the importance of the production of inflammatory molecules in kidneys in DN (204).

1.3.1.1   Polymorphisms rs2280788 and rs2107538 of the *CCL5* gene

The *CCL5* (*RANTES* or C-C motif chemokine ligand 5 gene) is located on chromosome 17 (17q11.2-q12), spans approximately 7.1 kb and has a typical conservative structure of β-chemokine family genes (3 exons and 2 introns) with a conserved position of introns to exons (205, 206). The human CCL5 promoter contains at least six transcription factor binding elements, and it was used as a model for studying the complex hierarchy of the gene transcription (207). Both polymorphisms of our interest are mapped on the promoter of the *CCL5* gene, rs2280788 (g.-28C>G) and rs2107538 (g.-403C>T). Both polymorphisms are described as functional and both increase the expression of the *CCL5* gene. Increased important components of inflammatory process could stimulate inflammation, with a less favourable course of the disease. Both polymorphisms have been studied in different diseases, like HIV-1 infection and acquired immune deficiency syndrome (AIDS), allergic diseases, autoimmune diseases, and chronic inflammatory diseases, such as atherosclerosis and DM (177).

The peripheral blood monocyte and CD4+ lymphocytes isolated from different people have a very different capability to secrete CCL5. Genetic analysis revealed that the change from cytosine to guanine on position -28 (counting from the genome start site) in promoter *CCL5* (g.-28C>G) (rs2280788) increases the expression of the *CCL5* gene (187). Liu and co-workers found that polymorphism rs2280788 (g.-28C>G) did not influence infection with HIV, but slowed down the progression to AIDS (187). It was supposed that the spread of HIV-1 in the body was slowed and the progression of the disease was prolonged because of the competition for CCR5. It is more likely that the down-regulation of CCR5 on host cell membranes with increased CCL5 tissue concentrations is more important (208, 209). The rs2107538 (g.-403C>T) polymorphism is caused by the substitution of cytosine for thymine at position -403 in the promoter region (210).

The polymorphism rs2107538 (g.-403C>T) can lead to an increased transcription of the *CCL5* gene and could be associated with increased levels of serum CCL5 (211, 212). Liu and co-workers describe that haplotype [-28G/G;-403T/T] has the lowest decline in the number of CD4+ lymphocytes before anti-HIV therapy, and it delayed progression to AIDS. Additionally, the assessment of serum CCL5 concentration did not show any differences. However, significant differences in CD4+ lymphocytes supernatant were found, confirming an increased production of CCL5 in these cells (187).

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Decreased infiltration of macrophages and lymphocytes in atherosclerotic lesions, and stabilization of atherosclerotic plaque were found after the blocking of CCR5 in ApoE-knockout animals on a high atherogenic diet (213, 214). Several studies showed a positive association between the rs2107538 (g.-403C>T) and CAD (215, 216, 217). Other studies are negative for this association (218, 219, 220, 221, 222).

Jang and co-workers found no association between CAD and rs2280788 (g.-28 C>G) in the Korean population. They included patients with CAD, with and without diabetes, and healthy control subjects (212). Similarly, no correlation was found between rs2280788 (g.-28C>G) and Caucasian T2DM patients on renal replacement therapy and all-cause and cardiac mortality (215).

Results from three German studies, MONICA/KORA Augsburg Case-Cohort, AtheroExpress, and CARDIoGRAM Study, revealed no association between the rs2107538 (g.-403C>T) polymorphism and CAD (218). Three meta-analyses were done to clarify the link between CAD and rs2107538 (g.-403C>T) polymorphism. Liu and co-workers included 8 studies and found no association with CAD. A sub-analysis by ethnicity indicates a possible increased risk for CAD only in Caucasians and a protective role for the Asian population (210). Wang and co-workers did not find any association between the rs2107538 (g.-403C>T) polymorphism and CAD. Again, sub-analyses showed the possibility of an increased risk for the Caucasian population (223). The third meta-analysis comprising 45 studies and 12 gene variants also did not find any association between coronary heart disease and rs2107538 (g.-403C>T) polymorphism (224). Additionally, several GWAS showed no signal for increased susceptibility to CAD near the *CCL5* gene locus (225, 226, 227).

The Tahakata study in Japan analysed the influence of the same polymorphisms in the *CCL5* gene on albuminuria in a non-diabetic population. Out of the SNPs in the *CCL5* gene only those with a minor allele frequency that was greater than 2.5% in the Japanese general population were chosen, which also included rs2107538 (g.-403C>T). Albuminuria increased with C allele in rs2107538 [rs2107538:C] from heterozygote to homozygote carriers and the conclusion was that this genetic influence plays an aggravating role in the development of albuminuria in the general population (228). Strong, nearly complete linkage disequilibrium (LD) was found for all four chosen alleles (rs2107538, rs2280789, rs3817655, and rs9909416).

Among genetic studies that analysed two polymorphisms of our interest, rs2280788 (g.-28 C>G) and rs2107538 (g.-403C>T), there are just four in the field of DN. Nakajima and co-workers analysed a possible influence of CCL5 promoter polymorphisms rs2280788 (g.-28 C>G) and rs2107538 (g.-403C>T) on DN in a population with T2DM in Japan (229). They included 261 patients with persistent microalbuminuria or macroalbuminuria and compared them to 355 normoalbuminuric T2DM patients. Frequencies of minor allele G [rs2280788:G] and T [rs2107538:T] in a population with T2DM were 13% and 34%, respectively. Significantly higher

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frequencies were found for the G [rs2280788:G] allele and genotypes including the G allele (GG + CG) in subjects with albuminuria. There were no significant differences in the frequencies of T [rs2107538:T] allele and genotypes including this allele. Only rs2280788 (g.-28C>G) polymorphism was associated with DN in a Japanese population with T2DM. Interestingly, they also found only 6 out of 9 possible genotypic combinations. This indicates a strong LD between SNPs (229). Strong LD, particularly between the two polymorphisms of our interest, has been reported in other studies (87, 208, 228). To confirm these findings, a longitudinal study was carried out in Japanese T2DM patients. Mokubo and co-workers retrospectively followed a group of 191 patients with normoalbuminuria at baseline for 10 years. Over the time participants were separated in two groups, one with persistence of normoalbuminuria and the other with progression to micro- or macroalbuminuria. At the end of the study, 120 patients were without DN and 71 patients progressed to DN. In the group with albuminuria progression frequencies of minor allele G [rs2280788:G] and genotype with this allele in polymorphism rs2280788 (g.-28C>G) were not significantly different compared to the non-progression group (230). This longitudinal retrospective study did not confirm the findings from cross-sectional studies, and the authors concluded that polymorphism rs2280788 (g.-28C>G) was not associated with DN (230). An interesting study was made in Korea to find out if there is any association between polymorphisms rs2280788 (g.-28C>G) and rs2107538 (g.-403C>T), and DN in subjects with T2DM. Patients with T2DM and ESRD on renal replacement therapy were compared to a control group. The control group consisted of 184 patients with T2DM for more than 15 years, with DR, no renal disease, preserved estimated glomerular filtration rate (eGFR) more than 60 ml/min/1,73m^2 and normoalbuminuria. Patient in the ESRD group had DR, no sign of any other renal disease and no familiar diabetic ESRD. They found only 6 genotypes out of 9 possible that were similar to the Nakajima report from Japan. Additionally, only three haplotypes of the CCL5 promoter region were found in the Korean population. Genotype frequencies for each polymorphism and also haplotype showed no significant differences between T2DM subjects with ESRD and the control group with T2DM without renal disease (231). In Ireland, *CCL5* and *CCR5* genes were re-sequenced by Pettigrew and co-workers. In the *CCL5* gene, 13 newly reported polymorphisms were found, 11 of them with minor allele frequencies (less than 5% in the population); rs2280788 (g.-28C>G) was among them. The allele frequency of rs2107538 (g.-403C>T) was 17%. The study showed no association between rs2280788 (g.-28C>G) and rs2107538 (g.-403C>T) and DN in the T1DM population of Caucasian origin (232).

The CCL5/CCR5 axis is an important element in acute and chronic inflammation because one of its functions is to maintain inflammation by recruiting and activating monocytes and lymphocytes. People infected with HIV, which are carriers of rs2280788 (g.-28C>G) and rs2107538 (g.-403C>T) polymorphisms, experienced delayed progression to AIDS because of the increased activity of *CCL5* gene. An increased concentration of CCL5 was proved in atherosclerotic plaque. Genetic

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studies analysing an association between atherosclerosis and rs2107538 or rs2280788 revealed contradictory results. Studies with both rs2280788 (g.-28C>G) and rs2107538 (g.-403C>T) are rare in the field of DN. Moreover, no study has been done with these polymorphisms in the Caucasian population with T2DM. Therefore, we decided to investigate the relationship between rs2280788 (g.-28C>G) and rs2107538 (g.-403C>T), and DN in subjects with T2DM.

1.3.2   CHEMOKINES RECEPTOR 5 (CCR5)

The primary function of chemokines is chemotaxis, directing inflammatory cells to the site of infection. Not only have chemokines very important roles in the activation of the immune system, but also in its maturation and maintenance (233). Genetic alteration in the chemokine system could have a significant impact on maintaining homeostasis and pathophysiological processes in different diseases. We focused our attention on chemokine receptor 5 (CCR5) or RANTES receptor. Mutations in the coding and promoter regions have been linked to a changed expression of CCR5 on cell membranes. An increased expression of CCR5 could be connected to increased inflammation also in the setting of chronic subclinical inflammation in DM and its chronic complication, such as DN.

CCR5 is a transmembrane receptor that has conserved a basic structure common to the GPCR composed of extracellular N terminal, seven hydrophobic transmembranes and cytoplasmic C terminal domains, and a calculated molecular mass of 40.6 kDa. The protein contains 352 amino acids and shares around 70% of the sequence identity with another common chemokine receptor, CCR2 (234, 235, 236). The extracellular part forms 3 loops with 2 disulphide bridges between cysteine residues that stabilize the tertiary structure. Interestingly, decreased membrane expression and loss of ligand binding were found with the mutational change of cysteine in extracellular portion of receptor.

Some mutational changes of amino acid residues changed tertiary protein structure that is important for CCR5 functions (237). Three intracellular loops are present and an additional pseudo loop, formed by the acetylation of cysteine residues on which the palmitic acid, which positions the cytoplasmic tail close to the cell membrane, is attached. The formation of the fourth loop facilitates signal transduction, receptor transport to the membrane, and is its endocytosis (236, 238). Besides CCL5, as the most important, other natural ligands for CCR5 include MIP1-α (CCL3), MIP1-β (CCL4) and monocyte chemotactic protein-2 (MCP-2, CCL8) (239).

Increased CCR5 was found in chronic inflammation, and a number of CCR5 positive cells correlates with histopathological changes and the severity of inflammation (240). Mice with target deleted CCR5 gene had partly insufficient macrophage function with reduced efficacy for clearing intracellular bacteria (241). CCR5 was practically undetected with either in situ hybridization or immunohistochemistry in normal kidney tissue and intrinsic renal cells (242). Cells in glomeruli

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and tubulointerstitium increased the secretion of chemokines after acute or chronic harmful influences that stimulate the infiltration of the kidney (243, 244, 245, 246, 247). After HIV-1 infection the expression of CCR5 was high and mainly restricted to mononuclear leukocytes infiltrating kidney. Interstitial lesions and urinary excretion of CCL5 correlated with the amount of CCR5 positive infiltrating cells (242). With the suppression of inflammation, glucocorticoids dramatically decreased the amount of cells positive for chemokine receptors (248).

Besides CCR2, CCR5 is the most studied and important chemokine receptor. CCR5 is important for leukocyte infiltration and endothelial transmigration. Its chemotactic role starts already in circulation. Its ligand, CCL5, embedded in endothelial glycocalyx, binds to CCR5 on leukocytes. This activates integrins on leukocytes in vascular lumen, predominantly in post-capillary venules, where diapedesis takes place. Activated integrins change the conformation to open extended molecule formations that enable a strong binding to their ligands. The consequence of this conformational change is the arrest of leukocytes on endothelial cells. In the tissue, CCR5 helps with navigation, chemotaxis and the activation of inflammatory cells (249). The CCR5 cooperates with other chemokines to stimulate T cells and enhance their responses and secretion. Together with CXCR4 they participate in the formation of immunological synapse, which is formed between T cell and antigen presenting cell. This is important for the augmentation of adaptive immune response (250). The role of CCR5 was displayed in coronary artery disease (251), bronchial asthma (252), SLE (253), multiple sclerosis (254), Alzheimer and Parkinson's disease (255).

1.3.2.1 The rs1799987 polymorphism *of the CCR5* gene

The *CCR5* (C-C motif chemokine receptor 5 gene) gene for CCR5 is located on the 3rd chromosome, cytogenetic location 3p21.31, and extends about 6kb. It consists of 3 exons and 2 introns, but the genetic structure and regulation is complex. The second exon is split into two parts: E2a and E2b. Intron 1 is located between exon 1 and exon 2, while intron 2 is positioned between exon 2 and the open reading frame (ORF). Actually, exon 3 consists of 3 parts: 11 base pairs in 5' untranslated region (5'UTR), ORF, and 3'UTR at the downward end of the gene. Two promoters regulate the transcription of CCR5 gene. The first or upstream promoter named Pu or Pr2 is located upstream or before exon 1 and induces two transcripts: CCR5A and CCR5B. The second promoter is downstream of exon 1, named Pd or Pr1, and consists of the whole intron 1 and exon 2 (239, 256, 257). The transcription in CCR5 starts in different places and the activation of different promoters can result in several different transcripts (256). Both promoters are important for CCR5 surface expression, especially upstream (Pr2) promoter is important in T cells after their activation (257). The polymorphism rs1799987 (g.-59029A>G or NC_000003.12:g.46370444A>G (GRCh38)) in the promoter region is one of the most studied SNPs of inflammatory cytokines in the field of HIV infection and AIDS. This polymorphism A allele [rs1799987:A] increases the expression of in vitro promoter activity (by about 45%) (258). Further, two times greater in vitro

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transcription was reported for A allele [rs1799987:A] in comparison to G allele [rs1799987:G] (259). An increased expression of CCR5 was found on CD14+ and CD3+ cells (260). Shieh and co-workers demonstrated an increased number of membrane CCR5 expression in carriers of rs1799987AA genotype on CD4+ cells (259). Similarly, a dramatical increase of CCR5 on peripheral blood monocyte cells (PBMC) membrane in carriers of A allele [rs1799987:A] after stimulation was found (257). Carriers of rs1799987AA genotype had increased susceptibility for HIV-1 infection and the progression of the disease (242, 261, 262). In comparison, carriers of rs1799987GG genotype also progressed to AIDS, but on average 3.8 years later (259).

The importance of the rs1799987 polymorphism was analysed in the haematological stem cell transplantation with human leukocyte antigen (HLA) compatible donors. A highly significant association between donors homozygous for the said SNP and the survival of recipients was found. The survival of recipients with donor carriers of rs1799987AA genotype was 20% lower in comparison with other genotypes in donors (233). A similar result was reported in a small Turkish study on renal transplant recipients (263). However, not all studies have been in consensus. In a bigger group of Tunisian renal transplant patients no differences were found between the groups with and without acute renal rejection, and distribution of the rs1799987 polymorphism allele variants in recipients. In recipients with the combination of CCR2-64I (rs1799864) and rs1799987A alleles, a higher risk for acute graft versus host disease (GVHD) was noted (264). A study from the USA revealed completely different results, namely a reduced risk for GVHD in renal transplant patients who were homozygous for the rs1799987A allele; this was against all expectations and difficult to explain (265).

Early studies in DN showed a possible association with the rs1799987A allele. Nakajima and co-workers found significantly more common rs1799987A alleles in Japanese T2DM patients with DN in comparison to T2DM patients without DN. Studies pointed to an independent association of rs1799987 polymorphism (229, 266). To confirm this finding, they conducted a retrospective study. The 10-year longitudinal retrospective study confirmed the association of rs1799987A allele with DN in T2DM patients (231). Similar positive results of an association between DN and rs1799987 polymorphism were found in a diabetic population in India (267, 268). Significantly fewer studies have been done on the Caucasian population of T2DM patients with DN.

A positive association between DN and rs1799987 polymorphism was found in T2DM patients in Poland (269). In patients with T1DM, the relation of this polymorphism was studied in a European EURAGEDIC (European rational approach for the genetics of diabetic complications) cohort. The study showed a positive association between DN and rs1799987 polymorphism (270, 271). Anyhow, not all studies are in consensus for this observed association. The group from the Joslin Diabetes Center in the USA presented completely opposite results. Only men with T1DM and the

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G allele [rs1799987:G] had an increased risk of developing DN (272). In an Irish population with T1DM, the rs1799987 polymorphism did not show any association with DN (234).

As mentioned, studies suggest that rs1799987 is a functional polymorphism, which may increase the activity of *CCR5* gene transcription. Consequently, this could increase inflammation per se, also in DN. In the very complex pathophysiology of DN this could be an additional factor in the tendency of kidney damage. Because of inconsistent findings in association studies in DM patients and the rarity of them in the Caucasian population, especially in T2DM patients, we included the rs1799987 polymorphism of the chemokine *CCR5* gene in our study. We anticipated that rs1799987 polymorphism might be an important factor in DN in our study population.

1.3.3 CHEMOKINE LIGAND 2 (CCL2) OR MONOCYTE CHEMO-ATTRACTANT PROTEIN-1 (MCP-1) AND CHEMOKINE RECEPTOR 2 (CCR2)

Migrations of immune cells are under the strict control of chemokines. MCP-1, also known as chemokine ligand 2 (CCL2), is a specific chemoattractant for monocytes and recruits monocytes/macrophages to the glomerulus and tubulointerstitium in proliferative glomerular diseases. Hyperglycemia, persistent proteinuria and locally increased concentration of angiotensin type II activate NF-κB, which stimulates inflammation with the up-regulation of chemokines, CCL2 and CCL5 (273). A significantly increased activity of NF-κB was found in cortical tubular cells, thus tubular epithelial cells are also the main source of CCL2 in patients with DN. Furthermore, intrarenal concentrations of CCL2 correlate with interstitial infiltration and the magnitude of proteinuria in humans (274). Normally, CCL2 is secreted in low levels by the tubular epithelium, but high levels were found in the kidney interstitium in DN (190, 275).

Chemokine receptors are expressed on target cells, like monocytes, lymphocytes, basophils and dendritic cells. Besides that, the CCR2 is expressed on non-hematopoietic cells, such as endothelial cells, fibroblasts and mesenchymal stem cells (276). Monocytes constitutively express CCR2. After the activation of monocytes, CCR2 expression increases similarly as the induced expression on lymphocytes after stimulation with IL-2 (171). Two protein isoforms of the *CCR2* gene are known as the result of the alternatively spliced *CCR2* gene: CCR2A and CCR2B. The predominant isoform in the human monocyte is CCR2B. This isoform represent 90% of all CCR2 receptors on cell membranes (277, 278). The other isoform, CCR2A, is predominantly found in the cytoplasm because its larger C terminal cytoplasmic tail represents a cytoplasmic retention signal, which impedes the passage to the membrane (277, 279). CCR2A expressed on membranes has a similar binding affinity for CCL2 as the CCR2B isoform (280). Additionally, the CCR2A in the cytoplasm binds and sequesters the immature form of CCR5, and consequently decreases their concentration on cell membranes (281).

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The CCR2 is the primary receptor for the CCL2 ligand, whereas the CCL2/CCR2 axis leads to the activation of intracellular signalling cascades with transient increased intracellular calcium and the inhibition of adenylyl cyclase. The primary function of CCR2 is mediating the chemotactic response of monocytes. Additionally, the CCL2/CCR2 axis plays a role in embryogenesis, the regulation of T helper 1 cells (Th1) and T helper 2 cells (Th2) cells differentiation, promotion of angiogenesis, the discharge of monocytes from bone marrow and metastasis in certain types of cancer (177, 282). Inflammation in bronchial asthma, chronic obstructive pulmonary disease, rheumatoid arthritis, psoriasis, obesity and atherosclerosis is importantly mediated through CCR2 stimulation (283, 284). Selectively knockout CCR2 receptor mice models of atherosclerosis developed markedly less lipid streaks, typical vascular lesions in early atherosclerosis compared to wild mice despite no change in plasma lipid levels in both groups of animals (285).

Similarly, in CCR2-knockout mice or when using CCR2 antagonist, decreased albuminuria and histological changes associated with DN, including a smaller amount of infiltrating macrophages, were found in experimental mice (282). Blocking the CCR2 in the T2DM model of mice decreased the development of DN and improved insulin sensitivity (286). The combined action of CCL2/CCR2 axis and TGF-β increased apoptosis of podocytes (287).

1.3.3.1 The rs1799864 polymorphism *of the CCR2* gene

The *CCR2* (C-C motif chemokine receptor 2 gene) is located on the short arm of chromosome 3 (3p21-p24) and consists of three exons that span over 7,207 bases (288). This region on chromosome 3 contains the CC chemokine receptor cluster, which contains chemokine receptor genes (*CCR1*, *CCR3*, *CCR4* and *CCR5*) (177, 289). The change of single nucleotide G at position 190 (counting from the ATG start codon) to A (**G**TC→**A**TC) represents a change of amino acid at position 64 from valine to isoleucine in the CCR2 protein (rs1799864:G>A or g.+46295G>A or c.190G>A or p.Val64Ile). This mutation in the second exon means a change in conservative first transmembrane domain and stabilizes the CCR2A isoform while it does not change the stability of the CCR2B isoform (290).

The levels of cell membrane expression of the CCR2A isoform with minor A allele [rs1799864:A], with isoleucine on position 64 (CCR2A-64Ile), are significantly greater than that of the usual CCR2A isoform with major G allele [rs1799864:G] (281). The envelope glycoprotein (gp120) of HIV-1 virus binds the main receptor (molecule CD4) and co-receptor (CXCR4 or CCR5) on the cell surface before the virus invades the host cell. Sometimes, the CCR2 functions as minor co-receptor (291). In comparison to the CCR2B, the predominant isoform on the membrane, this increase in the membrane expression of CCR2A-64Ile is probably clinically unimportant (292). However, natural resistance to the progression of HIV-1 infection was described for the *CCR2* rs1799864 polymorphism. The progression to overt AIDS is delayed for 2 to 4 years in patients

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with this polymorphism (293). For a clinical outcome an increased sequestration of immature CCR5 in the cytoplasm by CCR2A-64Ile is more likely to be an important mechanism with a more pronounced decrease in CCR5 on the cell membrane. As Smith and co-workers proposed, this could influence the progression to AIDS (281).

Batra and co-workers re-sequenced *CCR2* and *CCR5* genes in 20 people of Indian origin. Three SNPs were described in the exon part of the *CCR2* gene (rs1799864:A>G, rs1799865:C>T, rs743660:G>A), and one insertion/deletion in 5'UTR (rs3918356, -/CAA) 5kb upstream of the translation start site of the *CCR2* gene. Strong linkage disequilibrium was found between these four polymorphisms in the *CCR2* gene. Additional complete linkage disequilibrium was described (r2 = 1) between rs1799864 in the *CCR2* gene and rs1800024 in the *CCR5* gene (294).

The polymorphism rs1799864 was often analysed for a possible association with different diseases and different types of cancers (177, 295). Positive associations between the rs1799864 polymorphism and cerebral ischemic stroke (296), bronchial asthma (297), psoriasis (298), and CAD were found (292). A genetic study in patients with CAD younger than 65 years with the rs1799864 polymorphism revealed a positive association. Even when no increased calcium and coronary lesions were detected in coronary vasculature during coronarography, increased susceptibility for myocardial infarction was found. Increased vulnerability of relatively fresh, uncalcified atherosclerotic plaques was a possible explanation. This polymorphism could be connected with greater inflammatory cell infiltration and bigger production of inflammatory cytokines that makes atherosclerotic plaque more vulnerable and prone to rupture (299). However, not all studies presented the same results. The meta-analysis of 24 CAD studies revealed no association between CAD and the rs1799864 polymorphism (300). Also, no association was found between rs1799864 and ischemic stroke or Parkinson's disease (301, 302). Multiple studies showed that the distribution of rs1799864 polymorphism strongly depends on ethnicity (177). The frequency of the minor A allele [rs1799864:A] is about 10% in Caucasians and 25% in Asians, while frequencies in African Americans, Hispanics and Indians are in-between, with a range of 15 to 17% (303). With the exception of the study of Nakajima and co-workers, who found no association between DN and the rs1799864 polymorphism in Japanese T2DM patients, no other study in the field of DN was found (266).

Possible decrease in the CCR5 membrane expression mediated by the *CCR2* rs1799864 polymorphism, only one published study on the association between DN and rs1799864 polymorphism and no studies done in the Caucasian population were the main reasons to study this polymorphism in our population with T2DM.

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1.4 INTERLEUKINS

Interleukins are a subgroup of cytokines, which regulate and coordinate activities in the immune system, predominantly between leukocytes. These proteins are signalling molecules that constitute a complex, flexible and interconnected network that regulates immune cell proliferation, differentiation, activation, functions, growth and survival.

The name interleukin derives from "*inter*" and "*leukin*" or cross talk between leukocytes, where they were first discovered. Actually, the majority of interleukins are produced by helper T CD4+ cells, monocytes/macrophages and endothelial cells, but practically all cells can produce same interleukins and express different interleukin membrane receptors (304). Despite the fact that interleukins are redundant and pleiotropic in their functions, the group with predominately pro-inflammatory characteristics are IL-1, IL-12, IL-18, etc. (305). IL-4 and IL-10 are major anti-inflammatory cytokines that play a crucial role in silencing immune activity after stimulation. Normally, an inflammation that is necessary to eliminate pathogens is tightly controlled by anti-inflammatory mechanisms. Destructive effects and damage to tissue that is necessarily connected to the inflammation process are an obligatory toll in immune response and homeostasis restoration. After the peak of inflammatory process, it is important to limit and finally shut down the inflammation to enable tissue repair and anew-established homeostatic condition be established.

In general, the same cell types that activate and accelerate the inflammation also produce anti-inflammatory mediators. Triggers for the expression of anti-inflammatory mediators are nearly the same as for pro-inflammatory ones, only the signalling pathways and their kinetics are different. Usually there is a delay, six to eight hour difference, between the beginning of acute inflammation and the beginning of the silencing process (306).

1.4.1 INTERLEUKIN-12p40 (IL-12B)

Interleukin-12 (IL-12) is an important factor in cell-mediated immunity and one of the major regulators of Th1 immune response. IL-12 is a heterodimer cytokine of 70 kDa that consists of two subunits covalently linked with disulphide bond, namely IL-12p40 and IL-12p35. Thus, IL-12 could be marked as IL-12p40p35 or IL-12p70. Subunit IL-12p40 displays some homology with the extracellular domain of IL-6 receptors, while subunit p35 is structurally similar to IL-6 (307). The subunit IL-12p40 has conserved a hydrophobic pocket that functions as docking station for the IL-12p35 subunit with a prominent centrally located protruding arginine residue, which is important for heterodimerization (308). Both components are coded in different chromosomes: subunit IL-12p35 on chromosome 3 and subunit IL-12p40 on chromosome 5. Also, the regulations of both subunits are independent from each other. While the IL-12p35 subunit is constitutively produced in nearly all cell types, but cannot excrete on its own, because it has no excretory signal and it is excreted solely with IL-12p40 as heterodimer IL-12. Subunit IL-12p40 is produced in only

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those types of cells that actually secrete IL-12. Antigen presenting cells, like dendritic cells and macrophages, are major producers of IL-12 beside neutrophil and human B cells (309). In humans, the subunit IL-12p40 is secreted in excess in comparison to IL-12p35 that has a much smaller, rate-limiting production. Increased amounts of IL-12p40 in the cell are necessary for the stabilization, stimulation of transport and finally excretion of the IL-12p35 subunit. If IL-12p40 is not present, IL-12p35 subunit cannot proceed through the Golgi apparatus and undergo complex glycosylation. As Jalah and co-workers proved, a proper mutual relation of concentration is necessary for an optimal secretion of IL-12 (310, 311). In the past, all detected experimental activities have been attributed to IL-12, but later it was found that many of the described effects belong to related interleukins of an "IL-12 family" (IL-23, IL-27 and IL-35). All members of the family share subunits between them. For example, subunit IL-12p40 makes a disulphide bridge with subunit IL-23p19 and forms a functional IL-23. The main role of IL-23 is to stimulate the differentiation of T helper cells into Th17 cells that produce IL-17 (312). The receptor for IL-12 has two IL-12Rβ1 subunits that connect with IL-12p40 and two IL-12Rβ2 which connect with IL-12p35. Both receptor subunits are members of the cytokine receptor superfamily. Also, these subunits are coded separately, with genes on chromosome 1 and 19, and they are expressed on natural killer cells (NK) and T cells, predominately Th1 cells. The subunit IL-12Rβ2 has three tyrosine residues (in comparison, IL-12Rβ1 has none) on the C terminal that are phosphorylated with Janus kinase 2 (JAK2) and tyrosine-protein kinase TYK2 after binding to IL-12. This part of the intracellular tail of IL-12Rβ2 serves as a docking station for SRC homology domains (SH2) of the signal transducer and activator of transcription (STAT) molecules. STAT4 is important because after activation forms a homodimer or heterodimer in the cytoplasm, but in the nucleus it binds to appropriate transcription binding elements in the promoter regions of different IL-12 responsive genes (207, 307, 313).

In 1989, Kobayashi and co-workers discovered IL-12 as a natural killer cell stimulating factor (314). Actually, IL-12 increases proliferation and the cytotoxic effect of NK, cytotoxic CD8+T cells with stimulated production of perforins and granzymes. But the major functions are stimulating the differentiation of naïve T helper cells into Th1 cells with the stimulation of IFN-γ production. As we understand today, IL-12 does not have a direct effect on resting naïve T helper cells, but it stimulates pre-activated T cells and NK cells. The secretion of IL-12 is stimulated through TLRs and their agonists, predominately DAMP molecules in sterile chronic inflammation. The final effect of stimulation through TLRs is a strong stimulation of IFN-γ production by monocyte and macrophage. Additionally, a positive feedback is established with IL-12 stimulation of NK and T cells to secrete IFN-γ, which activate macrophage for further IL-12 production (315). IFN-γ increases the transcription of *IL-12p40* and *IL-12p35* genes. Interestingly, IL-4 and IL-13, both cytokines of the Th2 immune response stimulate IL-12 production. Cell-cell contact is also important for stimulating IL-12 production (310, 316). The control of production and secretion of

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IL-12 is transcriptional and translational to a certain extent. An extensive inhibition of the secretion of IL-12 is achieved by IL-10, TGF-β, TNF-α, INF-α, and INF-β. IL-10 and TGF-β are parts of the Th2 immune response; especially IL-10 is a strong opponent of IL-12 which blocks the transcription of *IL-12*, while TGF-β decreases the stability of *IL12B* mRNA. Even if INF-α and INF-β act similarly to IL-12 both inhibit IL-12 production (310, 317).

IL-12p40- and IL-12p35-knockout mice have a decreased immune response. The mice of the first type have major immune defect and lower resistance for intracellular bacteria, such as TBC and Staphylococcus. The reason is defective immune response in both Th1 and Th17 cell lines, because IL-12p40 is the subunit of IL-12 and IL-23 (307, 318). In the T1DM model of mice that are genetically predisposed to develop diseases, a decreased inflammation of the Langerhans islets was found after IL-12p40 was blocked (319). An increased expression of *IL12* mRNA and increased concentrations of proteins were reported in human atherosclerotic plaque (320). Animal experiments revealed the inflammatory function of the IL-12p40 subunit especially in the early phases of atherosclerosis with inflammation leading to Th1 and Th17 immunological activity. However, in advanced atherosclerosis other inflammatory cytokines overtake the leading role (321). The progression of atherosclerotic lesions was significantly slower in IL-12-knockout mice and the daily application of IL-12 drastically increased it (322, 323). Interestingly, the indirect involvement of IL-12p40 in vascular inflammation was confirmed in animal experiments with the repeated administration of a small dose of aspirin, which decreased IL-12p40 levels and formed more stable atherosclerotic plaque (324).

The function of IL-12 was studied in diseases like psoriasis and psoriatic arthritis, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease (IBD), T1DM, etc. (307). In psoriasis both Th1 and Th17 are important. Specific cell surface receptors direct the trafficking of lymphocytes into the skin and joint structures in the case of psoriatic arthritis. As the IL-12p40 subunit is shared between IL-12 and IL-23, antibodies to this subunit decrease the activity of both cell lineages. Ustekinumab is a fully human IgG1κ monoclonal antibody that inhibits IL-12p40 with high affinity and specificity. The program of clinical trials PHOENIX I, II (ClinicalTrials.gov #NCT00267969, #NCT00307437) and PSUMMIT-1 and 2 (ClinicalTrials.gov #NCT01009086, #NCT01077362) revealed a good clinical outcome in psoriasis and psoriatic arthritis (325). On the contrary, ustekinumab has a disappointing effect on multiple sclerosis and a much smaller effect on rheumatoid arthritis and IBD as it was foreseen in the first place (326, 327, 328, 329).

Despite the small number of studies, the role of IL-12 in atherosclerotic inflammation was confirmed. Arterial stiffness, one of the indicators of atherosclerosis, is positively associated with serum IL-12 in healthy volunteers (330). Increased serum levels of IL-12 were found in several studies in patients with CVD (331, 332, 333). The concentrations of serum IL-12 are also increased in T2DM patients and are even greater than in patients with CAD (334). A linear increasing

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concentration of serum IL-12 with statistically significant differences between healthy controls, T2DM patients without DN and T2DM patients with DN was shown in the Chennai Urban Rural Epidemiology Study (CURES) from South India (335). On the contrary, serum levels of IL-12p40 were significantly lower in patients with T2DM compared to control subjects in a study from the South-eastern part of Iran (336). The very complex control of production and secretion of IL-12 may contribute to this ambiguity. Interestingly, depending on the levels of analysed cytokines, Anand and co-workers found mixed Th1 and Th2 immunological response in T2DM patients without DN, but clear shift in Th1 cytokine secretion (IL-12, IL-2, IFN-γ) with established DN. This fact indirectly indicates the dominance of Th1 immune response in DN (335).

1.4.1.1   The rs3212227 polymorphism of the *IL12B* gene

The gene for IL-12p40 (*IL12B* or interleukin 12B gene) is on the distal end of the long arm chromosome 5 (cytogenetic locus 5q33.3) in the reverse orientation with respect to the centromere, and consists of 8 exons. The *IL12B* gene is unusual because it has untranslated exons on both ends (337). From the corresponding mRNA translation begins at the first codon in exon 2 and ends at the last codon in exon 7 (338, 339). Several SNPs associated with autoimmune diseases are present in the region, where the *IL12B* gene is located, despite the fact that the DNA region is highly conservative in this part. The majority of these SNPs are located upstream or downstream of the gene coding sequence and only the minority of SNPs are located in the intronic region of the gene. To our best knowledge, no polymorphism in the gene coding region was found. Huang and co-workers reported an interesting polymorphism with the A to C change in the position +1188A>C (rs3212227:A>C or c.+1188A>C or NC_000005.10:g.159315942T>G (GRCh38)) on the 3'UTR of *IL12B* (339). At first, this polymorphism was designated as "TaqI", because of restriction enzyme TaqI cleavages on its position. This enzyme, isolated from bacterium Thermus aquaticus in 1978, recognizes the 5'TCGA sequence and cuts between the T and C nucleotide. In the presence of an ancestral A allele no digestion occurs (5'--T**A**GA-- or 3'--AG**A**T--), but when C is present in the polymorphic allele C the enzyme creates restriction fragments (5'--T | **C**G A-- or 3'--A G**C** | T--) (340). In an Australian population, the prevalence of the A allele was 82%, and 80% in UK Caucasians, as reported by Huang and Hall, respectively (339, 341). Data from the 1000 Genomes Project, Phase3, revealed a frequency for ancestral major A allele in a European population, 77.7%, and 62.5% in a South Asian population (Japan 44.1%) (342).

Polymorphisms in the 3'UTR of the *IL12B* possibly have an influence on diseases with the dysregulation of immunity and the prevalence of the Th1 immune reaction, like psoriasis, psoriatic arthritis, rheumatoid arthritis, multiple sclerosis, T1DM, as well as in chronic inflammatory diseases like atherosclerosis, T2DM and different types of cancer. Analysing rs3212227 polymorphism Morahan and co-workers revealed its functionality. They found an increased production of *IL12B* mRNA in PBMC from rs3212227:AA genotype carriers. They analysed

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unstimulated PBMCs transfected with the Epstein-Barr virus (343). Davoodi-Semiromi and co-workers reported a 50% higher mRNA from stimulated PBMC in carriers of the A allele [rs3212227:A] in comparison to the C allele [rs3212227:C] (344). In analysing the functionality of the rs3212227 polymorphism, Bergholdt and co-workers found no association between the rs3212227 polymorphism and stimulated PBMC (345). Dahlman and co-workers showed an increased expression level of *IL12B* mRNA in 19 different cell lines, but they did not find any significant differences between the genotypes of the rs3212227 polymorphism (346). In healthy people, PBMC have no spontaneous secretion of IL-12 or IL-12p40 subunits. After the stimulation, a huge increase in the secretion of both components was found with levels of secretion in a wide range. Stanilova and co-workers found a significantly greater production of the IL-12p40 protein, in differently stimulated PBMC by carriers with rs3212227:AA genotype (347). On the contrary, Seegers and co-workers found an increased production and secretion of the IL-12 in vitro stimulated PBMC by carriers of the rs3212227:CC, but they did not find an increased secretion of the IL-12p40 subunit (348). Also, Bergholdt and co-workers found no association between the rs3212227 polymorphism and the production of IL-12 and IL-12p40 in PBMCs with different types of stimulation (345). Data in literature for protein IL-12 or subunit IL-12p40 secretion from PBMC are quite contradictory. Significant differences in the research results are probably the consequence of different experimental technics, from unstimulated to stimulated PBMC in different protocols with different substances, etc. (349).

In psoriasis and psoriatic arthritis, a number of studies showed a positive association with polymorphism rs3212227. Several meta-analyses confirmed the protective role of the minor C allele [rs3212227:C]. The meta-analysis by Lee and co-workers included 14 studies and showed an increased susceptibility for psoriasis in Caucasian (European) populations, while the meta-analysis by Zhu included 11 studies and they concluded that ethnicity probably was not so important despite an indicated tendency for a stronger association in Asian studies (350, 351). Meta-analyses that looked for a possible association between the rs3212227 polymorphism and rheumatoid arthritis as well as multiple sclerosis revealed no association, while a sub-analysis found no discrepancies in the different ethical populations (352, 353).

In 2001, Morohan and co-workers reported a positive association between T1DM and the rs3212227 polymorphism, which was named IDDM18, a new risk locus for T1DM. In their study the minor allele C [rs3212227:C] was a protective factor against T1DM in Australian patients and the same was confirmed in an American population. But many other studies conducted in Europe and North America did not confirm these results (343, 344, 345, 346, 355, 356, 357, 358, 359). Compared to healthy controls, an increased prevalence of the A allele [rs3212227:A] and the AA genotype was present in T2DM patients as found by Yaghini and co-workers. The stratification of patients with T2DM only depending on the rs3212227 polymorphism revealed no statistically

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significant difference (336). No association between the rs3212227 polymorphism and the outcome of kidney transplantation was found. More precisely, the association with acute, chronic and delayed renal graft rejection was analysed in a Polish and French population of patients with renal transplantation (360, 361). Also, in the field of atherosclerosis relatively few studies showed inconsistent outcomes. A retrospective Chinese study found that carriers of the minor C allele of the rs3212227 polymorphism are associated with an increased risk for intracranial aneurism in comparison to A allele (362). Mangino and co-workers did not find any association between myocardial infarction and the rs3212227 polymorphism in a large study in British Caucasians (363). Also, Momiyama and co-workers reported a failure to find an association between CAD and the rs3212227 polymorphism (364).

The basic inflammatory processes in atherosclerosis and chronic diabetic complications, such as DN, are similar, and the same parallels can be drawn. As already explained, quite contradictory results are found in literature for the association of the rs3212227 polymorphism with different pathologies. Only one article published in the field of DN for rs3212227 polymorphism was found. In Polish hemodialysis patients, Grzegorzewska and co-workers analysed a possible association between the rs3212227 polymorphism and patients with T2DM, but they found no significant association (365). Because of the high incidence of comorbidity, these hemodialysis patients die earlier than non-diabetic hemodialysis patients. Additionally, many T2DM with slowly progressive albuminuria and decreasing renal function die before they potentially develop ESRD (366). In view of the foregoing, the survival bias is very possible. Because of only one published research paper, we wanted to compare T2DM patients with and without DN with respect to rs3212227 and find out if a possible association with DN exists in our population of T2DM patients. The majority of them have preserved renal function.

1.4.2   INTERLEUKIN-18

Pro-inflammatory cytokine IL-18, also known as interferon-γ inducing factor, plays a crucial role in chronic inflammation. Similar to IL-1 in terms of structure, receptor utilization and cytokine processing, it is a member of the IL-1 family of cytokines, which are all synthesized as precursor proteins (367, 368). This inactive 23 kDa precursor molecule is processed into an active 18.3 kDa cytokine, by the enzyme caspase-1, before or after the release from the cell. IL-18 is constitutively expressed in renal tubular epithelia, infiltrating monocytes, macrophages, dendritic cells and keratinocytes; T cells, endothelial cells of interstitial vessels along with proximal renal tubular cells, are potential sources of this cytokine (369, 370). IL-18 is located on chromosome 11q22.2-q23.3 and several polymorphisms in its promoter region have been identified (371). IL-18 provides its biological function by binding to a specific receptor on the surface of target cells. IL-18 receptor binding sites are divided into the IL-18 receptor α chain (IL-18Rα) (also known as IL-1Rrp1, IL-18R1 or IL-1R5) and the IL-18 receptor β chain (IL-18Rβ) (also termed IL-18RacP, IL-18RII or

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IL-1R7). The binding sites of IL-18 to its receptors were identified: sites I and II are importantly specific to IL-18Rα and site III to IL-18Rβ (372). Its activity, however, is neutralized when it binds to IL-18-binding protein (IL-18BP), a naturally occurring, constitutively secreted, inhibitor of IL-18 (373). IL-18 has an impact on numerous inflammatory processes. The hallmark for IL-18 activity is its ability to induce IFN-γ in the presence of IL-2, IL-12 or IL-15, by driving Th1 polarization and priming NK cells, both resulting in a high-level production of IFN-γ (368, 373). IL-18 also harbours the unique property of inducing Fas ligand expression on NK cells, facilitating their killing of infected cells by Fas-mediated apoptosis (368). In addition, IL-18 leads the production of other pro-inflammatory cytokines, endothelial apoptosis, up-regulation of ICAM-1, and hyper-homocysteinemia (369). Recent studies have shown that pro-inflammatory cytokines contribute significantly to the development of diabetic complications, such as DN (139).

1.4.2.1 The rs187238 polymorphism of the *IL-18* gene

Polymorphism in the *IL-18* (interleukin 18 gene) has been shown to be associated with circulating IL-18 levels (374). A functional polymorphism in the *IL-18* promoter (rs187238, g.-137G>C) has been repeatedly found to be associated with the *IL-18* promoter transcription activity (374). To our knowledge, there are only a few studies investigating an association between the rs187238 polymorphism and DN (369, 375). Bai and co-workers have recently reported that individuals carrying the C allele of the rs187238 polymorphism showed a 2.16-fold higher risk for DN (375). Moreover, Elneam and co-workers compared the allele frequencies of the rs187238 polymorphism among patients with diabetes and without DN, and patients with DN. They revealed that the rs187238:G allele was significantly more common in patients with DN than the C allele (369). Therefore, the aim of this study was to investigate the association between the rs187238 polymorphism of the *IL-18* gene and DN among Caucasians with T2DM.

1.4.3 INTERLEUKIN-10

Interleukine-10 is one of the most important anti-inflammatory agents. This pluripotent cytokine, with predominantly anti-inflammatory and immunosuppressive actions, possesses also some stimulatory functions. Originally it was named cytokine synthesis inhibitory factor (CSIF), because of its ability to turn off cytokine productions by T cells as it was first revealed by Fiorentino and co-workers in 1989 (376).

Human IL-10 is a homodimer that consist of two non-covalently attached monomers, each about 18 kDa molecular weight. The protein of IL-10 has 160 amino acid residues and it is not glycosylated (377, 378). The receptor for IL-10 (IL-10R) consists of two subunits of IL-10R1 and two subunits of IL-10R2. The IL-10R1 binds IL-10 with high specificity and predominantly leukocytes express IL-10R1 subunits. IL-10R2, which is also found in other receptors of the IL-10 cytokine family, binds to the IL-10/IL-10R1 complex after the conformational change of the IL-10 molecule. The

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binding of IL-10 with its receptors provokes a signal transduction that starts with the activation of JAK1 and TYK2. The result of JAK-STAT signalling pathway is the release of the STAT. Dimmers of STAT3 or STAT1 in the nucleus bind to appropriate transcription binding elements in promoters of different IL-10 responsive genes (379, 380). One of these genes is a suppressor of the cytokine signalling 3 (*SOCS3*) gene. The STAT dimer binds on the promoter of *SOCS3* gene, which is a negative regulatory factor for many cytokine genes. Furthermore, IL-10 in this way negatively regulates its own production by binding STAT3 on the IL-10 promoter (381). Cytokines, hormones and derivatives of prostaglandins regulate IL-10 production (313).

Nearly all cells of the inflammatory system can produce and secrete IL-10. Interleukin-10 is mainly expressed in monocytes, stimulated macrophages and activated T and B cells. Between T cells the major sources are Th2 cells, Th1 cells, T helper type 17 cells (Th17) and some of the regulatory T (Treg) cells. Beside IL-10, the IL-10 cytokine family consists of twelve additional members (IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B and IL-29) and four viral homologs. The viral homologues indirectly show the importance of the immunosuppressive role of IL-10 in immune response; because they imitate it, viruses improve their survival in host organisms (380, 382). Most of its anti-inflammatory actions include inhibiting the antigen presentation to T cells. Dendritic cells and macrophages are the most important antigen presenting cells (APS). As professional APS, dendritic cells have the most versatile set of receptors for the recognition of pathogen-associated molecular patterns (PAMPs) and DAMPs. One of major function of dendritic cells is finding, processing and presenting antigens to naïve T cells in lymph nodes. By presenting antigens, expression co-stimulators and cytokines they activate T cells and start their differentiation into effector T cells (53). IL-10 inhibits the expression of co-stimulatory molecules and class II major histocompatibility complex (MHC-II) on the cell membrane of APS. The process of antigen presentation and the activation of naïve T cells require the formation of the immunologic synapse. This formation consists of MHC-II and presenting peptide, T cell receptor and a series of co-stimulatory molecules needed for signal transduction and the stabilization of the contact between APC and T cell. Without proper antigen presentation and naïve T cells polarization, no development of humoral immune response is possible (53). Additionally, IL-10 also decreases the production of IL-12, TNF-α, IL-1β and others cytokines necessary for the differentiation of the T cell (53, 313, 378). IL-10 can prevent the production of Th1 associated cytokines IL-2 and INF-γ and increase the production of two anti-inflammatory mediators, IL-1 receptor antagonist (IL-1RA) and soluble TNF-α receptors, which further inhibit inflammation (378, 383). Further, IL-10 inhibit proinflammatory cytokines, e.g. IL-1, IL-6, IL-12, TNF-α, chemokines, and also NF-κB (313). IL-10 inhibits the expression of ICAM-1 in intestinal epithelial cells. Together with IL-4 and IL-13, IL-10 down-regulates the production of CCL2 (MCP-1) in activated intestinal epithelial cells (384). Besides that, IL-10 inhibits leukocytes infiltration, because the endothelial expression of P- and E-selectins on endothelial cells is down-regulated (381). Some subgroups of cytotoxic CD8+ T

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cells and natural killer cells are stimulated into proliferation by IL-10 which acts like a growth factor. IL-10 co-stimulates B cell activation, inhibition of their apoptosis and regulation of Ig class (385).

The deliberate exclusion of the *IL10* gene in experimental mice revealed the development of chronic enterocolitis and disturbed expression of MHC-II on cell membranes (386). The absence of signalling through IL-10 leads to rare, intractable, inflammatory bowel disease which is usually manifested in humans in the first year of life (53). Administered as a systemic drug, recombinant IL-10 turns out to increase inflammation in autoimmune diseases. On the other hand, if administered locally it works as an anti-inflammatory (387). Despite the potential therapeutic use in inflammatory diseases, the practical applicability of IL-10 is of limited value because of its rapid proteolytic degradation and short half-life (388). Glucocorticoids up-regulate constitutive IL-10 production in human monocytes; moreover, a glucocorticoid response element was found in IL-10 promoter (389).

In normal kidneys, where immune cells infiltration is absent, the major source of IL-10 are mesangial cells; endothelial cells also secrete IL-10, but not tubular epithelial cells (390). IL-10 is an autocrine growth factor for mesangial cells and the application of recombinant IL-10 to animal kidney increased the number of glomerular cells (391). The pathophysiology of kidney disease with prominent mesangial proliferation, such as in DN, is associated with an increased local production of IL-10. The activation and proliferation of mesangial cells produce an increasing amount of growth factors leading to glomerular and tubular hypertrophy, thickening of GBM, accumulation of mesangial matrix and slow progression of glomerular and tubulointerstitial sclerosis to final fibrosis. Interestingly, IL-10 and TGF-β, both important profibrotic mediators, mutually stimulate the production of each other (387).

In patients with T1DM, Mysliwska and co-workers found very high levels of serum IL-10 in patients with DN. The serum levels were much smaller in T1DM patients without DN and practically undetectable in normal control subjects. They found a positive correlation between serum concentrations and levels of albuminuria (392). In another study, serum concentrations of IL-10 in healthy controls were undetectable, but they increased in patients with T2DM. The highest and statistically significant different concentrations of serum IL-10 were observed in patients with DN in comparison to patients without DN. They also found a correlation with the severity of albuminuria, but only in the DN group (393). Wu and co-workers found no statistical differences between serum concentrations of IL-10 in T2DM patients with and without DN. However, the study was small and only twelve patients were included in each group (206). In contrast to the aforementioned results, an opposite conclusion was made by Yaghini and co-workers. They found a lower concentration of IL-10 in patients with T2DM in comparison to healthy control participants, which was statistically significant (394). Due to its anti-inflammatory power, IL-10 is

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widely studied also in the domain of autoimmune diseases, including T1DM, SLE, rheumatoid arthritis, IBD and cancer (395, 396, 397, 398, 399).

1.4.3.1   The rs1800896 polymorphism of the *IL10* gene

The *IL10* (interleukin 10 gene) human gene is located on the long arm of 1 chromosome (1q31-32), in a locus often associated with immune diseases that spans 5.2 kb and comprises 5 exons and 4 introns (400, 401). The rs1800896 (g.-1082G>A) polymorphism affects the promoter region of the *IL10* gene. Turner and co-workers suggested that the presence of the rs1800896AA genotype is associated with a lower production of IL-10 probably because of the down-regulation of the human *IL10* promoter. Additionally, it was proved that the amount of secreted IL-10 depends directly on the production of mRNA and not on the stability of mRNA. This means that the production of IL-10 directly depends on transcriptional regulation (402). An association between the rs1800896AA genotype (low producer) and steroid-dependency has been shown for ulcerative colitis and Crohn's disease (403). In a study of monozygotic twins it was found that about 75%, whereas in dizygotic twins about 33% of variation in endotoxin induced IL-10 production is genetically determined (404).

Suarez and co-workers conducted a study in 128 healthy people from Northern Spain and revealed that the distribution of alleles and genotypes differed from the North and Central European studied subpopulations, but was similar to the Italian population. The main difference was the higher frequency of the A allele [rs1800896:A] and consequently about 50% of the population was a low producer of *IL10* mRNA (405). An increased frequency of the rs1800896GA and AA genotypes was reported in patients with rheumatoid arthritis, Wegener's granulomatosis, Crohn's disease, and ulcerative colitis (406, 407, 408). In a North Indian population, a positive association between the rs1800896 polymorphism and ESRD was found. Manchando and co-workers estimated that the risk for carriers of the rs1800896AA genotype to develop ESRD is three times greater in comparison to the rs1800896GG genotype (409). In the study of biopsy-proven glomerulonephritis allele frequencies of rs1800896 were similar between the patient and control groups; this did not apply to genotype frequencies. The patient group was divided to fast progressors and slow progressors to ESRD. Allele A [rs1800896:A] and GA/AA genotype, connected to the low producer of IL-10, were more frequent in the group of fast progressors, which also had a worse outcome of glomerulonephritis (382). Dialysis patients homozygous for the rs1800896:G allele have a 30% higher secretion of IL-10 compared to homozygous carriers of the rs1800896:A allele (410). The rs1800896:A allele that is associated with a low production of IL-10 was predictive for higher cardiovascular morbidity and mortality in patients on haemodialysis (411). In kidney transplanted patients, acute rejection rates were associated with polymorphism rs1800896 in an English population while there was no association in patients from Iran (412, 413). Li and co-workers found no association between the rs1800896 polymorphism and T2DM in a meta-analysis

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including 9 studies (414). An increased frequency of the rs1800896GG genotype, which is associated with higher IL-10 production, was found in the group of patients with ESRD in Germany; however, only 44 of these patients had T2DM and DN (415). Another small study from Brazil in T2DM patients revealed a loose positive association between DN and rs1800896, but additional analysis of haplotypes found no association (416).

Ezzidy and co-workers did not find an association between DN and rs1800896 in Tunisian T2DM patients (417). Similarly, Erdogan and Kung with co-workers found no association with DN in T2DM patients in a Turkish and Taiwanese population, respectively (418, 419). In patients with T2DM and DN, allelic frequencies of rs1800896:G were significantly higher in comparison with healthy control subjects, and the same was established for the GG genotype in South Indian patients (420). Two recent studies from China presented a positive association between DN and rs1800896 polymorphism (421, 422). Peng and co-workers made a meta-analysis of the rs1800896 polymorphism and DN in T2DM patients, which included 9 studies and revealed a possible increased risk for DN in carriers of the rs1800896AA genotype (423).

Therefore, in our study we intend to analyse a possible association between the rs1800896 polymorphism and DN in a population of T2DM patients, because no study on this association in the Caucasian population has been published so far.

1.4.4   INTERLEUKIN-4

Inflammatory cytokines, such as IL-1, IL-6, IL-12, IL-18, TNF-α, etc., have all been shown to be involved in the development and progression of DN (424). Cytokines help control and reduce inflammation in the immune system. Major anti-inflammatory cytokines include IL-1RA, IL-4, IL-6, IL-10, IL-11, IL-13 and TGF-β. Because of redundancy and pleiotropy, the IL-6 has both pro- and anti-inflammatory functions as well as many other (425). Equilibrium in the production of these inflammatory mediators depends on the polarization of T helper lymphocytes and subsequently phenotype polarization of macrophages. Both are probably the most important immune cells, especially in chronic inflammation (426).

IL-4 is an anti-inflammatory cytokine and is crucial in the regulation of the immune system. This protein has a compact tertiary structure, similar to other cytokines, with a molecular weight from 12 to 20 kDa, depending on post-translation modifications, especially glycosylation. It has an important role in regulating B cell and T cell differentiation (427).

IL-4 was discovered as a factor for the differentiation and stimulation of B cells. Today it is known that it has many different functions, such as regulating cell proliferation and apoptosis, Ig class switching, energy homeostasis, insulin resistance with the expression of multiple genes in lymphocytes, macrophages, fibroblasts, epithelial and endothelial cells. The most important functions are probably the stimulation of differentiation into the Th2 phenotype and the secretion of

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INF-γ (428, 429). Resting, naïve T cells differentiate towards either Th1 or Th2 cells and this process is mediated by cytokines (429). IL-4 promotes the differentiation of Th2 cells and inhibits the differentiation of Th1 cells (430). Consequently, IL-4 together with IL-10 strongly suppress the production of pro-inflammatory cytokines, like IL-1, IL-6, IL-8, IL-12 and TNF-α, as well as macrophage differentiation into the M1 phenotype. On the other hand, IL-4 stimulates an alternative way of macrophage differentiation to M2 phenotype with the production of IL-4δ2, IL-13, CCL18, etc. M2 macrophages induce the fibrotic process and when exaggerated they lead to diseases like, scleroderma, idiopathic pulmonary fibrosis, asthma, pulmonary sarcoidosis (431). IL-4 also regulates differentiation, proliferation, and apoptosis in some cell types of both hematopoietic and non-hematopoietic origin (432). The survival of B and T lymphocytes is increased because IL-4 works as a growth factor and probably decreases susceptibility to apoptosis. Probably the same influence of IL-4 increases the resistance of tumour cells during therapy. Under the influence of IL-4, TNF-α and INF-γ in endothelial cells increase VCAM-1 and decrease E-selectins expression which helps recruit T cells and eosinophils and decrease the number of other leukocytes (433). Th2 cells are the major producers of IL-4. Additional production is found in natural killer cells, basophiles, eosinophils, and mast cells. By preventing the necessary increase in cytosolic free calcium in the induction of signalling pathways, Cyclosporine A completely blocked the production of IL-4 (434). IL-4 and its signalling pathway were reported to be associated with the development of autoimmune and allergic diseases (435, 436). An association of allergic asthma with IL-4 was clinically demonstrated when the airway hyper-responsiveness significantly increased after the nebulized administration of IL-4 (436). The monoclonal antibody, designed to block IL-4, is used as a drug (dupilumab) in the treatment of atopic dermatitis and moderate to severe forms of asthma (434, 437). Additionally, allergic diseases were reported to be dependent on Th2 and the production of Th2 cytokines, whereas autoimmune diseases depend on Th1 and cytokines related to it (435). The role of IL-4 as an anti-inflammatory cytokine in autoimmune diseases can be speculated from its protective role in diabetes and rheumatoid arthritis (432, 438). The anti-inflammatory effect of IL-4 decreases imbalance in Th1 versus Th2 lymphocytes and decreases the secretion of Th1 cytokines together with matrix metalloproteinases in rheumatoid arthritis. Simultaneously, also the secretion of the cytokine antagonists, like soluble IL-1 and soluble TNF-α receptors, is increased (439). In two animal models of rheumatoid arthritis the overexpression of IL-4 prevented the development of arthritis (440). The experimental increased production of IL-4 prevented development of diabetes in non-obese diabetic (NOD) mice, an animal model of T1DM (441).

1.4.4.1   The rs2243250 polymorphism of the *IL4* gene

The *IL4* (interleukin 4 gene), located on the long arm of chromosome 5 (5q31), is 0.9kb long and contains 4 exons (442). This highly conserved region of chromosome 5 is a cluster of genes: *IL3*,

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IL4, *IL5*, *IL9*, *IL13*, *IL15*, and the gene for the granulocyte-macrophage colony-stimulating factor (GM-CSF) with gene *IL4* somewhere in the middle. The expression of *IL4* is exactly regulated at the level of gene transcription. The promoter for the *IL4* gene has multiple binding sites for transcription proteins, at least five for the nuclear factor of activated T-cells (NF-AT), signal transducer and the activator of transcription-6 (STAT-6), transcription factor GATA-3 and many others. It is not known, what transcription factors bind in the region near polymorphism rs2243250 (g.-590C>T, position relative to transcription start site) (433, 443). Rosenwasser reported that this polymorphism is functional, with a threefold higher promoter activity (444). Tindal and co-workers analysed the 5q31.1 region and reported a modest increased risk for prostate cancer with rs2243250 polymorphism in The Risk Factors for Prostate Cancer study population from Australia. Concentrations of mRNA in the prostate tissue and serum levels of IL-4 were not statistically significant (445). Opposite to previous reports, an increased activity of this polymorphism was not found in experiments of Sun and co-workers. They reported decreasing concentrations of *IL4* mRNA from genotype CC to CT, to TT. Genotype TT had the lowest level of *IL4* mRNA. Carriers of T allele [rs2243250:T] in the Chinese Han population have a significantly increased risk for rheumatoid arthritis (439). Researches show that the rs2243250 polymorphism could be linked to atopic dermatitis (446), multiple sclerosis, rheumatoid arthritis (447), and atopic asthma (448). Two studies found a positive link between DM and rs2243250 polymorphism. In the Egyptian study a significant difference in alleles and genotypes of the rs2243250 polymorphism was found between T2DM patients and healthy controls. Alsaid and co-workers proposed that rs2243250 polymorphism could be a susceptibility marker for the development of DM in Egyptian population (449). In a Taiwanese study a positive association between T2DM and the rs2243250 polymorphism was reported. Compared to the Egyptian study, the Taiwanese one was much larger with 425 T2DM patients and 148 healthy subjects (450).

In the field of DN only two studies were found and both showed a positive association with the rs2243250 polymorphism. The study from South-eastern Iran found an association between the rs2243250 polymorphism and T2DM in patients with proteinuria in comparison to healthy control subjects (451). In a Northern Indian population, Neelofar and co-workers carried out a study confirming the previous findings about a positive association between DN in patients with T2DM and the rs2243250 polymorphism. In the same study they did not find significant differences in the allele distribution between T2DM patients and healthy volunteers (452). In our study, we investigated a possible association between the rs2243250 polymorphism and DN in patients with T2DM.

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1.5   NUCLEAR RECEPTORS

Diabetes mellitus and its chronic complications are essentially chronic, sterile, low-grade inflammations. Metabolic control, energy balance and inflammation are tightly connected. Energy and metabolic homeostasis are to a larger extent controlled by gene transcription, which involves a close cooperation between transcription factors, co-factors and transcription apparatus. This complex regulatory mechanism increases the expression of genes involved in mitochondrial function and their biogenesis. The precise regulation is mainly under the control of peroxisome proliferator-activated receptor-γ coactivator-1α (PPARGC-1α or PGC-1α). An inappropriate function of this co-factor results in the disturbed function of mitochondria. Mitochondrial dysfunction is tightly connected to chronic inflammations like DM. Mitochondria are energy power stations of cells that produce ATP through oxidative phosphorylation. They are an important source of ROS in physiological and pathological conditions. Mitochondria are involved in the maintenance of the redox potential, calcium homeostasis, and have a role in apoptosis. Mitochondria dysfunction and oxidative stress are the background of many chronic and degenerative diseases. Recently they have been recognized as important signal organelles or centres where intracellular and indirectly extra-cellular influences are integrated. Mitochondria respond to such stimuli with changed metabolism and by activating stress-related signalling pathways (453). Mitochondrial dysfunction or damage leads to ROS production, reduction of cytoplasmic NAD+, potassium efflux, and aberrant calcium mobilization. The presence of ATP in an extracellular space is a sign of danger, a DAMP molecule that binds to TLR and other receptors of an innate immune response. Additionally, the release of mitochondrial DNA, cardiolipin release from inner mitochondrial membranes or cytochrome c also function as DAMP molecules that trigger innate immune response through the activation of NLRP3 inflammasome (454). In that way the injury of mitochondria or their dysfunction could start inflammation and maintain its chronic condition. Furthermore, stress enhances the energy demand of the cells. Dysfunctional mitochondria cannot cope with increased energetic demand and increase concentrations of AMP and adenosine. Adenosine binds to the adenosine monophosphate-activated kinase (AMPK) complex, which starts multiple signalling pathways (455). Most inflammatory cells dramatically increase metabolic rate after activation and differentiation in inflammation. A peroxisome, as the last discovered organelle in the cell, participates in the lipid metabolism and is a major source of ROS in the cell. This includes the participation in fatty acid α- and β-oxidation, phospholipid and bile acid synthesis, as well as in the metabolism of glyoxylate, polyamine and some other amino acids, which are part of the signalling processes that modulate innate immunity, inflammation and cell differentiation. Communication and cooperation between peroxisome and mitochondria are important for maintaining homeostasis of both organelles for the proper functioning of cells (456). The expression of genes involved in the peroxisome fatty acid oxidation managed by the members of the PPAR family (PPARα, PPARβ/δ and PPARγ). These nuclear receptors that form complexes

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with retinoid X receptors (RXRs) and attached ligands regulate many physiological processes in the cell (457). PPARs were detected and got their name as biological receptors for a group of fibrate-like substances that trigger the proliferation of peroxisomes (458).

1.5.1   PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-γ (PPARγ)

Peroxisome proliferator-activated receptor-gamma (PPARγ) belongs to a subfamily of the nuclear receptor superfamily of ligand-inducible transcription factors that regulate the expression of gene networks involved in adipogenesis, lipid metabolism, inflammation, and metabolic homeostasis (459). Moreover, PPARγ plays an important role in glucose homeostasis and is the molecular target of the thiazolidinedione class of insulin-sensitizing agents (460).

1.5.1.1   The rs1801282 polymorphism of the *PPARG* gene

The *PPARG* (peroxisome proliferator activated receptor gamma gene) is located on chromosome 3p25 and contains 11 exons that span more than 140,000 bases (461). There are two distinct isoforms of the PPAR-γ protein, i.e. PPARγ1 and PPARγ2, which originate from the same gene via alternative promoter usage and mRNA splicing (461). PPARγ2 contains 30 additional amino acids at the NH2-terminal end (460). Its expression is primarily restricted to the adipose tissue, where it is stimulated by insulin action (460). The most common functional polymorphism in the *PPARG* gene is a **C**CA to **G**CA missense mutation (rs1801282:C>G) in codon 12 of exon B, which results in the replacement of proline with alanine (p.Pro12Ala) in the N-terminal domain of the PPARγ2 isoform (462).

The rs1801282:G allele has been shown to reduce the transcriptional activity of the PPARγ2 by decreasing its binding affinity to DNA (463) and has been associated with a modestly decreased risk of T2DM (464).

Several association genetic studies have reported a relation between the *PPARG* rs1801282 polymorphism and DN (465, 466, 467, 468). However, the results of the association genetic studies published so far have not been entirely consistent (469). In two recent meta-analyses (470, 471), including 9,176 and 9,357 subjects, with T2DM the *PPARG* rs1801282 polymorphism was significantly associated with decreased risk of DN. When stratified by ethnicity, Caucasians with the *PPARG* rs1801282 polymorphism showed a decreased risk of DN in T2DM, while the Asians did not (470, 471). Recently, Lapice and co-workers showed that the *PPARG* rs1801282 polymorphism is protective against the progression of DN and deterioration of renal function in subjects with T2DM (567).

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1.5.2 PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-γ COACTIVATOR-1α (PGC-1α)

A peroxisome proliferator-activated receptor-γ co-activator-1α (PPARGC-1α or PGC-1α) is a powerful transcriptional co-activator of several nuclear receptors, including PPARG that plays a principal role in energy expenditure and glucose metabolism (472). The PGC-1α is a 91 kDa protein with binding sites for transcription and activating factors. When PGC-1α binds with estrogen-related receptor-γ (ERRγ), the N terminal part stabilizes with conformational changes, which is important for a proper tertiary structure of the ligand binding domain. The primal flexibility of the molecule enables the PGC-1α to bind a vast variety of ligands, link the transcription complex and co-activate transcription, despite the fact that PGC-1α alone does not bind to DNA (473). An additional astonishing feature is its capability to respond to a wide variety of physiological signals, generated intracellularly or coming from the environment. Furthermore, all PGC-1α effects are in tissue and cell specific manner or the same signal can trigger different effects in different cell types (474). Changes in the environment, like low temperatures, activities that require energy, such as exercises or inflammation, increase its concentration. Its concentration changes quickly because of the short half-life. The post-translation modification, with acetylation, phosphorylation and methylation, affects PGC-1α stability and activity. Ubiquitination leads to proteasome degradation (475).

PGC-1α has several functions. It is named "the master regulator of mitochondrial functions", meaning that it co-activates genes for mitochondrial biogenesis and enzymes for oxidative metabolism. It is involved in glucose metabolism on several levels, from influencing insulin secretion from β-cells, glucose uptake, insulin resistance, gluconeogenesis, to glucose utilization. Additional functions are fatty acid oxidation, thermogenesis, fat browning, endothelial cell migration, angiogenesis and fiber type switching in skeletal muscles (476). PGC-1α is connected to obesity, T2DM and its complications, cardiomyopathies, neurodegenerative diseases and aging (477). Usually, most co-activators are widespread but this does not apply to PGC-1α, which is found only in organs with high-energy demand, like the heart, brain, skeletal muscles, liver, adipose tissue and kidneys (478).

In animal models of DN, mRNA and protein PGC-1α are downregulated in renal tubular cells. With decreased concentrations and activity of PGC-1α also the mitochondrial function is decreased. The signalling pathway AMPK/SIRT1/PGC-1α is the main regulator in energy metabolism and mitochondrial biogenesis. A recent research linked the AMPK/SIRT1/PGC-1α signalling pathway with the progression of DN (476). The natural phenol resveratrol is produced in several plants as a response to injury and can be found in the skin of grapes and blueberries. In animal experiments resveratrol decreased the markers of glomerular inflammation, matrix expansion and albuminuria in db/db mice. It increased the phosphorylation of AMPK and induced

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signalling through the AMPK/SIRT1/PGC-1α signalling pathway. Decreased lipotoxicity and glucotoxicity lead to lower oxidative stress and reduced cells apoptosis (479). Metformin, a well-known antidiabetic drug and the first one to start antidiabetic drug therapy, also increases AMPK and subsequently PGC-1α (480). Telmisartan and fenofibrat mediate the renoprotective effect by increasing PGC-1α and decreasing oxidative stress (481, 482). As PGC-1α increases the transcriptional activity of PPAR-γ, defects in PGC-1-α expression and regulation may contribute to the pathophysiology of T2DM (483).

1.5.2.1   The rs8192678 polymorphism of the *PPARGC1A* gene

The *PPARGC1A* (*PGC-1α,* PPARG coactivator 1 alpha gene) is localized on chromosome 4p15.1–2 (484). The G to A substitution (**G**GT to **A**GT) at position 1444 in exon 8 of the *PPARGC1A* gene (rs8192678), resulting in the substitution of glycine with serine (p.Gly482Ser) in codon 482, has been associated with a lower gene expression and reduced PGC-1α protein activity. In addition, the half-life of the protein product of the allelic variant rs8192678:A is shorter than that of a more common allele variant [rs8192678:G] in humans (485).

In T2DM patients decreased concentrations of *PGC1A* mRNA were found in the skeletal muscle and β-cells as well as in adipocytes. These concentrations were even lower in carriers of the rs8192678:A polymorphism in comparison to homozygotes for allele rs8192678:G.

Polymorphism rs8192678 [rs8192678:A] bears a 34% increased risk for T2DM in the Danish population (486). A positive association between the rs8192678 polymorphism [rs8192678:A] and T2DM was found in three studies in North India (487, 488). A positive association was found in North Chinese, Iranian, Tunisian and Caucasian (Slovene) populations (489, 490, 491, 492). In the Slovene population, 305 patients with T2D and 240 healthy controls were analysed and a 1.9-fold higher risk for the development of T2DM was calculated for carriers of the rs8192678AA genotype (492). Studies with a negative association were done in French and British populations (Caucasians) (493, 494). Negative association studies were also found for Pima Indians, Colorado (USA), Japanese and Eastern Chinese Han populations (495, 496, 497, 498). The first meta-analysis, which included eight studies (8536 participants), revealed only a modest role of the rs8192678 polymorphism [rs8192678:A] for the risk of T2DM (467). The second meta-analysis, which included 23 studies (7539 T2DM patients and 9562 controls), showed a significant association between the rs8192678 polymorphism [rs8192678:A] and T2DM. In a sub-analysis, the significance for a positive association was greater for the Indian population than in a combined calculation, while no association was found for Caucasian and East Asian populations (499). A recent meta-analysis, which included eight studies (three from India (5499 T2DM patients and 5715 controls)), also found a significant association with T2DM (500). The rs8192678 polymorphism of the *PPARGC1A* gene was associated with DN in Asian Indians. The study

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included 255 T2DM patients without DN, 141 T2DM patients with DN and 571 healthy controls. The risk for DN was 2.14-fold higher in carriers of rs8192678GA and 8.01-fold higher for carriers of the rs8192678AA genotype (501). A significant association with DN in patients with T2DM was found also in a Korean population (502). A significant association between persistent albuminuria and rs8192678 polymorphism was reported in a Caucasian population with T2DM (Great Britain). Genotypes rs8192678AA and rs8192678GA had a 70% increased risk for DN in comparison to the rs8192678GG genotype. Minor A allele, [rs8192678:A] is associated with an approximately 50% increased risk for albuminuria. Interestingly, the study did not prove a statistical significant difference between rs8192678GG and rs8192678AA genotypes. It is not clear why (503).

The *PPARG* rs1801282 and the *PPARGC1A* rs8192678 polymorphisms seem to be related to T2DM and its complications also in the Slovene population. The rs8192678 polymorphism of the *PPARGC1A* gene was associated with an increased risk of T2DM (492), as well as with DR in subjects with T2DM (504). An association of both polymorphisms with waist circumference and progression of carotid intima-media thickness was demonstrated in individuals with T2DM of Slovenian origin (505, 506). The purpose of this study is to clarify whether common polymorphisms of the *PPARG* gene rs1801282 and the *PPARGC1A* gene rs8192678 are associated with DN in Slovenian patients with T2DM.

2 HYPOTHESIS AND AIMS OF THE RESEARCH

2.1 HYPOTHESIS

Polymorphisms of inflammatory genes may influence subclinical chronic inflammation and progression of DN and may therefore be implicated in the development of DN. We anticipated that a set of polymorphisms from our candidate genes could be associated with DN in patients with T2DM.

3 AIMS AND PURPOSE OF THE RESEARCH

3.1 GENERAL AIM

The general objective of this doctoral dissertation is to determine a possible association between chosen set of inflammatory polymorphisms with proposed influences on inflammatory process and DN in T2DM patients.

3.2   SPECIFIC AIMS

We wanted to know if:

1) There is an association between risk genotypes of chosen polymorphisms and DN in our set of patients with T2DM;

2) There is a difference in serum concentrations of cytokines (ICAM-1, PECAM-1, IL-18 and IL-10) in T2DM patients with and without DN;

3) Polymorphisms may affect the serum concentration of cytokines; were serum concentrations of sICAM-1, sPECAM-1, IL-18 and IL-10 affected by the belonging polymorphisms (*ICAM1* rs5498 and rs1799969; *PECAM1* rs668; *IL18* rs187238; *IL10* rs1800896) in our subset of subjects with T2DM?

4 SUBJECTS AND METHOD

4.1 SUBJECTS

In our retrospective association study we enrolled 651 unrelated Caucasians with T2DM of more than 10 years duration from outpatient clinics of the University Medical Centre Maribor and the General Hospitals of Murska Sobota and Slovenj Gradec.

The study group consisted of 276 subjects with DN (cases) and 375 subjects without clinical signs of DN (control group).

Patients were classified as having T2DM according to the current American Diabetes Association (507). Diagnosis of DN was made according to the WHO (World Health Organization) 1999 diagnostic criteria (508).

Exclusion criteria: no diabetes type 2, other known kidney diseases, except for DN, renal stones, haematuria, present urinary infection, clinical sign of cardiac decompensation (NYHA II-IV), poor glycemic control (glycated hemoglobin (HbA$_1$c) > 10.0%), age older than 80 years, alcoholism, and patients unable to participate in the study. To avoid the confounding effect of severely impaired kidney function, patients on dialysis were not enrolled in the study. After an informed consent for the participation in the study was obtained, a detailed interview was conducted. Information on smoking, presence, and family history of CVD, duration of arterial hypertension and T2DM, T2DM management and complications (DR, DN, and DF), therapy, and routine laboratory measurements were obtained from their medical records.

After the interview, the physicians physically examined all patients at their regular medical control at the study entry. Measurements of body weight, height and sitting blood pressure have been made. An examination of retinal fundi for the presence and staging of DR was done by an ophthalmologist for all participants. All data were registered in a single questionnaire.

All patients included in the study went to the central hospital laboratory in each clinical hospital three times over a period of three months, one visit each month.

Every laboratory testing was done in the morning, between 7:30 and 9:00 a.m. and in the fasting state. On the first laboratory visit, blood was take from the cubital vein for two 5 ml laboratory tubes with Na-EDTA and one classical 7 ml laboratory tube for biochemical analysis.

Second morning urine was collected from the participants during all three laboratory visits.

Urinalyses have been done for the exclusion of infection and hint on possible other kidney diseases.

In the morning sample urine, the amount of albumins has been determined and UACR was calculated.

Normoalbuminuria was defined when the UACR was < 3.0 mg/mmol in both sexes. Moderately increased urinary albumin excretion (microalbuminuria) is defined as range UACR in range 3.0 – 30 mg/mmol. A higher value was known as severely increased albuminuria (macroalbuminuria). Proteinuria is defined when the concentration of albumins in sample urine is > 300 mg/l (509).

Persistent normoalbuminuria or albuminuria was defined when at least 2 out of 3 urine samples were in the same group. Patients received information about the important parameters for proper urine collection and what influences analysis. In the cases of pathological results of urinalyses or signs of infection, the urine collection was repeated after several days. Cystatin C and MDRD study equation were used to calculate eGFR.

Patients were gathered in 2 groups, a group with persistent normoalbuminuria and a group with persistent microalbuminuria or macroalbuminuria, respectively. The group with microalbuminuria or macroalbuminuria formed cases, while the other group with norm-albuminuria was defined as controls.

The study was approved by the national medical ethics committee of Slovenia and was performed in compliance with the Helsinki Declaration.

4.2 METHODS

4.2.1 LABORATORY TESTS

Fasting glucose, HbA_1c, hemoglobin (Hb), urea, creatinine, cystatin C, total cholesterol, low-density lipoproteins (LDL), high-density lipoproteins (HDL) and triglycerides (TG) were determined in serum by standard biochemical methods. The UACR was determined in three urine samples for each patient, according to diagnostic criteria. The MDRD study equation and cystatin C were used to calculate eGFR).

4.2.2 DETERMINATION OF ICAM-1, PECAM-1, IL-10 AND IL-18

Levels of serum cytokines (sICAM-1, sPECAM-1, IL-10 and IL-18) were analysed in the Department of Laboratory diagnostics of the Maribor Clinical Centre by the enzyme-linked immunosorbent assay (ELISA).

Soluble PECAM-1 and IL-18 concentrations were analysed from serum samples using a manual ELISA method according to the manufacturer's instructions: Human sPECAM-1 ELISA (ALPCO, Salem New Hampshire, USA) and Human IL-18 ELISA (Medical & Biological Laboratories CO., LTD., Ina, Nagano, Japan), respectively. ELISA is a sandwich enzyme-linked immunosorbent assay based on two monoclonal antibodies directed against different epitopes on the analysed molecule. The microwells of the assay were pre-coated with the first antibody against human s-PECAM-1 or IL-18, where an analysed diluted tested serum was added. A second horseradish

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peroxidase (HRP) conjugated antibody was added to the analysed molecules. Following incubation, unbound HRP-conjugated antibodies were removed during washing procedures. Substrate solutions reactive with the HRP enzyme were added to the microwells and incubated according to the instructions. The reaction in which a coloured product was formed in proportion to the amount of tested molecules was terminated by the addition of acid. The absorbance of the coloured product was measured at a wavelength of 450 nm with a spectrophotometer (Magellan V6.6 TRA 2PC PAC Sunrise, TECAN, Austria)

The results were read from a standard curve (prepared from the standard dilutions of PECAM-1 or IL-18). The coefficients of intra-assay variation were 1.7% and 7.2% and 7.4% and 7.5% for the inter-assay variation for PECAM-1 and IL-18, respectively.

Soluble ICAM-1 and IL-10 serum concentrations were analysed using a multiplex method (LuminexÒ screening Assay, R&D systems, Minneapolis, Minnesota, USA). Analyte-specific antibodies were pre-coated onto colour-coded microparticles. Microparticles, standards, and serum samples were pipetted into wells and antibodies fixed on microparticles bound to the analysed molecules. After incubation and washing of unbound substances, the second set of specific biotinylated antibodies for analyses molecules was added to each well. Following incubation and washing, the streptavidin-phycoerythrin conjugate that binds to the biotinylated detection antibody was added. The final analysis was made with LuminexÒ analyser (Luminex 200TM xMAP Technology, Luminex corporation, Austin, Texas, USA) using two lasers for a separate detection of specific microparticles (which molecule is being detected) and the other for measuring the phycoerythrin-derived signal, which is in direct proportion to the amount of tested molecules.

4.3   GENETIC ANALYSIS

4.3.1   GENOMIC DNA EXTRACTION

Genomic DNA extraction was carried out in the Laboratory for Molecular Genetics at the Institute for Histology and Embryology, Medical Faculty in Ljubljana. Blood samples were collected in 6 mL hemogram vacutainers with 0.18M EDTA. The blood and body fluid spin protocol V3 for the removal of DNA from leukocytes in venous peripheral blood was carried out with the QIAcube robot machine (Qiagen GmbH, Hilden, Germany) following the manufacturer's DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) protocol: buffer AL, 96% ethanol, buffer AW1, buffer AW2, buffer AE and appropriate amount of proteases (285µL of proteases / 200µl of blood). According to the instructions, 4-12µg of genomic DNA should be extracted from 200µL of blood. Genomic DNA was then stored at -200C until genotyping.

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4.3.2   GENOTYPING

Genotyping was mainly performed by LGC Genomics from Queens Road, Teddington, Middlesex, United Kingdom, using their proprietary KASPar polymerase chain reaction technique (https://www.lgcgroup.com/genotyping/#.WvLojjhDvz4). Eight SNPs were analysed in the LGC Genomics Laboratory: *IL18* (rs187238), *IL12B* (rs3212227), *IL10* (rs1800896), *PPARG* (rs1801282), *ICAM1* (rs5498 and rs1799969), *PECAM1* (rs668), *CCR2* (rs1799864).

Five functionally tested SNPs were genotyped in the Laboratory for Molecular Genetics on the Institute for Histology and Embryology, Medical Faculty in Ljubljana: *IL4* (rs2243250/ C__16176216_10); *PPARGC1A* (rs8192678/ C__1643192_20); *CCL5* (rs2280788/ C__15874396_20, rs2107538/C__15874407_10); CCR5 (rs1799987/C__11988176_10). StepOne(TM) Real-Time PCR System running Software version 2.2 (Applied Biosystems, Foster City, California, USA) was performed for PCR cycling under the manufacturers' universal conditions for measuring fluorescence levels and scoring genotypes by analysing data for allele discrimination. The 5'Nuclease Assay with TaqMan probes are covered by patents owned by Roche Molecular Systems, Inc. and F. Hoffman-LaRoche Ltd. (Basel, Switzerland). Universal thermal cycling protocol for amplifying DNA involves 3 steps: strand denaturation, primer annealing, and primer extension, and is typically performed for 30 to 40 cycles. Universal thermal cycling conditions: 95ºC for 10 minutes activates the AmpliTaq Gold Polymerase; the PCR is performed at 95ºC for 15 seconds and an anneal/extend step at 60 ºC for 1 minute.

4.4   STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS program for Windows version 20 (SPSS Inc. Illinois). SNP was evaluated for Hardy-Weinberg equilibrium (HWE) by using an HWE calculator (http://ihg.gsf.de/). Continuous variables were compared by either unpaired Student's t-test or the Mann-Whitney. Chi-square test was used to compare discrete variables. Normal distribution of data was checked using the Kolmogorov-Smirnov test. Continuous variables were reported as mean ± standard deviation when normally distributed, and as median values (interquartile range (IQR)) when asymmetrically distributed, or as the number and percent of patients (categorical variables). Possible deviations from Hardy-Weinberg equilibrium were evaluated with Pearson's goodness-of-fit chi-square test (1 degrees of freedom). To assess the independent contribution of the genetic polymorphism to the risk of diabetic nephropathy, we used logistic regression analysis, which included the possible confounders. A $p < 0.05$ was considered statistically significant.

5　　　RESULTS

5.1　　　THE DEMOGRAPHIC AND CLINICAL CHARACTERISTICS

The demographic and clinical characteristics of the cases and control subjects are listed in *Table 1*. There were no significant differences between groups with respect to age, sex, duration of T2DM, diastolic blood pressure (DBP), body mass index (BMI), smoking status, family history of CVD, duration of DR, eGFR, serum hemoglobin (Hb), total cholesterol, HDL, and LDL cholesterol levels. On the other hand, statistically significant differences were observed in the following parameters: duration of hypertension, systolic blood pressure (SBP), presence of CVD, UACR, as well as serum fasting glucose, HbA1c, urea, creatinine, and TG levels. Cases also showed significantly more chronic diabetic complications, such as DR and DF, but not DNeuro.

Differences in parameters reflecting renal function (serum creatinine, cystatin C, eGFR, and UACR) confirmed chronic kidney disease in diabetic subjects with DN. Cystatin C was significantly higher in subjects with DN ($p < 0.001$) (*Table 1*).

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Table 1: Clinical and laboratory characteristics of cases and controls.

	Cases (DN+)	Controls (DN-)	p-value
No.	276	375	
Sex (M)	59.1%	52.4%	0.1
Age (years)	64.75±9.15	63.75±8.0	0.13
Duration of T2D (years)	14.0 (10.0-19.0)	13.5 (11.0-18.3)	0.84
Duration of hypertension (years)	10 (5-17)	10 (4-15)	0.06
SBP [mm Hg]	155.27±18.92	149.84±19.63	**<0.001**
DBP [mm Hg]	84.87±11.63	84.06±11.42	0.36
BMI	31.3±4.68	30.77±5.0	0.23
Active smokers	6.6%	8.9%	0.31
CVD	20.0%	12.2%	**0.007**
Family history of CVD	41.3%	58.7%	0.91
DR	37.8%	24.6%	**<0.001**
Duration of DR (years)	3.94±3.11	6.54±7.03	0.23
DNeur	9.1%	6.0%	0.38
DF	15.5%	8.1%	**0.03**
S-HbA1c [%]1	7.98±1.38	7.65±1.14	**<0.001**
S-fasting glucose [mmol/l]	9.03±2.76	8.51±2.53	**0.01**
S-Hb [g/l]	139.39±14.91	139.40±12.96	0.99
S-urea [mmol/l]	7.35±3.73	6.25±1.91	**<0.001**
S-creatinine [μmol/l]	81.0 (66.0-103.0)	76.0 (64.0-89.8)	**0.002**
male sex	92.0 (71.5-107.0)*	82.5 (69.0-95.0)*	**0.006**
female sex	70.5 (55.8-88.3) **	70.0 (59.0-81.0) **	0.7
eGFR [MDRD equation, ml/min]	72.6±19.74	75.22±15.16	0.22
male sex	71.97±19.45*	77.66±14.33*	**0.002***
female sex	74.31±20.72**	72.45±15.69**	0.13**
S-cystatin C [mg/l]	0.8 (0.7-1.1)	0.7 (0.6-0.9)	**<0.001**
S-Total cholesterol [mmol/l]	4.62 ± 1.17	4.55 ± 0.99	0.42
S-HDL [mmol/l]	1.23 ± 0.35	1.26 ± 0.36	0.29
S-LDL [mmol/l]	2.59 ± 0.95	2.57 ± 0.80	0.73
S-TG [mmol/l]	1.6 (1.1-2.5)	1.5 (1.0-2.3)	**0.04**
U-albumin/creatinine ratio [g/mol] - sample No. 1	9.4 (4.5-33.6)	1.0 (0.6-1.6)	**<0.001**
U-albumin/creatinine ratio [g/mol] - sample No. 2	10.6 (4.5-33.9)	1.0 (0.7-1.7)	**<0.001**
U-albumin/creatinine ratio [g/mol] – sample No. 3	9.5 (4.3-33.9)	1.1 (0.7-1.8)	**<0.001**

The values represent mean ± standard deviation. Bold indicates statistically significant results.
[1]The average value for hemoglobin A1c (HbA1c).
*Comparing eGFR in men with DN versus men without DN.
**Comparing women with DN versus women without DN

5.2 GENETIC ANALYSIS

5.2.1 ADHESION MOLECULES

5.2.1.1 Polymorphisms rs5498 and rs1799969 of the *ICAM1* gene

No significant differences in the frequencies of GG, GA and AA in rs5498, or AA, GA and GG in the rs1799969 polymorphism of the *ICAM1* gene. Similarly, no significant differences were observed in allele frequencies (*Table 2*).

A logistic regression analysis was used (*Table 3*) to evaluate whether these SNP were independently associated with DN after adjusting for duration of hypertension, SBP, CVD, DR, DF, HbA$_1$c, fasting glucose, urea, creatinine, cystatine C, and urine albumin/creatinine ratio. We did not find a statistically significant association of either rs5498 or rs1799969 with DN.

In a subgroup population of 120 diabetics with DN, the serum concentration of sICAM-1 was analysed according to different genotypes of rs5498 and rs1799969. We did not find any significant statistical differences (*Table 4*) with this analysis either.

Table 2: Distribution of rs5498 and rs1799969 polymorphism genotypes and alleles in patients with diabetic nephropathy (cases) and in those without diabetic nephropathy (controls).

		Cases (276)	Controls (375)	p-value
ICAM1 rs5498	GG	61 (22.2)	70 (18.6)	
	GA	136 (49.1)	191 (51.0)	0.5
	AA	79 (28.7)	114 (30.4)	
	G allele (%)	258 (46.7)	331 (44.1)	
	A allele (%)	294 (53.3)	419 (55.9)	0.4
	HWE	0.86	0.52	
ICAM1 rs1799969	AA	4 (1.5)	6 (1.6)	
	GA	59 (21.5)	65 (17.3)	0.4
	GG	213 (77.1)	304 (81.1)	
	A allele (%)	67 (12.1)	77 (10.3)	
	G allele (%)	485 (87.9)	673 (89.7)	0.3
	HWE	0.97	0.25	

HWE: p-values were computed using Pearson's goodness-of-fit chi-square test (1 df).

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Table 3: Association between the *ICAM1* rs5498 and rs1799969 polymorphisms and DN assessed by logistic regression analysis.

Inheritance model	Genotype	Cases (276)	Controls (375)	Adjusted OR, 95 % CI / p-value
rs5498 co-dominant	GG	61 (22.2)	70 (18.6)	1.29 (0.53-3.13)/0.6
	GA	136 (49.1)	191 (51.0)	1.02 (0.51-2.06)/0.9
	AA	79 (28.7)	114 (30.4)	reference
rs1799969 co-dominant	AA	4 (1.5)	6 (1.6)	0.24 (0.01-5.40)/0.4
	GA	59 (21.5)	65 (17.3)	1.46 (0.69-3.08)/0.3
	GG	213 (77.1)	304 (81.1)	reference

p-values were adjusted for duration of hypertension, SBP, CVD, DR, DF, HbA$_1$c, S-fasting glucose, S-urea, S-creatinine, S-cystatin, UACR. Odds ratio (OR); Confidence interval (CI)

Table 4: Serum ICAM-1 levels in a subpopulation of 120 diabetics with DN according to different genotypes of *ICAM1* rs5498 and rs1799969 polymorphisms.

Polymorphism	Genotype (number)	ICAM-1 (ng/ml)	p-value
rs5498	GG (34)	441.5±277.9	
	GC (53)	504.9±347.4	0.5
	CC (34)	414.6±390.2	
rs1799969	AA (2)	364.5±151.7	
	GA (29)	492.2±342.2	0.8
	GG (89)	457.6±346.8	

Values are mean ± SD. One-Way ANOVA

5.2.1.2   The polymorphism rs668 of the *PECAM1* gene

The genotype distribution and allele frequencies of rs668 (Leu125Val) polymorphism in subjects with DN (cases) and those without DN (controls) are presented in *Table* 2. Univariate analysis did not reveal significant differences in the genotype or allele frequencies between T2DM cases and controls (*Table 5*). The genotype distribution did not significantly deviate from the Hardy-Weinberg equilibrium (*Table 5*). Logistic regression analysis adjusted for different confounders did not reveal significant effect of the Leu125Val polymorphism on DN risk in subjects with T2DM (*Table 6*). No association was found between the Leu125Val polymorphism and serum sPECAM-1 levels in a subpopulation of 120 diabetics with DN (*Table 7*).

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Table 5: Distribution of *PECAM1* rs668 polymorphism genotypes and alleles in patients with DN (cases) and in those without diabetic nephropathy (controls).

Polymorphism		Cases (276)	Controls (375)	p-value
rs668	GG	56 (20.4)	64 (17.0)	
	GC	144 (52.0)	189 (50.3)	0.3
	CC	76 (27.6)	122 (32.7)	
	G allele (%)	256 (46.4)	317 (42.3)	0.2
	C allele (%)	296 (53.6)	433 (57.7)	
	HWE	0.42	0.53	

HWE: p-values were computed using Pearson's goodness-of-fit chi-square (1 df)

Table 6: Association between the *PECAM1* rs668 polymorphism and DN assessed by logistic regression analysis.

Inheritance model	Genotype	Cases (276)	Controls (375)	Adjusted OR, 95 % CI / p-value
rs668 co-dominant	GG	56 (20.4)	64 (17.0)	0.85 (0.34-2.11) / 0.7
	GC	144 (52.0)	189 (50.3)	1.65 (0.83-3.26) / 0.9
	CC	76 (27.6)	122 (32.7)	reference

p-values were adjusted for duration of hypertension, SBP, CVD, DR, DF, HbA1c, S-fasting glucose, S-urea, S-creatinine, S-cystatin, UACR. Odds ratio (OR); Confidence interval (CI)

Table 7: The serum *PECAM1* levels in a subpopulation of 120 diabetics with DN according to different genotypes of *PECAM1* rs668 polymorphism.

Polymorphism	Genotype (number)	PECAM-1 (ng/ml)	p-value
rs668	GG (25)	95.6±21.7	
	GC (61)	96.0±22.5	0.9
	CC (34)	96.8±20.8	

Values are mean ± SD. One-Way ANOVA

5.2.2   CHEMOKINES

5.2.2.1   Polymorphisms rs2107538, rs2280788 of the *CCL5* gene and the polymorphism rs1799987 of the *CCR5* gene

Distribution of rs2107538, rs2280788 and rs1799987 genotypes and alleles in patients with diabetic nephropathy (cases) and in those without diabetic nephropathy (controls) is shown in *Table 8*. There were no statistically significant differences in either genotype or allele distribution in cases and controls.

Logistic regression analysis was used (*Table 9*) to evaluate whether these SNPs were independently associated with DN after adjusting for duration of hypertension, SBP, CVD, DR, DF, HbA1c,

fasting glucose, urea, creatinine, cystatine C, UACR. We did not find a statistically significant association of rs2107538, rs2280788 and rs1799987 with DN.

Table 8: Distribution of rs2107538, rs2280788, and rs1799987 genotypes and alleles in patients with diabetic nephropathy (cases) and in those without diabetic nephropathy (controls).

Polymorphism		Cases (276)	Controls (375)	p-value
rs2107538	CC	181 (65.6)	235 (62.7)	
	CT	90 (32.6)	123 (32.8)	0.2
	TT	5 (1.8)	17 (4.5)	
	C allele	452 (81.9)	593 (79.1)	
	T allele	100 (18.1)	157 (20.9)	0.2
	HWE	0.1	0.8	
rs2280788	GG	253 (91.7)	347 (92.6)	
	GC	22 (7.9)	26 (6.9)	0.8
	CC	1 (0.4)	2 (0.5)	
	G allele	528 (95.7)	720 (96.0)	
	C allele	24 (4.3)	30 (4.0)	0.9
	HWE	0.5	0.06	
rs1799987	GG	42 (15.2)	75 (20.0)	
	GA	140 (50.7)	180 (48.0)	0.3
	AA	94 (34.1)	120 (32.0)	
	G allele	224 (40.6)	330 (44.0)	
	A allele	328 (59.4)	420 (56.0)	0.2
	HWE	0.4	0.6	

HWE: p-values were computed using Pearson's goodness-of-fit chi-square test (1 df).

Table 9: Association between the *CCL5* rs2107538, rs2280788, CCR5 rs1799987 polymorphisms and DN assessed by logistic regression analysis.

Inheritancemodel	Genotype	Cases (276)	Controls (375)	Adjusted OR, 95 % CI / p-value
rs2107538 co-dominant	CC	181 (65.6)	235 (62.7)	Reference
	CT	90 (32.6)	123 (32.8)	0.85 (0.57 – 1.28) / 0.4
	TT	5 (1.8)	17 (4.5)	0.38 (0.12 – 1.27) / 0.1
rs2280788 co-dominant	GG	253 (91.7)	347 (92.6)	Reference
	GC	22 (7.9)	26 (6.9)	1.45 (0.70 – 3.08) / 0.3
	CC	1 (0.4)	2 (0.5)	0.57 (0.05 – 6.54) / 0.7
rs1799987 co-dominant	GG	42 (15.2)	75 (20.0)	Reference
	GA	140 (50.7)	180 (48.0)	1.29 (0.76 – 2.19) / 0.3
	AA	94 (34.1)	120 (32.0)	1.24 (0.71 – 2.17) / 0.5

p-values were adjusted for duration of hypertension, SBP, CVD, DR, DF, HbA_1c, S-fasting glucose, S-urea, S-creatinine, S-cystatin and UACR. Odds ratio (OR); Confidence interval (CI)

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5.2.2.2   The polymorphism rs1799864 of the *CCR2* gene

Distribution of rs1799864 genotypes and alleles in patients with diabetic nephropathy (cases) and in those without diabetic nephropathy (controls) is shown in *Table 10*. There were no statistically significant differences in either genotype or allele distribution in cases and controls.

A logistic regression analysis was used (*Table 11*) to evaluate whether these SNP were independently associated with DN after adjusting for duration of hypertension, SBP, CVD, DR, DF, HbA$_1$c, fasting glucose, urea, creatinine, cystatine C, and UACR. We did not find a statistically significant association of rs1799864 (46295G/A) with DN.

Table 10: Distribution of rs1799864 genotypes and alleles in patients with DN (cases) and in those without DN (controls).

Polymorphism		Cases (276)	Controls (375)	p-value
rs1799864	AA	2 (0.7)	3 (0.8)	
	AG	65 (23.6)	82 (21.9)	0.9
	GG	209 (75.7)	290 (77.3)	
	A allele (%)	69 (12.5)	88 (11.7)	0.7
	G allele (%)	483 (87.5)	662 (88.3)	
	HWE	0.2	0.3	

HWE: p-values were computed using Pearson's goodness-of-fit chi-square test (1 df).

Table 11: Association between the *CCR2* rs1799864 polymorphism and DN assessed by logistic regression analysis.

inheritance model	Genotype	Cases (276)	Controls (375)	Adjusted OR, 95 % CI / p-value
rs1799864 co-dominant	AA	2 (0.7)	3 (0.8)	1.48 (0.20– 10.74) / 0.7
	AG	65 (23.6)	82 (21.9)	0.96 (0.63 – 1.47) / 0.9
	GG	209 (75.7)	290 (77.3)	Reference

p-values were adjusted for duration of hypertension, SBP, CVD, DR, DF, HbA$_1$c, S-fasting glucose, S-urea, S-creatinine, S-cystatin and UACR. Odds ratio (OR); Confidence interval (CI)

5.2.3   INTERLEUKINS

5.2.3.1   The polymorphism rs3212227 of the *IL12B* gene

Distribution of rs3212227 genotypes and alleles in patients with diabetic nephropathy (cases) and in those without diabetic nephropathy (controls) is shown in *Table 12*. There were no statistically significant differences in either genotype or allele distribution in cases and controls.

Logistic regression analysis was used (*Table 13*) to evaluate whether these SNP were independently associated with DN after adjusting for duration of hypertension, SBP, CVD, DR,

DF, HbA$_1$c, fasting glucose, urea, creatinine, cystatine C, and UACR. We did not find a statistically significant association of rs3212227 with DN.

Table 12: Distribution of rs3212227 polymorphism genotypes and alleles in patients with diabetic nephropathy (cases) and in those without diabetic nephropathy (controls).

Polymorphism		Cases (276)	Controls (375)	p-value
rs3212227	GG	10 (3.6)	19 (5.1)	
	GT	101 (36.6)	117 (31.2)	0.3
	TT	165 (59.8)	239 (63.7)	
	G allele (%)	121 (21.9)	155 (20.7)	0.6
	T allele (%)	431 (78.1)	595 (79.3)	
	HWE	0.3	0.3	

HWE: p-values were computed using Pearson's goodness-of-fit chi-square test (1 df).

Table 13: Association between the *IL12B* rs3212227 polymorphism and DN assessed by logistic regression analysis.

Inheritance model	Genotype	Cases (276)	Controls (375)	Adjusted OR, 95 % CI / p-value
rs3212227 co-dominant	GG	10 (3.6)	19 (5.1)	1.07 (0.45-2.56) / 0.9
	GT	101 (36.6)	117 (31.2)	1.31 (0.88-1.94) / 0.2
	TT	165 (59.8)	239 (63.7)	reference

p-values were adjusted for duration of hypertension, SBP, CVD, DR, DF, HbA$_1$c, S-fasting glucose, S-urea, S-creatinine, S-cystatin and UACR. Odds ratio (OR); Confidence interval (CI)

5.2.3.2 The polymorphism rs187238 of the *IL18* gene

The frequency of the rs187238 alleles in case and control group has been indicated in *Table 14*. As demonstrated in *Table 14*, the frequency of the G allele in DN patients was non-significantly higher (26.4%, p=0.5) than that in individuals without DN (24.5%).

As also shown in *Table 14*, the genotypic distribution of the rs187238 polymorphism in each group attained Hardy-Weinberg equilibrium (p> 0.05). The frequency of the GG, GC, and CC genotypes was 7.2, 34.7, and 58.1%, respectively, in the group without DN, compared with 6.2, 40.6, and 53.2%, respectively, in the group with DN.

Association between polymorphism rs187238 and DN was assessed by logistic regression analysis (*Table 15*) with adjustment for duration of hypertension, SBP, CVD, DR, DF, HbA$_1$c, S-fasting glucose, S-urea, S-creatinine, S-cystatin C, and UACR. We did not find a statistically significant association of rs187238 with DN according to co-dominant genetic model (*Table 15*).

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There is no statistically significant difference in serum IL-18 levels between patients with DN and without DN (p=0.2) (*Table 16*). Moreover, we divided patients with T2DM without DN into three groups according to their genotypes. There were no statistically significant differences in serum IL-18 levels between the three groups (p = 0.9) (*Table 16*).

Table 14: Distribution of rs187238 genotypes and alleles in T2DM patients with DN (Cases) and T2DM patients without DN (Controls).

Polymorphism		Cases (276)	Controls (375)	p-value
rs187238	GG	17 (6.2)	27 (7.2)	
	GC	112 (40.6)	130 (34.7)	0.3
	CC	147 (53.2)	218 (58.1)	
	G allele (%)	146 (26.4)	184 (24.5)	0.5
	C allele (%)	406 (73.6)	566 (75.5)	
	HWE	0.5	0.2	

HWE: p-values for the HWE were computed using Pearson's goodness-of-fit-chi-square (1df).

Table 15: Association between the rs187238 polymorphism and DN assessed by logistic regression analysis.

Inheritance model	Genotype	Cases (276)	Controls (375)	Adjusted OR, 95 % CI / p-value
rs187238 co-dominant	GG	17 (6.2)	27 (7.2)	0.74 (0.34-1.63)/0.5
	GC	112 (40.6)	130 (34.7)	1.25 (0.85-1.84)/0.3
	CC	147 (53.2)	218 (58.1)	reference

P-values were adjusted for duration of hypertension, SBP, CVD, DR, DF, HbA$_1$c, S-fasting glucose, S-urea, S-creatinine, S-cystatin and UACR. Odds ratio (OR); Confidence interval (CI)

Table 16: The serum levels IL-18 in a subpopulation of 165 T2DM patients without DN according to different genotypes of *IL18* rs187238 polymorphism.

Polymorphism	Genotype (number)	IL-18 (pg/ml)	p-value
rs187238	GG (9)	193.27 ± 53,3	
	GC (67)	204.20 ± 79.2	0.9
	CC (89)	199.20 ± 89.04	

Values are mean ± SD. One-Way ANOVA

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5.2.3.3 The polymorphism rs1800896 of the *IL10* gene

Distribution of rs1800896 genotypes and alleles in patients with diabetic nephropathy (cases) and in those without diabetic nephropathy (controls) is shown in *Table 17*. There were no statistically significant differences in either genotype or allele distribution in cases and controls.

Logistic regression analysis was used (*Table 18*) o evaluate whether these SNP were independently associated with DN after adjusting for duration of hypertension, SBP, CVD, DR, DF, HbA1c, fasting glucose, urea, creatinine, cystatine C, and UACR. We did not find a statistically significant association of rs1800896 (*IL10* -1028G/A) with DN.

Table 17: Distribution of rs1800896 genotypes and alleles in T2DM patients with DN (Cases) and T2DM patients without DN (Controls).

Polymorphism		Cases (276)	Controls (375)	p value
rs1800896	CC	45 (16.3)	52 (13.9)	
	CT	131 (47.5)	188 (50.1)	0.6
	TT	100 (36.2)	135 (36.0)	
	C allele (%)	221 (40.0)	292 (38.9)	0.7
	T allele (%)	331 (60.0)	458 (61.1)	
	HWE	0.8	0.3	

HWE: p-values for the HWE were computed using Pearson's goodness-of-fit-chi-square (1df).

Table 18: Association between the rs1800896 polymorphism and DN assessed by logistic regression analysis.

Inheritance model	Genotype	Cases (276)	Controls (375)	Adjusted OR, 95 % CI / p-value
rs1800896 Co-dominant	CC	45 (16.3)	52 (13.9)	1.33 (0.76-2.33) / 0.3
	CT	131 (47.5)	188 (50.1)	1.03 (0.69-1.54)/0.9
	TT	100 (36.2)	135 (36.0)	reference

p-values were adjusted for duration of hypertension, SBP, CVD, DR, DF, HbA1c, S-fasting glucose, S-urea, S-creatinine, S-cystatin, and UACR. Odds ratio (OR); Confidence interval (CI)

There is no statistically significant difference in serum IL-10 levels between patients with DN and without DN (p=0.7) (*Table 19*). Moreover, we divided patients with T2DM without DN into three groups according to their genotypes. There were no statistically significant differences in serum IL-10 levels between the three groups (p = 0.9) (*Table 20*).

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Table 19: IL-10 serum levels in diabetics with DN and without DN.

	Cases (DN+)	Controls (DN-)	p-value
IL-10 [pg/ml]	148.17 ± 65.86	151.19 ± 68.73	0.7

Values are mean ± SD. One-Way ANOVA

Table 20: IL-10 serum levels in diabetics with DN and without DN according to different genotypes.

Polymorphism	Genotype	IL-10 (pg/ml)	p-value
rs187238	CC	151.61 ± 64.95	
	CT	147.42 ± 70.00	0.9
	TT	151.65 ± 65.49	

Values are mean ± SD. One-Way ANOVA

5.2.3.4 The polymorphism rs2243250 of the *IL4* gene

Genotype and allele distributions for rs2243250 polymorphism of the *IL4* gene are demonstrated in *Table 21*. The genotype frequencies were in Hardy-Weinberg equilibrium (*Table 21*). There were no statistically significant differences in the genotype distribution between cases and controls (*Table* 21). A logistic regression analysis was used in *Table 22* to evaluate whether the rs2243250 polymorphism was independently associated with DN after adjusting for gender, age, diabetes duration, and glycosylated haemoglobin concentration. The results indicated no relationship of rs2243250 polymorphism with DN risk (*Table 22*).

Table 21: Distribution of rs2243250 genotypes and alleles in patients with diabetic nephropathy (cases) and in those without diabetic nephropathy (controls).

Polymorphism		Cases (276)	Controls (375)	p-value
rs2243250	TT	9 (3.2)	10 (2.7)	
	CT	81 (29.4)	129 (34.4)	0.4
	CC	186 (67.4)	236 (62.9)	
	T allele (%)	99 (17.9)	149 (19.9)	
	C allele (%)	453 (82.1)	601 (80.1)	0.4
	HWE	0.9	0.1	

HWE: p-values for the HWE were computed using Pearson's goodness-of-fit-chi-square (1df).

Table 22: Association between the rs2243250 polymorphism and DN assessed by logistic regression analysis.

Inheritance model	Genotype	Cases (276)	Controls (375)	Adjusted OR, 95 % CI / p-value
rs2243250 co-dominant	TT	9 (3.2)	10 (2.7)	1.06 (0.37-3.05)/0.9
	CT	81 (29.4)	129 (34.4)	0.79 (0.54-1.17)/0.2
	CC	186 (67.4)	236 (62.9)	reference

p-values were adjusted for gender, age, diabetes duration, and glycosylated hemoglobin concentration Odds ratio (OR); Confidence interval (CI)

5.2.4 NUCLEAR RECEPTORS

5.2.4.1 The polymorphism rs1801282 of the *PPARG* gene and the polymorphism rs8192678 of the *PPARGC1A* gene

The distribution of rs8192678 and rs1801282 genotypes and alleles in subjects with DN (cases) and those without DN (controls) is presented in *Table 23*. Univariate analysis didn't reveal significant differences in the genotype or allele frequencies between TD2M cases and controls (*Table 23*). The genotype distribution did not significantly deviate from the Hardy-Weinberg equilibrium (*Table 23*).

A logistic regression analysis was used (*Table 24*) to evaluate whether the SNPs were independently associated with DN after adjusting for gender, age, diabetes duration, duration of hypertension, SBP, CVD, DR, DF, HbA$_1$c, fasting glucose, urea, creatinine, cystatine C, and UACR. We did not find a statistically significant association of either rs8192678 or rs1801282 with DN according to co-dominant genetic model (*Table 24*) The studied subjects with GG genotype of the rs1801282 polymorphism had 3.80 higher risk for DN, which was borderline statistically significant.

Table 23: Distribution of rs8192678 and rs1801282 genotypes and alleles in patients with diabetic nephropathy (cases) and in those without diabetic nephropathy (controls).

		Cases (276)	Controls (375)	p-value
rs8192678	TT	29 (10.5)	31 (8.2)	
	TC	107 (38.8)	145 (38.7)	0.6
	CC	140 (50.7)	199 (53.1)	
	T allele (%)	165 (29.9)	207 (27.6)	0.4
	C allele (%)	387 (70.1)	543 (72.4)	
	HWE	0.2	0.5	
rs1801282	GG	10 (3.6)	5 (1.3)	
	GC	68 (24.7)	101 (26.9)	0.1
	CC	198 (71.7)	269 (71.8)	
	G allele (%)	88 (15.9)	111 (14.8)	0.6
	C allele (%)	464 (84.1)	639 (85.2)	
	HWE	0.2	0.1	

HWE: p-values for the HWE were computed using Pearson's goodness-of-fit-chi-square (1df).

Table 24: Association between rs8192678 and rs1801282 polymorphisms and DN assessed by logistic regression analysis.

Inheritance model	Genotype	Cases (276)	Controls (375)	Adjusted OR, 95 % CI / p-value
rs8192678 co-dominant	TT	29 (10.5)	31 (8.2)	1.37 (0.73 – 2.58)/0.3
	TC	107 (38.8)	145 (38.7)	1.08 (0.73 – 1.59)/0.7
	CC	140 (50.7)	199 (53.1)	Reference
rs1801282 co-dominant	GG	10 (3.6)	5 (1.3)	3.80 (0.99 – 14.55)/0.05
	GC	68 (24.7)	101 (26.9)	1.14 (0.75 – 1.73)/0.6
	CC	198 (71.7)	269 (71.8)	Reference

p-values were adjusted for duration of hypertension, SBP, CVD, DR, DF, HbA$_1$c, S-fasting glucose, S-urea, S-creatinine, S-cystatin and UACR. Odds ratio (OR); Confidence interval (CI).

5.3 SERUM CONCENTRATIONS IN PATIENTS WITH AND WITHOUT DN

We did not find differences in ICAM-1, PECAM-1, IL-10 and IL-18 serum levels between DN patients and the control group (*Table 25*).

Table 25: Calculation of cytokines serum concentrations in patients with diabetic nephropathy (120 cases) and in those without diabetic nephropathy (110 control subjects).

	Cases (DN+)	Controls (DN-)	p-value
IL-10 [pg/ml]	148.17 ± 65.86	151.19 ± 68.73	0.7
ICAM-1 [ng/ml]	$386.9 \pm 195.2.6$	404.7 ± 211.6	0.5
PECAM-1 [ng/ml]	96.9 ± 19.9	93.5 ± 18.9	0.2
IL-18 [pg/ml]	212.1 ± 67.1	206.7 ± 67.6	0.5

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6   DISCUSSION

Low-grade inflammation is expected to play an important role in the pathogenesis of DN. The set of genetic polymorphisms was chosen from the inflammatory genes in our group of patients with T2DM of Caucasian origin.

6.1   DEMOGRAPHIC AND CLINICAL CHARACTERISTICS

Demographic and clinical characteristics of the cases and control subjects did not show any significant differences between the groups with respect to age, sex, duration of T2DM, DBP, BMI, smoking status, family history of CVD, duration of DR, eGFR, serum haemoglobin, total cholesterol, HDL, and LDL cholesterol levels. On the other hand, statistically significant differences were observed in the following parameters: duration of hypertension, SBP, presence of CVD, UACR, as well as serum fasting glucose, HbA$_1$c, urea, creatinine, and TG levels. As expected, cases had significantly more chronic diabetic complications, such as DR and DF, but not DNeuro.

Differences in parameters reflecting renal function (serum creatinine, cystatin C, eGFR and UACR) confirmed chronic kidney disease in diabetic subjects with DN. Cystatin C was significantly higher in subjects with DN ($p < 0.001$). Cystatin C was a better marker for the estimation of renal function than eGFR (MDRD equation, ml/min) (511, 512).

6.2   GENETIC POLYMORPHISMS OF ADHESION MOLECULES

6.2.1   POLYMORPHISMS rs5498 AND rs1799969 OF *ICAM1* GENE AND DIABETIC NEPHROPATHY

In this study we were unable to find an association between the tested polymorphisms rs5498 (p.Lyz469Glu) and rs1799969 (p.Gly241Arg), and DN. Moreover, we did not find any significant differences in the levels of sICAM-1 across different genotypes of the tested polymorphisms rs5498 and rs1799969.

In DN, chronic subclinical inflammation is generated and sustained. Adhesion molecules play an important role, especially ICAM-1, along with VCAM-1, which are considered to be the most important adhesion molecules (513). Differences in the gene structure, such as SNPs, and possible dependent changes in the function of adhesion molecules may have an impact on inflammation and the development of DN. We chose polymorphisms rs5498 and rs1799969 because of the reported functional effect and a small number of studies in the field of DN. Polymorphisms rs5498 and rs1799969 are located in exon 6 and 4, which encode Ig-like domains 5 and 3, respectively (127,

514). Both Ig-like domains are involved in the dimerization of ICAM-1 molecules. Changes in domain 5 and 3 possibly interact with dimer formation. Extracellular parts of ICAM-1 molecules are like bend rods. The bend is between domain 3 and 4. The amino acid change in rs1799969 in domain 3 could influence the bending. Polymorphism rs5498 changes amino the acid sequence in domain 5, the closest one to the cell membrane, which is important for the orientation of the extracellular part. The conformational change in this position may interact with dimer formation. ICAM-1 dimers had greater binding affinity for LFA-1 and two-times greater cell-cell adhesion in comparison to monomers. Additionally, dimers had better position of binding sites on domain 1 for attachment to LFA-1, as revealed by the crystal structure analysis (515, 516). Even small spatial distortion of third Ig-like domain could decrease binding affinity for Mac-1 that binds on this domain (94, 95). With these changes, both SNPs can potentially influence the inflammatory process (95, 98, 127, 128). Thus far, only four articles dealing with rs5498 polymorphisms and DN have been reported (one article is in Chinese and inaccessible) (113, 132, 133, 517). Seman and co-workers found an association between the major A allele of rs5498 and DN in subjects with T2DM in the Malaysian population (113). Ma and co-workers did not find a significant association with DN in Swedish patients with T1DM (132). A replication of the study was conducted on a greater GoKinD population (T1DM patients, nearly 92% of European descent), where the A allele [rs5498:A] was associated with a greater risk for DN, but only in female patients with T1DM (133).

Major allele A was associated with DR in studies on Japanese and Indian populations (169). Three Chinese studies confirmed positive associations between DR and A allele (518, 519, 520). On the contrary, major A allele reduced the risk for DR in Slovenian (Caucasian) and Indian populations with T2DM (130, 521). The major A allele significantly increases the risk for diabetic microvascular complication in the meta-analysis. However, only two out of seven studies in this analysis enrolled subjects with DN (131). An ethnic-specific sub-analysis confirmed the association between the rs5498 polymorphism and diabetic microvascular complications in the Asian population, but not in Caucasians (131).

The results of the studies looking for a possible association between the rs5498 polymorphism and DN are inconsistent in patients with DM and indicate possible ethnic differences. Because of the paucity of studies in the field of DN, an overview of studies with the rs5498 polymorphism in another chronic diabetic complication (DR) was done. Comparisons between DR studies also pointed to possible ethnic differences. Thus, genetic variations depending on ethnicity could be the cause for inconsistency among results. More studies will be needed in the future to elucidate possible ethnic differences and the role of the rs5498 polymorphism as a susceptibility marker for DN and other diabetic complications.

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In our study, the second selected polymorphism, rs1799969 (rs1799969:G>A) in the *ICAM1* gene, was also not associated with DN in T2DM patients. This is the first report assessing the potential link between the rs1799969 and the DN in T2DM.

In a Swedish and in a larger GoKinD population of American patients with T1DM with mostly European ancestors, no association between the rs1799969 polymorphism and DN was found (132, 133). According to Nejentsev and co-workers, the commonly transmitted major G allele [rs1799969:G] is probably associated with T1DM in a predominantly white GoKinD population, but no information about other ethnic populations is available (522).

Proliferative DR, another serious DM complication, in the Caucasian (Slovene) population was not associated with rs1799969 polymorphism (130). Three studies from China also found no association between DR and the rs1799969 polymorphism in patients with T2DM (513, 518, 519).

Despite a very small number of published studies concerning the rs1799969 polymorphism in the field of DN and also in the field of DR in populations of patients with DM, the majority showed no association with the rs1799969 polymorphism. Similarly, the results of two studies in the Slovene (Caucasian) population of patients with T2DM are negative for a possible association.

In our study serum sICAM-1 levels were not associated with different genotypes of rs5498 and rs1799969 polymorphisms in a subpopulation of 120 patients with DN.

Bielinski and co-workers demonstrated a 5% increase in the concentration of sICAM-1 for each minor allele G of the rs5498 polymorphism [rs5498:G], but only in the white tested Americans (127). A statistically significant stepwise increase in sICAM-1 serum levels in people without DM compared to T2DM patients without DN, and T2DM patients with DN was found in a Malaysian population. Moreover, they found an increased concentration of sICAM-1 in association with the genotype AA of the rs5498 polymorphism (rs5498:AA) in control subjects without T2DM in comparison to heterozygous genotype (113). Additionally, a reduced plasma concentration of sICAM-1 was found in carriers of the minor G allele of the rs1799969 polymorphism [rs1799969:G] in healthy white women in the white Stanislas Cohort and also in the white population of patients with T1DM (128, 134, 522). In patients with DR, increased concentrations of sICAM-1 were found in association with the GG genotype of the rs1799969 polymorphism (rs1799969:GG) (130). Akman and co-workers did not find any differences in the serum concentrations of sICAM-1 in carriers of different genotypes of the rs5498 polymorphism, as well as for the rs1799969 polymorphism in the Turkish population (523).

No reports about serum ICAM-1 levels in subjects with DN and rs1799969 polymorphism have been published so far.

In conclusion, in our T2DM study population we did not find an association between either rs5498 or rs1799969 of the *ICAM1* gene and DN in Caucasians (Slovenian population). Additionally, we

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did not find any statistically significant differences in serum sICAM-1 levels in different genotypes of rs5498 and rs1799969 polymorphisms in subpopulations of subjects with DN.

6.2.2 THE POLYMORPHISM rs668 (RS281865545) OF *PECAM1* GENE AND DIABETIC NEPHROPATHY

In the study of subjects with T2DM, our studying group failed to confirm an association between the rs688 (rs281865545, p.Lyz469Glu) polymorphism of *PECAM1* gene and DN. Likewise, no association was found between the rs688 polymorphism and serum sPECAM-1 levels in a subpopulation of 120 diabetics with DN.

DN is considered an inflammatory disease. Inflammatory cells are implicated at every stage of renal impairment, and the extent of inflammatory cell accumulation in the kidney is closely related to DN (139). A 130 kDa adhesion molecule PECAM-1 is a crucial mediator of leukocyte migration through intercellular junctions of vascular endothelial cells (145) and may thus contribute to micro- and macrovascular inflammatory complications of T2DM. Hyperglycemia and oxidative stress have been shown to promote transendothelial migration of monocytes through phosphorylation of PECAM-1 (524, 525), whereas hyperinsulinemia enhanced neutrophil transendothelial migration by increasing endothelial PECAM-1 expression via mitogen activated protein kinase activation (MAPK) (526). Abnormal angiogenesis may also play a significant role in the pathogenesis of DN (527). PECAM-1 is involved in endothelial cell-cell and cell-matrix interactions and signal transduction, which are essential during angiogenesis (528). Further, Kondo and co-workers have demonstrated that PECAM-1 is a critical modulator of endothelial cell adhesion, migration, and capillary morphogenesis in kidneys (142). In normal kidneys, PECAM-1 is expressed on endothelial cells of glomerular and peritubular capillaries, whereas its expression is reduced in obliterated glomeruli with endothelial cell destruction, such as in diabetic glomerulosclerosis (529). In animal models with reversible kidney injury, such as anti-Thy-1 treatment in rats, PECAM-1 expression increases during the recovery phase (530). Thus, it seems that under pathological conditions, compensatory PECAM-1 modulation may enable glomerular endothelial cell survival (531). Recently, Cheung and co-workers showed that PECAM-1 signalling is both necessary and sufficient to prevent inflammation-induced endothelial cell death and confer immune privilege to the vascular endothelium (532). As a peculiarity, Baelde and co-workers have studied the messenger RNA expression profiles of diabetic glomeruli and *PECAM1* gene was found to be upregulated among other ninety-six overexpressed genes in comparison to the glomeruli from healthy individuals (533). PECAM-1/PECAM-1 homophilic interactions, which are mediated by the first two IgD of NH2-terminal Ig homology domains, are primarily responsible for leukocyte transmigration and play a vital role in the regulation of the endothelial barrier function (145, 147, 148). The first IgD of PECAM-1 is encoded by the third exon of *PECAM1* gene that contains the rs668 polymorphism (154). A single amino acid mutation of valine to leucine may affect the

homophilic binding capability and therefore influence PECAM-1-mediated cellular interactions. The rs668 (L/V) polymorphism is in strong linkage disequilibrium with amino acid polymorphisms in exon 8 at codon 563 altering a serine to an asparagine (S/N) and in exon 12 at codon 670 altering an arginine to a glycine (R/G) (154). Goodman and co-workers have described an association between the LSR and VNG haplotypes and leukocyte/endothelial interaction (158). Namely, they showed that LSR/VNG heterozygous monocytes adhere better to endothelium under conditions of flow than LSR/VNG homozygous cells (158). In accordance with the possible functional effect, the rs668 polymorphism has been shown to be associated with a number of cardiovascular and cerebrovascular diseases (157, 162, 163, 164, 165) However, the results of the association genetic studies published so far have not been unequivocal. Similar to our study, Kamiuchi and co-workers were unable to prove an association between *PECAM1* rs688 polymorphism and the presence of DR in subjects with T2DM (169), whereas Bazzaz and co-workers found no correlation between *PECAM-1* rs668 polymorphism and microangiopathic complications in subjects with T1DM (534). In Japanese subjects with T2DM, *PECAM1* rs668 polymorphism was not associated with chronic kidney disease (535). However, a possibility of a weak association of *PECAM1* rs668 polymorphism with chronic kidney disease was found in Japanese individuals with both T2DM and arterial hypertension (535). The results from a recent meta-analysis suggested that the rs668 polymorphism in the *PECAM1* gene is not a susceptibility marker of coronary heart disease (160).

Contrary to our findings, several studies have reported an association between the circulating sPECAM-1 levels and the rs668 polymorphism (162, 164, 165, 167, 168). The soluble plasma sPECAM-1 exists in two distinct forms: a trans-membrane-less 120 kDa form and a truncated 90 kDa form (536). The transmembraneless sPECAM-1 is formed by alternative splicing of the transmembrane segment-encoding (exon 9) transcript upon cell activation, while the truncated form is generated by PECAM-1 proteolytic cleavage at the cell surface and shedding of the extracellular portion of PECAM-1 into the plasma (536, 537). Soluble forms of PECAM-1 can act as competitive inhibitors of membrane-bound PECAM-1 and by this regulate the transmigration of leukocytes (538). It has been suggested that due to the localization of rs668 in the first loop of the extracellular domain of PECAM- 1 protein, the valine to leucine mutation may facilitate the cleavage of sPECAM-1 from the cell surface and thus increase the serum sPECAM-1 level while decreasing the endothelial barrier function of PECAM-1 (168).

The inconsistencies among the results of our and other genetic association studies may be explained by differences in their phenotype definition, the variation in the genetic or environmental background of the populations studied, the possibility of various gene-gene and gene-environment interactions, or an insufficient sample size (539). Although the number of subjects included in our study was relatively small, all the participants were recruited from a rather homogenous genetic

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and environmental background. In addition, the strength of the study is a rather long duration of T2DM in both cases and control subjects.

To conclude, we were not able to prove an association between the rs668 polymorphism of the *PECAM1* gene and DN in subjects with T2DM, indicating that the rs668 single nucleotide polymorphism is not a genetic marker for susceptibility to DN in Caucasians with T2DM.

6.3 GENETIC POLYMORPHISMS OF CHEMOKINES

6.3.1 POLYMORPHISMS rs2280788 AND rs2107538 OF *CCL5* GENE AND DIABETIC NEPHROPATHY

In our study we did not confirm an association between rs2280788 (g.-28C>G) and rs2107538 (g.-403C>T) polymorphisms in the promoter of the *CCL5* gene and DN in our study population of T2DM patients.

The CCL5, first describes as RANTES, is a small protein and belongs to the β or CC family of chemokines with a potent chemotactic function (171). The main role of *CCL5* in inflammation is to recruit monocytes and lymphocytes to the site of inflammation or injury and to activate lymphocyte T cells (176, 181).

Our results are in consensus with a study in the Irish population done by Pettigrew and co-workers. Their analysis, conducted in a population with T1DM, did not find any association between DN and both polymorphisms rs2280788 (g.-28C>G) and rs2107538 (g.-403C>T). The reported frequencies for rs2280788:G (g.-28>G) allele were below 5% and 17% for the rs2107538:T (g.-403>T) allele (234). Data from 1000 Genome Project Phase3 reveal 1.19% allele frequencies for rs2280788:G (g.-28>G) and 16.1% for rs2107538:T (g.-403>T) allele (540).

Opposite to our results, Nakajima and co-workers reported an association for the rs2280788 (g.-28C>G) polymorphism in a population with T2DM. Their study showed a positive association between DN in Japanese T2DM patients and rs2280788 (g.-28C>G) polymorphism (229). Mokubo and co-workers tried to confirm these data in a longitudinal retrospective study in T2DM patients also in a Japanese population. This longitudinal study did not confirm an association between the rs2280788 (g.-28C>G) polymorphism and DN in T2DM (232). Another study in a South Asian population was carried out in Korea in T2DM patients with DN and ESRD on renal replacement therapy, the final stage of development of progressive DN. No association between the rs2280788 (g.-28C>G) polymorphism and DN was found (233). The frequencies of the G allele of the rs2280788 (g.-28>G) polymorphism are about 1% in both the Caucasian and Asian populations (534). The frequency of C and G alleles of the rs2280788 (g.-28C>G) polymorphism in our study population are 4% and 96%, respectively. The frequency of the C allele [rs2280788:C] in our population is higher than reported in the 1000 Genome Project, Phase3.

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Frequencies of C and T alleles of the rs2107538 (g.-403C>T) polymorphism in our study population are about 80% and 19%, respectively. Reported frequencies for the T allele [rs2107538:T] for the same polymorphism in a South Asian population from the 1000 Genome Project are 30.88% and for the Caucasian population about 16% (534). These data for the Asian population match the results from the report of Nakajima and co-workers for rs2107538:T (g.-403>T), where T allele was found in 34% of Japanese T2DM patients. Similar to our result, their study showed no significant differences in the frequency of alleles and genotypes of the rs2107538 polymorphism. Additionally, no associations between DN with ESRD in T2DM patients and the rs2107538 (g.-403C>T) polymorphism were found in a Korean study (233). The strong linkage dimorphism between both rs2280788 (g.-28C>G) and rs2107538 (g.-403C>T) polymorphisms was reported. According to some researchers both of them are practically always inherited together. This was pointed out by Nakajima, Pettigrew, An, Liu, Konta, Herder, Gonzalez and others in their articles (187, 208, 211, 218, 228, 229, 234).

Recently, two meta-analyses in the field of DN were published, which included association studies with both rs2280788 (g.-28C>G) and rs2107538 (g.-403C>T) polymorphisms. Nazir and co-workers found no significant association with the SNPs we observed and DN among 34 studies and 11 genetic variants (541). Tziastoudi and co-workers made a similar meta-analysis and a systematic review with emphasis on candidate genes involved in six inflammatory and immune pathways. In the study, they included 103 genetic association studies with 443 gene variants from 75 genes. Again, no association between DN and rs2280788 (g.-28C>G) and rs2107538 (g.-403C>T) polymorphisms was found (542).

Between association studies in DN for rs2280788 (g.-28C>G) only one was positive, but a longitudinal retrospective study in the same Japanese population did not confirm the result of the first study. All published association studies in DN for the rs2107538 polymorphism were negative. Our results are in line with the above findings.

To conclude, no association was found between rs2280788 (g.-28C>G) and rs2107538 (g.-403C>T) polymorphisms of the *CCL5* gene and DN in our study population of T2D patients.

6.3.2   THE POLYMORPHISM rs1799987 OF THE *CCR5* GENE AND DIABETIC NEPHROPATHY

In our study we did not confirm an association between the rs1799987 polymorphism (rs1799987:A>G or g.-59029A>G) in the promoter of the *CCR5* gene and DN in our population of T2DM patients.

Few studies analysing the association between the rs1799987 polymorphism and DN have been conducted in T1DM patients with inconsistent findings. Nearly all these studies were performed on the Caucasian population (270, 271, 234). Two studies in a T1DM population are in contrast to our

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results. The study from the Joslin Diabetes Center included 496 T1DM patient with overt proteinuria or end-stage renal disease, and 298 control subjects with normoalbuminuria for at least 15 years. Only men with the rs1799987:G variant showed a significant association with DN and the risk for them was nearly two-fold in comparisons with non-carriers. In this case, the rs1799987:A allele had a protective role, but this applies only to men, as women had no association. Interestingly, co-analysed *CCR5* 32-base pair deletion (rs333) revealed a risk role, with more than two-fold risk of DN for men carriers of both SNPs (rs333 and rs1799987) versus non-carriers (272). Both results are consistent, but unexpected and difficult to interpret. As a reminder: polymorphism *CCR5* 32-base pair deletion (rs333) means no expression of *CCR5* because the defective protein and allele G [rs1799987:G] of the rs1799987 polymorphism leads to a less active *CCR5* promoter and lower expression of CCR5 on cell surface. In addition, the results are consistent with the most recent animal experiment in RAAS-dependent hypertension and CKD with an unexpected increase in the infiltration of leukocytes and fibrosis in CCL5-knockout animals (201). Furthermore, no other studies in the field of DN found sex differences, although theoretical possibilities exist (543). In the second study by Yang and co-workers in T1DM patients of Caucasian origin in the USA, the G allele [rs1799987:G] was slightly increased in patients with microvascular DM complication in comparison to patients without complication (544).

The results of these studies are completely contrary to other studies. EURAGEDIC (European rational approach for the genetics of diabetic complications) is a big study conducted in T1DM patients in north Europe with a large cohort, which showed a negative association between the rs1799987 polymorphism and DN (270, 271). This study comprised 1176 case subjects with overt DN and 1323 control subjects with normoalbuminuria for 15 years. The primary results showed a nominal significant association for the rs1799987 polymorphism, but it turned into a non-significant after correction for multiple testing (270, 271). Pettigrew and co-workers re-sequenced the gene for CCL5 and CCR5 and identified 58 variant alleles. Eight efficient haplotype tag SNPs were selected and evaluated for an association with DN. In their Irish T1DM population, the rs1799987 polymorphism did not show any association with DN (234).

Several studies in populations with T2DM have been carried out to discover an association between the rs1799987 polymorphism and DN, the majority of them in Asian populations. Nakajima and co-workers published two papers that deal with the same population of T2DM patients in Japan. They found that the A allele [rs1799987:A] was significantly more common in T2DM patients with DN in comparison to T2DM patients without DN. Studies pointed to an independent association of the rs1799987 polymorphism with DN (229, 266). To confirm an association between the rs1799987 polymorphism and DN from cross-sectional studies, they made a 10-year retrospective longitudinal study in the same population. All participants had normoalbuminuria at the start of the study, but a greater frequency of allele A [rs1799987:A] was found in the group that developed

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microalbuminuria or overt proteinuria in comparison to the group that persisted in normoalbuminuria (232). In Asian Indians with T2DM and progressive chronic renal insufficiency, among nine tested polymorphisms only the rs1799987 showed a significant association (267). A study from India that analysed two cohorts of patients with T2DM, from the northern and southern parts of the country, confirmed a positive association between established DN and the rs1799987 polymorphism in both cohorts (268). Another study from India also confirmed this association. The A allele [rs1799987:A] and AA genotype were more frequent in T2DM patients with DN in comparison to T2DM patients without DN and healthy subjects (545). A study in a Caucasian population analysed the rs1799987 polymorphism and genotype differences between healthy subjects and a population with T2DM. No difference in frequencies of alleles and genotypes of this SNP was found. Interestingly, a sub-analysis in a population with T2DM revealed an increased frequency of A allele [rs1799987:A] and AA genotypes in diabetics with in comparison to patients without DN. They concluded that the rs1799987 polymorphism was significantly associated with DN in T2DM patients (269). The results of our study are inconsistent with all mentioned studies in populations with T2DM.

Four meta-analyses in the field of DN and different SNPs have been done in a span of 7 years (from 2010 to 2017). All of them fund a significant correlation between the rs1799987 polymorphism and DN. The first study of Mooyaart and co-workers included in the meta-analysis all genetic variants with a significant association in primarily study and the subsequent reproduction of results in another independent study (different inclusion criteria). They included 21 genetic variants. The rs1799987 polymorphism was not associated with DN in the whole group of nine studies, but it was associated with DN in the subset of studies with an Asian background (546).

Nazir and co-workers included 34 studies and 11 genetic variants showed a positive association with DN. They included studies where cases had DM with overt proteinuria or biopsy proved DN, and diabetic control cases with normoalbuminuria for at least 10 years. The rs1799987 polymorphism was positively associated with DN and the pooled odds ratio was 1.29 (95% CI 1.20-1.38). In the sub-group analysis, a positive association was reproduced for Asian and Caucasian ethnic populations and T2DM but not for T1DM (541). The meta-analysis of Tziastoudi and co-workers is interesting in its approach because it includes genetic variants from six inflammatory pathways as classified by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, with the idea to explain the influence of the immune system on the development and progress of the DN. They included 443 polymorphisms from 75 candidate genes and found a significant association with DN for 66 variants in 18 genes. In a sub-analysis of eight studies based on results indicating positive association with DN, the rs1799987 polymorphism was excluded because it was not in Hardy-Weinberg equilibrium (542). The last meta-analysis was conducted

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just for the rs1799987 polymorphism and included 13 studies (3 studies in Caucasians and 5 studies in Asians). This polymorphism dramatically increased the susceptibility for DN in the total analysis and sub-group with Asian ethnicities and T2DM. The authors draw attention to the small number of studies included, which were mainly based on the Asian population, the significant heterogeneity of studies (probable phenotypic differences), and a very likely publication bias (studies with negative results are seldom published) (547). The results of studies indicate possible differences between ethnicities in the case of an association of the rs1799987 polymorphism and DN. The Asian background has a distinct association, while this is not present in the Caucasian background.

Our study is in contradiction with the only study published in T2DM patients with a Caucasians ethnic background. A Polish study showed no differences in the frequencies of A allele [rs1799987:A] and AA genotype between T2DM and healthy subjects, but presented differences between T2DM patients with and without DN (269). Our study has a smaller number of included subjects, it has no healthy control group and we defined DN with persistent micro- and macroalbuminuria, whereas the Polish study included just T2DM patients with persistent macroalbuminuria or proteinuria. Because Slovenia is a small country, our population of T2DM patients is homogenous and the enrolment of participants was very precise in terms of phenotypes.

To conclude, no association was found between the rs1799987 polymorphism of the *CCR5* gene and DN in our study population of T2D patients.

6.3.3   THE POLYMORPHISM rs1799864 OF THE *CCR2* GENE AND DIABETIC NEPHROPATHY

In our study we did not confirm an association between the rs1799864 (g.+46295G>A) polymorphism of the *CCR2* gene and DN in our study population.

After analysing diabetic chronic complications in T1DM patients, Yang and co-workers did not find any association between them and the rs1799864 polymorphism (544). In Korean diabetics with T2DM, no difference in the frequency of genotypes of the rs1799864 polymorphism between patients with ESRD and the control group of diabetics without nephropathy was found (233). Similarly, negative results were revealed in a study among T2DM patients in India. No association between the rs1799864 polymorphism and chronic renal insufficiency was found (267).

In the field of DN and rs1799864 polymorphism only one genetic study was published. Our result is in consensus with the study of Nakajima and co-workers. They found no statistical significant difference in allele frequency between patients with and without DN. The frequency of minor A allele [rs1799864:A] of the rs1799864 polymorphism was 28% in Japanese T2DM patients, similarly to healthy Japanese people. In our study population of T2DM patients, frequencies of A and G allele are about 12% and 88%, respectively. For a comparison: the frequency of minor A allele [rs1799864:A] is 8.6% in the European and 9.8% in the Southeast Asian population,

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according to the 1000 Genomes Project, Phase 3 (548). Despite higher frequencies of A allele [rs1799864:A] in the Japanese in comparison to our population with T2DM, the results of both studies are the same.

Several authors report strong linkage disequilibrium between polymorphisms in *CCR2* genes, as well as between the *CCR2* and *CCR5* genes. Both genes are in close proximity; *CCR2* is positioned 15 kb upstream of the *CCR5* gene (256, 294). Nakajima and co-workers reported strong linkage disequilibrium between *CCR2* rs1799864 and *CCR5* rs1799987 (g.-59029A>G). Additionally, they analysed an insertion/deletion polymorphism of the angiotensin-converting enzyme (ACE I/D, rs4646994) in parallel and excluded the possible confounding influence on DN (266). Furthermore, *CCR2* rs1799864 and *CCR5* rs1800024 are in strong linkage disequilibrium as found in a multi-ethnic study (549). Thus, different linkage disequilibrium may have additional influence on these results. In the population of T2DM patients with DN, only two studies have been carried out. Larger studies are needed in the future to validate the results of these studies.

To conclude, no association was found between the rs1799864 polymorphism of the *CCR2* gene and DN in our study population of T2D patients.

6.4   GENETIC POLYMORPHISMS OF INTERLEUKINS

6.4.1   THE POLYMORPHISM rs3212227 OF *IL12* GENE AND DIABETIC NEPHROPATHY

In this case-control study we found no association between the rs3212227 (c.+1188A>C) polymorphism and DN in patients with T2DM.

Transcription is the first line control of IL-12p40 production, but additional levels of control are in translation. Beside the classic process of translation initiation, elongation and termination, additional factors control the start of initiation, termination and even the length of elongation. The majority of this control elements are in 5'UTR and 3'UTR, but 3'UTR of mRNA is much longer, usually about 600 nucleotides and contains multiple binding sites for trans-acting regulatory RNA proteins and micro RNA (miRNA). This kind of control is fast and economical for the cell and can integrate environmental influences. A disturbed regulation of a specific mRNA translation could be the consequence of genetic mutation or environmental pressure and could lead to increased or decreased protein production (550, 551, 552). The polymorphism rs3212227 of *IL12B* lies in 3'UTR. Morahan and co-workers found an increased production *IL12B* mRNA in PBMC from carriers of the rs3212227:AA genotype (343). Davoodi-Semiromi and co-workers confirmed this result, but Bergholdt and Dahlman with co-workers did not (344, 345, 346). Stanilova found an increased production of protein IL-12 in carriers of the rs3212227:AA genotype, but Seegers and co-workers found an increased IL-12 protein production in carriers of the rs3212227:CC genotype (347, 348). An increase production of subunit IL-12p40 may have consequences and could

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participate in different pathologic conditions, like psoriasis, rheumatoid arthritis, multiple sclerosis, T1DM, but also T2DM and atherosclerosis. Meta-analyses confirmed the association between rs3212227 in psoriasis and psoriatic arthritis (350, 351). Similar analyses were negative for rheumatoid arthritis and multiple sclerosis (352, 353). In a review of published studies, Tang and co-workers did not find an association between T1DM and T2DM and rs3212227 polymorphism (553).

In Polish hemodialysis patients, Grzegorzewska and co-workers analysed a possible association between the rs3212227 polymorphism and patients with T2DM, but they found no significant association (365). The main difference between two cross-sectional studies, ours and that of Grzegorzewska, is the positioning of the time point of both studies DN progress to ESRD. Our patients had incipient and developed DN with preserved renal function in comparison to patients with ESRD on renal replacement therapy in Grzegorzewska's study. The survival bias is possible in long-standing patients with DN and ESRD because of competing death risk from cardio-vascular events (8, 9). Many T2DM patients with persistent albuminuria and decreasing renal function die before potentially developing ESRD and this could bring differences in observed allele frequencies. Additionally, we compared patients with and without DN. Grzegorzewska and co-workers made a comparison with healthy controls and patients on hemodialysis because of reasons other than DN.

Studies with a bigger number of included patients will be needed for a final conclusion of a possible association between the rs3212227 polymorphism in T2DM patients. Discrepancies found in studies with risk for T1DM show the need to study also different ethnic populations. Interestingly, no study was found for the rs3212227 polymorphism in patients with T1DM and DN. This indicates the need for a research of the rs3212227 polymorphism and DN susceptibility in populations with T1DM.

To conclude, the polymorphism rs3212227 of the *IL12* gene was not associated with DN in our study population of T2D patients.

6.4.2 THE POLYMORPHISM rs187238 OF THE *IL18* GENE AND DIABETIC NEPHROPATHY

In the retrospective case control study, including 651 Caucasian subjects with T2DM, we failed to confirm an association between the rs187238 (g.-137G>C) polymorphism of the *IL18* gene and DN. Moreover, we did not find a statistically significant difference in serum IL-18 levels between T2DM patients with DN and without DN.

The leading role in kidney disease, as well as DN, is played by inflammatory cytokines (139). Several studies highlight the important relationship between IL-18 and DN (373, 554, 555). IL-18 is expressed on tubular epithelial cells, while it has increased values in patients with DN (369). Nakamura and co-workers recorded elevated IL-18 values in the blood and urine of patients with

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DN compared with patients without DN (373). In a second study, Donate-Chorea and co-workers also showed a significant increase in IL-18 values in patients with DN, suggesting that the IL-18 might be a predictor of the progression of DN (139). In addition, the inhibition of inflammatory cell recruitment into the kidney was reported to be protective in experimental DN, indicating that IL-18 might be a potential therapeutic target for DN treatment (393).

The closest to our study is the report by Cilenšek and co-workers who investigated the effect of IL-18 on the development of DR in Caucasians with T2DM, where they found no difference in serum IL-18 levels between patients with DR and without DR (556); a similar result was reported in our current study. Serum concentration of IL-18 was not different between the T2DM patients with DN and T2DM patients without DN. The statistically significant differences that were observed during our research in the UACR confirmed chronic kidney disease in patients with DN. Moriwaki and co-workers reported that IL-18 levels were significantly elevated in diabetic patients with microalbuminuria compared to patients with normoalbuminuria (557). Polymorphisms in the *IL-18* gene have been shown to be associated with circulating IL-18 levels (374). The functional polymorphism in the *IL18* promoter rs187238 has been repeatedly found to be associated with the *IL18* promoter transcription activity (374). To our knowledge, there are only a few studies investigating an association of *IL18* rs187238 polymorphisms and DN (369, 375). Bai and co-workers have recently reported that individuals carrying the C allele of the rs187238 polymorphisms showed a 2.16-fold higher risk for DN in T2DM Chinese Han population (375). Moreover, Elneam and co-workers compared the allele frequencies of the rs187238 polymorphism among patients with diabetes and without DN and patients with DN in Saudi Arabia population. They revealed that G allele [rs187238:G] was significantly more common in patients with DN than C allele [rs187238:C] (369). However, our results are not in accordance with previous studies (369, 375). We found no association between polymorphism rs187238 and DN. We speculate that a different genetic background could influence the results. According to the 1000 Genomes Project Phase3, minor allele G frequency of the rs187238 polymorphism varies across different ethnic groups, from 12% in East Asia, 18% in South Asia, 28% in Europe to 31% in America (558). The average frequency of the G allele [rs187238:G] in the whole study population in our study was 25.3%, which was slightly lower than that observed in Caucasians according to the 1000 Genomes Project data.

The limitation of our study that may lead to inconsistencies between our results and other studies are basically the rather small number of patients included in the study and the possible heterogeneity of the genetic and environmental background of the subjects. However, participants in our research were carefully selected so that homogeneity in terms of ethnicity, age and type of diabetes would be achieved. Similarities in the genetic and environmental background of our patients are another strength of the present study.

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To conclude, we did not find an association between the rs187238 polymorphism of the *IL18* gene and DN in Caucasians with T2DM.

6.4.3 THE POLYMORPHISM rs1800896 OF THE *IL10* GENE AND DIABETIC NEPHROPATHY

In this study we failed to demonstrate an association between the *IL10* rs1800896 polymorphism and DN in our T2D patients. Moreover, we did not find a statistically significant difference in serum IL-10 between patients with and without DN.

DN is the consequence of a chronic inflammatory process that inevitably leads to renal damage together with constant hyperglycaemia. The main infiltrating cells are macrophages and T-cells that produce IL-10. Among renal residential cells, the major producer of IL-10 are mesangial cells (387, 560). IL-10 is an important anti-inflammatory cytokine that reduces the progression of inflammation and the activation of adaptable immune response by blocking antigen presentation to T helper cells and the inhibition of differentiation, activation and effector function of T cells, but also macrophages and a variety of other cells (53).

Our results are in accordance with the studies of Ezzidy, Erdogan and Kung with their co-workers. Ezzidy, Erdogan and Kung with their co-workers did not find an association between the rs1800896 polymorphism and DN in T2D patients in the Tunisian, Turkish and Taiwanese population, respectively (417, 418, 419). Recent studies from China found a positive association between DN and the rs1800896 polymorphism in T2DM patients. These results are in contradiction with our and the aforementioned studies. In the first study, Yin and co-workers analysed genotype results for 172 overt DN patients with T2DM and 344 healthy control subjects. Carriers of rs1800896:AA genotype had a statistically significantly increasing risk for DN in the co-dominant model in comparison to carriers of the GG genotype. Significant positive results were also found in the dominant (GA + AA versus GG) and recessive (AA versus GG+GA) logistic regression statistical model (421). The second study confirmed the results of the previous one. The rs1800896:AA genotype also significantly increased the risk for DN in T2DM patients (422). The third study of Wu HC and co-workers written in Chinese, found no significant association between rs1800896 polymorphism and ESRD in patients with T2DM in comparison to healthy controls (563). Peng and co-workers conducted a meta-analysis of the rs1800896 polymorphism and DN in T2DM patients. The analysis showed a positive association between this polymorphism and DN. The rs1800896:AA genotype might increase the risk for DN more than AG and GG genotypes. Interestingly, among all included articles none of them concerned the rs1800896 and DN in T1DM patients (423). Some inconsistencies were noted in this meta-analysis. The included Arababadi's study, which actually analysed the rs1800872 (*IL10*, *g.-592*C>A) polymorphism, whereas the published article did not include any data about the rs1800896 polymorphism (561). Additionally German study was very small and it was more of a pilot study, because it included only 44 patients

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with T2DM. In this study a positive association between the rs1800896:GG genotype and ESRD was found (415).

However, the results of studies between the rs1800896 polymorphism and DN in patients with T2DM differ in different populations. In general, the results are negative in the Caucasian and Arab populations, and positive in the Chinese population. For example, ancestral A allele [rs1800896:A] of the rs1800896 polymorphism is found in 54.6% of Europeans, 75.7% of South Asians and 96.6% of North Chinese (562). The average frequency of the major allele A of rs1800896 in the whole study population in our study was 60%, which was slightly higher than that observed in Caucasians according to the 1000 Genomes Project data. Ethnic differences are probably the main reason for the different results of genetic researches conducted in different populations. Additionally, other polymorphisms that may combine with rs1800896 may increase the risk of DN in T2DM patients. This results indicates that DN-associated variants have emerged independently in different populations and that distinctive steps in disease-associated pathways are altered by genetic risk factors in the different populations.

The comparison of IL-10 serum concentrations between our patients with and without DN revealed no significant differences in frequencies of alleles and genotypes of the rs1800896 polymorphism. Data in literature are contradictory for serum concentrations in DM and healthy people. Additionally, serum concentrations of IL-10 in healthy people are often very low or undetectable. Notwithstanding this, Suarez and co-workers found a group of healthy people with relatively high serum IL-10 concentrations. Carriers of the rs1800896:GG genotype also produce a significantly greater concentration of IL-10 in PBMC stimulated with lipopolysaccharide (405). In patients with DM, serum concentrations are increased as reported by Mysliwska and co-workers for T1DM patients and Wong and co-workers for T2DM patients (392, 393). On the contrary, Yaghini and co-workers revealed that serum levels of IL-10 were lower in patients with T2D in comparison to healthy control subjects (394, 563). In a small pilot study, Wu and co-workers found no differences, but the number of included participants was really small (12 patients) (204). It is expected that diabetics, due to their underlying disease, have elevated IL-10 serum concentrations. However, DN is a long-standing inflammatory process, probably with flare-ups and reductions or subliminal relapses and remissions of inflammation. This could influence serum and urine levels of inflammatory cytokines. Additionally, a long-lasting inflammation in DN goes through different stages and slows down to overt DN, overt proteinuria and developed histological changes (559). In the final stage of ESRD, the concentration of IL-10 could be lower than before (560). More studies are needed to accumulate more knowledge to properly explain these differences.

To conclude, we found no association between the *IL10* rs1800896 polymorphism and DN in T2DM patients of Caucasian ethnicity. Furthermore, we also found no association between serum levels of IL-10 and rs1800896 polymorphism in our study population.

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6.4.4 THE POLYMORPHISM rs2243250 OF *IL4* GENE AND DIABETIC NEPHROPATHY

In our study, we did not found an association between the rs2243250 (g.-590C>T) of the *IL4* gene and DN in patients with T2DM.

Chronic inflammation is the underlying cause for DN. Immune cells, including T helper cells, are vital in the process of inflammation (58). IL-4 is involved in the differentiation of T cells, and a change in the gene structure caused by a SNP might cause a structural or functional change that may modify its anti-inflammatory function, and therefore influence the pathogenesis of DN (429). So far, only two studies reporting an association of the rs2243250 polymorphism and DN have been published. Kazemi Arababadi and co-workers compared T2DM patients with DN to a healthy control group in a Southeastern Iranian subset of population (451). They concluded that the rs2243250 polymorphism had an important role in the development of DN in subjects with T2DM (451). Another study conducted by Neelofar and colleagues also found an association between the rs2243250 polymorphism and chronic kidney disease in patients with T2DM in the North Indian population (452). We speculate that different populations represent a different genetic background, and the differences between populations (the Iranian and Indian subset of subjects versus Slovene subjects) could be the cause for the varying results concerning the association between the studied polymorphisms and DN. Proliferative DR is another microvascular complication of T2DM. Cilenšek and co-workers investigated the association between rs2243250 and proliferative DR in Caucasians with T2DM and determined that rs2243250 is not a risk factor for proliferative DR (564). In the current report on DN as well as in our previous report in subjects with proliferative DR, we failed to demonstrate an association with either phenotype (564). The rs2243250 polymorphism was analysed in two additional reports on Iranian and Egyptian subjects with T2DM (452, 449). In the Egyptian study, an association between rs2243250 and T2DM was reported, indicating that the rs2243250 polymorphism is functional (449).

To conclude, we did not find an association between rs2243250 of the *IL4* gene and DN in Caucasians (Slovene population) with T2DM. This implies that the rs2243250 polymorphism cannot be used as a genetic marker for DN in Caucasians with T2DM.

6.5 GENETIC POLYMORPHISMS OF NUCLEAR RECEPTORS

6.5.1 POLYMORPHISMS rs1801282 OF THE *PPARG* GENE AND rs8192678 OF *PPARGC1A* GENE AND DIABETIC NEPHROPATHY

In the present case-control study, which included 651 subjects with T2DM, we were not able to confirm an association between the rs1801282 polymorphism of the *PPARG* gene or the rs8192678 polymorphisms of the *PPARGC1A* gene and DN.

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The frequency of the *PPARG* gene rs1801282:G allele as reported in the 1000 Genomes Project Phase3, varies considerably among different ethnic groups, being low in African (0.5%) and East Asian (2.6%) and higher in European (12%) populations. In our subjects with T2DM, the frequency of the rs1801282:G allele was similar to the frequency described in the German diabetes population (15.3% vs. 14%) (467). The frequency of the *PPARGC1A* gene rs8192678:A allele in our population was 28.6%, while in a British study the reported frequency in European subjects with T2DM was 33% (503). Again, considerable variation in A allele [rs8192678:A] frequency was observed in different ethnic groups, from 1% in the African to 66% in the Asian diabetic population (503). The exact mechanisms by which PPAR-γ and its genetic variation might actually protect against DN are not completely clear. PPAR-γ plays an important role in regulating insulin sensitivity, which has been closely associated with glomerular filtration rate and albuminuria (565).

The PPAR-γ agonists were shown to improve urine albumin excretion and slow the progression of DN in both animals and humans (565). Besides improving insulin sensitivity, the inhibition of inflammation and oxidative stress is among the major renoprotective mechanisms of PPAR-γ activation (566). Carriers of the G allele [rs1801282:G] of *PPARG* gene have improved insulin sensitivity (463) and increased resistance to hyperglycaemia-induced oxidative stress (565). In a recent study, the rs1801282:C>G (p.Pro12Ala) polymorphism was associated with a significantly lower progression of albumin excretion rate and with lower decline in the glomerular filtration rate as compared to the wild type rs1801282:CC (Pro12Pro) genotype (567). Several studies showed that PPAR-γ agonists benefit all kinds of kidney cells including the glomerular mesangial cells, endothelial cells, podocytes, and tubular epithelial cells under the diabetic condition (565). The activation of PPAR-γ can directly improve DN through the inhibition of mesangial cell growth, reduction of mesangial matrix, and cytokine production of glomerular cells (568). Furthermore, PPAR-γ activation protects glomerular capillaries against injury by promoting podocytes and endothelial cell survival (568).

PGC-1α is a tissue-specific transcriptional co-activator involved in the regulation of genes implicated in the whole body energy expenditure and glucose metabolism (467). This is actually a versatile protein with many roles. It is important for the homeostasis of mitochondria, fatty acid oxidation, thermogenesis and angiogenesis (472, 474). It is found in organs with high-energy consumption, including kidneys (478). This big protein has multiple binding sites for different ligands, like transcription factors, activators and hormones. As a co-activator, it augments or represses signals from differed ligands and translates them to transcriptional complex (475, 483). In the most common polymorphism, rs8192678:G>A, A substitute G at position +1444 in exon 8 which results in the substitution of glycine with serine (Gly482Ser) in codon 482 (484, 569).

In addition, it plays an important role in the regulation of the ROS defence system, including the antioxidant enzymes, such as glutathione peroxidase-1 and superoxide dismutase-2 (570). PGC-1α

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co-activates nuclear respiratory factor-1, which by binding to PPAR-γ induces the expression of mitochondrial uncoupling protein-2, which in turn protects against cellular oxidative damage (503). The rs8192678:A allele of the *PPARGC1A* gene results in lower protein levels and reduced activity of the PGC-1α protein in the muscle tissue of subjects with T2DM (571) and is associated with lower levels of glucose and fatty acid oxidation (572). In the animal models of DN, decreased mRNA and protein concentrations of PGC-1α were found in mesangial cells (573). Hyperglycemia influences the expression of *PGC-1α* with metilation of DNA in the region of its promoter, hindering the access to the promoter (569). This results in the reduction of PGC-1α and increase in the dynamin-related protein 1 (DRP-1). The final part of mitochondria fission is controlled by DRP-1. Overproduction of DRP-1 leads to more fragmented mitochondria, their structure deranged, whereas the production of ROS is dramatically increased (574). It is well known that the overproduction of ROS and consequent metabolic stress drives biochemical and morphological changes of DN and other diabetic complication (573). Moreover, carriers of the rs8192678:A allele reveal increased levels of DNA damage (570).

A lot of studies showed a positive association between T2DM and rs8192678:G>A polymorphism. Studies were carried out in Danish (486), northern Indian (488), northern Chinese (489), Iranian (490), Tunisian (491), and Slovenian populations (492). Opposite findings for this association between T2DM and rs8192678:G>A polymorphism were revealed by other studies (495, 496, 497, 498). All three meta-analyses of the association between T2DM and the rs8192678:G>A polymorphism show positive results (467, 499, 500). On the other hand, Yang and co-workers reveal in their sub-analysis a positive association just for the Indian population, and no association for Caucasian and East Asian populations (499).

 The reasons for these deviations in the outcomes of the studies that address the association of T2DM and rs8192678:G>A polymorphism are numerous. The differences in the number of involved participants and the phenotypic characterisation of participants may not be the same. The ethic background has a strong influence. Studies with negative results are from Japan, China and the USA. Positive studies are from Europe, India, Africa (Tunis), Iran and China. The majority of Chinese studies were made in the northern or eastern parts in the Han population. However, these studies are inconsistent. Studies from India, mostly from northern and north-western parts of the country, showed a positive association between T2DM and rs8192678:G>A polymorphism. However, smaller studies are not equivocal as Sharma and co-workers presented by analysing the haplotypes of six polymorphisms of the *PPARGC1A* gene, which included rs8192678:G>A. For example, the same haplotype that presents an increased risk for T2DM in the Bania ethnic group has protective function in Brahmin ethnic group (500). The differences between diverse ethnic populations in the minor allele A of polymorphism rs8192678:G>A, as it results from the 1000 Genome Project Phase3, are not big. In the European population, the frequency of minor allele A is

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36.0% and 29.0% in South Asians (575). On the other hand, the calculated risks in different populations for T2DM are in a wide range. The risk for T2DM in Caucasian carriers of the rs8192678:G>A polymorphism is 10 to 80% greater than for non-carriers (569). In the Slovene population, the risk for T2DM is around 90% greater for carriers of this polymorphism (492). This risk is modestly increased in the Chinese (OR 1.46 to 1.85), but is greater in the Tunisian (1.17 to 2.98) and Indian (1.64 to 5.0) population (487, 488, 489, 491, 500). The biggest reported risk is from Iran, OR 9.0 (490). Such a large risk range for T2DM linked to this polymorphism may be in the additive or synergistic effect of other genes or specific factors of a particular environment.

In this study we did not find any significant differences in the allele and genotype frequencies of the rs8192678:G>A polymorphism between T2DM patients with and without DN. Our outcomes differ from the results of Gayathri and Jung with co-workers. Both studies from Asia showed a positive association with DN and the rs8192678:G>A polymorphism (501, 502). The only study in the field of DN in T2DM linked to rs8192678:G>A polymorphism in the Caucasian population (Great Britain) showed a positive association. In comparison to the rs8192678GG genotype, rs8192678AA/GA genotypes had 1.58-fold higher risk for DN after adjustments (503). Comparing rs8192678GG to the GA genotype, the risk was 1.7-fold higher for the GA genotype also after adjustments. We expected significant differences between the two poles of homozygous genotypes, that is GG vs. AA. Surprisingly, the comparison between the rs8192678GG and rs8192678AA genotype revealed no statistically significant difference, odd ratio 1.20 ([0.66–2.16], p = 0.56). It is difficult to explain this outcome. One possible explanation, as the authors proposed, could be in the case of a significantly increased risk for cardio-vascular events in patients with the rs8192678AA genotype and increased mortality. Significant survival bias could be connected to a higher proportion of small dense LDL particles and the association with atherosclerosis. The study analysed the lipid status and markers of oxidative stress (oxidized – LDL particles and plasma total anti-oxidant status (TAOS)). Small dense LDL particles were in significant association with the rs8192678AA genotype and the presence of albuminuria, but neither was associated with TAOS. As no increased markers of oxidative stress were found in the study, the probability of this explanation was low (503).

Possible explanations for the discrepancies among the results of our and other genetic association studies are numerous and may comprise differences in phenotype definition, the variation in the genetic or environmental background of the populations studied, the possibility of various gene–gene and gene–environment interactions, or an insufficient sample size (539). The negative result of our study could also be due to the fact that environmental risk factors overshadowed the modest effect of studied genetic polymorphisms. After adjustment for several possible confounders (duration of hypertension, SBP, CVD, DR, DF, HbA1c, S-fasting glucose, S-urea, S-creatinine, S-cystatin C, and UACR), a logistic regression analysis showed a 3.8-fold higher risk for DN in

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T2DM subjects with the rs1801282:GG genotype of *PPARG* gene, which proved to be borderline statistically significant. The diabetic patients included in our study did not use anti-diabetic drugs from the thiazolidinedione group. However, the majority used ACE inhibitors, which showed a beneficial effect in improving kidney function (576). It has been recently suggested that the efficacy of ACE inhibitors to protect diabetic patients from microalbuminuria may be related to the *PPARG* gene variability (577).

To summarize, in our study we did not confirm an association between the rs1801282:C>G polymorphism of the *PPARG* gene and the rs8192678:G>A polymorphisms of the *PPPARGC1A* gene and DN. Polymorphisms rs1801282 and rs8192678 are not genetic markers for the susceptibility to DN in Slovenian subjects with T2DM.

6.6   SERUM CONCENTRATIONS IN PATIENTS WITH AND WITHOUT DN

In our study we found no statistical significant differences in the concentrations of sICAM-1, sPECAM-1, IL-10 and IL-18 between patients with and without DN.

Similar to our results for sICAM-1, Fasching and co-workers did not find significant differences in sICAM-1 concentrations between T2DM patients with and without microvascular diabetic complications. The majority of their patients in this small study had DR and DN (110). Additionally, no significant difference in sICAM-1 was found between T2DM patients with and without DN in a small Egyptian study. The study included only 22 T2DM patients with DN and 17 without DN (578). Opposite to our results, Mastej and co-workers found significant differences in sICAM-1 concentrations between T2DM patients with and without microvascular DM complications (579). A comparison between two groups of T2DM patients with microvascular complications and without complications revealed significant greater serum sICAM-1 concentrations in the study group with DM complications. Abu Seman and co-workers also found significant differences in serum sICAM-1 between T2DM patients with and without DN in a Malaysian population. But, as they pointed out, the result was valid only for lean participants (113). Other studies found no correlation between obesity and sICAM-1 concentrations. No correlation was found between BMI and sICAM-1 concentrations in Japanese T2DM patients that use similar BMI criteria as the Malaysians regarding obesity (106). In a North American population, the Atherosclerosis Risk in Communities (ARIC) Study found no correlation between BMI and sICAM-1 concentrations (580). Additionally, the study of Abu Seman was relatively small and patients in the DN group had overt DN with macroalbuminuria and ESRD and lower average levels of eGFR in comparison to our patients with DN. A lower average level of renal function in Malaysian T2DM patients is probably not problematic, because the big prospective Hoorn Study from Netherlands did not find any association between sICAM-1 and renal function (318), despite

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older studies reported a positive association between sICAM-1 concentrations and serum creatinine concentrations in patients with chronic renal failure not receiving dialysis (581, 582).

In the literature review, I did not find any another article on the concentration of sPECAM-1 in T2DM patients with and without DN, except our article from present study (583).

In our study, no significant difference was found between patients with and without DN regarding serum IL-18 concentrations, but practically all accessible literature showed opposite results (373, 554, 555, 557). Araki and co-workers presented serum IL-18 concentrations that progressively increased from normoalbuminuria to microalbuminuria and proteinuria. Elevated serum IL-18 in T2DM patients with UAER in the upper level of the normal range could be predictive markers for the development of DN (555). All four studies were done on a Japanese population and no published study was done in the Caucasian or any other populations. The reasons for these discrepancies regarding the concentration of IL-18 could be attributed to differences in laboratory methods, the number of patients studied, the level of metabolic control, comorbidities and therapy (statins) (584). Furthermore, ethnicity may have influence. For example, susceptibility for DN or DR is different across different ethnic populations. Because such diabetic complication comprises inflammation, it is possible that also some components of the inflammatory process are influenced by ethnicity (559, 585, 586, 587).

Similarly to our result, Wu and co-workers found no differences regarding IL-10 serum concentrations between patients with T2DM with and without DN. It should be said that the part of the study where cytokines were determined was more like a pilot sub-study (each group included only 12 patients) (206). Contrary to our findings, two studies (from China and Egypt) have reported significant differences between T2DM patients with and without DN regarding IL-10 serum concentrations (393, 578). Also, two studies (from Poland and India) in T1DM showed differences between patients with and without DN regarding IL-10 serum concentrations (392, 588). Again, not all studies in the field of DN and serum IL-10 concentrations in T1DM patients are consistent. Prestana and co-workers found no significant differences in serum IL-10 concentrations between Brazilian patients with and without DN (589).

The inconsistencies among results of our and other studies are difficult to explain. The reason for differences may be different study sizes, differently defined phenotypes of patients, different environmental factors or metabolic regulation, and even ethnical differences.

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7 CONCLUSIONS

In this retrospective study, 651 Caucasians with T2DM of more than 10 years' duration were enrolled. They were divided into two groups: patients with DN (276 subjects) and patients without DN (375 subjects).

- Patients with DN had significantly higher systolic blood pressure, duration of arterial hypertension, serum creatinine, urea and cystatin-C levels in comparison with patients without DN.

- Moreover, patients with DN had a poorly regulated T2DM, a higher number of cardiovascular events, and were more likely to suffer from DR compared to controls.

In our retrospective association study, the distribution of genotypes of different polymorphisms between cases and controls was compared.

- Polymorphisms rs5498 and rs1799969 of *ICAM1* gene were not associated with DN.

- The polymorphism rs688 of the *PECAM1* gene was not associated with DN in our study population of T2DM patients.

- No association was found between rs2280788 and rs2107538 polymorphisms of the *CCL5* gene and DN in our study population of T2D patients.

- No association was found between the rs1799987 polymorphism of the *CCR5* gene and DN in our study population of T2D patients.

- No association was found between the rs1799864 polymorphism of the *CCR5* gene and DN in our study population of T2D patients.

- The rs3212227 polymorphism of the *IL12* gene was not associated with DN in our study population of T2D patients.

- The rs187238 polymorphism of the *IL18* gene was not associated with DN in our study population of T2D patients.

- The rs1800896 polymorphism of the *IL10* gene was not associated with DN in our study population of T2D patients.

- The rs2243250 polymorphism of the *IL4* gene was not associated with DN in our study population of T2D patients.

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- The rs1801282 polymorphism of the *PPARG* gene was not associated with DN in our study population of T2D patients.

- The rs8192678 polymorphism of the *PPARGC1A* gene was not associated with DN in our study population of T2D patients.

Serum concentrations of ICAM-1, PECAM-1, IL-18 and IL-10 were determined and compared with regard to genotypes of appropriate polymorphisms.

- No association was found between different genotypes of either the rs5498 or rs1799969 polymorphism of the *ICAM1* gene and serum sICAM-1 levels in a subpopulation of 120 diabetics with DN.

- No association was found between different genotypes of the rs668 polymorphism of the *PECAM1* gene and serum sPECAM-1 levels in a subpopulation of 120 diabetics with DN.

- No association was found between different genotypes of the rs187238 polymorphism of the *IL18* gene and serum IL-18 levels in a subpopulation of 120 diabetics with DN.

- No association was found between different genotypes of the rs1800896 polymorphism of the *IL10* gene and serum IL-10 levels in a subpopulation of 120 diabetics with DN.

Moreover, serum concentrations of ICAM-1, PECAM-1, IL-18 and IL-10 did not differ between patients with DN and those without DN.

To conclude, genetic polymorphisms from our set of genes involved in the inflammatory response were not associated with DN in Caucasians with T2DM. For this reason, they may not be considered as potential markers for DN in a population of Caucasians with T2DM.

Moreover, serum levels of sICAM-1, sPECAM-1, IL-18 and IL-10 were not associated with different genotypes of corresponding selected polymorphisms in a subgroup of patients with DN.

As chronic inflammation is the background for the initiation and progress of DN, and genetic susceptibility for DN is known, additional genetic studies with a much higher number of participants and more polymorphisms of different genes included in inflammation processes will be needed to elucidate which inflammatory genetic markers could be useful for an early detection of patients who progress to DN.

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8   SAŽETAK

Polimorfizam upalnih gena kao mogući predskazatelj diabetičke nefropatije u bolesnika sa šećernom bolesti tipa 2

Matej Završnik, 2019

Cilj: Dijabetička nefropatija (DN), edna od težih kroničkih komplikacija šećerne bolesti (DM), posljedica je kronične upale niskog stupnja. Cilj istraživanja bio je utvrditi povezanost gena "kandidata" koji mogu biti važni za razvoj upale i DN u bijelaca s tipom 2 DM.(T2DM).**Metode i bolesnici:** Bolesnici su bili podijeljeni u skupine s DN (276 bolesnika) i bez DN (375 bolesnika). Obavljena je single nucleotide polymorphism (SNP) genotipizacija za 13 SNPs za četiri područja upale: adhezijske molekule: *ICAM1* geni (rs5498, rs1799969), *PECAM1* (rs668); kemokine: *CCL5 (*rs2280788, rs2107538), *CCR5* (rs1799987) i *CCR2 (*rs1799864); interleukine (IL): rs3212227, rse87238, rs1800896, rs2243250 geni za *IL12B*, *IL18*, *IL10* i *IL4*; nuklearne receptori: *PPARG* (rs1801282) i *PPARGC1A* (rs8192678).

Rezultati: Nije pronađena povezanost SNPs odabranih adhezijskih molekula (rs5498, rs1799969, rs668) i kemokina (rs2280788, rs2107538, rs1799987 i rs1799864) za DN u bolesnika s T2DM. Slično tome, nije pronađena povezanost sa SNP interleukina (rs3212227, rs187238, rs1800896 i rs2243250) i nuklearnih receptora (rs1801282, rs8192678) s DN. Razine sICAM-1, sPECAM-1, IL-18 i IL-10 u serumu nisu se razlikovale između različitih genotipova odgovarajućih odabranih polimorfizama.

Zaključci: Nije utvrđena povezanost za genski polimorfizmi naše odabrane skupine gena koji sudjeluju u upalnom odgovoru s DN u bijelaca s T2DM i zato se ne može smatrati mogućim biljezima za nastanak DN u toj populaciji. Također nisu utvrđene razlike u razini sICAM-1, sPECAM-1, IL-18 i IL-10 u serumu u različitim genotipovima odgovarajućih gena.

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9   ABSTRACT

Polymorphisms of inflammatory genes as potential predictors of diabetic nephropathy in patients with type 2 diabetes

Matej Završnik, 2019

Objectives   One of the more devastating chronic complications of diabetes mellitus (DM) is diabetic nephropathy (DN), with a chronic low-grade inflammation occurring in the background. The aim of this study is to examine the relationship between chosen candidate genes with potential functional importance in inflammation and DN among Caucasians with type 2 (T2DM) as markers to identify patients who are more likely to develop DN.

Methods and patients Participants were divided into two groups: patients with DN (276 subjects) and without DN (375 subjects). SNP genotyping for 13 SNPs from four fields of inflammation were performed: Adhesion molecules: *ICAM1* gene (rs5498, rs1799969), *PECAM1* (rs668); Chemokines: *CCL5 (*rs2280788, rs2107538), *CCR5* (rs1799987) and the *CCR2 (*rs1799864); Interleukins (ILs): rs3212227, rs187238, rs1800896, rs2243250 of *IL12B*, *IL18*, *IL10* and *IL4* genes, respectively; Nuclear receptors: *PPARG* (rs1801282) and *PPARGC1A* (rs8192678).

Results No associations were found between the SNPs of chosen adhesion molecules (rs5498, rs1799969, rs668) and chemokines (rs2280788, rs2107538, rs1799987, rs1799864) and DN in T2DM patients. Similarly, no associations were found for SNPs from interleukins (rs3212227, rs187238, rs1800896, and rs2243250) and nuclear receptors (rs1801282, rs8192678) even with DN. Serum levels of sICAM-1, sPECAM-1, IL-18 and IL-10 did not differ between different genotypes of corresponding selected polymorphisms.

Conclusions Genetic polymorphisms from our set of genes involved in the inflammatory response were not found to be associated with DN in Caucasians with T2DM and may not be considered as potential markers for DN in this population. Additionally, no differences were found in serum levels of sICAM-1, sPECAM-1, IL-18 and IL-10 according to different genotypes of corresponding genes.

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11 CURRICULUM VITAE

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